Neuropeptide Levels as well as Neprilysin Activity Decrease in Renal Cell Carcinoma

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Abstract Calcitonin Gene-related Peptide (CGRP), Vasoactive Intestinal Peptide (VIP) and Substance P (SP) are sensory neuropeptides which may alter cancer growth through modulation of chronic inflammation. We recently reported that SP suppresses breast cancer growth and metastasis through neuroimmune modulation. These neuropeptides are hydrolyzed by Neprilysin (NEP) to bioactive fragments. Decreased activity of NEP was reported in clear cell and chromophobe type renal cell carcinoma (RCC). It is however not known how the levels of neuropeptides hydrolyzed with NEP changes in RCC. Decrease activity of SP and CGRP containing sensory nerve endings was previously reported to increase cancer metastasis in animal models. It is however not known how peptidergic nerve endings are altered in RCC. Hence we here evaluated the levels of neuronal and non-neuronal neuropeptides and NEP activity in RCC including papillary type as well as neighboring uninvolved kidney. A cross-sectional study was conducted in 57 patients undergoing radical nephrectomy and diagnosed with RCC. NEP activity, levels and expression were determined using fluorogenic substrate, western blot and qPCR respectively in freshly-frozen tissues. Immunohistochemical analyses were also performed.

Neuronal and non-neuronal levels of CGRP, SP and VIP levels were determined using two-step acetic acid extraction. Levels and activity of NEP were markedly decreased in RCC regardless of subtype. Similar levels of VIP were detected in first and second extractions. VIP levels were higher in clear cell and papillary RCC compared to nearby kidney tissue. VIP levels of neighboring kidney tissue of papillary type RCC was significantly lower compared to kidney samples from clear cell RCC. CGRP levels were higher in second extraction. Similar to VIP levels, CGRP levels of neighboring kidney tissue from clear cell and chromophobe type RCC was significantly lower compared to corresponding tumor samples, an effect observed in the second extraction. VIP and CGRP levels of nearby kidney tissue varied subtype dependently demonstrating that different subtypes of RCC alter their local environment differently. Furthermore NEP-induce hydrolysis of VIP creates selective VPAC-1 receptor agonist which has anti-proliferative and anti-inflammatory effects. Hence loss of NEP activity may prevent anti-tumoral effects of VIP on RCC.

Keywords Renal cell carcinoma · Neutral endopeptidase · CGRP · VIP

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Introduction

Neutral endopeptidase (NEP, neprilysin, enkephalinase, CD10), a transmembrane metalloendopeptidase, cleaves neuropeptides such as Substance P (SP), calcitonin gene-related peptide (CGRP) and vasoactive intestinal peptide (VIP) [1–3]. Loss of NEP activity is associated with cancer formation [4]. Anti-tumoral effects of NEP not only arise from its peptidase activity but also its direct effect on cell growth, survival and migration [5].

Vasoactive intestinal peptide (VIP) acts as a neurotransmitter and is widely distributed in central and peripheral nervous system. Nerve fibers immunoreactive for VIP were demonstrated in human kidneys [6]. VIP exerts its effects by binding to two different receptors namely VPAC1 and VPAC2 [7]. Degradation of VIP by NEP generates an active metabolite selective for the VPAC1 Receptor [3] which is found in the glomeruli and activation of VPAC1 induces anti-inflammatory response [8, 9]. VIP is also produced by various immune cells, acting as a potent anti-inflammatory factor [10]. VIP was recently found to have antitumoral effects in human renal cell carcinoma cells [11]. CGRP, widely expressed in central and peripheral nervous systems, [12] also exerts potent anti-inflammatory effects [13] which may decrease cancer formation [14]. In accordance loss of CGRP expression in human colon cancer was reported [15]. SP is found in both neuronal and extraneuronal cells [16] and we recently demonstrated that SP through neuroimmune modulation decreases breast cancer metastasis and enhances the therapeutic effects of radiotherapy [17]. Recent findings suggest that hydrolysis of SP by NEP produces bioactive fragments which inhibit tumor cell growth [18]. These neuropeptides, particularly CGRP and SP, are also found in capsaicin-sensitive sensory nerve fibers which may act to inhibit cancer metastasis as demonstrated in animal models [17–20].

NEP/CD10 is considered as a potential diagnostic marker in RCC [21]. There are however only few studies examining the possible functional role of NEP activity and actual levels of NEP in RCCs [22, 23]. In these studies, decreased enzymatic activity of NEP was shown in certain types of RCC: It is however not known how decreased NEP activity affects neuropeptide levels in RCC. Up to our knowledge neuropeptide content of RCC as well as uninvolved neighboring kidney tissue was not examined before. The goal of this study was to determine the neuronal and non neuronal levels of CGRP, VIP, SP as well as levels and activity of NEP in RCC which may help us to understand microenvironmental changes in RCC.

Materials and Methods

This cross-sectional study was conducted in 57 patients with RCC undergoing radical nephrectomy at the Department of Urology, Medical Faculty of Akdeniz University. Among these patients 35 were diagnosed with clear-cell RCC (61,4 %); 13 (22,8 %) were diagnosed with papillary RCC and 9 (15,8 %) were diagnosed with chromophobe RCC. Distant metastasis at the time of diagnosis was observed in 4 (11,4 %) and 3 patients (23,1 %) with clear and papillary RCC respectively. None of the patients with chromophobe RCC had distant metastasis. Thirty-eight of these patients

(66.67 %) were male. The average age was 56.75 ± 11.89 (SD). The study protocol was approved by the Committee of Ethics at the Faculty of Medicine, Akdeniz University, Antalya, Turkey (Protocol number: B.30.2.AKD.0.01.00.00). Written informed consent was obtained from each patient. Procedures were conducted according to the principles expressed in the Declaration of Helsinki and informed consents with guarantees of confidentiality were obtained.

Measurement of Substance P, VIP and CGRP Levels

Samples taken from fresh tumors as well as uninvolved kidney tissue after nephrectomy were frozen in liquid nitrogen and kept in -80°C until analysis. Neuronal levels of peptides were measured using sequential acetic acid extraction as described before [16, 24]. Briefly, tissue samples were cut into small pieces and kept in 1 ml of 2 % acetic acid at 95°C for 10 min. Sequential collections of supernatants were performed in which the first 10 min extraction included neuropeptides predominately found in capsaicin-sensitive sensory neurons while the second extraction re-incubated in 1 ml of 2 % acetic acid at 95°C for 50 min yielded the both the neuronal and non-neuronal compartment of the peptide [16].

Supernatants were dried completely then reconstituted in 150–300 μl of sample buffer from the SP EIA kit (Cayman Chem., Catalog No. 583,751). From each sample, 25 and 50 μl were used for immunoassay, which gave results within confidence interval (%95). Tissue extractions were also used for quantifying VIP (Bachem-Penninsula Laboratories, cat. no.: S1183) and CGRP (Phoenix Pharmaceuticals, cat. no.: EK-015-02) by immunoassay.

Dansyl-d-Ala-Gly-p-nitro-Phe-Gly, dansyl-d-Ala-Gly and fosforamidon were purchased from Sigma (St. Louis, MO). Measurement of NEP activity was performed as described previously with some modifications [5]. Because we could detect very low levels of SP in first 30 samples, SP measurement were not continued.

Measurement of Neprilysin (NEP) like Activity NEP-like activity in snap-frozen tissues was measured using Dansyl-d-Ala-Gly-p-nitro-Phe-Gly, Dansyl-d-Ala-Gly and fosforamidon as described before [19]. Briefly snap-frozen tissues (50–75 mg) were weighed and sonicated 5 times on ice for 15 s in ice-cold 50 mM Tris-HCl, (pH 7.4) buffer which included 1 % Triton-X100. Samples (10 μl) were pre-incubated with enalapril, an angiotensin-converting enzyme (ACE) inhibitor, to prevent cleavage of the fluorogenic substrate (N-dansyl-d-Ala-Gly-p-nitro-Phe-Gly) by ACE, in the presence or absence of fosforamidon, a specific NEP inhibitor. Following this pre-incubation, the fluorogenic substrate was added, and samples were incubated for an

additional 2 h at 37 °C. The final concentrations were 16.5 µM for enalapril, 16.5 µM for phosphoramidon, and 200 µM for the substrate, in a reaction volume of 160 µl. After 2 h of incubation, fluorescence absorbance was recorded using a BIOTEC FX800 Reader. The amount of product was estimated by measuring its fluorescence intensity at 562 nm with excitation at 342 nm. Arbitrary fluorescence units for each sample were compared with a standard curve prepared using Dansyl-d-Ala-Gly (cleavage product) to determine NEP-specific activity per mg tissue.

Immunohistochemical Analysis of NEP (CD10) Paraffin embedded tissues were stained with routine immunohistochemical techniques in Department of Pathology. Intensity and localization of staining were evaluated using immunoreactivity scale. Specifically the staining intensity of CD10 (Thermo Scientific cat no: MS-728-S1) were evaluated with a four-tier grading system (0 – negative, 1 – weak, 2 – moderate, 3 – strong staining intensity). Besides intensity, area of staining was also graded as follows: Percentage scale: 0- no stained cells, 1- >0–25 % stained cells, 2- >25–50 % stained cells, 3- >50–75 % stained cells, 4- >75–100 % stained cells).

The immunoreactivity score was calculated using the formula below.

$$IRS = SI \times PS, IRS : \text{Immunoreactivity score}; PS$$

: score for percent of staining, *SI*, Staining intensity

Western Blot Analysis Freshly frozen tissues were homogenized and were separated on 10 % polyacrylamide gels under denaturing conditions. The proteins were then transferred to Polyvinylidene Difluoride (PVDF) membrane at 40 V overnight. NEP and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels were determined using rabbit polyclonal anti-CD10 (sc9149, Santa Cruz) and mouse monoclonal anti-GAPDH antibody (sc-365,062, Santa Cruz) respectively.

Quantitative Polymerase Chain Reaction (qPCR) Level of gene expression of NEP was determined using snap-frozen tissues. RNAs were isolated using High Pure RNA Isolation Kit (ROCHE cat no: 11,828,665,001) followed by cDNA synthesis (Transcriptor High Fidelity cDNA Synthesis kit. ROCHE Cat no: 05,081,963,001). PCR reactions were performed using LightCycler 480 Probes Master kit (ROCHE cat no: 04,707,494,001) and the appropriate primers for CD10 (Assay ID: 109,438, amplicon length 77 bps, 1347–1423 bps) and GAPDH (Assay ID. 101,128, amplicon length 112 bps, 647–712 bps) obtained from Roche.

Assays were performed in Roche LightCycle 480 96 well plate system.

Statistical Analysis Statistical analyses were performed using Instat software (Graph-Pad). The difference in immunostainings among different groups was analyzed via Mann–Whitney Test. Depending on the data, differences in peptides and enzyme activity levels were determined either using Student's *t* tests with or without Welch correction. Results were presented as mean ± standard error. *P* < 0, 05 was considered significant.

Results

Neprilysin Activity and Levels significantly Decreased in Renal Cell Carcinoma with Greatest Decrease in Papillary Type

NEP like activity decreased markedly in all types of RCC compared to neighboring uninvolved kidney tissue. There were however type-dependent differences in NEP activity and expression (Fig. 1a–d and Table 1). Specifically NEP like activities as well as tissue expression of NEP were significantly lower in papillary RCC compared to clear cell RCC. Western blot analysis also documented lack of NEP expression in clear cell RCC. These results were further validated using qPCR analysis of NEP expression. Immunohistochemical analysis demonstrated diffuse and intense expression of NEP in proximal tubules of uninvolved neighboring kidney which was both cytoplasmic and membranous. NEP expression in clear cell and chromophobe RCC was more membranous and markedly lower compared to renal tissue and it was mostly absent in papillary RCC.

Changes in VIP and CGRP Levels

Peptide levels were measured using two-step acetic acid extraction. Using Substance P we previously documented that majority of peptides in capsaicin-sensitive sensory nerve fibers were found in the first extraction. Second extraction likely to contain peptides found in other nerve fibers and in non-neuronal tissue [16]. We could here detect very low levels of Substance P in only few tissue extracts (assay verification was made using skin tissue).

Similar levels of VIP were detected in first and second extractions. VIP levels were higher in clear cell and papillary RCC compared to nearby kidney tissue. VIP levels obtained in first and second extractions of neighboring kidney tissue of papillary type RCC was significantly lower compared to kidney samples from clear cell RCC (Fig. 2).

Fig. 1 Changes in NEP/CD10. Panel **a** type dependent changes in NEP activity, Panel **b** Changes in NEP levels determined by using western blot. First 3 lanes demonstrate the samples from papillary RCC, Panel **c**

expression of NEP determined by using qPCR. Panel **d** tissue expression of NEP, A denotes 20× and B denotes 40× magnification

CGRP levels were higher in second extraction. Similar to VIP levels, CGRP levels of neighboring kidney tissue from papillary RCC was significantly lower compared to kidney samples from clear cell RCC in

second extraction. CGRP levels found in second extraction were significantly lower in papillary RCC compared to clear cell RCC (Fig. 3).

Table 1 Immunohistochemical analysis of NEP expression

	CLEAR CELL	PAPILLARY	CHROMOPHOBE
Kidney	10,54 ± 0,56	11,89 ± 0,11	9,67 ± 1,5
Tumor	2,21 ± 0,62 **	0,11 ± 0,11	3,0 ± 1,92

The immunoreactivity scores were shown which was calculated using the formula: IRS = SI × PS, IRS; Immunoreactivity score; PS: Score for percent of staining, SI: Staining intensity.

**Significantly different than papillary RCC

Discussion

Previously it was demonstrated that NEP activity and levels are decreased in subset of clear-cell and chromophobe RCC and in renal oncocytomas [22, 25]. Our results obtained after detailed examination of NEP levels with activity assays, qPCR, immunohistochemistry and western blot analysis have further confirmed and extended these findings. Specifically we have demonstrated for the first time that activity of NEP in papillary type RCC is decreased compared to clear-cell

Fig. 2 Changes in VIP levels. VIP levels in first and second extractions are seen and significantly different compared to papillary renal cell carcinoma

RCC. Furthermore decreased levels of CGRP and VIP were found in neighboring kidney tissue of papillary type RCC for the first time. We also found that SP levels are below detection limit in both tumor tissue and neighboring kidney. Because similar levels of NEP activity was present in neighboring kidney tissue of all the patients, lower levels of neuropeptides in papillary type suggest decrease sensory innervations of kidney tissue invaded by papillary RCC.

We here used two-step acetic acid extraction to evaluate levels in sensory neurons and in non-neuronal tissues. Although similar levels of VIP were found in both first and second extractions, CGRP was mostly confined to the second extraction which mainly represents levels in non-neuronal tissue. Although we previously documented that first extraction is sufficient to yield the peptides found in capsaicin-sensitive sensory nerve fibers, second extraction may also include peptides found in large diameter nerve fiber but more detailed studies required to clarify this part [16]. VIP and CGRP are also found in immune cells [26]. Hence part of VIP and majority of CGRP in our samples might be originated from infiltrating immune cells. It was previously shown that loss of

peptidergic sensory neurons sensitive to capsaicin increases metastasis of breast carcinoma inducing aggressive phenotype and possibly stemness and resistance to treatment [18, 20, 27]. Although it was not possible to measure peptide levels in normal kidney tissues (not involved with a tumor), comparing kidney samples of different subtypes demonstrated the loss of peptidergic innervation was most obvious in kidney neighboring papillary RCC.

NEP is considered to be a tumor suppressor protein [5, 28] and demonstrates an anti-angiogenic effect. Degradation of VIP by NEP generates an active peptide [3] which may have direct anti-tumoral effects [29]. We here demonstrated that NEP expression and activity decreases in all major types of RCC, lowest levels being in papillary RCC. Levels of VIP and CGRP in tumor tissue were higher compared to neighboring kidney tissue which might be due to decreased enzymatic cleavage of the peptides.

Increased inflammatory markers systemically or locally is associated with poor survival, metastasis and resistance to treatment in RCC [14]. For example IL-8, an inflammatory mediator, was found to be higher in primary renal tumors and

Fig. 3 Changes in CGRP levels. CGRP levels in first and second extractions are seen and significantly different compared to papillary and chromophobe renal cell carcinoma

it may contribute to sunitinib resistance [30]. Immune suppression is observed over the course of inflammatory diseases [31]. Anti-inflammatory molecules with immune modulatory activities may actually inhibit tumor growth and metastasis [32]. Chronic inflammation-induced local immune suppression may not only increase metastasis but may also decrease response to immunotherapy [33]. Both CGRP and VIP are involved in local immune regulation providing significant anti-inflammatory response [13]. Hence decreased levels of neuropeptides in neighboring tissue of certain subtypes of RCC may have an etiological role. Recently it was demonstrated that papillary type in RCC signifies resistance to immunotherapy [34]. We here found that lowest levels of CGRP and VIP in kidney tissues were found in papillary RCC. In addition to its direct anti-tumoral effects [29], VIP and analogues are considered for treatment of autoimmune and inflammatory diseases [35]. Hence local inhibition of peptidergic system in papillary type RCC may partly be responsible from resistance to immunotherapy. Furthermore NEP-induce hydrolysis of VIP creates selective VPAC-1 receptor agonist which has anti-proliferative and anti-inflammatory effects [36]. Hence loss of NEP activity may prevent anti-tumoral effects of VIP on RCC. Analogues of VIP selective for VPAC-1 and CGRP as well as NEP might be effective as adjuvant therapy in addition to chemimmunotherapy.

Chromophobe RCCs have better prognosis than clear cell RCC or papillary RCC, with fewer than 5 % of the cases being metastatic at the time of diagnosis [37]. We had similar finding here such that none of the patients with chromophobe RCC had distant metastasis at the time of diagnosis. Genetic and epigenetic differences of different subtypes of renal cell carcinoma were also previously demonstrated [38]. In accordance we here observed that adjusted level of CGRP was not decreased in chromophobe RCC while it was markedly diminished in clear and papillary RCC demonstrating possible protective effects of CGRP.

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