A PILOT STUDY ON SOLUBLE (PRO)RENIN RECEPTOR IN SYSTEMIC SCLEROSIS

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Abstract

Background. Wnt signaling is involved in fibrosis, but the mechanisms of cross-talk with other pathways, like TGF-β, are not fully understood. (Pro)renin receptor (PRR) functions as an accessory protein to a V-ATP-ase responsible for acidic pH maintenance in intracellular compartments, and recent data indicate that PRR could be a regulator of Wnt signaling. A circulating fragment (sPRR) is generated by enzymatic cleavage of the extracellular domain and can be quantified in plasma, serum and urine.

Objectives. This is a pilot study, to explore the serum concentrations of the soluble (pro)renin receptor (sPRR) in patients with systemic sclerosis (SSc).

Patients and methods. Serum samples from 29 subjects with a confirmed diagnosis of SSc have been tested using an ELISA method. Clinical and laboratory parameters have been analysed for associations with sPRR serum levels.

Results and conclusion. Serum levels of sPRR were higher in patients with a history of digital ulcers (p 0.041, Mann-Whitney U-test) and those with digital pitting scars (p 0.037, Mann-Whitney U-test). Serum levels of sPRR correlated with serum creatinine (r 0.424, Spearman test), in line with previously reported data. Our results indicate the need for a larger study to assess the sPRR changes in patients with SSc, including more cases with severe disease and excluding a possible impact of angiotensin-receptor blockers.

Keywords: systemic sclerosis, scleroderma, soluble (pro)renin receptor, renin-angiotensin system

INTRODUCTION

Wnt signaling is essential in embryonic development and carcinogenesis and recent findings suggest that Wnt is also a central pro-fibrotic signal in systemic sclerosis (SSc) (1,2,3). Activation of canonical Wnt signaling via β-catenin is a necessary step for TGF-β mediated fibrosis (4). Further studies are required to fully elucidate the intracellular mechanisms involved in this cross-talk. TGF-β would act via p38 MAPK (mitogen-activated protein-kinase), releasing the pro-fibrotic Wnt signal through inhibition of Dkk1 (a natural inhibitor of Wnt signalling) (4).

(Pro)renin receptor (PRR) is a multifunctional transmembrane protein, with a wide tissue distribution, conserved in evolution. Initially the receptor has been characterized as a component of the renin-angiotensin system (RAS) and it was suggested to have a role in the end-organ damage in diabetes and hypertension, through direct and indirect (angiotensin II-mediated) pro-fibrotic actions (5,6,7). PRR binds renin and its precursor pro-renin, inducing their activation via a conformational change and this led to the hypothesis that PRR may *indirectly* exert pro-fibrotic effects through the pro-fibrotic activity of angiotensin II (generated through renin's proteolytic activity) (5). In addition, in cell models, *direct* PRR-mediated signaling (independent of angiotensin II), results in activation of MAPKs ERK1/2 (extracellular signal-regulated kinase 1/2), with up-regulation of pro-fibrotic genes (TGF-β, PAI-1, collagen, fibronectin), COX-2 and p38 MAPK (6). However,

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the occurrence of this effect in vivo is under questioning: one study indicated that to achieve this angiotensin-independent phosphorylation of ERK1/2, (pro)renin concentrations in a nanomolar range are required, while in vivo concentrations are in the picomolar range (8).

PRR is also the full-length of a smaller protein (ATP6ap2), which functions as an accessory protein of the vacuolar H-ATPase (V-ATPase), a proton-pump responsible for acid pH maintenance in intracellular compartments (7,9). Totally independent of (pro)renin binding, PRR proved recently to be involved in the control of intracellular pH, via its interaction with V-ATPase, and was somewhat unexpectedly found to be necessary for Wnt receptor Frizzled (Fz) endocytosis and its intracellular traffic: in association with V-ATPase, PRR is essential for the endocytosis of PRR-bound LRP5/6 (Wnt co-receptors) in endosomes, creating the acidic pH necessary for LRP5/6 phosphorylation and activation of downstream canonical Wnt (β-catenin mediated) pathway (10). Also, PRR might be necessary for interaction with Frizzled (Fz) in the non-canonical PCP (planar cell polarity) Wnt signaling (11,12).

PRR significant role in development through Wnt signalling, with lethal impact on knock-out models, made generation of murine models nearly impossible and thus the interpretation of its physiological roles is quite difficult (7,9). The mechanisms of interaction between PRR and Wnt pathway components at molecular level are not precisely known. The first selective knock-out models (at cardiac and renal level) lend support to an alternative hypothesis, of PRR involvement in multiple signaling pathways through interference with the autophagy process (9).

PRR is organized in three domains: an extracellular (EC) domain, a transmembrane domain (TM) and an intracellular domain (IC). A truncated form of the PRR (composed of TM, IC and a short portion of the EC domain) was firstly described, associated with the V-ATPase (the 'accessory protein') (13). The ectodomain was thereafter identified as a soluble form (sPRR) in conditioned medium, but also in the human plasma (14,15), and is now quantifiable with an ELISA method (16).

OBJECTIVES

An imbalance of RAS system (17) and canonical (β-catenin) Wnt activation (2-4) were both reported in patients wit SSc, but PRR relevance has not been previously evaluated. We therefore aimed to screen in a pilot study for a potential involvement of soluble (pro)renin receptor (sPRR) in this disease, by evaluating the correlations of serum sPRR levels with clinical and lab parameters in patients with SSc.

PATIENTS AND METHODS

29 patients with a confirmed diagnosis of SSc have been included in this study, all being part of a study cohort in the evidence of a centre specialized in the study of scleroderma. All patients consented in writing for participation in this cohort and the approval of the institution ethics committee was in place for this research. All patients included in the study fulfilled the ACR criteria for classification of SSc (18) or LeRoy criteria for early or limited SSc (19). All patients also fulfilled the 2013 classification criteria applied in retrospect (20). One serum sample was collected and stored at -70^{o}C until processing. Clinical and laboratory data have been retrieved, by using a standardized approach, including general data, duration and subtype of the disease, history of the disease and comorbidities (including any diagnosis of arterial hypertension, congestive heart failure, scleroderma renal crisis or decreased renal function), objective assessments, functional tests, serum creatinine and lab values, status of scleroderma-specific autoantibodies and previous and current treatments, including antihypertensive medication.

Serum concentrations of sPRR have been measured by ELISA method, with a 'sandwich' type test which uses two specific anti-sPRR antibodies (soluble (Pro)renin Receptor Assay Kit, IBL International GmbH, Hamburg, Germany) for quantitative detection of the receptor in serum, EDTA plasma and urine. For levels above 8,000 pg/mL at the initial determination, samples have been retested after dilution in accordance with manufacturer instructions. Serum concentrations corresponding to optical density readings at 450 nm have been calculated using a standard curve generated by the same operator at each testing.

All statistical analysis have been conducted by using SPSS 20.0 software. For continuous variables normally distributed, differences between groups have been tested by independent samples t-test; for continuous variables non-normally distributed, differences between groups have been tested with Mann-Whitney U test. For categorical variables, dif-

ferences have been tested by Pearson chi-square (with continuity correction) or Fischer's exact test. *p* values < 0.05 were considered significant.

RESULTS

Patients characteristics are described in Table 1. Depending on skin extent of disease, our patient group included 24 patients (83%) with a limited form of SSc and 5 patients (17%) with a diffuse form. 26 patients (89%) were females and all patients were of Caucasian race, without a history of scleroderma renal crisis and with serum creatinine levels within normal ranges, except one patient with a chronic kidney disease diagnosis and serum creatinine level of 2.0 mg/dL. We carried a comparative analysis depending on disease subtype and we did not find statistically significant differences between the two subgroups, except the modified Rodnan skin score (which was higher in the subgroup of patients with a diffuse form) and the objective presence of digital pitting scars (again more frequent in the subgroup of patients with a diffuse form). No significant differences have been noted in relation to smoking status, body mass index, disease duration (considered from the first non-Raynaud symptom), pulmonary function tests, scleroderma-specific autoantibodies, or presence of arterial hypertension. Laboratory parameters analysed, including creatinine and serum inflammation tests, did not show differences among subgroups. sPRR levels (between 102 and 25,389 pg/mL) have been found within the ranges reported by other investigators (16,21,22) and were similar in patients with diffuse or limited subtype of disease (*p* 0,051; Mann-Whitney U-test),

TABLE 1. *Patients characteristics, dcSSc (diffuse cutaneous systemic sclerosis), lcSSc (limited cutaneous systemic sclerosis)*

	Total (n = 29)	dcSSc (n = 5)	lcSSc (n = 24)	*p*-value*
Age, years	56 (42-60)	45 (44-52)	56 (41-60)	0.12
Female, n (%)	26 (89.7)	4 (80.0)	22 (91.7)	0.44
Smoker, n (%)	10 (35.7)	3 (60)	7 (30.4)	0.31
BMI	24.5 (3.8)	23.9 (4.4)	24.6 (3.8)	0.74
Disease duration, months	70 (39.5-149)	115(39-150)	69(40-125)	0.72
modified Rodnan Skin Score	3 (2-7)	7 (7-9)	3 (2-6)	0.01
Arterial hypertension, n (%)	7 (24.1)	1 (20.0)	6 (25.0)	0.81
Pulmonary function tests**				
FVC, % of predicted value	95.8 (20.5)	84.3 (17.2)	99.0 (20.6)	0.16
DLCO, % of predicted value	57.8 (25.5)	57.6 (24.8)	57.9 (26.4)	0.98
KCO, % of predicted value	77.1 (17.7)	71.5 (25.4)	78.6 (15.6)	0.43
Digital ulcers history	15 (51.7)	4 (80.0)	11 (45.8)	0.33
Digital pitting scars	13 (44.8)	5 (100)	8 (33.3)	0.011
SSc-specific autoantibodies **, n (%)				
Anti-Scl70	14 (56%)	4 (100)	10 (47.6)	0.10
Anti-centromere	7 (28.0)	0 (0.0)	7 (33.3)	0.29
Laboratory values				
creatinine, mg/dL	0.68 (0.60-0.73)	0.67 (0.58-0.70)	0.68 (0.60-0.74)	0.48
Hb, g/dL	12.9 (12.1-13.5)	13.5 (13.0-13.6)	12.8 (12.0-13.3)	0.11
C-reactive protein, mg/dL	2.8 (1.3-6.5)	2.9 (2.8 – 10.2)	2.8 (1.3-4.5)	0.38
Treatment, n, %				
ACEIs/ARBs	3 (10.3)	1 (20.0)	2 (8.3)	0.44
Anticoagulants	2 (6.9)	1(20.0)	1(6.9)	0.32
Calcium blockers	19(65.5)	3 (60.0)	16 (66.7)	0.77
NSAIDs	8 (27.6)	2 (40.0)	6 (25.0)	0.59
Azathioprine	3 (10.3)	2 (40.0)	1 (4.2)	0.06
Methotrexate	4 (13.8)	1 (20.0)	3 (12.5)	0.55
Corticosteroids (low dose)	7 (24.1)	1 (20)	6 (25)	0.71
Cyclophosphamide	9 (31)	3 (60)	6(25)	0.28
sPRR, pg/mL	8764 (2250-16319)	18944 (9032-22682)	5011 (1998-9982)	0.051

*Mann-Whitney U test or independent Student t-test

**Missing values in 13-20% cases

BMI – body mass index; FVC – forced vital capacity; DLCO – diffusion lung capacity for carbon monoxide; KCO – carbon monoxide transfer coefficient; Hb – haemoglobin; ACEIs – angiotensin-converting enzyme inhibitors; ARBs – angiotensin-receptor blockers; NSAIDs – Non-steroidal anti-inflammatory drugs.

with the observation that the cohort included only a small number of patients with the more severe form of skin disease.

Correlations analysis of sPRR levels and multiple clinical and biological parameters expressed as continuous variables, showed a moderate correlation of sPRR with serum creatinine levels (Table 2) (Spearman correlation coefficient *r* 0.424), similar to previous reports (22). We did not find significant correlations of sPRR with age, disease duration, body mass index, systolic and diastolic blood pressure values, NYHA class for congestive heart failure, modified Rodnan score for cutaneous fibrosis, pulmonary function tests or serum markers of inflammation (ESR, C-reactive protein).

By analysing the sPRR levels in subgroups of patients, depending on the presence of certain clinical parameters, we could not find differences based on gender, smoker status, or diagnosis of arterial hypertension. We also could not find differences based on some disease-related features: presence versus absence of diffuse oedema of hands, sclerodactily, skin and joint contractures, calcinosis, telangiectasia, history of diffuse extension of skin disease, articular disease or pulmonary fibrosis. No differences were noted based on concomitant medications (with the observation that only two patients were on anticoagulant treatment). However, we found significantly higher sPRR values in patients with a history of digital ulcers (*p* 0,041; Mann-Whitney U-test), and in those with objective presence of digital pitting scars, compared with patients without digital scars (*p* 0,037; Mann-Whitney U-test). By excluding the patients under RAS blockers from analysis, we could not find differences among groups.

DISCUSSION

Studying potential factors with an influence on sPRR levels in 121 subjects, Nguyen *et al* (21) reported sPRR serum concentration of 23.5 ng/mL (20.9-26.5 ng/mL), with significantly lower levels in black subjects compared to those of Caucasian race. No differences related to diabetes, age, circadian rhythm or hormonal status (in non-pregnant women) have been noted. In non-treated hypertensive patients the levels were similar to those in non-hypertensive subjects. The authors found higher levels in hypertensive patients treated with medications with an impact on RAS, by approximately 12%, compared to non-hypertensive subjects. No correlations could be found with pro-renin or aldosterone concentrations in healthy subjects, nor in patients with a pathological condition characterized by RAS activation (primary aldosteronism or Gittelmann syndrome). In summary, this lack of impact of RAS on sPRR levels suggested that sPRR might not contrib-

TABLE 2. *Spearman correlations (rho) between serum levels of sPRR and clinical and lab parameters*

	sPRR	Age	Disease duration	BMI	Rodnan score	SBP	DBP	FVC	TLC	DLCO	KCO	PAPs	Hb	CRP	creatinine	CHF NYHA
sPRR	1.000	-.130	-.039	-.229	.301	-.192	-.254	-.313	-.185	-.128	.108	.036	.061	-.059	.424*	.202
Age	-.130	1.000	.276	.346	-.081	.518**	.027	.313	.260	.159	.162	.457*	-.663**	-.032	.249	.394*
Disease duration	-.039	.276	1.000	.331	.076	-.001	-.202	.263	.292	.197	.120	.012	-.303	-.135	-.226	.316
BMI	-.229	.346	.331	1.000	.272	-.035	-.440*	.420*	.314	.349	.237	.292	-.177	.180	.186	.236
Rodnan score	.301	-.081	.076	.272	1.000	-.270	-.276	-.085	-.008	-.066	.025	-.308	-.175	.377*	.279	.297
SBP	-.192	.518**	-.001	-.035	-.270	1.000	.512**	.144	.099	.003	.007	.120	-.391*	-.135	.047	.009
DBP	-.254	.027	-.202	-.440*	-.276	.512**	1.000	-.084	-.101	.070	-.048	-.233	-.067	-.152	-.151	-.364
FVC	-.313	.313	.263	.420*	-.085	.144	-.084	1.000	.872**	.672**	.192	-.336	-.290	.270	-.216	-.010
TLC	-.185	.260	.292	.314	-.008	.099	-.101	.872**	1.000	.548*	.050	-.087	-.266	.226	-.058	.076
DLCO	-.128	.159	.197	.349	-.066	.003	.070	.672**	.548*	1.000	.540**	-.266	.051	-.020	-.260	-.243
KCO	.108	.162	.120	.237	.025	.007	-.048	.192	.050	.540**	1.000	.027	.012	-.223	.000	-.129
PAPs	.036	.457*	.012	.292	-.308	.120	-.233	-.336	-.087	-.266	.027	1.000	-.055	-.224	.437*	.243
Hb	.061	-.663**	-.303	-.177	-.175	-.391*	-.067	-.290	-.266	.051	.012	-.055	1.000	-.107	-.146	-.411*
CRP	-.059	-.032	-.135	.180	.377*	-.135	-.152	.270	.226	-.020	-.223	-.224	-.107	1.000	.276	.295
creatinine	.424*	.249	-.226	.186	.279	.047	-.151	-.216	-.058	-.260	.000	.437*	-.146	.276	1.000	.474**
CHF-NYHA	.202	.394*	.316	.236	.297	.009	-.364	-.010	.076	-.243	-.129	.243	-.411*	.295	.474**	1.000

* Correlation is significant at 0.05 level (2-tailed); ** correlation is significant at 0.01 level (2-tailed)

BMI – body mass index; SBP – systolic blood pressure; DBP – diastolic blood pressure; FVC – forced vital capacity; TLC – total lung capacity; DLCO – diffusion lung capacity for carbon monoxide; KCO – carbon monoxide transfer coefficient; PAPs – systolic pulmonary artery pressure (by echocardiography); Hb – haemoglobin; CRP – C-reactive protein; CHF NYHA – congestive heart failure NYHA class.

ute to regulation of blood pressure (21). In other studies, the authors found with multiple regression analysis a negative correlation of sPRR with glomerular filtration rate (eGFR) (22); a modulation of sPRR with renal function in chronic kidney disease was reported as well (23).

The results of our pilot study are in line with the data reported in literature on sPRR serum levels in healthy subjects, and with previous studies describing the impact of other pathological conditions on sPRR levels. We found a moderate correlation with serum creatinine levels confirming the modulating influence of renal function on the soluble fragment of prorenin receptor, as found by other authors (22, 23). As in the study by Nguyen *et al*, we did not find significant correlations with general patient's characteristics or the presence of arterial hypertension (21). In relation to disease features specific for SSc, we found significantly higher levels of sPRR in subjects with a history of digital ulcers and in those with objective presence of digital pitting scars, an intriguing finding considering the role of Wnt in wound healing and regeneration (24). Only a limited number of patients had a current digital ulcer, therefore we did not conduct a separate analysis based on it. Also, only a few patients were on anticoagulant treatment, so the impact of this medication type has not been analysed. Our study is not without limitations: our cohort did not include consecutive patients, we enrolled more subjects with a limited form of the disease, and we did not exclude subjects treated with antihypertensive medications with an impact on RAS.

Identifying the roles of soluble (pro)renin receptor requires further studies, however the current data point toward a regulatory role of (pro)renin receptor in Wnt signaling (9). The availability of an ELISA test becomes a step forward for studying the role of the soluble fragment in various disease conditions and as a potential biomarker for Wnt signaling activation (9,21). To confirm the relevance of sPRR in scleroderma, our preliminary results indicate a need for further studies.

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