Adrenomedullin mediates lung metastasis of colorectal cancer through CRLR receptor

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Abstract

**Purpose:** The current study investigated adrenomedullin (ADM) as a potential autocrine regulator of colorectal cancer (CRC) cells.

**Methods:** The expression levels of ADM and calcitonin receptor–like receptor (CRLR) mRNA in 26 primary colorectal tumor and adjacent non-tumor tissues were analyzed by quantitative real time (qRT)-PCR. And CRLR expression in 7 CRC cell lines was tested by qRT-PCR and Western blot analysis. Cell migration, invasion and lung metastasis were detected in vitro and in vivo after exogenous ADM stimulation and silencing of CRLR.

**Results:** We found that the expression levels of ADM and CRLR were significantly higher in colon cancers than those in adjacent nontumorous tissues. In addition, the expression levels of ADM and CRLR in CRC were correlated with UICC stage, lymph node metastasis and differentiated degree. Exogenous ADM promoted the migration of RKO and HT-29 cells, and RNA silencing of CRLR in colon cancer cells blocked ADM-induced migration and invasion, suggesting that CRLR might be involved in the autocrine actions of ADM. Xenograft tumors with CRLR knockdown had dramatically reduced lung metastasis compared to control cells.

**Conclusions:** This study describes the important roles of ADM in potentiating the aggressive characteristics of CRC, and the CRLR receptor was identified as being critical for these effects. These observations provide significant insights into the multifaceted role of ADM in CRC. Combined with the known importance of ADM as an angiogenic factor, these data generally support the further development of CRLR as a potential therapeutic target of CRC.

Introduction

CRC is the third leading cause of cancer-related death in North America, with an estimated 49,190 deaths from the disease in 2016 (1). Advanced CRC with distant metastasis (mCRC) had poor prognosis, though over the last decade the availability of combination chemotherapy and biological agents has improved the median survival time of those patients (2, 3). The mechanisms underlying CRC progression have not been fully understood. Many studies have demonstrated that mutations of KRAS, p53, SMAD4 and BRAF play prominent roles in CRC distant metastasis and may be both potential biomarkers of CRC metastasis as well as therapeutic targets (2, 3). Additionally the epithelial-to-mesenchymal transition (EMT) serves as a prominent molecular event in the early stages of distant metastasis. During EMT, epithelial cancer cells lose the 'epithelial phenotype', leading to a loss of apical–basal polarity, thus gaining mesenchymal cell-like properties, detaching from the primary tumor mass and migrating to the new location for colonization and proliferation (2, 3). Moreover tumor hypoxic microenvironment may result in the up-regulation of hypoxia inducible factor (HIF), which leads to the elevation of vascular endothelial growth factor VEGF production and angiogenesis. With the continuous development of basic research and clinical trials, targeted therapy of CRC has gradually become individualized and refined. Currently,
epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR) are the most commonly-used molecular targets for CRC therapy. Studies have shown that KRAS and other gene mutations are associated with resistance to EGFR targeted therapy (2, 3). The mechanisms of drug resistance usually include target self-change, activation of bypass pathway, activation of downstream effectors or crossover through related pathways, etc. As no advantages for targeted therapy have been displayed on aspects of clinical efficacy and drug resistance compared with conventional chemotherapy, new potential molecular targets are required to find out. We have previously showed that Adrenomedullin (ADM) was upregulated in CRC patients carried with mutant K-ras and hypoxic condition. Knockdown of ADM blocked angiogenesis and suppressed tumor growth, which suggest that ADM may serve as a therapeutic target depending on the hypoxia tumor microenvironment in CRC (4).

ADM is a multifunctional peptide with properties ranging from inducing vasorelaxation to acting as a regulator of cellular growth and angiogenesis (5-7). Recent studies showed that ADM was widely expressed in various types of tumor including lung, breast, glioblastoma, kidney, prostate and pancreas tumors (8-14). The expression of ADM was upregulated in CRC tissues when compared with that in adjacent normal tissues (15-17). The expression of ADM was correlated with HIF-1 and VEGF in CRC (18). There was a positive correlation between the expression level of MMP-9 and ADM in colorectal adenocarcinomas (15). In addition, ADM could promote cellular proliferation in a penal of cancer cell lines including lung, breast, glioblastoma, kidney, prostate and pancreas tumors (8-14). Treatment of colon cancer cells HT-29 with synthetic ADM promoted cellular proliferation and invasion, while incubation with anti-ADM antibody (aAM) or ADM antagonist AM22–52 inhibited cell proliferation (19).

ADM also acts as a peptide ligand that activates receptors on the cell. ADM binds to and mediates its activity through the G-protein-coupled receptor (CRLR). Receptor activity modifying protein (RAMP) binding to calcitonin receptor-like receptor (CRLR) causes the relocation of CLR to the plasma membrane thus leading to subsequent elevation of cAMP activation of protein kinase A the opening of ATP-sensitive K+ channels and vasodilatation (19) which is now considered as an initial mechanism of tumor formation. However, the expression of specific receptor of ADM (CRLR) and its roles in the development and progression of CRC were not clarified. In the current study, we aimed to systemically examine the expression of ADM and CRLR on human CRC cells and tissues, and investigated whether the promoting effect on cell invasion and distant lung metastasis mediated by ADM was dependent on its receptor CRLR.

**Methods**

**Cell culture and CRC samples**

The human CRC cell lines (SW480, SW620, RKO, HT-29, DLD-1, HCT116, and LoVo) were obtained from Riken Gene Bank (Japan) and American Type Culture Collection (Manassas, VA, USA). HCT116 cell line was cultured in McCoy’s 5A medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum. LoVo cell line was cultured in F12K medium (Invitrogen), SW480 and SW620 cell
lines in L-15 medium (Invitrogen), supplemented with 10% fetal bovine serum. All other cell lines were cultured in DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum. All cell lines were incubated at 5% CO\textsubscript{2}, 37°C and 95% humidity.

Twenty-six samples of colorectal adenocarcinoma and adjacent nontumor specimens were obtained from Sir Run Run Shaw Hospital, School of Medicine Zhejiang University. Specimens were immediately frozen in liquid nitrogen and stored in −80°C until further processing. The study protocol was approved by the Clinical Research Ethics Committee of Sir Run Run Shaw Hospital.

RT-PCR and quantitative real-time PCR

Total RNA (1μg) was reversely transcribed into cDNA with the high-capacity cDNA reverse transcription system (Promega, Madison, WI, USA). The expression level of CRLR was determined by conventional polymerase chain reaction (RT-PCR) with GoTaq polymerase (Promega) and quantitative RT-PCR (qPCR) using SYBR Green Master Mix Kit (TaKaRa) in an ABI 7500 PCR system. Glyceraldehyde-3-phosphatedehydrogenase (GAPDH) was used as an internal control. The relative expressions of ADM and CRLR in primary colorectal tumor and adjacent nontumor tissues were normalized to GAPDH using the 2^\(\Delta\Delta^{Ct}\) method. Primers designed for ADM were F: 5′-GTTCGCTCGCCTTCCTA and R: C5′-ACATCCGCAGTTCCCTC. Primers designed for CRLR were F: 5′-GATACCCATCCTCCTCTACAT and R: 5′-AAAGACCCTGGAAGTG.

Western Blot Analysis

Total proteins were extracted from cells using RAPI lysis buffer. Lysates were resolved on SDS-PAGE gel and transferred to PVDF membranes (Millipore). Primary antibodies ADM (1:500, Abcam), CRLR (1:500, Abcam) and GAPDH (1:2000, Abcam) were used. The blots were developed using a chemiluminescence with Las-4000 Imaging System (Fujifilm, Tokyo, Japan).

Cell Migration and Invasion Assays

Cell migration and invasion were assessed by modified Boyden transwell chambers (Corning, NY, USA) coated without or with BD Matrigel. Briefly, 1×10\textsuperscript{5} cells/well were plated into 250 μl of 5% FBS medium in the upper chamber, and 500μl of medium containing 15% FBS were added to the lower chamber. After being incubated for 24h, the nonmigratory cells in the upper chamber were removed with cotton swabs. The cells on the bottom of the membrane were fixed and stained with DAPI or iodine. The number of visible cells was counted by fluorescence microscope in five random high power fields.

Lentiviral silencing of CRLR

Knockdown of CRLR was achieved in CRC cell lines using lentivirus infection. Lentiviral plasmid vectors-control and CRLR shRNA (Genechem Shanghai, CHN) were co-transfected with packaging vectors and lentivirus was produced in 293T cells by the calcium transfection method. Lenti-CRLR shRNA was titrated
and CRC cell lines were infected using 25 AL viral supernatant/mL of medium mixed with polybrene (4 Ag/mL medium). Cells were then selected by puromycin (1 mg/mL) to develop stably transfected cells.

Lung metastasis models

Animal experimental protocols were approved by the Committee of Animal Ethics, Zhejiang University. Female athymic nude mice (nu/nu) (3 to 4 wks old) were purchased from Shanghai Laboratory Animal co Ltd (SLAC, Shanghai, China). After preparation with colon cancer cells stably knockdown of CRCL. Lung metastasis were developed by tail vein injection of $4 \times 10^6$ cells/100 μl (n =5) per mouse. After 15 weeks, mice were euthanized and tissues were harvested. Routine histological processing and HE stain to check the metastasis in lung was performed.

Statistical Analysis

The differential expressions of ADM and CRLR mRNAs between CRC and normal tissues was analyzed by the Wilcoxon matched pairs test. Mann-Whitney U test was used to analyze categorical variables, and Student's test was used in calculating two independent data points. All data were analyzed using SPSS 15.0 software and graphs were generated with GraphPad Prism 6.0 software. A value of $P < 0.05$ was applied for statistical significance.

Results

The expression of ADM and CRLR is upregulated in human CRC

To investigate the relationship between ADM/CRLR and its clinical relevance, we analyzed the expression level of ADM and CRLR mRNA in primary colorectal tumor and adjacent non-tumor tissues (n = 26) by quantitative real time (qRT)-PCR. The results showed that ADM and CRLR mRNA levels were significantly upregulated in tumor tissues as compared to the adjacent non-tumor tissues (Figure 1A, 1B). The expression level of ADM and CRLR mRNA were also significantly enhanced in CRC patients with advanced stage (UICC III+IV), or with lymph node metastasis, and poorly or undifferentiated ($p < 0.05$, Table 1). In addition, after examining CRLR expression in a panel of CRC cell lines (n=7) by qRT-PCR and Western blot analysis, we found that CRLR was highly expressed in all CRC cell lines tested (Figure 1C, 1D).

ADM promotes the migration and invasion of CRC cells via its receptor CRLR

Interestingly, we found that exogenous administration with ADM (50 nM) significantly stimulated the migration of RKO and HT-29 cells ($p < 0.01$). However, a higher concentration of ADM (200 nM) was not able to exert a stronger effect on cell migration (Figure 2).

Next, we determined whether CRLR was responsible for the effects of ADM on CRC cell migration and invasion. RKO and HT29 cells stably transfected with CRLR shRNA showed a significant reduction in the expression of CRLR when compared with that of control shRNA-bearing cells (Figure 3A,
3B). ADM stimulation showed a significant increase in migration, whereas silencing of CRLR significantly reduced cell migration (Figure 3C, 3D, 3E and 3F) and invasion (Figure 4A, 4B, 4C and 4D) in both HT29 and RKO cells.

**Silencing of CRLR inhibited lung metastasis in CRC xenograft tumors**

To directly evaluate the role of CRLR in CRC lung metastasis in vivo, we conducted separate studies in nude mice by tail vein. In contrast, there was an obvious increase of the body weight in shCRLR mice when compared to shControl in 9, 12, and 15 weeks (Figure 5A). In addition, there was no cancer lobe observed in the lung after CRLR knockdown (Figure 5B). HE staining showed that silencing CRLR could reduce the incidence of lung metastasis (4/5 Vs 0/5) (Figure 5C and D). These data indicate that ADM can act as a metastasis inducer of CRC cells via the receptor CRLR.

**Discussion**

CRC is a common cause of cancer death worldwide due to late tumor presentation and rapid progression (1, 20, 21). Currently, studies are investigating the etiology, pathogenesis and epidemiology of CRC (22-25). However, their molecular mechanisms remain poorly understood. Classical chemical drugs that prevent mitotic progression have been successful in the clinical treatment of cancer, but side effects limit their clinical application (26-32). We previously reported that ADM was highly expressed in CRC tissues and it regulated CRC cell invasion in vitro (4). Also, knockdown of ADM in colon tumor xenografts blocked angiogenesis and stimulated apoptosis, resulting in tumor suppression. In the current study, we further investigated the expression of ADM and its receptor CRLR in CRC, and observed that ADM and CRLR were present in nearly all colorectal tumors and cell lines and that ADM stimulated CRC cell migration and invasion through CRLR in vitro.

We previously reported that ADM was higher in CRC when compared with normal colon tissues (4). In the current study, we examined ADM together with its receptor CRLR, and confirmed their presences in different types of CRC cell lines. Besides, ADM and CRLR mRNA levels were upregulated significantly in tumor tissues in relative to adjacent non-tumor tissues. Also, qPCR assay revealed a high mRNA levels of ADM and CRLR in CRC of higher UICC stage, positive lymph nodes and poor differentiation. The marked expressions of ADM and CRLR in CRC tissues strongly suggest that ADM and CRLR might be involved in CRC progression and metastasis.

To examine the possibility of an autocrine effect of ADM on CRC cells, exogenous ADM was added, and a significantly increased number of migrated CRC cells was observed. Combined with the observation that the cells normally express and secrete ADM, these data strongly support the conclusion that ADM can act in an autocrine manner in CRC.

ADM has complex physiological actions mediated by its receptor (33). It has been reported that endothelial cells expressed CRLR (5) and CRLR is the main mediator of ADM effects on the vasculature (34, 35). For this reason, there is a concern that systemic inhibition of ADM may have harmful effects on
normal physiology. Therefore, rather than ADM itself, we were interested in the possibility of targeting its receptor. Currently little is known about the role of CRLR in the regulatory effects of ADM on CRC. The current study showed that CRLR-silenced cell lines showed a significant reduction on ADM-stimulated migration and invasion of RKO and HT-29 cells in vitro. These data indicate that ADM act as a migration and invasion inducer of CRC cells and these effects of ADM on cancer cells are mediated via the receptor CRLR. Having identified CRLR as a critical ADM receptor for CRC, we examined the effect of CRLR-knockdown specifically in cancer cells by stably silencing CRLR in human CRC cells in vitro and then using them to develop metastatic tumor. We found that silencing CRLR reduced the incidence of lung metastasis as measured by pathological staining. These studies supported that CRLR might act as a metastasis inducer and a potential therapeutic target of CRC.

In conclusion, this study describes the important roles of ADM in potentiating the aggressive characteristics of CRC, and the CRLR receptor was identified as being critical for these effects. These observations provide significant insights into the multifaceted role of ADM in CRC. Combined with the known importance of ADM as an angiogenic factor, these data generally support the further investigation of CRLR as a potential therapeutic target of CRC.

Declarations

Authors’ contributions


Conflict of Interest: None

Ethical Approval

not applicable

Competing interests

The authors declare no competing interests.

Authors’ contributions


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Availability of data and materials

not applicable

References


Tables

Table 1. Relationships between ADM/CRLR expression and clinicopathologic characteristics
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**Figures**
Figure 1

Adrenonedullin and CRLR are expressed in CRC. A: ADM mRNA expression was determined by quantitative realtime PCR in 26 paired operation samples from primary colorectal tumor and adjacent nontumor tissues. The data were described as relative log10 value, and analyzed by Wilcoxon matched pairs test. B: CRLR mRNA level was significantly higher in CRC than in adjacent nontumor tissues ($p<0.01$). C: The expression profile of CRLR was analyzed in seven colorectal cancer cell lines, as well as in two of normal stomach tissues by conventional RT-PCR. GAPDH was used as the internal control. D: The expression profile of CRLR was analyzed in seven colorectal cancer cell lines by Western Blot. GAPDH was used as the internal control.
Figure 2

Exogenous ADM stimulates CRC cells migration in vitro. A: Cell migration assays were performed in modified Boyden transwell chambers assay. RKO cells were plated and different concentrations of ADM (0, 50, 200nmol/L) were added into the upper chamber. 24h later, the migratory cells on the bottom of the membrane were fixed and nucleus stained with DAPI. B: The mean number of visible RKO cells was counted by fluorescence microscope in five random high power fields. C: Cell migration assays were performed in HT29 cells. D: The mean number of visible HT29 cells was counted by fluorescence microscope in five random high power fields. (**) representative of p<0.01)
Cell migration respond to ADM was mediated via its receptor CRLR. A: CRLR was silenced using a lentiviral shRNA technique, and the mRNA expressions of CRLR in RKO and HT-29 were detected by PCR. B: ShCRLR significantly reduced the protein expression of CRLR in RKO and HT-29 cells. C: Cell migration assays. RKO cells stably transfected with shCRLR or control shRNA, and then treated with ADM (50nmol/L) or not. D: The mean number of visible RKO cells was counted by fluorescence microscope. E: Cell migration assays were performed in transwell chambers assay in HT29 cells. F: The mean number of visible HT29 cells was counted. (** representative of p<0.01)
Figure 4

Cell invasion respond to ADM was mediated via its receptor CRLR. A: Cell invasion assays were performed in modified Boyden transwell chambers assay. RKO cells stably transfected with shCRLR or control shRNA, and then treated with ADM (50nmol/L) or not. B: The mean number of visible RKO cells was counted by fluorescence microscope. C: Cell invasion assays in HT29 cells D: The mean number of visible HT29 cells. (** representative of p<0.01)
Figure 5

Effects of CRLR on metastasis. RKO cells bearing luciferase gene stably transfected with shControl or shCRLR cells were injected into the tail vein to measure lung metastasis. Animals bearing CRLR silenced cells showed reduction in the incidence of lung. A: Body weight of mice treated with shControl or shCRLR showed obvious difference after a period of 9 weeks, 12 weeks and 15 weeks. B: cancer was not found in the lung. C: Stable silencing of CRLR reduced the number of metastatic foci on lung when compared to shControl. Pictures of pathological staining showed the reduction on number of metastatic foci in lungs. (** representative of p<0.01)