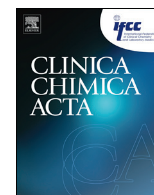
Clinica Chimica Acta 439 (2015) 84–90

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim



Circulating intact and cleaved forms of the urokinase-type plasminogen activator receptor: Biological variation, reference intervals and clinical useful cut-points

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ARTICLE INFO

Article history:
Received 25 July 2014
Received in revised form 17 September 2014
Accepted 2 October 2014
Available online 13 October 2014

Keywords:
Cancer biomarker
Urokinase receptor
Reference intervals
Age- and gender-dependent variation
Colorectal cancer
Time-resolved fluorescence immunoassay

ABSTRACT

Background: High levels of circulating forms of the urokinase-type plasminogen activator receptor (uPAR) are significantly associated to poor prognosis in cancer patients. Our aim was to determine biological variations and reference intervals of the uPAR forms in blood, and in addition, to test the clinical relevance of using these as cut-points in colorectal cancer (CRC) prognosis.
Methods: uPAR forms were measured in citrated and EDTA plasma samples using time-resolved fluorescence immunoassays. Diurnal, intra- and inter-individual variations were assessed in plasma samples from cohorts of healthy individuals. Reference intervals were determined in plasma from healthy individuals randomly selected from a Danish multi-center cross-sectional study. A cohort of CRC patients was selected from the same cross-sectional study.
Results: The reference intervals showed a slight increase with age and women had ~20% higher levels. The intra- and inter-individual variations were ~10% and ~20–30%, respectively and the measured levels of the uPAR forms were within the determined 95% reference intervals. No diurnal variation was found. Applying the normal upper limit of the reference intervals as cut-point for dichotomizing CRC patients revealed significantly decreased overall survival of patients with levels above this cut-point of any uPAR form.
Conclusions: The reference intervals for the different uPAR forms are valid and the upper normal limits are clinically relevant cut-points for CRC prognosis.

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1. Introduction

Cancer invasion requires breakdown of the extracellular matrix and one of the main enzyme systems involved in this breakdown is the plasminogen activation system [1]. Two key molecules of this system are the protease urokinase-type plasminogen activator (uPA) and its cell surface receptor (uPAR). Binding of uPA to uPAR localizes uPA to the cell surface, where it activates plasminogen to plasmin, which, apart from activating pro-uPA, degrades the extracellular matrix. Several studies have shown that uPAR and uPA are prognostic biomarkers, when measured in blood and tumor tissue from cancer patients [2–5], indicating essential roles of these proteins in cancer progression. Intact uPAR [uPAR(I–III)], consisting of three protein domains connected by two linker regions, is anchored to the cell surface by a glycolipid-anchor on domain III [6]. uPAR(I–III) can be cleaved by uPA between domains I and II, liberating domain I [uPAR(I)], while the cleaved uPAR(II–III) remains bound to the cell surface [7]. As the glycolipid-anchored uPAR forms can be shed from the cell surface, soluble uPAR(I–III) and uPAR(II–III) in addition to uPAR(I) are found in blood from cancer patients [5]. The combined amount of all the soluble uPAR forms in blood has been quantified using different immunoassays and found to be a strong prognostic marker in various types of cancer [8–10]. Increased protease activity is considered as being a hallmark of aggressive cancer and the two cleaved soluble uPAR forms were accordingly early hypothesized to be stronger cancer biomarkers than intact soluble uPAR(I–III) [11]. To enable specific and accurate quantification of the individual uPAR forms, we have designed time-resolved fluorescence immunoassays (TR-FIAs) for quantification of the intact and cleaved uPAR forms [11,12]. Using these assays, a clearly significant correlation between high levels of the cleaved uPAR forms measured in blood and tumor tissue and poor prognosis of patients with colorectal (CRC),

Abbreviations: uPA, urokinase-type plasminogen activator; uPAR, uPA receptor, all forms; uPAR(I–III), intact uPAR; uPAR(I), uPAR domain I; uPAR(II–III), cleaved uPAR domains II–III; TR-FIA, time-resolved fluorescence immunoassay; CRC, colorectal cancer; CV, coefficient of variation; HR, hazard ratio; CI, confidence interval; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases.

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http://dx.doi.org/10.1016/j.cca.2014.10.004
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ovarian, prostate, and lung cancer has been demonstrated [12–17]. In addition, the cleaved uPAR forms have been demonstrated to be promising diagnostic and predictive biomarkers [15,17,18]. Collectively, the results from these studies underscore the great potential of the cleaved uPAR forms as biomarkers in cancer. To enable clinical use of the individual soluble uPAR forms it is, however, a prerequisite to define the reference levels as well as the normal biological variation of these in circulation in healthy individuals. We here provide data on within and between individual variation, diurnal variation, as well as reference intervals of the uPAR forms in citrated plasma and EDTA plasma samples from healthy individuals. We have furthermore tested the validity of the reference intervals by dichotomizing a cohort of CRC patients by the normal upper limit of the established reference intervals of the uPAR forms and hence providing data suggesting clinically relevance of these cut-points in CRC prognosis.

2. Materials and methods

2.1. Subject cohorts

Intra- and inter-individual variation, diurnal variation, as well as reference levels of all soluble uPAR forms were determined in citrated plasma and EDTA plasma samples from the following cohorts (see also Table 1): Reference levels were established on the basis of cohort A1 (citrated plasma) and A2 (EDTA plasma). Cohort A1 consisted of 200 men and 200 women (median age 48 years (21–85)) randomly selected from a group of subjects, who were found to have "no findings" at endoscopic examination as part of a multi-center cross-sectional study conducted at six Danish hospitals [19]. Eligible for inclusion in cohort A1 were individuals (aged 18 + years) undergoing endoscopic examination following symptoms related to CRC; all without registered comorbidity and use of medication [19]. Cohort A2 was a randomly selected subset of cohort A1 consisting of 100 men and 100 women (median age 48 years (24–82)). Biological variation (within and between individual variations) were determined in cohort B1 (citrated plasma) and B2 (EDTA plasma). The subjects in cohorts B1 and B2 are identical and consisted of 10 males and 10 females (median age 47 years (23–63)) recruited from the hospital staff, with 5 samples from each individual taken with one week intervals. Diurnal variations were determined in cohort C1 (citrated plasma) and C2 (EDTA plasma). The subjects in cohorts C1 and C2 are identical and consisted of 6 males and 6 females (median age 46 years (24–58)) recruited from the hospital staff with samples taken three times a day at 8 am, 12 am, and 3 pm. The discriminatory power of the cleaved uPAR forms in CRC was determined by analysis of cohort D which included 298 CRC patients identified in the multi-center cross sectional study described in details in [12]. The protocol for the multi-center cross sectional study was approved by the National Ethics Committee (KF 01-080/03) and the Data Protection Agency (2003-41-3312) and the study was carried out in accordance with the Helsinki Declaration II.

2.2. Plasma samples

Blood samples were collected prior to large bowel endoscopy in all subjects in cohorts A1, A2, D and E, following a standard operating procedure [19]. The blood samples from cohorts B1, B2, C1 and C2 were collected at moderate tourniquet pressure using the same procedure. For preparation of citrated plasma, blood was collected in 4 ml citrate-coated tubes (Vacutainer Becton-Dickinson, Mountain View, CA, USA), and spun for 10 min at 2500 *g* and 4 °C within 1 h following collection. For preparation of EDTA plasma, blood was collected in 10 ml EDTA-coated tubes (Vacutainer Becton-Dickinson, Mountain View, CA, USA) and spun for 10 min at 2500 *g* and 4 °C within 30 min following collection. Plasma was collected and samples were immediately stored at −80 °C.

2.3. Immunoassays

Intact soluble uPAR(I–III) was measured by TR-FIA 1 [11], soluble uPAR(I–III) and soluble uPAR(II–III) were collectively measured by TR-FIA 2 [11], and liberated uPAR(I) was measured by TR-FIA 4 [12]. TR-FIA 1 and 2 only measures uPAR forms without uPA bound to it and both assays have previously been validated for use in 20% citrated plasma and 20% EDTA plasma [17,20]. TR-FIA 4 has been validated for use in 20% citrated plasma [12] and herein we used the same standard operating procedure to validate the use of the assay in 20% EDTA plasma. The uPAR forms in citrated plasma from 298 CRC patients in cohort D were previously measured [12].

2.4. Statistics

Descriptive statistics are presented by the median and range; rank correlations (Spearman) are used as a measure of association. 95% reference levels have been estimated for all uPAR forms using linear modeling with the uPAR levels log transformed with gender and age as explanatory variables. The lower and upper reference limits have been calculated based on this model. Tests for interaction between gender and age have been performed for all analyses. The assumptions of linearity and normally distributed data have been assessed using conventional methods; in particular the latter assumption was fulfilled if the dependent variable was log transformed. The intra- and inter-variation for each uPAR form have been done using mixed modeling with the uPAR levels on the log scale. Thus, the variance components were done on the log scale and coefficient of variation (CV) was therefore calculated as $\sqrt{e^{\sigma^2}-1}$, where σ^2 is the variance. The analysis of diurnal variation was done using a linear model with time of day as a fixed factor and individual as a random effect. This analysis was also done on

Table 1
Summary of the study cohorts.

	Reference intervals		Intra- and inter-individual variation		Diurnal variation		CRC discrimination
Cohort	A1	A2	B1	B2	C1	C2	D
Plasma type	Citrated	EDTA	Citrated	EDTA	Citrated	EDTA	Citrated
n	400	200 (subset from cohort A1)	20	20	12	12	298
Gender	200 males, 200 females	100 males, 100 females	10 males, 10 females, 5 samples from each individual taken with one week intervals	10 males, 10 females, 5 samples from each individuals taken with one week intervals	6 males, 6 females, samples taken three times a day at 8 am, 12 noon, and 3 pm	6 males, 6 females, samples taken three times a day at 8 am, 12 noon, and 3 pm	179 males, 119 females
Median age (min, max)	48 (21–85)	48 (24–82)	47 (23–63)	47 (23–63)	46 (24–58)	46 (24–58)	70 (33–93)
Reference	[19]	[19]					[12]

Reference intervals were established in cohorts A1 (citrated plasma) and A2 (EDTA plasma), intra- and inter-individual variations were determined in cohorts B1 (citrated plasma) and B2 (EDTA plasma), and circadian variation in cohorts C1 (citrated plasma) and C2 (EDTA plasma). Discriminatory power of the uPAR forms was investigated by a pooled analysis of cohorts A1 and D.

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