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Research Article

Quantitative Analysis of Hypothalamic Proline-Rich Peptide-1 in Blood Serum of Patients With Malignant Tumors

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Abstract: The proline-rich-peptides are a class of neurosecretory hypothalamic bioactive polypeptides derived from cattle and comprise the content of bovine posterior pituitary neurosecretory granules. These peptides are co-validated through a single precursor protein neurophysin-vasopressin-associated glycoprotein. Proline-Rich-Polypeptide-1 (PRP-1) is a 15-amino acid peptide with anti-neurodegenerative, immunoregulatory, hematopoietic, antimicrobial, and antitumor activity. The aim of this study, accordingly, is to elaborate an assay for the determination of PRP-1 and its defined amount in the blood samples from patients with breast, lung, stomach, and colonic cancer, as well as cancer of any type localized/circulated predominantly in genitourinary organs. Control for this experiment is the blood serum of normal healthy patients, which is compared to cancer patients' serum. We have employed an Enzyme-Linked Immunosorbent Assay (ELISA) using a polyclonal antibody directed against synthetic PRP-1 for this purpose and have analyzed blood serum diluted 1:1. The ELISA analysis has revealed no statistically significant differences in PRP-1 levels between the analyzed types of neoplasms, while a significant difference was observed when comparing all tumors with control serum. This work investigates the content of endogenous PRP-1 in patients who have received chemotherapy as treatment for different periods. Its decrease may be associated with the fact that the patients' blood was drawn almost a week after chemotherapy. The expected result of an increased level of PRP-1 observed in various pathologies is not detected, most likely due to the proteolytic breakdown of endogenous PRP-1 in two days after it enters the blood.

Keywords*:* Proline-Rich-Polypeptide-1 (PRP-1), Breast Cancer, Lung Cancer, Gastrointestinal Cancer, Genitourinary Cancer

Introduction

PRP-1 (also known as Galarmin) belongs to a recently identified group of peptide neurohormones composed of approximately 10 to 15 amino acids. It is produced by the hypothalamic supraoptic and paraventricular nuclei (SON and PVN) (Galoyan, 2004; 2008), as well as found in neurosecretory granules of bovine neurohypophysis. The nature of these peptides is the fact that they are products of neuropeptide precursors of neurophysin–vasopressin–associated glycoprotein, genetically programmed proteolytic processing that occurs during axonal transport (Srapionyan *et al*., 2014). The peptides of this class widely manifested biological activities as neuroprotective (Galoyan *et al*., 2005; 2007), immunoregulatory (Galoyan, 2004; 2008), hematopoietic (Galoyan and

Aprikyan, 2002), antimicrobial (Galoyan *et al*., 2016), and antitumor effects (Galoian *et al*., 2011b).

The rationale and interest in PRP-1 level detection in the serum of oncology patients stemmed from the observations that PRP-1 is an antineoplastic factor. Results about this activity of PRP-1 in second common cancer of bone-chondrosarcoma, which is refractory to radiation and chemotherapy were described earlier (Galoian *et al*., 2009a). The stimulating effect of PRP-1on tumor suppressors is by increasing their expression and tumor suppressor miRNAs as well as inhibiting the expression of oncogenes and OncomiRNAs (Galoian *et al*., 2009b; 2014). Introduced by our group for the first-time detection of innate immunity receptors in human chondrosarcoma cells PRP-1 is an mTORC1 inhibitor and also is a ligand for Toll-like receptors 1, 2, and 6 (Galoian *et al*., 2014; 2011a). In other words, PRP-1 upregulates the expression of these receptors by binding to them, and signal transduction events that result in the antiproliferative activity of peptides (Galoian *et al*., 2017). In this regard, PRP-1 has also been shown to have cytostatic and antiproliferative effects on MDA MB 231 triple-negative breast carcinoma cell lines (Galoian *et al*., 2011b). Most recently, it has been shown that PRP-1 is also a potent epigenetic modulator (Galoian *et al*., 2015a-b; Moran *et al*., 2020). PRP-1 upregulates the unfolded protein response (Galoian *et al*., 2022) and downregulates inflammation in chondrosarcoma (Galoian *et al*., 2016).

In the case of PRP-1, Granger and colleagues have established that its cytotoxic effects rely on the targeting of Cancer Stem Cells (CSCs) and suppressing CSC markers ALDH1A1 in sarcomas (Granger *et al*., 2020). A morphofunctional study of antitumorigenic and potential cytotoxic effects of PRP-1 has been carried out in mouse Ehrlich ascites carcinoma (EAC) model (Abrahamyan *et al*., 2020). Characteristics of apoptosis include cell shrinkage, with the formation of membrane blebs, chromatin condensation, and nuclear fragmentation. These characteristics have indeed been induced in EAC cells by PRP-1, consistent with an apoptotic effect. The treatment with a single dose of PRP-1 (0.1 μg/ml) have decreased viable tumor cells by 44% at day 11 post-inoculation, compared with a decrease by 2%, observed at day 7 post-inoculation. Annexin V-Cy3 detection kit apoptosis assays have shown a remarkable increase in apoptotic cell, i.e., 50.33% of cells entered into apoptosis after incubation for 24 hrs. with PRP-1 at 0.1 μg/ml on day seven post-inoculating compared to the control group which had an 8.33% apoptosis rate (Abrahamyan *et al*., 2020).

The impact of PRP-1 on Protein Tyrosine Kinase (PTK) activity was investigated before in a rat leukemia model induced by chemotherapy Cyclophosphamide (CPA) treatment (Kirakosova *et al*., 2012). The results have shown increased PTK activity in the first 5 days of administration of CPA. PRP-1 co-administration with CPA decreased the PRP-1-mediated CPA-induced increase in PTK activity. Interestingly, PRP-1 has not enhanced PTK activity on days 3 and 5 ($p = 0.226$ and $p = 0.170$, respectively), but significantly has reduced the activity of PTK by day 7 ($p = 0.01$), suggesting that PRP-1 may act as an inhibitor of PTK and implicating a potential therapeutic role for this peptide in CPA-induced toxicity. In addition, the antitumor action of PRP-1 is dose-dependent and organ- and disease-specific (Galoian *et al*., 2015a).

The above data have led us to detect and quantify PRP-1 in the serum of patients with malignant tumors. Our previous experiments addressed the issue of detection and quantification of PRP-1 in blood samples from untreated rats and treated with a single PRP-1 injection. The concentration of PRP-1 in this fluid was 1.5 ng/ml (Tumasyan *et al*., 2024).

In previous studies were tested 5 hrs. and 2 days of PRP-1 i/p administration (Abrahamyan *et al*., 2014).

The minimum detectable concentration of PRP-1 was 1.78 ng/ml in the concentrated serum from healthy rats. Five hours after the injection, PRP-1 level increased significantly; however, at day 2 post-injection period peptide concentration decreased to nearly control value. This decrease was associated with the proteolytic degradation of PRP-1 by Dipeptidyl peptidases (DPPs) which resulted in a shorter half-life of PRP-1 in blood serum (Antonyan *et al*., 2011).

Proline-containing neuropeptides, including PRP-1, undergo proteolytic processing mediated by proline bond-specific proteases (Yaron *et al*., 1993; Mentlein, 1999; Lambeir *et al*., 2001). DPPs, particularly Dipeptidyl peptidase-4 (DPP-4) use chemokines, hormones, neuropeptides, and growth factors as substrates (Lambeir *et al*., 2001). As a result, we could also identify PRP-1 as a natural substrate for the multifunctional DPP-4 (Brandt *et al*., 2006).

In various traumatic and stressful conditions, also 5 hours after the administration of PRP-1, immunohistochemical analysis revealed an increased number of PRP-1-Ir cellular structures in the Bone Marrow (BM) both in the sinusoids and in lymphocytes grouped into islets in the stroma, which indicates the presence of hematopoiesis processes in the BM. (Abrahamyan *et al*., 2011; Tumasyan *et al*., 2024). In addition, biosynthesis of the investigated peptide *in vitro* in intact lymphocytes stimulated by appropriate activators has also been shown, thus confirming the suggestion of stress-induced PRP-1 biosynthesis *in vivo*.

Furthermore, stress-induced neuronal activation is also indicated by the appearance of cell nuclei with high phosphatase activity and PRP-1-specific immunoreactivity and the gene c-fos in several brain

regions following labyrinthectomy, vibration of the vestibular nucleus, and immobilization stress (Abrahamyan *et al*., 2011). We propose that it can also function as a transcription factor similar to c-fos.

According to these data, the aim of this study is the analysis of PRP-1 serum content in a group of healthy human individuals and cancer patients using laboratory modified ELISA method which has been produced in our laboratory with application polyclonal antibodies against synthetic PRP-1.

Materials and Methods

Inclusion criteria for this study are oncological patients (aged 45-60 years) divided into the following groups: breast (BC) (n = 10), lung (LC) (n = 10), gastrointestinal (GIC) (n = 10) and genitourinary (GUC) (n = 10) cancers. The blood serum of healthy patients has been served as the control and has been compared to the serum of patients with cancer.

All the patients with oncologic disease were treated before by chemotherapy. ELISA method has been employed to detect and quantitate PRP-1 in blood serum samples (Engvall, 1980). A polyclonal antibody developed against synthetic PRP-1, in our laboratory, has been used (Abrahamyan *et al*., 2014). The concentration of PRP-1 in serially two-fold diluted PRP-1 standard solution has been 1.5 ng/ml (Tumasyan *et al*., 2024) as was given earlier. Then, the PRP-1 concentration in patient blood serum samples has been calculated.

The conditions found are the best for detection of PRP-1 in the blood of humans: 25 ng/ml PRP-1 coated with a coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$) in wells of Falcon ELISA plates (Becton Dickinson, NJ, USA). The primary anti-rabbit antibody against synthetic PRP-1 has been used at a dilution of 1:500. The secondary biotin-conjugated anti-rabbit antibody and peroxidase-conjugated extravidin have been used at a dilution of 1:1000. The wells have been immobilized with 100 µl of a 25 ng/ml of PRP-1 in coating buffer and incubated overnight. Afterwards, 1 h blocking has been conducted in 200 µl/well of blocking buffer (1% BSA in washing buffer), to prevent nonspecific binding of the peptides. One hundred microliters of either a two-fold serially diluted PRP-1 standard solution (starting from 25 ng/ml) or patient blood serum samples (diluted 1:1 or 1:2), plus 50 µl of the 1:250 antibody dilution (final dilution 1:500) were added to each well. The samples have been incubated at room temperature (90 min). After each incubation step, the plates have been washed 3-5 times with 0.01 M Phosphate-Buffered Saline (PBS) at pH 7.4 containing 0.1% Tween-20. The antiserum without PRP-1 has been used to determine maximum binding (Bmax). After that, plates have been incubated for 90 min at room temperature with 100 µl/well of biotinylated goat anti-rabbit IgG secondary antibody (Sigma, Cat. No. B8895) diluted to 1:1000, followed by additional incubation for 60 min with 100 µl/well of Horseradish Peroxidase (HRP)-conjugated extravidin (Sigma, Cat. No. E8386) diluted to 1:1000. Then, 100 µl substrate mixture (8 mg of orthophenylenediamine diluted in 12 ml citric acid and 5 µl of 30% H_2O_2) has been added to the wells. The reaction has been incubated in the dark for 30 min. After that, the reaction has been stopped by adding 50 µl of 1 N sulfuric acid (H_2SO_4) in each well. Optical density (OD) has been measured at 450 nm by an ELISA reader (Rayto, RY-2600C, China).

PRP-1 Antiserum Production

Four male wild rabbits (Oryctolagus cuniculus, Linnaeus, 1758) of the Californian breed, 1 to 1.5-year-old, each weighing approximately 2 kg, for immunization have been used. Immunization has been carried out under pentobarbital anesthesia (Nembutal; serial number 71308321, registration number 0285003) at a dose of 30 mg/kg. The antiserum against synthetic PRP-1 is produced according to a method in a well-established study. More completely, 0.2 mg/ml of Freund's Complete Adjuvant (catalog number F5881; Sigma-Aldrich Co., Ltd.) is mixed with PRP-1-bovine serum albumin (PRP-1-BSA) conjugate to form a homogeneous emulsion (Bret-Dibat *et al*., 1994). This emulsion, divided in equal parts, is then injected into both popliteal lymph node regions of the rabbits. A month later, re-immunization is done by using freshly prepared emulsion in accordance with the above recipe (1 mg PRP-1-BSA in 1 ml PBS, pH 7.4), and injecting the following quantities into the left popliteal lymph node (0.4 ml), the auricular vein (0.2 ml), and right side intramuscularly (0.4 ml). Following reimmunization, blood samples are taken from auricular vein on days 7, 9, and 11, pooled and stored at 4^0C for future use. Then 10 ml of this mixture is lyophilized. The specificity of the resulting antiserum is determined by immunodiffusion and ELISA testing.

PRP-1 Antiserum Affinity Chromatography for Purification

The antiserum against PRP-1 was affinity-purified using the AminoLink™ Plus Immobilization Kit (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions.

Statistical Analysis

Data have statistically been analyzed in the Institute of Colloid and Water Chemistry after A. Dumanskyof NAS, Ukraine. Blood serum samples taken from patients have been used for measuring the minimum detectable concentration of PRP-1. In the statistical processing of PRP-1 concentrations, the software package Statgraphics Centurion 16.2 (StatPoint Technologies, Inc.) has been used. The statistical significance between male and

female cancer patients for every cancer type plus the control group has been estimated using Student's t-test. Data from Mann-Whitney U-test assays have been used to compare medians between control and tumor groups. Data are expressed as medians ± standard deviation, and the p-value was considered significant at <0.05. Formal testing for normality was not conducted. In light of this, we used the Mann-Whitney U-test for group comparisons where data skewness or outliers could not be ruled out. The Student's t-test was used for gender comparisons under the assumption that the Central Limit Theorem would apply given the sample sizes.

Results

Studies to detect and determine PRP-1 have been conducted in the patients with various types of cancer, such as BC, LC, GIC, GUC, as well as in relatively healthy individuals who did not have malignant tumors. PRP-1 concentration in human blood samples with 1:1 and 1:2 dilutions has been detected via measuring absorbance in Optical Density units (OD) at 450 nm wavelength.

Table 1 shows no significant difference by sex within each group of patients. The data from both males and females are given. In the case of GIC (1:2 dilution), we have found a significant difference between sexes using Student's t-test ($p = 0.004$). In BC, this analysis has not been performed because only female patients are included in the study.

Figure 1 shows that according to the results no significant differences has been found between the OD levels at 1:2 dilution in the control group compared to the studied cancer types: OD of the control group is 0.98±0.04, versus BC 0.93±0.01 ($p = 0.31$), LC 0.98±0.01 ($p = 0.23$), GIC 0.97±0.03 ($p = 0.78$), GUC 0.96±0.02 ($p = 1.0$), that is why a 1:1 dilution of blood serum has been chosen for the research.

In order to quantitatively determine PRP-1 in the serum of the patients, the competitive ELISA has been conducted as shown in (Fig. 2). The calibration curve is shown in red, on which the ODs of various concentrations of PRP-1 are outlined (red circles). The green circles indicate the PRP-1 concentrations in the control and pathology groups.

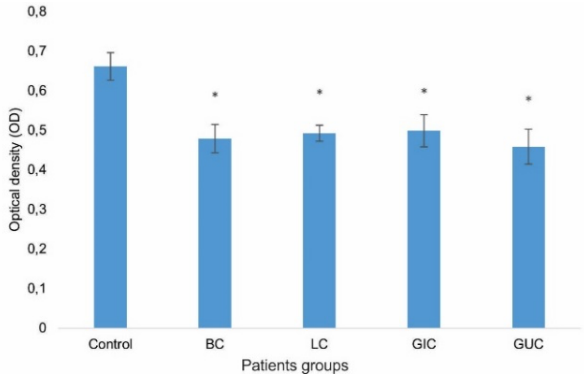

Fig. 1: Differences between the Optical Densities of Proline Rich Polypeptide-1 of the investigated cancer types compared to control group. Values are shown as median ± Standard Deviation. * p<0.05—significant difference compared to control group

Table 1: PRP-1 detection in the blood serum samples of the patients with the Breast, Lung, Gastrointestinal and Genitourinary cancers

Blood serum dilution / Patients group	Dilution 1:1		Dilution 1:2	
	Male	Female	Male	Female
Control group	0.66 ± 0.06	0.67 ± 0.04	1.01 ± 0.09	0.95 ± 0.06
Breast cancer (BC)	-	0.48 ± 0.03	-	0.93 ± 0.02
Lung cancer (LC)	0.47 ± 0.06	0.49 ± 0.04	0.97 ± 0.06	0.99 ± 0.09
Gastrointestinal cancer (GIC)	0.49 ± 0.06	0.47 ± 0.03	1.01 ± 0.09	0.91 ± 0.07*
Genitourinary cancer (GUC)	0.46 ± 0.05	0.43 ± 0.03	0.96 ± 0.09	0.97± 0.09

Discussion

The results have shown that the concentration of endogenous PRP-1 detected in serum of patients with various types of cancer has decreased compared to the PRP-1 concentration of non-cancer patients. Two post-injection periods (5 hrs. and 2 days) after PRP-1 administration were previously tested (Abrahamyan *et al*., 2014).

In previous experiments, the data have shown that the minimal detectable amount for PRP-1 in concentrated blood serum of intact rats is 1.78 ng/ml. The concentration of PRP-1 in the serum has significantly increased in 5 hours after the i/p injection, however, after 2 days it has approximately decreased to the control level. It might be caused by the proteolytic breakdown of peptide by DPPs (Antonyan *et al*., 2011).

The proline containing neuropeptides and hormones proteolytic processing from the precursors are processed by the proteases specific to the proline bonds (Mentlein, 1999; Lambeir *et al*., 2001). DPPs, as a family of serine proteases, remove the X-Pro and X-Ala dipeptides from the N-terminus of the substrate (Brandt *et al*., 2006). DPP-

4 (E.C. 3.4.14.5), is a more widespread and extensively studied member of the DPP family that utilizes chemokines, hormones, neuropeptides and growth factors as substrates (Lambeir *et al*., 2001; Brandt *et al*., 2006). Multifunctional DPP-IV represents a novel natural substrate for PRP-1 (Yaron *et al*., 1999).

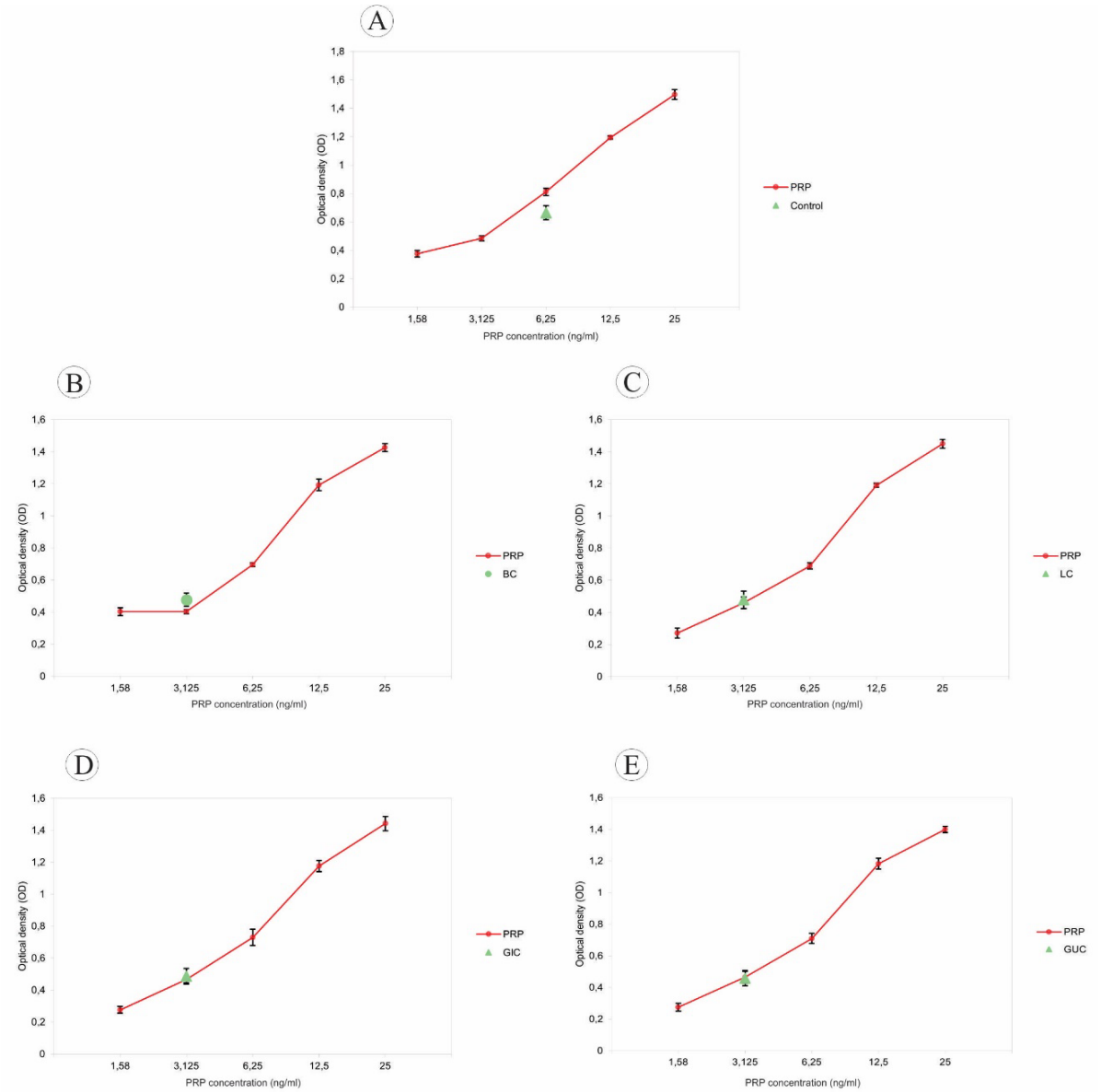

Fig. 2: Calibration curve for detecting the PRP-1 concentration in the diluted 1:1 of blood samples, obtained using a calibrator PRP-1 standard solution and the tested blood samples of the (A) healthy people (control). (B) BC people. (C) LC people. (D) GIC people. (E) GUC people. *X* axis—logarithmic scale of the PRP-1 concentration, *Y* axis—linear scale of the OD means

To explore the PRP-1 antitumor and possible cytotoxic effects, a morpho functional investigation of the effect of the peptide on a mouse EAC model was conducted (Abrahamyan *et al*., 2020). Morphological changes in EAC cells have confirmed the apoptotic nature of PRP-1 for this model. More research will be necessary, therefore, before it can be concluded that PRP-1 is effective against EAC in animal experiments (Abrahamyan *et al*., 2020). Morphological changes in EAC cells have been induced by PRP-1 causing shrinkage of cells, blebbing of membranes, pyknosis and nuclear fragmentation. Thus, the effect of PRP-1 at 0.1 µg/ml on the number of tumor cells and their viability in trypan blue stained samples with 24 h incubation have led to a 44% reduction of viable cells on day 11 post-inoculation vs. 22% inhibition of viable cells after PRP-1 treatment on day 7 post-

inoculation. Apoptosis experiments using an Annexin V-Cy3 apoptosis detection kit indicated that caused a significant increase in the number of apoptotic cells, reaching 50.33%, compared to 8.33% in the sample control on day 7 post-inoculation (Abrahamyan *et al*., 2020).

Previously, PTK activity was studied in the spleen of rats with a model of leukemia caused by CPA, without and with PRP-1 exposure (Brandt *et al*., 2006). The increase of PTK activity was shown after 5 days of CPA administration. The induction of PRP-1 after CPA administration has shown the reduction of the effect of CPA. According to results, PRP-1 has acted as a natural inhibitor of PTK.It is noteworthy that PRP-1 effect on the CPA has shown no increase of PTK activity on the 3rd day (P = 0.226) and 5th day (P = 0.170). However, at later stages of intoxication, starting from the 7th day, it significantly decreases (P = 0.01); this serves as one of the grounds for considering PRP in the treatment of CPA intoxication.

Based on the data on PRP-1 levels reported above under stress injury conditions (Abrahamyan *et al*., 2011; Tumasyan *et al*., 2024; Galoyan *et al*., 2007) and the antitumor effect of PRP-1 (Galoyan *et al*., 2009a-b; 2014; 2011a-b; 2017; 2015a-b; 2022; 2016; 2020; Moran *et al*., 2020), we have expected the release of the latter from the hypothalamic nuclei SON and PVN and a subsequent increase in the amount of PRP-1 in patients with different cancer (BC, LC, GIC, GUC) compared to healthy people.

This study has revealed a decrease in the amount of endogenous PRP-1 in the patient's blood of all the studied groups (3.1-3.3 ng/ml) in comparison to control (6.2-6.3 ng/ml). Since this work has examined the content of endogenous PRP-1 in patients who received chemotherapy as treatment at different periods, its decrease may be associated precisely with the negative effect of chemotherapy on the content of PRP-1or other reasons yet to be defined.

Conclusion

T-test analysis has shown no significant differences in PRP-1 levels between male and female patients within most cancer groups. The exception has been GIC at a 1:2 dilution (p = 0.00371). Therefore, most results are presented with genders combined

No significant differences in OD levels (and thus PRP-1) have been observed at a 1:2 dilution between the control group and any of the cancer types. Specific OD values and p-values are provided for each comparison. Because the 1:2 dilution has not shown significant differences, the 1:1 dilution is selected for the analysis. The rationale given is that the 1:1 dilution has shown significant differences between control and cancer groups ($p < 0.05$)

The study has found that endogenous PRP-1 levels are lower in the blood serum of cancer patients compared to the control group. This reduction has been observed across all cancer types studied

In cancer patients, PRP-1 levels have ranged from 3.1 ng/ml to 3.3 ng/ml. In the control group (healthy individuals), PRP-1 levels have ranged from 6.2 ng/ml to 6.3 ng/ml

The decreases of PRP-1 levels in cancer patients might be linked to the negative effects of chemotherapy treatment. This is because the cancer patients in the study had received chemotherapy at various points

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Authors Contributions

Narine Tumasyan: Methodology, funding acquisition, writing-original draft1

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Nune Kocharyan: Validation, formal analysis, resources1.

Zoya Paronyan: Resources, investigation, visualization.

Armen Avagyan: Methodology, resources, writing-review and editing.

Lilit Harutyunyan: Writing-original draft, resources, resources, investigation, data curation.

Anna Simonyan: Software, formal analysis.

Inesa Sahakyan: Investigation, writing-review and editing, visualization.

Karina Galoian: Validation, supervision, project administration.

All authors have read and agreed on the submitted version of the manuscript.

Ethics

This study has been approved by the Ethics Committee of Yerevan State Medical University after Mkhitar Heratsi (N 3-3/2021) for the use of patients' blood samples.

Conflict of Interest

Dr. Karina Galoian is the inventor of the PCT patent of University of Miami PCT/US23/73277. "TYR PEPTIDE COMPOSITION AND METHOD OF USE" WO2O24050501. The authors have no other competing interests or affiliations with any organization or entity on the subject matter or materials discussed in the manuscript, apart from those disclosed.

Declaration

We declare that the manuscript intitled "Quantitative analysis of proline-rich polypeptide-1 in blood samples from the patients with various malignant tumors" is original, that all statements asserted as facts are founded in our own investigation and research. The manuscript has not been published in whole or in part, and is not being submitted or considered for publication, in whole or in part.

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