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Substance P and Neprilysin in Chronic Pancreatitis

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Key Words
Chronic pancreatitis · Neuroimmune interaction · Substance P · Neprilysin · MicroRNAs

Abstract
Background/Aims: We aimed to analyze substance P (SP) and neprilysin (NEP), the membrane metallopeptidase that degrades SP, in chronic pancreatitis (CP). ***Methods:*** SP and NEP mRNA levels were analyzed by qRT-PCR in tissue samples from 30 patients with CP and 8 organ donors. In addition, SP serum levels were determined before and after surgery in the same patients, by means of a competitive ELISA assay. Genetic and epigenetic analyses of the NEP gene were also performed. ***Results:*** SP mRNA expression levels were higher in CP tissues compared to controls ($p = 0.0152$), while NEP mRNA showed no significant differences between CP and healthy subjects ($p = 0.2102$). In CP patients, SP serum levels correlated with those in tissue, and after surgical resection SP serum levels were reduced compared to the preoperative values. Failure of NEP to overexpress in CP tissues was associated with significant miR-128a overexpression ($p = 0.02$), rather than with mutations in the NEP coding region or the presence of hypermethylation sites in the NEP promoter region. ***Conclusion:*** Tissue and serum levels of SP were increased in CP, while NEP levels remained unaltered. In an SP/NEP-mediated pathway, it would appear that NEP fails to provide adequate surveillance of SP levels. Failure of NEP to overexpress could be associated with miRNA regulation.

Introduction

Chronic pancreatitis (CP) is an often painful inflammatory disease that leads to pancreatic insufficiency [1, 2]. The pathophysiology of pain, the main symptom in 75% of patients with CP, is still poorly understood. First Keith et al. [3] and later Bockman et al. [4] suggested that neural and perineural alterations might modulate pain pathogenesis in CP. Further studies showed that levels of the growth-associated protein-43 (GAP-43), a well-established marker of neuronal plasticity, as well as the neuropeptide substance P (SP) and its receptor neurokinin-1 (NK-1R), significantly correlated with pain in CP patients [5–8]. The neuropeptide SP is involved in neural transmission of sensory information, smooth muscle contraction, and nociception, with a wide range of functional

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effects, not only in the central and peripheral nervous systems, but also in the so-called 'neuroimmune cross-talk' between the nervous and the immune systems [9]. It is also known that the SP/NK-1R pathway is regulated by neprilysin (also known as neutral endopeptidase, NEP), a major membrane-bound metalloenzyme responsible for degrading SP in human tissues [10]. Recently, Michalski et al. [11] showed that NEP levels were not increased in patients with CP. Therefore, evaluation of the possible mechanisms responsible for NEP gene regulation may be important for clarification of the SP-mediated pathway in CP. So far, gene promoter hypermethylation and microRNA (miRNA, miR)-mediated regulation of gene expression represent two important mechanisms of gene silencing that might explain NEP disturbance in CP. As reported by Usmani et al., hypermethylation of specific CpG islands was associated with the loss of NEP expression in prostate cancer [12]. On the other hand, miRNAs represent a new class of noncoding RNA molecules of approximately 22 nucleotides in length, which bind the 3′UTR of target mRNAs and mediate translational repression in animals. MiRNAs have been shown to regulate developmental processes, such as self-renewal of stem cells and differentiation of cancer and neuronal cells [13]. In particular, miR-128a was reported to play a central role in the nervous system since it was found to be enriched in the brain during neural cell differentiation and involved in neuronal differentiation and synaptic formation and plasticity [14, 15]. Furthermore, in brain tissue from patients with Alzheimer's disease, a direct correlation between miR-128 expression and down-regulation of NEP levels, the major amyloid-beta peptide catabolic enzyme, was observed [16, 17].

In the present study we evaluated SP expression in CP to confirm that neuroimmune interaction is an important factor in chronic inflammation of the pancreas. In addition, we investigated whether there is a relationship between SP levels in tissue and serum in patients with CP. NEP expression levels were also analyzed in order to evaluate the role of this enzyme in the SP-mediated pathway. Finally, we performed genetic and epigenetic analysis of the NEP gene and tested the possible involvement of specific miRNA in regulation of NEP gene expression.

Patients and Methods

Patient Population and Tissue Sampling

Tissue samples were obtained from 30 patients (22 males, 8 females; median age 50 years, range 30–72) undergoing pancreatic resection for CP. The primary cause of CP was alcohol abuse in all patients. In all cases, the surgical procedure consisted of duodenum-preserving pancreatic head resection performed for medically intractable recurrent abdominal pain. Normal human pancreatic tissue samples were obtained through an organ donor program from 8 previously healthy individuals (5 males, 3 females; median age 43 years, range 35–60). In all cases, freshly removed tissue samples were fixed in paraformaldehyde solution for 12–24 h, followed by paraffin embedding for histological analysis. Furthermore, part of the tissue samples destined for RNA extraction and for protein extraction was frozen in liquid nitrogen immediately upon surgical removal and maintained at –80°C. In addition, blood samples were collected from CP patients on the day prior to surgery and on day 3 postoperatively. This early measurement of SP serum level was performed to evaluate the immediate variation in the level of this neuropeptide after the removal of the head of the pancreas. Blood samples from 8 healthy individuals were also collected to obtain the normal value of SP serum levels. Samples were allowed to clot for 30 min before centrifugation for 10 min at 1,000 *g*, and then serum samples were transferred to new plastic tubes and stored at –80°C until evaluated for SP levels.

The present study excluded patients with medical histories of other chronic inflammatory diseases (asthma, rheumatoid arthritis), or major depression. The study was approved by the ethics committees of the University of Heidelberg, Heidelberg, Germany (No. 301/01), and Hospital Casa Sollievo della Sofferenza, San Giovanni Rotondo, Italy (No. 8450/08).

Quantitative RT-PCR

All reagents and equipment for mRNA and cDNA preparation were purchased from Roche (Roche Applied Science, Mannheim, Germany). RNA was prepared by automated isolation using the MagNA Pure LC instrument and isolation kit (specific for tissues). RNA was reverse transcribed into cDNA using the First Strand cDNA synthesis kit, according to the manufacturer's instructions.

Real-time qRT-PCR was performed with the Light Cycler Fast Start DNA SYBR Green kit, as described previously [18, 19]. The standard curve method is used to assess the amount of SP and NEP mRNA, and expression values were normalized to the housekeeping genes cyclophilin B and hypoxanthine-guanine phosphoribosyltransferase. All primers were obtained from Search-LC (Heidelberg, Germany).

Enzyme-Linked Immunosorbent Assay

The quantitative measurement of SP in serum was determined using a commercially available ELISA kit (R&D Systems Inc., Minneapolis, Minn., USA), following the standard conditions recommended by the manufacturer. This immunoassay is based on the competitive binding technique, in which SP present in a sample competes with a fixed amount of alkaline phosphatase-labeled SP for sites on a rabbit polyclonal antibody. Immediately following color development, the absorbance is read at 405 nm. The sensitivity of the SP assay is less than 8.0 pg/ml.

NEP Coding Region Analysis

Primers were designed, using the Primer 3 program, to specifically amplify the entire Open Reading Frame of the NEP gene (RefSeq accession No. NM_000902). The PCR reaction was run on each exon with a total sample volume of 25 μl containing 50–100 ng of DNA, 0.10 μM of each respective primer, 0.25 mM of each

Table 1. Details of the CpG islands within the NEP promoter region (total length: 1,847 bp) and forward and reverse primer sequences for methylation-specific PCRs of NEP-Met1 and NEP-Met2 CpG islands

CpG island	Size	Start–end	Forward oligonucleotide sequence (5′–3′)	Reverse oligonucleotide sequence (5′–3′)
NEP-Met1	137 bp	971–1110	GGTTTTGTAGCGGCGAGGCGGG	TCCCGCGCCAAAAATTAAACGAC
NEP-Met2	157 bp	1163–1320	AGGTGTTTCGTCGTTTTACGG	TAAACGCACGAAACCGAATAAC

dNTP, 1× buffer, and 1 U of AmpliTaq Gold® DNA Polymerase (PE Applied Biosystems, Foster City, Calif., USA). The PCR amplification was performed with an initial denaturing step at 95°C for 10 min, followed by 35 cycles consisting of denaturation at 94°C for 30 s, annealing at melting temperature for 30 s, extension at 72°C for 30 s, and ending with a final extension step at 72°C for 7 min. PCR products were checked on 2% agarose/TBE gels and were purified using Illustra™ GFX PCR and Gel Purification Kit (GE Healthcare Life Sciences, Buckinghamshire, UK) to remove unused primers and precursors according to the manufacturer's instructions.

Amplicons were then screened for sequence variants by denaturing high-performance liquid chromatography (DHPLC) on a WAVE DNA fragment analysis system (Transgenomic, San Jose, Calif., USA) according to the manufacturer's protocol. The running conditions for each amplicon were determined by the Wavemaker 3.4.4 software (Transgenomic) based on the DNA sequence. PCR product was denatured by heating it to 94°C for 5 min, followed by cooling to 50°C over a 25-min period to enhance heteroduplex formation. DHPLC variants displaying variant elution peaks were confirmed by direct sequencing with the Big Dye™ terminator cycle sequencing ready reaction kit (Applied Biosystems, Inc., Foster City, Calif., USA) on an ABI Prism™ 3100 DNA Sequencer under the standard conditions recommended by the manufacturer. Sequences obtained were aligned and compared to wild-type sequences using AB Navigator Analysis Software (PE Applied Biosystems).

NEP Promoter Methylation Assay

Two CpG islands (NEP-Met1 and NEP-Met2) were identified in the promoter region of the candidate NEP gene using the UCSC genome browser (http://genome.ucsc.edu/). Specific primers were designed on the sense filament of the NEP promoter gene using the MethPrimer Software (http://www.urogene.org/methprimer/) (table 1).

First, 1–2 μg of DNA was chemically treated using the Epitect Bisulfite Kit (Qiagen, GmbH, Germany) following the manufacturer's protocol. This bisulfite-converted DNA was amplified for the housekeeping β-actin *(ACTB)* gene to estimate the efficiency of chemical modification. Then, one-step methylation-specific PCRs (MSPs) were performed to specifically amplify bisulfite-converted sequences of NEP-Met1 and NEP-Met2. The PCR reaction was run in a total sample volume of 20 μl containing 50–100 ng of bisulfite-modified DNA using Platinum Taq DNA Polymerase (Invitrogen™, Carlsbad, Calif., USA) according to the manufacturer's instructions, and amplification was performed following the touch-down PCR method. MSP products were visualized in a 2% agarose/TBE gel. Each MSP was repeated in triplicate, with each assay including both water blanks and in vitro methylated DNA as positive controls for the methylation status of DNA. This positive control was obtained by treating the DNA from lymphocytes of healthy volunteers with *Sss*I methyltransferase (New England Biolabs, Beverly, Mass., USA) according to the manufacturer's instructions and based on the results of previous studies [20].

miRNA Expression Analysis

We selected the miR-128a gene from mirBase using the Sanger Center miRNA Registry (http://microrna.sanger.ac.uk/sequences/index.shtml). mirBase is one of the most well-known target prediction databases, and it identifies NEP as a specific miR-128a target.

MiRNAs were isolated from fresh pancreatic tissues of CP patients (n = 13) and healthy donors (n = 8) using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions.

TaqMan MicroRNA Assay for miR-128a, available through Applied Biosystems (P/C: 4395327, Applied Biosystems), was used for quantitative determination of mature miR-128a using the standard curve method, and expression values were normalized to the endogenous control U6B small nuclear RNA (RNU6B) (P/C: 4373381, Applied Biosystems). TaqMan MicroRNA™ Assays include RT and real-time PCR with only two steps. Therefore, 10 ng of total RNA was first reverse transcribed into cDNA with a final reaction volume of 15 μl using the TaqMan MicroRNA Reverse Transcription Kit (P/N: 4366596, Applied Biosystems) and a 5× specific stem-loop RT primer (P/N: 4395327 and 4373381, Applied Biosystems) according to the manufacturer's instructions. Next, PCR reactions were assembled with a final reaction volume of 10 μl (using 384-well plates), using 20× TaqMan Assay (P/N: 4395327 and 4373381, Applied Biosystems) and Universal MasterMix without UNG (P/N: 4324018, Applied Biosystems) according to the manufacturer's instructions. Real-time PCR was performed with an Applied Biosystems 7900HT Sequence Detection System, using FAM™ dye as the reporter. All reactions, including a no-template control and the RT negative control, were run in triplicate, and the average Ct values were used to perform subsequent analyses, discarding any outliers (>2 standard deviations).

Statistical Analysis

Results are expressed as mean ± SD. Statistical analysis was carried out using the software GraphPad (Prism, Inc., San Diego, Calif., USA). The comparative statistical evaluations among groups were done using the Mann-Whitney U test. Correlation analysis was performed using the Pearson correlation coefficient. A $p < 0.05$ was considered statistically significant.

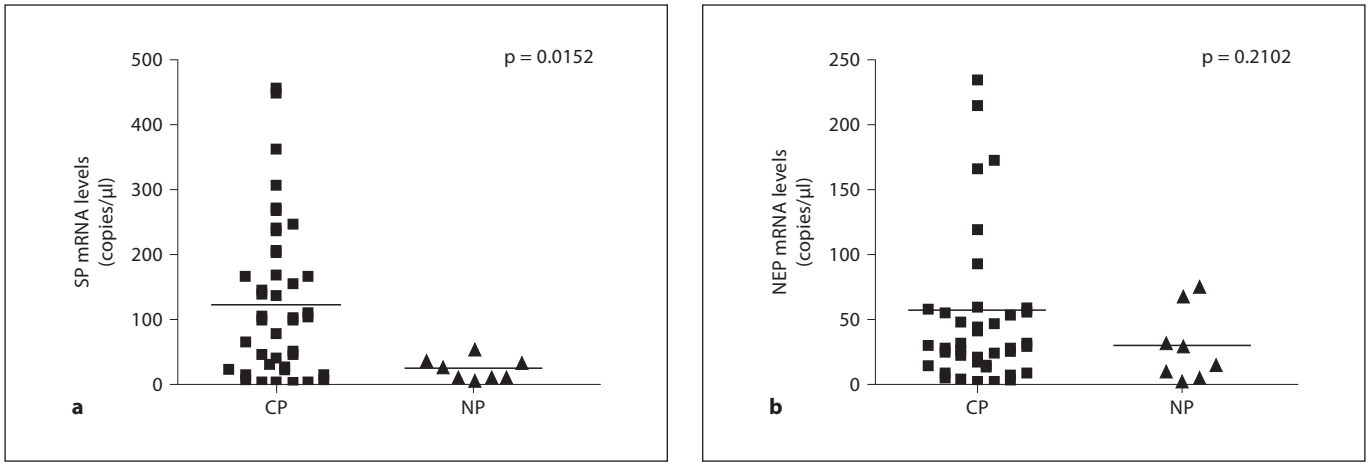

Fig. 1. a Analysis of SP mRNA in normal pancreas (NP) and in CP. There was a significant difference in SP mRNA expression levels between CP patients and NP controls. **b** NEP mRNA expression levels in NP and in CP patients. No significant difference was observed between the two groups.

Results

Quantitative RT-PCR

SP mRNA expression levels ranged from 6 to 55 copies/μl (mean = 24.75, SD = 16.184 copies/μl) in normal pancreas samples, while a larger detectable range from 0 to 452 copies/μl (mean = 94.06, SD = 117.536 copies/μl) was observed in CP tissues. Looking at SP mRNA expression in CP patients compared to normal controls, we observed that SP levels were significantly higher in CP than in normal tissue samples ($p = 0.0152$) (fig. 1a). Conversely, no significant differences were observed in NEP mRNA expression levels between CP (range = 0–233, mean = 55, SD = 62 copies/μl) and normal samples (range = 0–75, mean = 26.5, SD = 26 copies/μl; $p = 0.2102$) (fig. 1b). Correlation analysis showed no significant linear association between SP and NEP expression levels among the 30 CP tissue samples ($r = 0.03$, $p = 0.015$). Therefore, the mean level of SP mRNA in control groups was used as a cutoff value to distinguish patients with high SP levels from those with low expression of SP. Similarly, the mean NEP mRNA level observed in healthy subjects was used as a cutoff value to separate patients with high NEP expression from those with low NEP levels. Correlation analysis, performed in the subgroups of samples with low NEP mRNA and high SP mRNA expression levels, showed a significant inverse correlation between NEP and SP expression levels ($r = -0.96$, $p < 0.01$).

Enzyme-Linked Immunosorbent Assay

In normal subjects, SP serum levels ranged from 35 to 91 pg/ml (mean = 59.62, SD = 22.803 pg/ml). Preoperative SP serum levels were increased in 80% (24/30) of CP patients compared to normal controls (range = 10–1,182, mean = 222.23, SD = 237.637 pg/ml). Interestingly, SP serum levels observed in CP patients were 4 times higher than those in normal subjects ($p = 0.0058$) (fig. 2a).

In addition, 58% (14/24) of patients with elevated preoperative SP serum levels (range = 139–561, mean = 270.5, SD = 140.3 pg/ml) exhibited a significant decrease in SP serum level 3 days after surgery (range = 64–421, mean = 185.1, SD = 125.9 pg/ml; $p = 0.0416$) (fig. 2b).

Screening of NEP Coding Region

Screening of the NEP coding region did not reveal any mutation of interest; it revealed only the presence of 7 intronic polymorphisms located downstream or upstream of several exons (fig. 3). In particular, 2 of these 7 intronic polymorphisms were located 28 bases downstream of exon 4 (c.439 + 28G>T) and 20 bases downstream of exon 14 (c.1497 + 20A>G), respectively, and were observed only in 1 patient. They were not present in donors and had not been described before.

NEP Promoter Methylation Assay

This analysis was performed only in the subgroup of CP patients (n = 13) with SP mRNA levels higher than those in healthy controls (n = 8), since in these patients we expected to find a failure in NEP gene expression.

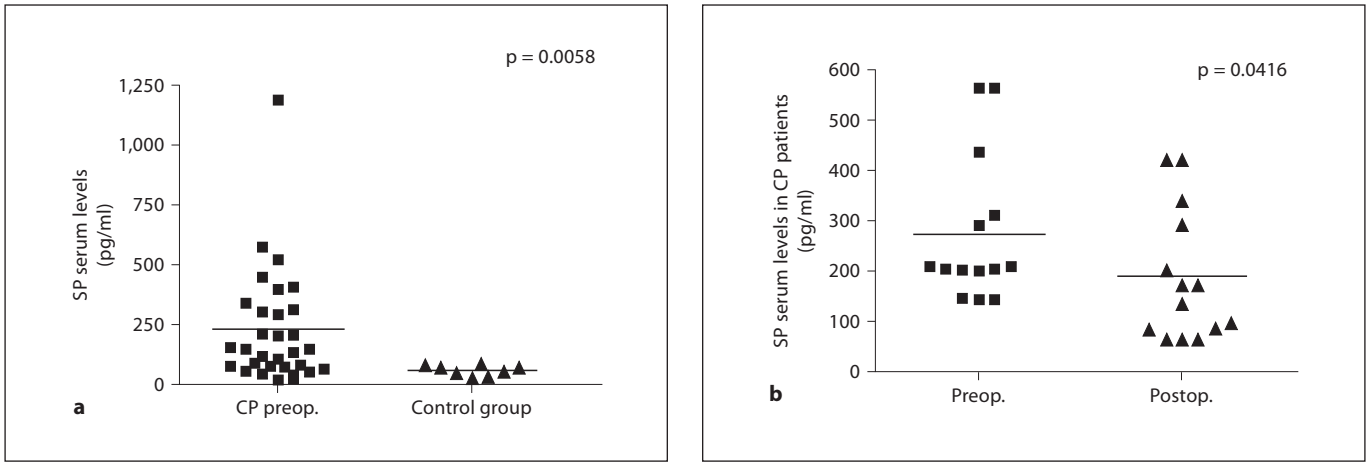

Fig. 2. a In 80% (24/30) of patients with CP, preoperative SP serum level was increased 4-fold when compared to normal controls. **b** In 58% (14/24) of patients with elevated preoperative SP levels, significantly reduced SP serum levels were observed 3 days after surgery.

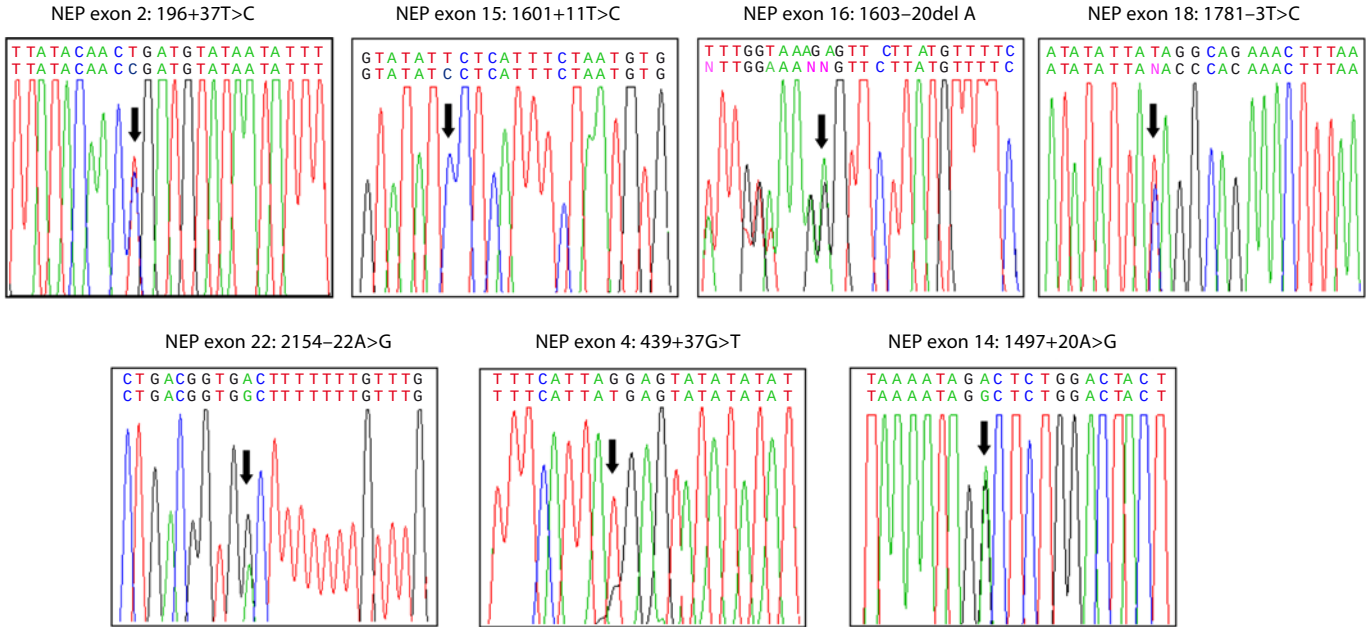

Fig. 3. Excerpts of DNA sequencing traces corresponding to exon 2, exon 4, exon 14, exon 15, exon 16, exon 18 and exon 22 of the NEP gene. Each box portrays the normal sequence (above) versus the sequence with nucleotide change (below).

Amplification of the *ACTB* housekeeping gene underlined a good efficiency of sodium-bisulfite modification (fig. 4a). However, the methylation status of both NEP-Met1 and NEP-Met2 islands was substantially unchanged in CP samples compared to healthy donors (fig. 4b, c).

miR-128a Expression Analysis

As for the NEP promoter methylation assay, this analysis was performed only in the subgroup of CP patients with increased SP expression levels (n = 13) compared to the group of healthy donors (n = 8).

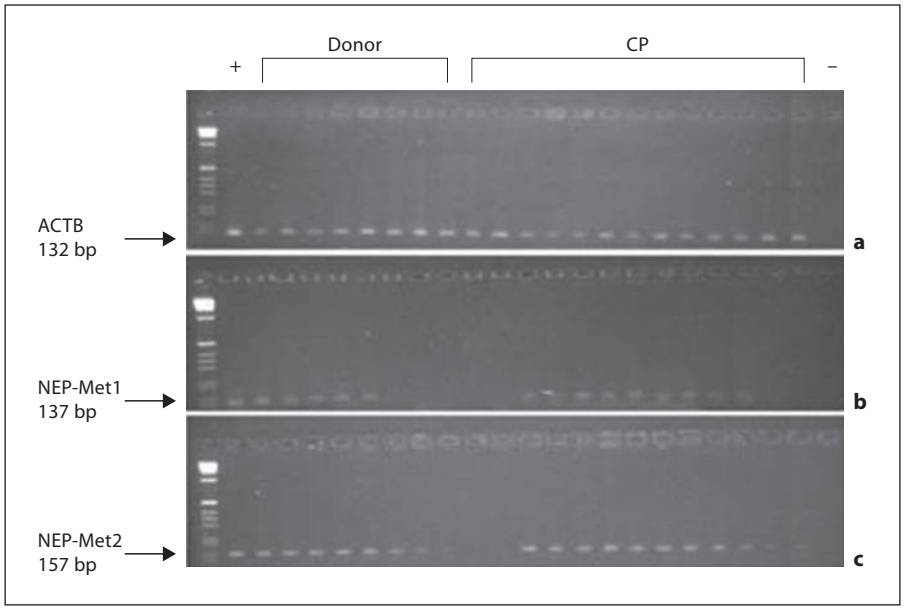

Fig. 4. MSPs. Bisulfite-converted DNA derived from CP and control group patients were amplified with primer annealing to the ACTB gene (**a**) and to methylated CpG islands in the promoter region of the NEP gene, NEP-Met1 (**b**) and NEP-Metp2 (**c**). ACTB amplification underlined the efficiency of sodium-bisulfite modification (**a**). Methylation status of NEP-Met1 and NEP-Met2 islands was substantially unchanged in CP samples when compared with the healthy donor group (**b**, **c**).

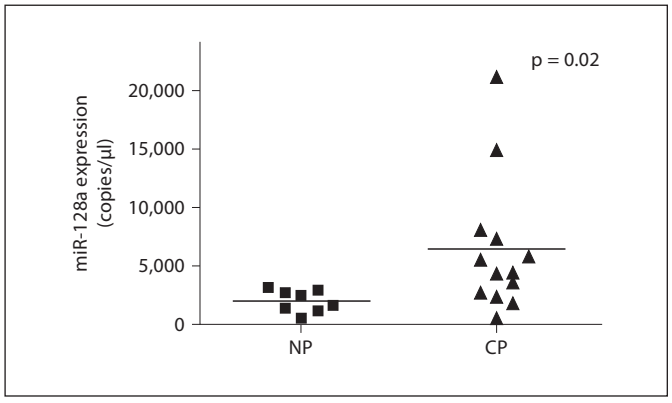

Fig. 5. miRNA-128 analysis measured by qRT-PCR. miR-128a expression, normalized to the endogenous control RNU6B, is significantly overexpressed in CP patients compared to the donor group.

Expression levels of mature miR-128a ranged from 478 to 3,085 copies/µl in healthy donor samples (mean = 1,990, SD = 5,496 copies/µl) and from 678 to 21,128 copies/µl in tissues from CP patients (mean = 6,386, SD = 888 copies/µl). Interestingly, miR-128a expression levels were significantly higher in CP samples than in samples from healthy donors ($p = 0.02$) (fig. 5). Furthermore, miR-128a values were evaluated in correlation with both NEP and SP mRNA expression levels. Interestingly, we found that miR-128a was negatively correlated to NEP expression levels ($r = -0.8$, $p = 0.016$), while a direct association between miR-128a and SP mRNA ($r = 0.9$, $p < 0.01$) was observed in CP tissue samples.

Discussion

Over the past decades, evidence has been obtained regarding the interactions between the immune and nervous systems. Proinflammatory neuropeptides, released from central and peripheral nerve fibers, can modulate the chronic inflammation response by activating the immune cells, such as macrophages, lymphocytes, and neutrophiles [21]. This response plays a role in many inflammatory conditions, such as chronic appendicitis [9], ulcerative colitis, and Crohn's disease [21], as well as asthma and arthritis [22].

In the present study we focused on the SP-mediated pathway in CP. Expression levels of SP and NEP were analyzed in tissue samples from patients undergoing pancreatic resection due to painful CP. Our results confirmed that SP mRNA levels were increased in CP tissues compared to normal controls, suggesting that SP can be synthesized not only outside of the pancreas by extrapancreatic ganglia but also by immune cells present in the inflamed pancreatic tissues [23]. Interestingly, analysis of preoperative SP serum levels showed a significant increase in SP patients compared to normal subjects. Three days after pancreatic resection, following removal of the

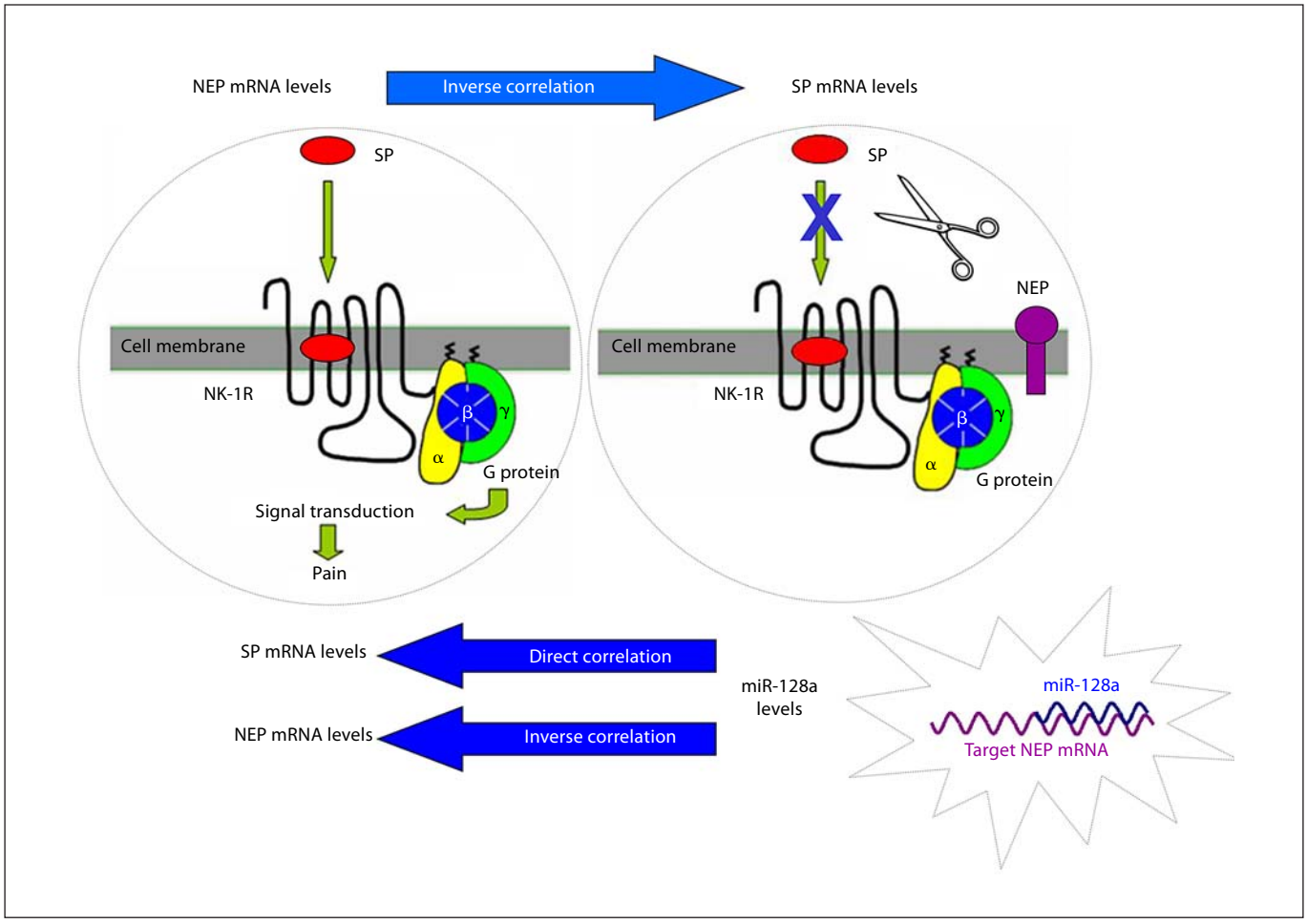

Fig. 6. Hypothetical model of SP, NEP and miR-128a involvement in CP.

pain trigger, 58% of the patients exhibited a significant reduction in SP serum levels. In addition, there was a significant inverse correlation between SP and NEP mRNA levels in a subgroup of CP tissue samples. These data allow us to hypothesize that NEP might fail to provide adequate surveillance of SP levels. Indeed, in the absence of this degrading enzyme, accumulation of SP can take place, and in turn sustain inflammation and pain (fig. 6). Looking at different mechanisms for NEP gene regulation, we performed both genetic and epigenetic analysis in order to identify possible mutations and/or potential sites of hypermethylation in the promoter region. However, no mutation was found in the NEP gene coding region, and MSP analysis showed that methylation status of NEP-Met1 and NEP-Met2 islands was substantially unchanged in CP samples when compared with samples of healthy donors. Thus we considered the possibility of miRNA-mediated post-transcriptional regulation of NEP gene expression. Genes with long 3′UTRs are more prone to miRNA-mediated regulation than genes with short 3′UTRs, which tend to be specifically deprived of miRNA target sites [24]. Interestingly, the full-length NEP transcript includes a long 3′UTR region of 3,270 bases (information obtained from http://genome.ucsc.edu/) suitable to be targeted by specific miRNAs. miR-128a was selected from the Sanger Center miRNA Registry and based on all the available evidence on this miRNA reported in the literature [16, 17, 25, 26]. Interestingly, in tissues from CP patients with high SP amounts and no increase in NEP expression levels, we found significant overexpression of miR-128a compared to tissues from normal controls. In addition, we observed that miR-128a levels were significantly correlated to both SP and NEP expression levels.

These data allow us to hypothesize for the first time that NEP expression might be associated with miRNA deregulation. Although this hypothesis may be considered speculative in the absence of functional studies, we can at least suppose that miR-128a plays a role in the modulation of the SP/NEP-mediated pathway (fig. 6). Further studies are needed on this topic. Understanding the pathophysiology of pain generation and inflammation in CP could highlight new strategies for pain treatment in CP patients, and could help us to select the best surgical procedure for these patients, with significant repercussions on the economic management and quality of life of these patients.

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Disclosure Statement

All authors declare that they have no conflicts of interest. They certify that they have no financial arrangements with a company whose product figures prominently in the submitted manuscript or with a company making a competing product, unless disclosed in a separate attachment.

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