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Characterization of novel 3′untranslated regions and related polymorphisms of the gene *NPPC*, encoding for the C-type natriuretic peptide

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ABSTRACT

Elevated plasmatic levels of C-type natriuretic peptide (CNP) were found in patients with chronic heart failure (CHF), but its use as sensitive and specific clinical bio-marker is still controversial. In fact, high levels of CNP were also observed in patients classified in low severity New York Heart Association (NYHA) classes. CNP is encoded by a gene poorly studied (*NPPC*, natriuretic-precursor peptide C), where the regulatory regions are not well defined and the role of single nucleotide polymorphisms (SNPs) poorly ascertained. In the present work, we focused on the characterization of the 3′untranslated region (3′UTR) of the gene, using Rapid Amplification of cDNA 3′-End (3′ RACE), and we identified two novel transcript isoforms (L-3′UTR; S-3′UTR; accession number JF420840, HQ419060 respectively). Since it could be hypothesized that genetic variations could explain the observed inter-patients differences, we searched for novel SNPs, by the use of High Resolution Melting (HRM). The results showed a complete lack of genetic variations among our series of samples. Moreover, a preliminary evaluation, using literature information and bioinformatic prediction allowed us to predicted the putative relevant microRNAs binding to the novel 3′UTRs that could modulate the post-transcriptional regulation of *NPPC* and affect the plasmatic levels of CNP. We obtained 750 and 1024 predicted miRNAs targeting the S- and L-3′UTRs, respectively.

1. Introduction

The 22-aminoacids vasodilator C-type natriuretic peptide (CNP), released by the vascular endothelium and by the heart [2,8], belongs to a family that includes also the urodilatin, and the ANP (atrial), BNP (brain), and DNP (dendroaspis) natriuretic peptides [6,11,26,27,29,30]. In patients with chronic heart failure (CHF), plasma levels of ANP and BNP are elevated in relation to the clinical severity (measured according to the classes issued by the New York Heart Association, NYHA) and have diagnostic and prognostic relevance [4,5,23]. On the other hand, the significance of CNP is still controversial [15]. In fact, also CNP shows elevated levels in CHF patients in relation to the functional and clinical severity [7,9,10,12,16,17,25]. However, high levels of CNP were also observed in some patients with low NYHA classes, whereas others present low levels although with a severe disease, preventing its use as sensitive and specific clinical bio-marker [12].

It could be hypothesized that genetic variations could explain the observed inter-patients differences. In fact, CNP is encoded by a gene poorly studied (NPPC, natriuretic-precursor peptide C) where the regulatory regions were not well defined and the role of single nucleotide polymorphisms (SNPs) was never ascertained. In a single previous work Ono et al., studied four SNPs within NPPC and one of them, the variant G2628A (rs5268), hypothesized to be located within the putative 3′UTR, was found associated with essential hypertension [20]. However, it should be noticed that the 3′UTR described by the authors showed a strange inconsistency when compared to the orthologous 3′UTRs of other mammals. The polymorphism rs5268 was considered to belong to the 3′UTR, simply because it was located 82 base pairs downstream of the stop codon. Experiments aimed to characterize the region were not performed and there was not any evidence that the SNP was actually located within it. Thus, in the present work, we focused on the characterization of the 3′UTR and we identified two novel transcript isoforms. Moreover, to study the role of microRNAs in the post-transcriptional regulation of NPPC we carried out a preliminary evaluation using literature information together

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with the use of different algorithms to obtain bioinformatic prediction.

2. Materials and methods

2.1. Sample collection

Human cardiac tissues. Patients undergone to left ventricular assist device (LVAD) implantation and at the end-stage heart failure (HF) (NYHA class III and IV; age: 57 ± 11 yrs; LVEF% <20), provided biopsies necessary for the study. The cardiac biopsies ($n = 5$) were collected from left ventricle (LV) at the time of the heart transplantation, thanks to the collaboration with the Institute of Clinical Physiology (IFC)-CNR, Niguarda Ca' Granda, Milan. Immediately after collection, samples were frozen in liquid nitrogen and stored −80 °C until sample preparation. The study protocol was conform to the principles outlined in the Declaration of Helsinki and was approved by the local Ethics Committee of the Niguarda Ca' Granda Hospital. All subjects gave written informed consent to participate in the study.

Plasma samples. Plasma samples were collected from 63 patients (mean age 61 ± 1 years) with a diagnosis of chronic heart failure (CHF) provided by Institute of Clinical Physiology, CNR-Milan. Blood samples (10 ml), collected in ice-chilled disposable polypropylene tubes containing aprotinin, 500 KIU/ml, and EDTA, 1 mg/ml, were rapidly separated by centrifugation for 15 min at 4 °C, and stored frozen at −20 °C in ml aliquots in polypropylene tubes until assay, performed within 1 month from sampling.

Blood samples, used as controls, were obtained from 63 volunteers without heart disease and were provided by a routine biochemical analysis laboratory of the region. Plasma and blood samples were matched for the age of the donors. All subjects were informed and gave written consent to participate to the study and to allow their biological samples to be genetically analysed, according to the Helsinki declaration.

Neuroblastoma cells. The human neuroblastoma SKNBE cells (kindly donated by the National Research Council, Genova, Italy) were grown to confluence in RPMI 1640 medium (Lonza, Switzerland), containing 10% heat-inactivated fetal calf serum (Sigma Aldrich Corp. St. Louis, MO, USA), 1% glutamine (Lonza, Switzerland), 1% PEN-STREP (Lonza, Switzerland), at 37 °C in a 5% CO_2 humidified atmosphere.

2.2. Nucleic acid extraction and quantification

Total RNA was extracted by acid guanidinium thiocyanate-phenol-chloroform method from tissue samples obtained from human cardiac tissue with Rneasy Midi kit (Qiagen MI, Italy). Briefly, the tissue was homogenized with a Mixer Mill MM300 (Qiagen MI, Italy). In order to remove contractile proteins, connective tissue and collagen, which can interfere with the procedure, the protocol was modified to include proteinase K digestion. Samples were lysed in a guanidine-isothiocynate-containing lysis buffer. After dilution of the lysate, the samples were treated with proteinase K. Debris was pelleted by centrifugation. Ethanol was added to the cleared lysate and RNA was bound to the Rneasy membrane. Contaminants were washed away, and total RNA was eluted in Rnase-free water. The presence of proteinase K and the tissue homogenization by Mixer Mill MM300 allowed us a high yield in RNA extraction. For the extraction of total RNA from the human neuroblastoma cell line, cells were washed twice with DPBS-A, treated with trypsin-EDTA (Invitrogen MI, Italy), and counted in a haemocytometer. Three millions cells were pelleted by centrifugation at 200 × *g*, then the QIAgen Rneasy Mini Kit (Qiagen MI, Italy) was used according to the manufacturer's protocol for all large scale samples. RNA concentration was determined spectrophotometrically (Beckman DU 640) at 260 nm. The ratio of readings at 260 nm and 280 nm (A_{260}/A_{280}) provided an estimate of the purity of RNA with respect to contaminants that absorb in the UV spectrum, such as proteins and DNA. The integrity and purity of total RNA was also detected by electrophoresis of samples on ethidium bromide agarose gels. Only samples that showed clear and distinct 28S and 18S ribosomal RNA bands and had spectrophotometric OD 260/280 ratios of 1.9–2.1 were used. A known amount of total RNA (Ambion Inc. Austin, USA) was used as marker. DNA was extracted using the commercially available kit QIAmp DNA Mini and Blood Mini kit (Qiagen MI, Italy). Quantification of all experimental DNA samples was assessed using Qubit® Fluorometer (Invitrogen, MI, Italy). Briefly, total gDNA was extracted using the recommended protocol. Proteinase K (20 μl) was added to the sample and the mixture was further incubated at 56 °C for 1 h. Lysis buffer (200 μl) was added to the sample, mixed thoroughly by vortexing, and incubated at 56 °C for 10 min. Ethanol (95–100%, 200 μl) was then added to the sample, mixed thoroughly by vortexing, and the whole mixture was applied to a QiAmp spin column with a collection tube. The mixture was allowed to bind to the column by centrifugation at 6000 × *g* for 1 min. The QiAmp spin column was washed with buffers followed by centrifugation at 6000 × *g* for 1 min to remove excess ethanol. The spin column-bound gDNA was eluted with the elution buffer (50 μl) and was incubated at room temperature for 5 min, and then was centrifugated at 6000 × *g* for 1 min. DNA was incubated at 65 °C for 1 h.

2.3. DNA sequences alignment and miRNA target prediction

The alignment between the available human consensus sequence (RefSeq) and its orthologous of mouse, rat, cow, pig, goat, and guinea pig was performed with UCSC Genome Browser (URL: http://genome.ucsc.edu/). The sequences obtained from our experiments were aligned against the human genome as well as against mammals' genomes using Nucleotide BLAST (URL: http://blast.ncbi.nlm.nih.gov/Blast.cgi). Putative miRNA-binding sites within the 3′UTRs of NPPC were identified by means of miRanda (http://www.microrna.org). This algorithm evaluates the sequence complementarity between the mature miRNA and the target site, locates the binding position on the target, and calculates the binding free energy (expressed as ΔG, Gibbs free energy, in kcal/mol). The miRanda software was ran locally, with a threshold of $\Delta G \leq -15.00$ kcal/mol, using the two identified isoforms as target sequences and the most updated list of human mature miRNAs as query. MiRNAs were downloaded from miRBase a public database, available at the URL: http://www.mirbase.org/.

2.4. Rapid amplification of cDNA 3′-end (3′-RACE)

Total RNA was reverse-transcribed with the commercially available kit Cloned AMV reverse transcriptase (Cloned AMV First-Strand cDNA Synthesis Kit, Invitrogen, MI, Italy), following the protocol suggested by the produced. For the identification of the 3′end, a rapid amplification of cDNA ends (3′-RACE) was performed. Briefly, the cDNA was synthesized from 4 μg of total RNA extracted from human cardiac tissue and 5 μg of RNA extracted from human neuroblastoma cells, using an universal primer (RACE-1) containing an anchor sequence, in a 20 μl reaction. Reaction protocol started with 42 °C for 1 h 30 min and followed by 85 °C for 5 min. cDNA was precipitated with glycogen, sodium acetate 3 M, pH 5.2, and absolute ethanol. cDNA was then amplified using a gradient PCR as follows: an initial denaturation step (8 min at 95 °C) followed by 35 amplification cycles (30 s at 95 °C, 30 s at 53–60 °C, 1 min and 30 s at 72 °C). The sequences of the primers used are reported in Table 1, where the forward primer (RT-F) was gene-specific, whereas the