Research Article

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Saliva proteomic analysis reveals possible biomarkers of renal cell carcinoma

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Abstract: Early diagnosis is a key to improve the prognosis of renal cell carcinoma (RCC); however, reliable RCC biomarkers are lacking in clinical practice. In this study, we used isobaric tags for relative and absolute quantification-based mass spectrometry to identify salivary proteins as biomarkers for the diagnosis of RCC. The objective of this study is to discover biomarkers from saliva by utilizing high-throughput quantitative proteomics approaches. Saliva proteins from 124 RCC patients and healthy individuals were identified and quantified. RCC putative biomarkers were verified by real-time polymerase chain reaction or enzyme-linked immunosorbent assay in a prevalidation sample set. Seventy-one differentially expressed salivary proteins were identified. Serotransferrin, haptoglobin, KRT9, and S100A9, which in previous studies were found to be most closely related to cancers, were selected as putative RCC biomarkers. Haptoglobin and S100A9 were significantly elevated in RCC compared with healthy control samples, although the expression of serotransferrin and KRT9 did not differ between the groups. Furthermore, receiver operating characteristic curves with a cut-off value of 75.49 ng/mL for S100A9 revealed a sensitivity of 87.10% and a specificity of 91.94% for discriminating RCC patients from healthy individuals. Salivary haptoglobin differentiated RCC patients from healthy controls with a sensitivity of 85.48% and specificity of 80.65% (cut-off value 43.02 µg/mL). These results provide experimental

evidence to support S100A9 and haptoglobin as potential novel, noninvasive biomarkers for the diagnosis of RCC.

Keywords: saliva proteomics, renal cell carcinoma, diagnosis, S100A9, haptoglobin

1 Introduction

Renal cell carcinoma (RCC) is a significant public health issue worldwide. According to the National Central Cancer Registry of China 2015 annual report, the overall incidence of RCC was 3.35 per 10⁵ population [1]. Currently, RCC detection relies on imaging technologies and traditional disease manifestations. However, as RCC often presents with few signs, symptoms, or laboratory abnormalities, the disease is frequently diagnosed at an advanced stage, when the prospect for a cure is limited. Therefore, the identification of efficient biomarkers for detecting RCC in clinical practice is the key to improving treatment and survival.

Using saliva as a diagnostic tool to monitor an individual's health or disease is an appealing objective for healthcare research. Saliva is one of the most complex and important body fluids, containing water, minerals, nucleic acid, electrolytes, and proteins and reflecting a large range of physiological information [2]. Indeed, the research has shown that saliva can be used for the diagnosis of clinical diseases due to its origin, composition, function, and interaction with substances that compose plasma [3]. With the advantages of simple, noninvasive, and cost-effective methodologies, saliva-based diagnostics can be used for laboratory investigations, including basic and clinical studies of oral to systemic diseases [4]. Proteomics, a relatively new platform for research into tumor hallmarks, facilitates high-throughput screening processes in clinical diagnosis. Thus, saliva proteomics has been used extensively to detect altered expression of various substances, with varying degrees of sensitivity and specificity in a range of cancers.

The aim of the present study is to investigate and identify potential biomarkers in human saliva to facilitate the diagnosis of RCC at early and intermediate stages.

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We employed the isobaric tag for the relative and absolute quantification (iTRAQ) technique to compare salivary protein profiles between RCC patients and healthy subjects. Candidate protein biomarkers were selected from the iTRAQ results, and further clinical validation was performed by the real-time polymerase chain reaction (PCR) and the enzyme-linked immunosorbent assay (ELISA) using an independent sample cohort. The functions of putative biomarkers in various cancer types are only minimally understood, and the application of salivary analysis for RCC remains unclear.

2 Material and methods

2.1 Patients and sample collection

This trial was conducted and approved by the Committee on Human Research of the Second People's Hospital of ShenZhen. A total of 62 patients with RCC and 62 age- and sex-matched healthy individuals were enrolled. Patients diagnosed with another malignancy, undergoing chemotherapy or radiotherapy, or with a cardiovascular disorder, immunodeficiency, autoimmune disorder, hepatitis or human immunodeficiency virus (HIV) infection were excluded. The characteristics of the RCC patients with respect to age, sex, histopathological type, and tumor size and stage were collected for analysis.

2.2 Sample collection

Unstimulated whole saliva was collected from each consenting participant, essentially as described previously [5]. Briefly, participants were asked to refrain from eating, drinking, or brushing teeth for at least 8 h before collection at night. Whole saliva was collected in the next morning and centrifuged at 18,000 rpm for 10 min at 4°C. The supernatant was removed, treated with protease inhibitor cocktail (10 µL/mL) (Solarbio, Beijing, China) to minimize protein degradation, and stored at -80°C.

2.3 iTRAQ analysis

The total protein concentration of the soluble fraction of saliva was quantified using the bicinchoninic acid (BCA) assay (Thermo Fish Scientific). Proteins in each sample

(100 µg) were precipitated with ice-cold acetone, and the precipitated proteins were digested with trypsin at 37°C overnight. iTRAQ reagent (Applied Biosystems, CA, USA) labeling was performed, yielding healthy control sample iTRAQ reagent 113 and RCC sample iTRAQ reagent 114. The samples were incubated at room temperature for 4 h and then desalted and dried. The iTRAQ-labeled peptides were fractionated by the high-performance liquid chromatography (HPLC) with the strong cation exchange (SCX) chromatography (Agilent Technology, CA, USA). The samples were subsequently loaded onto a C18 column (Waters Corporation, MA, USA). The components produced by the SCX chromatography were subjected to the mass spectrometry (MS) analysis.

The raw protein data were examined using the Mascot algorithm and subjected to a search with the human International Protein Index database (version 3.45) using ProteinPilot software (Applied Biosystems, SCIEX, MA, USA). To reduce false-positive data, a strict cut-off value for protein identification was applied with an unused ProtScore >1.3 and at least one peptide with 95% confidence limits [6,7]. A ratio of >1.5 or <0.7 for protein expression between the two groups was considered significant.

The gene ontology (GO) analysis was performed using the online program DAVID (<https://david.ncifcrf.gov/>). Differentially expressed proteins were classified according to their biological process, molecular function, and cellular component.

2.4 RNA isolation and quantitative reverse transcription PCR (RT-qPCR)

Total RNA was isolated from the salivary samples using the mirVana™ PARIS™ kit (Life Technologies). After treatment with DNase I, the RNA was reverse transcribed into cDNA with the Thermo Scientific Maxima First-Strand cDNA Synthesis kit for mRNA detection. Quantitative PCR was performed using the Applied Biosystems 7500 PCR instrument. Relative standard curves were constructed for S100A9, KRT9, haptoglobin, serotransferrin, and an endogenous reference (GAPDH) using serial dilutions of cDNA, and these standards were included in each run. Samples of unknown concentration were quantified relative to these standard curves. S100A9, KRT9, haptoglobin, and serotransferrin mRNA levels were normalized to that of GAPDH to account for cDNA loading differences. Each sample was analyzed in triplicate, and average values were used for the subsequent statistical analysis. Primer sequences were obtained from the primer

bank (<https://pga.mgh.harvard.edu/primerbank/>), as follows: S100A9 sense 5'-GGT CAT AGA ACA CAT CAT GGA GG-3' and anti-sense 5'-GGC CTG GCT TAT GGT GGT G-3'; KRT9 sense 5'-GGG GCC GAT TCA GCT CTT C-3' and anti-sense 5'-CTA CTG GCA CTA AAA CCA CCC-3'; haptoglobin sense 5'-CAG CAC AGT CCC CGA AAA GAA-3' and anti-sense 5'-CAG TCG CAT ACC AGG TGT CC-3'; serotransferrin sense 5'-GGT GGC AGA GTT CTA TGG GTC-3' and anti-sense 5'-ACA GTA AAG TAA GCC TAT GGG GA-3'; and GAPDH sense 5'-ACA ACT TTG GTA TCG TGG AAG G-3' and anti-sense 5'-GCC ATC ACG CCA CAG TTT C-3'.

2.5 ELISA

ELISA was employed to quantify salivary S100A9 and haptoglobin concentrations. The human CircuLex S100A9 ELISA kit (MBL, Nagoya, Japan; detection limit of 6.55 pg/mL and dilution factor of 1:50) and the human haptoglobin ELISA kit (Abcam, Cambridge, USA; detection limit of 0.078 µg/mL and dilution factor of 1:2,000) were used. ELISA was also applied to assess saliva protein levels in the RCC ($n = 62$) and healthy control ($n = 62$) groups. ELISA was performed according to the instructions provided in each kit. Concentrations of proteins were analyzed based on a four-parameter logistic curve. The R -squared (R^2) values for our ELISA standard curves were typically in the range of 0.99 to 0.999.

2.6 Statistical analysis

Statistical analysis was performed using SPSS software version 19.0 and GraphPad Prism version 5.0. All variables under a normal distribution are presented as the mean $\pm$ standard deviation. Qualitative variables were analyzed with Student's t -test. A P value of less than 0.05 was considered significant. Receiver operating characteristic (ROC) curves were utilized to assess the discriminatory value of S100A9 and haptoglobin for differentiating between the two groups.

3 Results

3.1 Clinical characteristics of RCC patients

From 2012 to 2015, 62 RCC patients and 62 healthy individuals were enrolled in this study. The demographic

Table 1: Clinicopathological information of the enrolled participants

	RCC patients ($n = 62$)	Healthy individual ($n = 62$)
Age (years)	52.3 $\pm$ 8.5	47.8 $\pm$ 10.2
Sex (n , %)		
Male	47 (75.81)	49 (79.03)
Female	15 (24.19)	13 (20.97)
Histology		
ccRCC	35 (56.45)	
non-ccRCC	27 (43.55)	
TNM stage		
I + II	46 (74.19)	
III + IV	16 (25.81)	
C reactive protein		
>10 mg/L	29 (46.77)	0 (0.00)
<10 mg/L	33 (53.23)	62 (100.00)

and clinical information of the subjects are shown in Table 1. The mean age of the RCC patients (52.3 years) was not notably different from that of the healthy controls (47.8 years). Among the RCC patients and healthy controls, 75.81% and 79.03%, respectively, were males. More than half of the patients had stage I + II disease (74.19%).

3.2 Analysis of the salivary proteome

To investigate differentially expressed proteins as potential biomarkers of RCC in saliva, we used an iTRAQ-based MS method to assess ten RCC patients and ten healthy control samples. A total of 1,467 proteins were quantified by MS. Fold changes in protein expression were calculated from the intensities of iTRAQ reporter ions between the groups. A total of 71 significantly differentially expressed proteins were identified in the saliva of RCC patients. Specifically, 36 proteins were downregulated (<0.7-fold) and 35 were upregulated (>1.5-fold) (Table 2). Moreover, we categorized the differentially expressed proteins according to biological processes, molecular functions, and cellular components using the GO database. Regarding biological processes, 27% of the proteins are involved in cell motion or migration, whereas 36% are involved in defense responses and inflammatory defense. The most common molecular functions identified included protein binding (53%), enzyme regulation (41%), and structural constituent of the cytoskeleton (6%). In addition, 57% of the proteins are extracellularly located (Figure 1a–c). A network of protein–protein interactions is presented in Figure 1d.

Table 2: Salivary protein biomarker candidates

Accession	Gene symbol	Description	RCC/control	P value
(A) Downregulated protein biomarkers				
P68032	ACTC1	Actin, alpha cardiac muscle 1	0.214	0.004
A0A087WSV8	NUCB2	Nucleobindin2	0.261	0.009
H0YLF3	B2M	Beta-2-microglobulin	0.265	0.004
P23280	CA6	Carbonic anhydrase 6	0.342	0.032
C9JPQ9	FGG	Fibrinogen gamma chain	0.345	0.029
F5GZQ4	LDHA	L-Lactate dehydrogenase A chain	0.381	0.005
Q5T0I0	GSN	Gelsolin	0.388	0.005
P25815	S100P	Protein S100-P	0.391	0.002
P06737	PYGL	Glycogen phosphorylase	0.407	0.013
P19961	AMY2B	Alpha-amylase 2B	0.415	0.020
G3V1A4	CFL1	Cofilin 1 (Non-muscle)	0.45	0.005
P68036	UBE2L3	Ubiquitin-conjugating enzyme E2 L3	0.465	0.017
D3DSM0	ITGB2	Integrin beta	0.475	0.002
Q9GZZ8	LACRT	Extracellular glycoprotein lacritin	0.495	0.002
P43490	NAMPT	Nicotinamide phosphoribosyltransferase	0.501	0.001
P30520	ADSS	Adenylosuccinate synthetase isozyme 2	0.503	0.004
P35579	MYH9	Myosin-9	0.503	0.000
P30740	SERPINF1	Leukocyte elastase inhibitor	0.522	0.047
Q9NP55	BPIFA1	BPI fold-containing family A member 1	0.538	0.040
A0A0C4DGN4	ZG16B	Zymogen granule protein 16 homolog B	0.544	0.006
O75083	WDR1	WD repeat-containing protein 1	0.549	0.004
P02774	GC	Vitamin D-binding protein	0.558	0.010
P52566	ARHGDIB	Rho GDP-dissociation inhibitor 2	0.561	0.047
P07737	PFN1	Profilin-1	0.566	0.012
P31146	CORO1A	Coronin-1A	0.573	0.020
P55058	PLTP	Phospholipid transfer protein	0.575	0.032
P52790	HK3	Hexokinase-3	0.579	0.049
P30044	PRDX5	Peroxiredoxin-5, mitochondrial	0.582	0.012
P20700	LMNB1	Lamin-B1	0.592	0.004
O15144	ARPC2	Actin-related protein 2/3 complex subunit 2	0.608	0.006
P26038	MSN	Moesin	0.611	0.001
B0YJC4	VIM	Vimentin	0.619	0.002
P0CF74	IGLC6	Ig lambda-6 chain C region	0.62	0.009
Q32Q12	NME1-NME2	Nucleoside diphosphate kinase	0.628	0.037
F5H265	UBC	Polyubiquitin-C	0.653	0.004
P14618	PKM	Pyruvate kinase PKM	0.662	0.046
(B) Upregulated protein biomarkers				
Q9HC84	MUC5B	Mucin-5B	1.501	0.001
A0A0B4J2B5	IGHV3OR16-9	Protein IGHV3OR16-9	1.541	0.003
Q9NQ38	SPINK5	Serine protease inhibitor Kazal-type 5	1.587	0.018
P02766	TTR	Transthyretin	1.602	0.015
P28799	GRN	Granulins	1.609	0.030
A0A0A0MTQ6	IGKV2D-28	Protein IGKV2D-28	1.625	0.022
O75594	PGLYRP1	Peptidoglycan recognition protein 1	1.629	0.010
V9GYG9	APOA2	Apolipoprotein A-II	1.649	0.010
Q6UW32	IGFL1	Insulin growth factor-like family member 1	1.681	0.031
Q01469	FABP5	Fatty acid-binding protein, epidermal	1.738	0.026
Q14624	ITIH4	Inter-alpha-trypsin inhibitor heavy chain H4	1.749	0.010
P80188	LCN2	Neutrophil gelatinase-associated lipocalin	1.772	0.020
P06702	S100A9	Protein S100-A9	1.791	0.001
A0A0B4J1X8	IGHV3-43	Protein IGHV3-43	1.792	0.025
C9J9T0	RPE	Ribulose-5-phosphate-3-epimerase	1.873	0.006
A0A0G2JPR0	C4A	Complement C4-A	1.876	0.030
P35527	KRT9	Keratin, type I cytoskeletal 9	2.004	0.002
A0A0C4DGV7	RBP4	Retinol-binding protein 4	2.102	0.004

Table 2: continued

Accession	Gene symbol	Description	RCC/control	P value
P04217	A1BG	Alpha-1B-glycoprotein	2.154	0.010
A0A0A0MS11	IGHV3-13	Protein IGHV3-13	2.176	0.017
P02790	HPX	Hemopexin	2.22	0.008
O95274	LYPD3	Ly6/PLAUR domain-containing protein 3	2.26	0.010
E9PIT3	F2	Prothrombin	2.26	0.033
Q14508	WFDC2	WAP four-disulfide core domain protein 2	2.269	0.017
A0A075B6N7	IGHA2	Ig alpha-2 chain C region	2.39	0.025
P00738	HP	Haptoglobin	2.426	0.001
Q07654	TFF3	Trefoil factor 3	2.427	0.001
A0A075B7B8	IGHV3OR16-12	Protein IGHV3OR16-12	2.443	0.035
P02760	AMBP	Protein AMBP	2.578	0.020
P02787	TF	Serotransferrin	2.677	0.005
P59666	DEFA3	Neutrophil defensin 3	2.696	0.020
P02768	ALB	Serum albumin	3.07	0.003
P31151	S100A7	Protein S100-A7	3.594	0.001
A0A087WYE1	IGHG1	Ig gamma-1 chain C region	4.64	0.050
P55000	SLURP1	Secreted Ly-6/uPAR-related protein 1	5.588	0.000

3.3 Examination of S100A9 and haptoglobin as potential RCC biomarkers

Among the 71 differentially expressed salivary proteins, four well-documented proteins (S100A9, serotransferrin, haptoglobin, and KRT9) most closely associated with solid cancers in previous studies were selected as putative protein biomarkers. We first used the discovery sample set ($n = 20$) to confirm that the relative expression levels of these four genes were consistent with the iTRAQ results, with significant differences between cancer patients and healthy subjects based on RT-qPCR. As shown in Figure 2, S100A9 and haptoglobin were validated (≥ 2 -fold change, $P < 0.0001$; $n = 124$) in RCC saliva samples, whereas serotransferrin and KRT9 expression did not exhibit differences between the groups. The expression patterns of the validated biomarkers were consistent with the iTRAQ results.

3.4 Prediction pattern of the validated biomarkers

To evaluate the clinical utility of S100A9 and haptoglobin in patients, we employed ELISA for further validation of individual saliva samples of RCC patients ($n = 62$) and healthy controls ($n = 62$). The saliva concentrations of S100A9 in the healthy controls and RCC patients were 50.79 ± 21.78 and 131.61 ± 56.61 ng/mL, respectively, and the concentration of haptoglobin in these two groups was 35.84 ± 9.86 and 56.25 ± 12.43 μ g/mL,

respectively. The saliva levels of S100A9 and haptoglobin in the RCC group were markedly increased compared with the control group. The expression trend of the validated biomarkers was consistent with the proteomics results (Figure 3a).

ROC curves were used to define the discriminatory power of the validated biomarkers between RCC patients and healthy individuals. As shown in Figure 3b and Table 3, with a cut-off value of 75.49 ng/mL, salivary S100A9 exhibited a sensitivity and specificity of 87.10% and 91.94%, respectively, in discriminating between RCC patients and normal individuals. If the cut-off value was set to 43.02 μ g/mL, salivary haptoglobin differentiated RCC patients from healthy controls with a sensitivity of 85.48% and specificity of 80.65%.

4 Discussion

RCC accounts for 2–3% of all adult malignancies. According to the Globocan data released in 2015, the prevalence and mortality rates of RCC worldwide were 4.4 and 1.8 per 10^5 population, respectively [8]. RCC cases are frequently incidentally diagnosed during the imaging examination for nonspecific symptoms or other abdominal disease, which now accounts for half of all new RCC cases [9]. Some reports have identified that molecules such as TRAF-1 [10], Hsp27 [11], MMPs [12] or NMP-22 [13], which exhibit altered levels in serum or urine in RCC patients, may provide useful diagnostic information. However, no standardized serum or urine

Figure 1: Bioinformatics analysis of differentially expressed proteins. (a) Biological process. (b) Molecular function. (c) Cellular component. (d) Network of proteins analyzed.

biomarkers are currently available for the accurate diagnosis of RCC in early and intermediate stages. Therefore, a more efficient diagnosis technique or a biomarker is urgently needed.

Serum molecules that are also present in saliva were first discovered in the 1970s [14]. Subsequently, other molecules, such as hormone-binding globulin and monoclonal gammopathy-related paraproteins, have been reported as found in human saliva, suggesting a mass transport mechanism between serum and saliva in a nonspecific and active manner. Although the exact roles of salivary biomarkers in specific systemic diseases remain unclear, changes in salivary biomacromolecule profiles during the development of cancer have been demonstrated *in vivo* [15].

Quantitative proteomics coupled with the bioinformatics analysis can provide novel insight into changes

occurring at the molecular level in cancer. Therefore, the purpose of this work is to explore saliva-based non-invasive and cost-effective methods for the detection of RCC. Prior to their clinical use, we must first address whether identified biomarkers can be validated in different sample cohorts and also assess the robustness of their discriminatory power for RCC differentiation. In this study, we verified that S100A9 and haptoglobin proteins exhibit the discriminatory power for differentiating RCC patients from healthy controls, with sensitivities and specificities greater than 80% (Table 3). These results strongly suggest that the abnormal expression of S100A9 and haptoglobin in saliva is closely related to RCC.

S100A9 is a posttranslation-modified, calcium-binding protein with multiple ligands that is involved in the pathogenesis of inflammatory events and the

Figure 2: Discovery and verification of candidate salivary biomarkers. S100A9, haptoglobin, serotransferrin and KRT9 levels in the saliva of RCC patients ($n = 62$) and healthy controls ($n = 62$) were assessed by real-time PCR.

Figure 3: Efficacy of S100A9 and haptoglobin in discriminating RCC. (a) Box plots denoting 25 to 75th percentiles for the expression of S100A9 and haptoglobin in the saliva of RCC patients and healthy controls by ELISA. (b) The ROC curve analysis for salivary S1A9 and haptoglobin in the diagnosis of RCC.

Table 3: The discriminatory power of salivary S100A9 and haptoglobin for the detection of RCC

Protein	P value	AUC	95% confidence interval	Sensitivity (%)	Specificity (%)	Std error ^a
S100A9	0	0.941	0.899–0.983	87.1	91.94	0.026
Haptoglobin	0	0.909	0.858–0.960	85.48	80.65	0.021

^aUnder the nonparametric assumption.

initial development of cancer cells [16–19]. According to the previous reports, S100A9 is associated with poor overall survival of non-small-cell lung cancer (NSCLC) patients [20,21]. TGF- β signaling has been shown to cooperate with S100A9 to influence cancer metastasis [22,23]; NF- κ B binds to the S100A9 promoter and activates reactive oxygen species-dependent signaling pathways, protecting hepatocellular carcinoma cells from the apoptotic death [24]. S100A9 is also involved in the invasive phenotype at invasive margins of human colorectal carcinomas [25]. In breast cancer, S100A9 has been associated with high-grade disease and negative-ER and -PR status and high Ki67, p53, ERBB2, and EGFR expression [26,27]. Furthermore, S100A9 is abnormally expressed in many types of tumors, indicating that it has high value for cancer diagnosis. Our study also demonstrated that S100A9 levels were significantly increased in patients compared with healthy controls. Given that S100A9 is abnormally expressed in a variety of cancers, S100A9 in saliva may be a useful indicator for diagnosis of cancer, including RCC.

Haptoglobin is an acute-phase protein biosynthesized by the liver, and its expression is regulated by cytokines such as IL-1, IL-6 or TNF- α [28]. The level of haptoglobin is significantly increased in pathological conditions such as infection, acute inflammation, trauma, and tumors. Haptoglobin is involved in cell migration and extracellular matrix degradation, which may contribute to cancer angiogenesis or metastasis [29]. In addition, clinicopathological variables, TNM state and poor overall survival in non-small-cell lung carcinoma (NSCLC) patients have been associated with the levels of haptoglobin [30]. Recent research has further shown increased significance of co-detection of haptoglobin and CYFRA21-1 for the diagnosis of lung squamous carcinoma [31]. Thus, it has been suggested that haptoglobin may be used as a new tumor marker with a high clinical application value for cancer. In our study, haptoglobin was evaluated in the saliva of RCC patients compared with healthy controls, with an area under the ROC curve of 0.909, demonstrating high diagnostic value for RCC.

C-reactive protein (CRP) is a prognostic marker related to RCC diagnosis. We also analyzed the potential

existence of a salivary biomarker with better discriminating power in detecting RCC than that of serum CRP. According to international criteria, the upper limit of serum CRP for a healthy individual is 10 mg/L. In the present study, 29 of 62 RCC patients showed CRP levels exceeding this limit, indicating that serum CRP had a sensitivity of 46.77% for the detection of resectable RCC (Table 1). Compared with the healthy individuals, the RCC patients with serum CRP levels >10 mg/L showed significantly increased S100A9 and haptoglobin levels.

Our study demonstrates that patient-based saliva proteomics is a promising model for detecting biomarkers for RCC. In particular, our findings support the putative diagnostic value of S100A9 and haptoglobin in distinguishing RCC patients from normal healthy controls. However, several limitations of this study should be noted. First, the patient data were obtained from a single center. Approval for the use of a set of biomarkers for a given clinical decision relies on the results of large multicenter trials to adjust for possible biases; thus, the value of salivary proteins as biomarkers of RCC or other types of cancers (the potential salivary biomarkers of RCC was identified in this study, but the study can only assure their potential as cancer biomarkers, rather than biomarkers for the specific case of RCC) should be further evaluated in larger studies. In addition, as RCC is typically detected at an advanced stage, it is crucial to detect RCC as early as possible to improve the rate of survival. However, the clinical stages of the RCC patients in our study were not available in subcategories, and some of the cases were in intermediate and advanced stages (III + IV, 25.81%). Finally, the exact mechanism responsible for S100A9 and haptoglobin overexpression in cancer patients must be explored in further studies.

Conflict of interest: The authors declare no conflict of interest.

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References

- [1] Pang C, Guan Y, Li H, Chen W, Zhu G. Urologic cancer in China. *Jpn J Clin Oncol.* 2016;46:497–501.
- [2] Lima DP, Diniz DG, Moimaz SA, Sumida DH, Okamoto AC. Saliva: reflection of the body. *Int J Infect Dis.* 2010;14:e184–8.
- [3] Chiappin S, Antonelli G, Gatti R, De Palo EF. Saliva specimen: a new laboratory tool for diagnostic and basic investigation. *Clin Chim Acta.* 2007;383:30–40.
- [4] Lee YH, Kim JH, Zhou H, Kim BW, Wong DT. Salivary transcriptomic biomarkers for detection of ovarian cancer: for serous papillary adenocarcinoma. *J Mol Med.* 2012;90:427–34.
- [5] Kumar SV, Hurteau GJ, Spivack SD. Validity of messenger RNA expression analyses of human saliva. *Clin Cancer Res.* 2006;12:5033–9.
- [6] Xiao Z, Li G, Chen Y, Li M, Peng F, Li C, et al. Quantitative proteomic analysis of formalin-fixed and paraffin-embedded nasopharyngeal carcinoma using iTRAQ labeling, two-dimensional liquid chromatography, and tandem mass spectrometry. *J Histochem Cytochem.* 2010;58:517–27.
- [7] Ye H, Sun L, Huang X, Zhang P, Zhao X. A proteomic approach for plasma biomarker discovery with 8-plex iTRAQ labeling and SCX-LC-MS/MS. *Mol Cell Biochem.* 2010;343:91–9.
- [8] Guo J, Ma J, Sun Y, Qin S, Ye D, Zhou F, et al. Chinese guidelines on the management of renal cell carcinoma (2015 edition). *Ann Transl Med.* 2015;3:279.
- [9] Gupta K, Miller JD, Li JZ, Russell MW, Charbonneau C. Epidemiologic and socioeconomic burden of metastatic renal cell carcinoma (mRCC): a literature review. *Cancer Treat Rev.* 2008;34:193–205.
- [10] Rajandram R, Yap NY, Pailoor J, Razack AH, Ng KL, Ong TA, et al. Tumour necrosis factor receptor-associated factor-1 (TRAF-1) expression is increased in renal cell carcinoma patient serum but decreased in cancer tissue compared with normal: potential biomarker significance. *Pathology.* 2014;46:518–22.
- [11] Katsogiannou M, Andrieu C, Rocchi P. Heat shock protein 27 phosphorylation state is associated with cancer progression. *Front Genet.* 2014;5:346.
- [12] Di Carlo A. Matrix metalloproteinase-2 and -9 in the sera and in the urine of human oncocytoma and renal cell carcinoma. *Oncol Rep.* 2012;28:1051–6.
- [13] Kaya K, Ayan S, Gokce G, Kilicarslan H, Yildiz E, Gultekin EY. Urinary nuclear matrix protein 22 for diagnosis of renal cell carcinoma. *Scand J Urol Nephrol.* 2005;39:25–9.
- [14] Landman AD, Sanford LM, Howland BE, Dawes C, Pritchard ET. Testosterone in human saliva. *Experientia.* 1976;32:940–1.
- [15] Gao K, Zhou H, Zhang L, Lee JW, Zhou Q, Hu S, et al. Systemic disease-induced salivary biomarker profiles in mouse models of melanoma and non-small cell lung cancer. *PLoS One.* 2009;4:e5875.
- [16] Leanderson T, Liberg D, Ivars F. S100A9 as a pharmacological target molecule in inflammation and cancer. *Endocr Metab Immune Disord Drug Targets.* 2015;15:97–104.
- [17] Srikrishna G. S100A8 and S100A9: new insights into their roles in malignancy. *J Innate Immun.* 2012;4:31–40.
- [18] Buttler RM, Bagci E, Brand HS, Heijer MD, Blankenstein MA, Heijboer AC. Testosterone, androstenedione, cortisol and cortisone levels in human unstimulated, stimulated and parotid saliva. *Steroids.* 2018;138:26–34.
- [19] Koh HM, An HJ, Ko GH, Lee JH, Lee JS, Kim DC, et al. Prognostic role of S100A8 and S100A9 protein expressions in non-small cell carcinoma of the lung. *J Pathol Transl Med.* 2019;53:13–22.
- [20] Kawai H, Minamiya Y, Takahashi N. Prognostic impact of S100A9 overexpression in non-small cell lung cancer. *Tumour Biol.* 2011;32:641–6.
- [21] Huang H, Huang Q, Tang T, Gu L, Du J, Li Z, et al. Clinical significance of calcium-binding protein S100A8 and S100A9 expression in non-small cell lung cancer. *Thorac Cancer.* 2018;9:800–4.
- [22] Massague J, Blain SW, Lo RS. TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell.* 2000;103:295–309.
- [23] Hiratsuka S, Watanabe A, Aburatani H, Maru Y. Tumour-mediated upregulation of chemoattractants and recruitment of myeloid cells predetermines lung metastasis. *Nat Cell Biol.* 2006;8:1369–75.
- [24] Németh J, Stein I, Haag D, Riehl A, Longerich T, Horwitz E, et al. S100A8 and S100A9 are novel nuclear factor kappa B target genes during malignant progression of murine and human liver carcinogenesis. *Hepatology.* 2009;50:1251–62.
- [25] Stulík J, Koupilova K, Osterreicher J, Knížek J, Macela A, Bures J, et al. Protein abundance alterations in matched sets of macroscopically normal colon mucosa and colorectal carcinoma. *Electrophoresis.* 1999;20:3638–46.
- [26] Arai K, Takano S, Teratani T, Ito Y, Yamada T, Nozawa R. S100A8 and S100A9 overexpression is associated with poor pathological parameters in invasive ductal carcinoma of the breast. *Curr Cancer Drug Targets.* 2008;8:243–52.
- [27] Cormier K, Harquail J, Ouellette RJ, Tessier PA, Guerrette R, Robichaud GA. Intracellular expression of inflammatory proteins S100A8 and S100A9 leads to epithelial-mesenchymal transition and attenuated aggressivity of breast cancer cells. *Anticancer Agents Med Chem.* 2014;14:35–45.
- [28] Moshage H. Cytokines and the hepatic acute phase response. *J Pathol.* 1997;181:257–66.
- [29] Bashyam MD. Understanding cancer metastasis: an urgent need for using differential gene expression analysis. *Cancer.* 2002;94:1821–9.
- [30] Lu J, Wang Y, Yan M, Feng P, Yuan L, Cai Y, et al. High serum haptoglobin level is associated with tumor progression and predicts poor prognosis in non-small cell lung cancer. *Oncotarget.* 2016;7:41758–66.
- [31] Wang B, He YJ, Tian YX, Yang RN, Zhu YR, Qiu H. Clinical utility of haptoglobin in combination with CEA, NSE and CYFRA21-1 for diagnosis of lung cancer. *Asian Pac J Cancer Prev.* 2014;15:9611–4.