Original Article
Hydroxylated collagen peptide in urine as biomarker for detecting colorectal liver metastases

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Abstract: The clinical efficacy of carcinoembryonic antigen (CEA) as a marker of colorectal liver metastasis is limited, motivating a search for new biomarkers. Recently, urine proteomic analysis revealed AGPP(-OH)GEAGKP(-OH) GEQGVP(-OH)GDLGAP(-OH)GP (AGP), a promising peptide for this application. This study aimed to determine whether combining urine AGP testing with serum CEA analyses improves the sensitivity of detecting colorectal liver metastases. Urine samples from 100 patients with CRLM were collected prospectively and compared to three control groups: healthy kidney donors, patients who were relapse-free for 24 months after curative CRLM surgery, and primary colorectal cancer patients. A stable isotope labeled peptide standard was used to quantify the abundance of AGP in urine samples by selective reaction monitoring. Combined testing of urine AGP levels and serum CEA levels revealed a significantly increased sensitivity compared to CEA alone (85% vs. 68%, P<0.001; specificity 84% and 91%, respectively). No correlation was found between CEA and AGP-positive test results within individual patients ($r^2 = 0.08$). Urine AGP testing was negative in the three control groups. These results indicate that collagen-derived urine AGP peptide with a specific hydroxylation pattern combined with serum CEA levels may significantly improve the detection of colorectal liver metastases in patients at risk.

Keywords: Colorectal liver metastases, urine, collagen, biomarker, mass spectrometry

Introduction

Colorectal cancer (CRC) represents one of the most common malignant diseases, with 1.2 million new cases a year worldwide [1]. Even after curative surgical resection of the primary tumor, 25-40% of CRC patients will develop colorectal liver metastases (CRLM) [2-5]. Follow-up aims to detect metastases at an early stage, offering additional treatment and survival benefit [6]. Early detection of CRLM leads to better results with the application of surgery or local ablation [2]. Although surgery may offer the best outcome, 80% of all patients with CRLM are not considered candidates for resection due to advancement of the disease beyond curative treatment options [7]. Therefore, the ASCO guidelines recommend an intensive follow-up every 2-3 months during the first 2 years after surgery [8].

Although follow-up protocols for patients who undergo curative resection for CRC differ worldwide, all advise ultrasound (US), computed tomography (CT), and/or carcinoembryonic antigen (CEA) testing [5]. Ultrasound has a sensitivity of approximately 57% and a specificity of 91%. Computed tomography performs slightly better (sensitivity approximately 68% and specificity 96%) [9]. A positive CEA test has a sensitivity of ~64% for detecting CRLM [10]. The low sensitivity of CEA is due to the fact that not all colorectal tumors and their metastases produce CEA, leading to false negatives [10, 11]. However, CEA has a high specificity, as it is rarely elevated in the absence of CRC [12].

Recently, we performed urine proteome analysis and demonstrated two promising peptides to detect colorectal liver metastases, both being part of collagen type a1(I): AGPP(-OH)GE-

AGKP(-OH)GEQGVP(-OH)GDLGAP(-OH)GP (AGP) and KGNSGEP(-OH)GAPGSKGDTGAKGEP(-OH) GPVG (KGN). These peptides had a sensitivity of approximately 88% and a specificity of 88%. When AGP and KGN were combined, they had a sensitivity of 85% and a specificity of 92% in a discovery setting [13]. As AGP and KGN showed a strong correlation with a better performance for AGP we continued our studies with urine AGP analysis.

In this study, we determined the additional value of urine AGP screening in addition to serum CEA levels to identify patients with CRLM.

Material and methods

Ethics statement

The use of patient materials was approved by the medical ethics committee of Erasmus MC (MEC-2008-062). Written informed consent was obtained from all patients and controls for our prospective observational case-control study.

Sample vials were de-identified by numerical coding; only the people involved had access to patient information.

Patient selection

A total of 100 adult patients (age ≥18 years) with radiologically confirmed CRLM who were planned to undergo surgical resection of metastatic liver lesion(s) (ICD 10 C18-C19) were prospectively selected. Patients with the primary tumor in situ or concomitant malignant diseases were excluded. The diagnosis of CRLM was confirmed by the pathologist in the resection specimens of all patients.

Three groups of control subjects were used. The first group of controls consisted of 100 healthy kidney donors (HKDs) who had a complete blood examination and abdominal CT imaging prior to donation and were considered healthy. The second group of controls consisted of 20 patients who underwent liver surgery for CRLM and were relapse-free for at least 24 months (relapse-free controls, RFCs). The third group of controls included 18 patients with primary CRC in situ without CRLM as demonstrated by CT-imaging (primary colorectal cancer controls, PCCs). For all patients, the diagnosis of CRC was confirmed by the pathologist. None of the patients received neoadjuvant chemotherapy or radiotherapy.

Study design

Serum and urine samples were prospectively collected in three teaching hospitals in the city of Rotterdam, the Netherlands. Urine sampling was designed to mimic the clinical setting as much as possible. Urine was collected randomly during the day and the time to aliquoting and freezing the urine varied depending on the routine schedule of the hospital, but it was always within 4 hours of withdrawal. Clinical data were retrieved from (electronic) medical records, including age, gender, body mass index (BMI), number of lesions, size of the largest lesion, and serum creatinine.

Specimen characteristics

Midstream urine samples (50 ml) were collected from all patients and controls the day before surgery, or in case of the RFC group at the time of inclusion. Samples were stored as 10 ml aliquots in 15 ml BD Falcon tubes at -80°C within 4 hours of sample withdrawal. No additives were added prior to sample processing. Freeze-thaw cycles were kept to a minimum, with a maximum of two cycles per sample prior to sample processing. CEA measurements were part of the standard follow-up of patients with CRC and were extracted from the (electronic) patient files.

An additional serum sample was taken from the controls to determine serum CEA levels using the Elecsys CEA quantitative electrochemiluminescence immunoassay (Roche, Switzerland).

Chemicals

UHPLC solvents were obtained from Biosolve (Valkenswaard, the Netherlands) and all other chemicals from Sigma Aldrich (Zwijndrecht, the Netherlands). The stable isotope-labeled peptide was obtained from Pepscan (Lelystad, the Netherlands). The hydroxylation pattern of the stable isotope-labeled peptide on MS^2 spectra was compared to that of AGP. Both spectra had a high overlap and similar hydroxylation pattern that confirmed the hydroxylation pattern of the identified human peptide (**Figure 1**).

Sample preparation

An automated sample preparation method that was cross-validated with the previous method published by Bröker et al. was applied (data not shown) [13]. Unless otherwise stated, samples
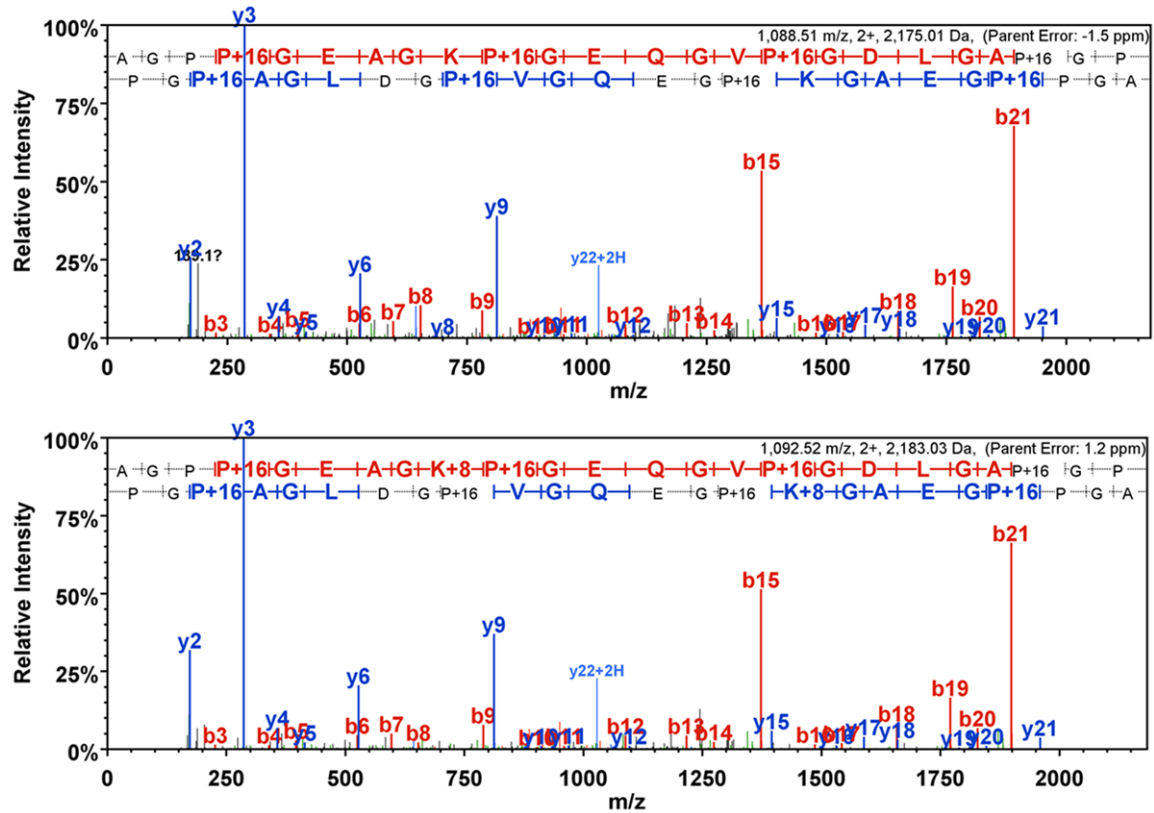
Figure 1. The hydroxylation pattern of AGP was confirmed by MS^2. The high resolution spectra of endogenous (top) and stable isotope-labeled peptides (bottom) were compared and found to be identical.

were processed at room temperature. Prior to sample preparation the urine samples were thawed, first at room temperature for 1 h, and then in a 37°C water bath for 15 min. The samples were then vortexed for 5 s. Subsequently, 1.4 µl of a 5 µM internal standard and 0.5 ml of urine were transferred to a 96 deep well plate. The samples were frozen at -32°C, lyophilized overnight, and dissolved by adding 200 µl of 10 M urea. Samples were shaken on a plate shaker (Eppendorf) for 30 s at 800 rpm and then centrifuged for 5 min at 2500 g to precipitate insoluble particles. One hundred microliters of sample was transferred from each well to a 96-well plate, which was sealed with adhesive aluminum foil to prevent evaporation and contamination of the samples. The samples (40 µl) were separated on an mRP C-18 Hi-Recovery Protein Column (4.6 × 50 mm) (Agilent, Amstelveen, the Netherlands) installed in an Ultimate 3000 (Dionex, Amsterdam, the Netherlands). Solvents A and B were 0.1% TFA in water and 0.1% TFA in acetonitrile, respectively, with a flow rate of 750 µl/min. The column was kept at a constant temperature of 80°C. The gradient was started with 100% sol-

vent A and reduced after 4 min in a 0.4 min step to 75% solvent A. At 6.0 min, solvent A was increased in 0.1 min to 80% and then kept constant for 2.4 min, followed by a decrease in solvent A over 24 s to 30%. It was then kept constant for 1.5 min. At 10.5 min, solvent A was further decreased over 15 s to 5%. Finally, at 13.3 min the column was equilibrated for 6.45 min with 100% solvent A.

A portion of the flow was collected in a 96 deep well plate; the fractionation was started at 4.9 min and stopped at 6.5 min. The solvent was evaporated using a SpeedVac. The samples were then dissolved in 50 µl of 0.1% TFA. Each sample (5 µl) was injected and analyzed in a nanoAcquity Xevo-TQS mass spectrometer (Waters, Milford, Massachusetts, USA). The samples were measured in a randomized order. Urine AGP levels were expressed as the analyte/internal standard (IS) ratio.

Quantitative mass spectrometry

The liquid chromatography and mass spectrometry settings were similar to those published by Bröker et al. [13].

Table 1. The three transitions and collision energies used for detection of AGP and corresponding stable isotope-labeled internal standard

Peptide	Parent mass (*m/z*)	Fragment (*m/z*)	Collision energy (V)
AGP P(-OH)GEAGK P(-OH)GEQGV P(-OH)GDL (z = +2)	1088.51	527.28 (y6)	37
		812.38 (y9)	33
		1364.64 (b15)	33
AGP P(-OH)GEAGK* P(-OH)GEQGV P(-OH)GDL (z = +2)	1092.52	527.28 (y6)	37
		812.38 (y9)	33
		1372.66 (b15)	33

*Stable isotope-labeled lysine (K) (+8Da).

A selective reaction monitoring (SRM) method was developed and optimized on a Waters nanoAcquity Ultra Performance LC connected online to a Xevo TQS mass spectrometer (Waters, Milford, Massachusetts, USA). The sample was trapped on a Symmetry C18 nano-Acquity column (5 mm × 180 μm × 20 mm) (Waters, Milford, Massachusetts, USA) for 5 min and washed with a solution of 99% A and 1% B (solvent A, 0.1% formic acid in water; solvent B, 0.1% formic acid in acetonitrile) at a flow rate of 8.00 ml/min, followed by separation on an Acclaim PepMap100 C18 3 μm column (75 μm × 150 mm) at a flow rate of 0.3 ml/min and gradient starting with 98.5% solvent A reduced over 30 min to 60%. In 0.10 min it was further decreased to 20% solvent A and kept constant for 5 min, followed by an increase over 0.10 min to 98.5% and kept constant for 20 min. Ions were produced by a Z-spray nanoflow source under atmospheric pressure using a capillary voltage of 3.00 kV, cone voltage of 50 V, and a source offset of 50 V. The source temperature was maintained at 70°C. For AGP and the stable isotope-labeled peptide, three transitions with different collision energies were chosen (**Table 1**). Fragmentation was induced by collision dissociation with argon gas inserted with a flow rate of 0.15 ml/min. The following parameters were taken into account for the selection of AGP for the final quantitative assay: no interference in SRM transitions, co-elution of the peptide and IS, linearity of response in measured concentration range, symmetry of peak shape, and a signal intensity at least 10 times the average observed background. A chromatogram of a sample with the lowest AGP level (ratio = 0.170) is shown in **Figure 2A**; peaks are measured properly with a high signal to noise ratio and a symmetrical peak shape. The selected transitions were relatively free of interference.

Power calculation

In a previous pilot study we demonstrated a sensitivity of 85% and specificity of 92% for the combination of two collagen urine peptides, AGP and KGN, in urine [13]. With the availability of urine samples from 100 patients with CRLM followed over a 3-year period, the 95% confidence interval (CI) and estimated the sensitivity and specificity was calculated. The CIs for the estimated sensitivity and specificity of 70, 80, and 90% were 60-79%, 71-87%, and 82-95%, respectively. We judged these CIs to be sufficiently small and used a control group of 100 normal subjects (healthy living kidney donors; HKDs).

Quality controls

One quality control (QC) was inserted per row of the 96-well plates. A total of 18 QC samples were measured with an average ratio of 0.232 and 14.6% CV. The QC AGP level was in the lower region of the measured concentrations, which are generally more receptive to variation; therefore, the CV indicated good reproducibility.

Statistical analysis

The peak analysis was performed using Skyline v1.4.0.4421 (MacCoss Lab, University of Washington, WA, USA). Skyline is an open source, freely available application which can be used to refine targeted methods for large-scale quantative mass spectrometry studies in life sciences. Next, ROC-curves were used to generate cut-off values and to determine sensitivity and specifity. These analyses were performed using Microsoft Excel 2007 (Redmond, WA, USA) and GraphPad Prism v5.00 (GraphPad Software, San Diego, CA, USA).

Differences in basic patient characteristics and treatment outcomes were assessed using the
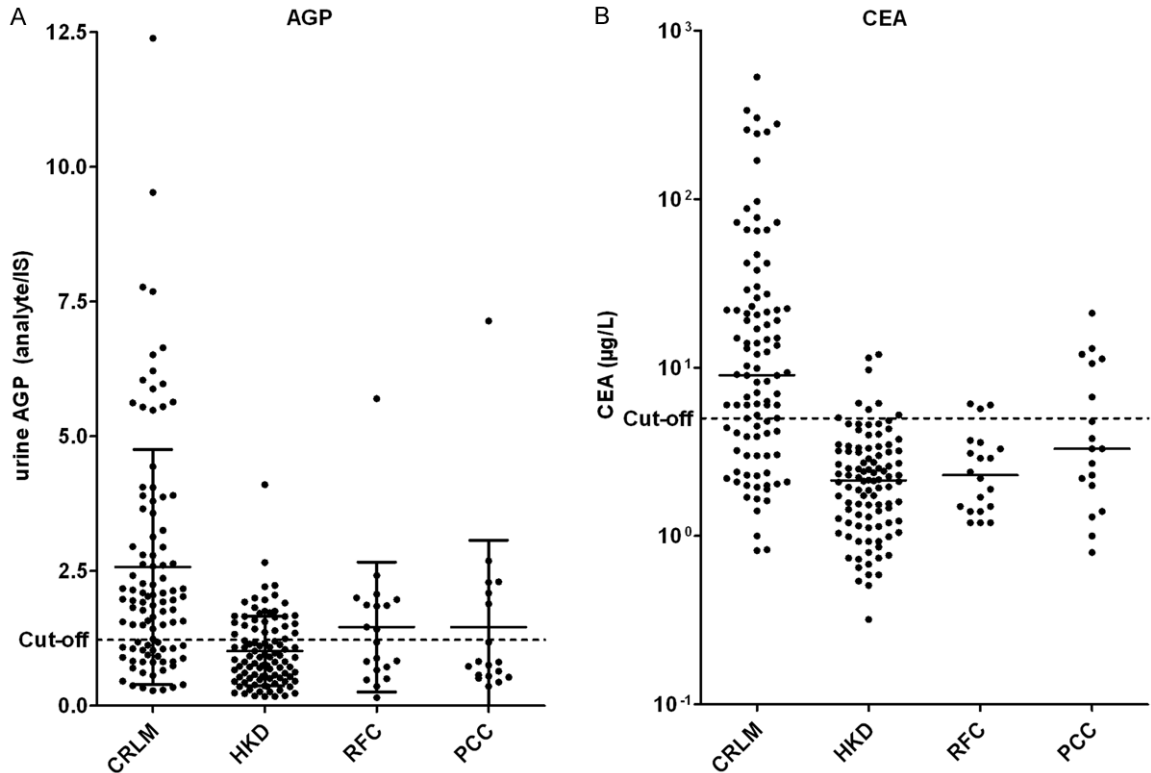
Figure 2. A and B. Distribution of data points based on a) the urine AGP ratios and b) serum CEA levels. AGP cut-off = 1.223, CEA cut-off = 5 µg/L. HKD = healthy kidney donors; RFC = relapse-free controls; PCC = primary colorectal cancer controls.

unpaired t-test and chi-squared test. Correlations between markers were assessed using the Pearson correlation coefficient. A *p*-value <0.05 was considered significant. Missing data were supplemented using multiple imputation [14]. The basic characteristics were analyzed using SPSS (Version 21.0. Armonk, NY: IBM Corp.).

To investigate the capabilities of CEA and AGP to discriminate between controls and patients with CRLM the distinctiveness of serum CEA and urine AGP levels were investigated separately (**Figure 2A** and **2B**).

Next, a multivariate logistic regression analysis was performed. Potential non-linear effects of CEA and AGP in the log odds of having CRLM were looked for using restricted cubic splines with three internal knots. The *p*-values for non-linearities were calculated based on the Wald test. In a second stage, the discriminative ability of the fitted model quantified by the RN^2 index of NagelKerke and Somers' Dxy rank correlation between predicted probabilities and observed responses (in other words, the

model's ability to distinguish patients with CRLM from controls) was investigated [15]. For both measures, values close to one indicate good predictive performance [16]. These measures were validated to account for possible over-fitting using the Bootstrap method taking 500 re-samples. Results and conclusions are based on the corrected (i.e., validated) RN^2 and Dxy indexes. The regression analysis and model fitting were performed in the R programming language (version 3.1.3).

Since the group sizes were unequal, the Tukey's contrasts test was used to test for significant differences between the four groups. Tukey's contrasts test was also performed in the R programming language (version 3.1.3.).

Results

Basic characteristics

The basic characteristics of both patients and controls are presented in **Table 2**. The median age and percentage of males was significantly different between the four groups (both P<

Table 2. Basic characteristics of patients with colorectal liver metastases (CRLM) and controls

	CRLM (n = 98)	HKD (n = 100)	RFC (n = 20)	PCC (n = 18)	*P*-value ANOVA
Age	64 (57-70)	52 (43-63)	72 (63-81)	73 (69-81)	<0.001
Gender, male	71 (72%)	37 (37%)	11 (55%)	13 (72%)	<0.001
BMI	26 (25-28)	25 (23-28)	27 (23-29)	26 (23-28)	0.541
No. of lesions	2 (1-4)	-	-	-	-
Size of largest lesion, cm	2.7 (1.8-4.0)	-	-	-	-
Serum creatinine >115 μM/L	4 (4%)	0 (0%)	2 (10%)	1 (6%)	0.062

HKD = healthy kidney donors; RFC = relapse-free controls; PCC = primary colorectal cancer controls; BMI = body mass index. Data are presented as a median with the interquartile range (25th-75th percentile) or n (%).

Table 3. Serum CEA levels and urine AGP levels in patients with colorectal liver metastases (CRLM) and controls

	CRLM (n = 98)	HKD (n = 100)	RFC (n = 20)	PCC (n = 18)
Serum CEA (ng/ml)	9.05 (3.90-22.93)	2.14 (1.22-3.21)	2.3 (1.48-3.38)	3.3 (2.05-9.63)
Serum CEA >5 ng/ml	65 (66%)	8 (8%)	3 (17%)	6 (33%)
Urine AGP (analyte/IS)	1.96 (1.06-3.23)	0.9 (0.53-1.44)	1.3 (0.71-1.9)	0.78 (0.56-2.04)
Urine AGP >1.223 (analyte/IS)	66 (67%)	27 (27%)	10 (50%)	6 (33%)
Mult. Var.	10.38 (1.73-158.42)	0.19 (0.08-0.47)	0.32 (0.19-0.89)	0.44 (0.12-2.93)
Mult. Var. >0.6278	83 (85%)	16 (16%)	6 (33%)	8 (44%)

HKD = healthy kidney donors; RFC = relapse-free controls; PCC = primary colorectal cancer controls; IS = internal standard; CEA: carcinoembryonic antigen; AGP: (AGPP(-OH)GEAGKP(-OH)GDLGAP(-OH)GP). Urine AGP ratios were used in calculations. The lowest and highest quartile (ratio of 0.53-3.23) represents a concentration range of 7.4-45.2 nmol/L (16-98 μg/L). Data are presented as the median with the interquartile range (25th-75th percentile) or n (%).

0.001). No difference in BMI was found between the groups (P = 0.541). The median number of liver lesions was 2 (IQR 1-4), with a median diameter of 2 cm (IQR 1.8-4.0 cm). The serum creatinine level was >115 μmol/L for several subjects (CRLM n = 4, HKD n = 0, RFC n = 2, PCC n = 1), indicating impaired renal function.

CEA and AGP

A relatively large proportion of patients in the CRLM group (34%) had serum CEA levels below the cut-off value (5 ng/ml, **Table 3**). In the control groups, several subjects had elevated CEA levels (HKD n = 8, RFC n = 3, PCC = 6). As shown in **Table 4**, the serum CEA levels in the CRLM group significantly differed from the serum CEA levels in all control groups individually, whereas the controls did not significantly differ from each other. The sensitivity and specificity was 66% (95% CI 56-76%) and 92% (95% CI 85-96%), respectively, when comparing the CRLM and HKD groups.

Urine AGP levels were measured in all CRLM samples except for two in which no signal was observed. In the CRLM group, 33% of the urine AGP levels were below the cut-off value of 1.223, which was the optimal cut-off point calculated with an ROC curve (**Table 2**). In the control groups, several subjects had elevated urine AGP levels (HKD n = 27, RFC n = 10, PCC = 6). Urine AGP levels in the CRLM group significantly differed from the urine AGP levels in all control groups individually, but the controls did not significantly differ from each other (**Table 3**). The AGP test had a sensitivity and specificity of 68% (95% CI 58-77%) and 69% (95% CI 59-78%), respectively. No significant correlations were found between AGP and the size of the liver lesion, the number of lesions, or liver enzyme values for alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma glutamyl transferase (γ-GT).

Serum CEA levels and urine AGP levels did not correlate ($r^2 = 0.08$) and, therefore, were complementary.

Multivariate logistic regression model

A multivariate logistic regression model was created (Formula 1).

Formula 1:

Odds of being sick = $e^{3.9090 + 1.1213 \cdot AGP + 1.622 \cdot \ln(CEA)}$

Table 4. Univariate and multivariate group comparisons using regression analysis

Group comparison	AGP *p*-value	CEA *p*-value	Mult Var *p*-value
CRLM-CRC	0.028	<0.001	0.001
HKD-CRC	0.671	0.127	0.098
RFC-CRC	1.000	0.572	0.798
HKD-CRLM	<0.001	<0.001	<0.001
RFC-CRLM	0.020	<0.001	<0.001
RFC-HKD	0.640	0.932	0.615

CRLM = colorectal liver metastasis; HKD = healthy kidney donors; RFC = relapse-free controls; PCC = primary colorectal cancer controls. *P*-values were calculated using Tukey's contrasts.
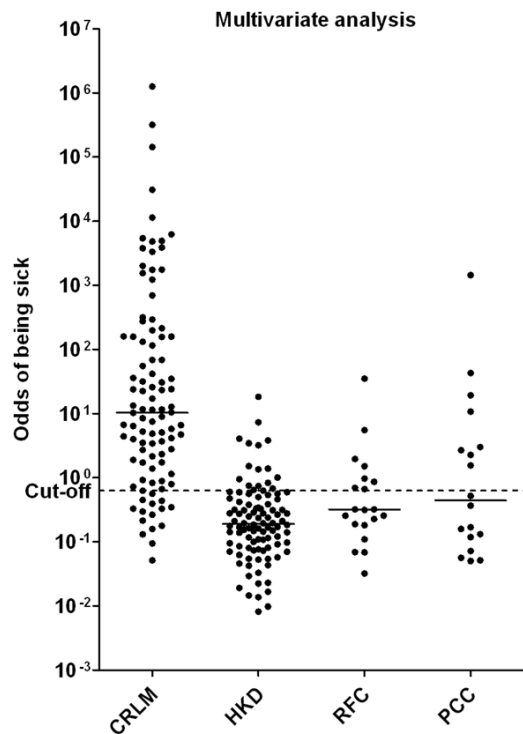

Figure 3. Distribution of data points based on the final multivariate regression analysis combining serum CEA and urine AGP levels. HKD = healthy kidney donors; RFC = relapse-free controls; PCC = primary colorectal cancer controls.

Based on this model, the combined value of serum CEA and urine AGP was significantly different for the CRLM group compared to all individually tested control groups, whereas the combined values in the controls groups did not significantly differ (**Table 3**). The final model produced with an AUC of 0.9139 (95% CI 0.8745-0.9532) resulted in a sensitivity of 85% (95% CI 76-91%) and specificity of 84% (95% CI

75-91%) with a corresponding optimal cut-off value of 0.6278 (**Figure 3**).

Discussion

The current study demonstrates that collagen-derived peptide AGP has a very specific hydroxylation pattern that can be reliably measured in urine using mass spectrometry. When combined with serum CEA levels, urine AGP demonstrates to be a promising biomarker with a sensitivity of 85% (95% CI 76-91%) and specificity of 84% (95% CI 75-91%).

Clinical proteomics using mass spectrometry has yielded early and positive results in different diseases [17]. These results have the potential to detect patients with a specific disease but need to be confirmed in large-scale studies [18]. Large-scale validation is essential for assessing the value of biomarkers, as large independent validation studies have often shown less promising results than small discovery sets [17, 19]. Due to the cost and time required for prospective sample collection, preliminary results are often presented. In a small discovery study consisting of 24 patients, we reported a sensitivity and specificity of 88% for AGP [13]. In the current study with 100 patients, the sensitivity decreased to 68% for AGP alone. This decrease may be attributed to the increased variation in patients and sampling conditions, since both males and females were included, urine was collected randomly during the day and the time to aliquoting and freezing the urine varied depending on the routine schedule of the hospital. However, even with these more variable conditions, the sensitivity of AGP combined with CEA clearly exceeded that of CEA alone (85% vs. 68%), which is similar to the sensitivity of CEA combined with liver imaging [5].

This study focused on AGP, a naturally occurring hydroxylated peptide that is part of collagen type α1(I) [13]. Collagens are the most abundant proteins in the animal kingdom. In the human body, 80-90% of the total collagen is collagen type I, II, or III [20]. In our study, not the amino acid sequence, but more the hydroxylation pattern for AGP found appears to be very specific since the chance that this specification hydroxylation occurs at the specific positions can be estimated to be 0.00072% based on a chance process described by Rapaka et

al. [21]. The location from which the AGP peptide with the specific hydroxylation pattern found in the urine of patients with CRLM is derived remains unclear. However, it is tempting to suggest that it is derived from the liver, either from the metastasis or the metastasis surrounding tissue. One may reason that it is not likely that AGP originates from more central, hypoxic regions of the tumor as the lack of oxygen inhibits the hydroxylation of proline by prolyl 4-hydroxylase. This assumption is supported by the previously described decrease in hydroxylation of hypoxia-induced factors inhibiting degradation in hypoxic regions [22] and suggests the formation of collagen with a limited number of hydroxylations in hypoxic tumor regions, whereas AGP is fully hydroxylized. A more plausible origin may be the activity at the invasion front, including increased tissue remodeling and production of collagen, thus enabling tumor progression with enhanced production of matrix metalloproteinase and an increase in urine AGP levels with a specific hydroxylation pattern, as observed in this study.

CEA, the standard biomarker used for CRLM, is known for its high specificity. Ten subjects in the control groups (7%) had serum CEA levels >5 ng/ml. Other factors that have been linked to an increase in CEA include smoking, the use of paroxetine (a selective serotonin reuptake inhibitor, SSRI), metabolic syndrome, and alcoholic liver disease [23-28]. Of the 10 subjects, four were medium to heavy smokers (15-25 cigarettes per day), three used an SSRI at the time of CEA determination, one was diagnosed with metabolic syndrome, and one was diagnosed with alcoholic liver disease, leaving only one control subject with an unexplained elevation of CEA.

From a clinical perspective, the main goal of our study was to increase the sensitivity of CEA to more accurately identify CRLM in patients with a medical history of resected CRC. When comparing CEA alone and combined with the urine peptide, the sensitivity increased from 68% to 85% and specificity decreased from 91% to 84%.

Further research is needed to evaluate the potential of using the combined biomarkers to detect CRLM at an earlier stage, possibly resulting in more effective interventions. Longitudinal sampling is expected to be of value and may improve the sensitivity, as an increase within one patient can be observed. More research

should be performed in patients with CRLM who have a false negative AGP. Whether these false negative patients are positive after multiple testing at various time points should be investigated. To answer these questions and to validate the added value of the AGP peptide, a large follow-up study should be performed in which urine AGP levels are determined in addition to the regular follow-up tests (CEA and US and/or CT). Ultimately, a test may be constructed in which the urine AGP test and serum CEA test are combined and routine imaging is needed less frequently.

In conclusion, the collagen-derived urine AGP peptide with a very specific hydroxylation pattern can be measured reliably using mass spectrometry and may be a promising biomarker to reliably identify CRLM in combination with serum CEA levels.

Disclosure of conflict of interest

None.

Abbreviations

CRC, Colorectal cancer; CRLM, Colorectal liver metastases; US, Ultrasound; CT, Computed tomography; CEA, Carcinoembryonic antigen; BMI, Body mass index; QC, Quality control; SSRI, Selective serotonin reuptake inhibitor; RFC, Relapse-free control; PCC, Primary colorectal cancer control; HKD, Healthy kidney donor; AGP, AGPP(-OH)GEAGKP(-OH)GEQGVP(-OH)GD-LGAP(-OH)GP (peptide); KGN, KGNSGEP(-OH)GAPGSKGDTGAKGEP(-OH)GPVG (peptide); MS, Mass spectrometry; CV, Coefficient of variation.

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