SUPPORTING INFORMATION

for

Identification of Two-Dimensional Copper Signatures in Human Blood for Bladder Cancer with Machine Learning

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Contents

1. Experimental Section

Chemicals and Reagents

Nitric acid was purchased from Merck (Darmstadt, Germany). Hydrochloric acid was from Beijing Chemicals Works (Beijing, China). Sub-boiled distilled nitric acid, hydrochloric acid, and water were produced by a distilling apparatus. Hydrogen peroxide (30%) was from Sinopharm Chemical Reagent Co. (Shanghai, China). Ultrapure water (18.2 MΩ·cm) was produced from a Milli-Q Gradient system (Millipore, Bedford). The copper isotopic standard material ERM-AE633 was purchased from the Institute for Reference Materials and Measurements (GEEL, Belgium), and another standard material CAGS-Cu was provided by Chinese Academy of Geological Sciences (Beijing, China). The element calibration standard solution for ICP-MS was from Agilent (Santa Clara, CA). No unexpected or unusually high safety hazards were encountered.

Study participants

All blood samples of BCa patients (n = 41) and benign patients (n = 17) were collected in the Second Hospital of Tianjin Medical University during 2013-2015 (Tianjin, China). The BCa group included different types, malignancy grades, and cancer stages. The benign diseases included cystitis, benign prostatic hyperplasia, ureteral calculus, renal calculus, vesical calculus, renal hamartoma, renal cyst, adrenal cortical adenoma, stress incontinence, and bladder mucosa mild epithelial dysplasia. The patients were classified by clinical diagnosis. The blood samples of healthy subjects were collected around the Tianjin city, China. Thirty age-matched healthy adults (n = 30) and eighteen healthy young adults were recruited (n = 18). The healthy subjects presented no clinical evidence of diseases. The demographics of all study participants are given in Table S1, and the blood biochemical indexes of patients and healthy subjects are given in Table S6-S8. Ultra-clean disposable devices were used for sample collection. After collection, the blood samples were immediately separated into plasma and red blood cells (RBC) by centrifugation and stored separately at -80 °C in the dark before analysis.

All participants provided informed consent, and the study protocol was approved by the Ethics Committee of the Second Hospital of Tianjin Medical University (No. KY2018K082) and compliant with all relevant ethical regulations for studies involving human subjects.

Sample preparation

The sample preparation procedure included two steps, digestion and isolation of target element. For digestion, 1 mL of plasma or RBC sample was transferred to a Teflon tube containing 7.5 mL of 14 M HNO_3 and 1 mL of 30% H_2O_2. The mixture was completely digested in a microwave reaction system (Anton Paar). After that, the sample was put in a Teflon beaker followed by heating to dryness, and the residue was redissolved with 8 M HCl/0.001% H_2O_2 to 1 mL.

For MC-ICP-MS analysis of copper isotopes, we used an anion exchange chromatographic method to purify the digested samples as reported previously.[1] AG-MP-1M anion exchange resin (Bio-Rad, 100-200 mesh) was activated for more than 12 h prior to use. The resin (1.8 mL) was loaded into a Bio-Rad column, and 10 mL of 0.7 M HNO_3 and 10 mL of H_2O were used to clean the resin. Then, 10 mL of 8 M HCl/0.001% H_2O_2 was loaded onto the top of resin bed. After that, the sample solution was loaded to the column. The elution was performed as follows: 8 mL of 8 M HCl/0.001% H_2O_2 for elution of matrix, 12 mL of 5 M HCl/0.001% H_2O_2 for elution of copper, 7 mL of 0.6 M HCl for elution of Fe, and finally 8 mL of 0.7 M HNO_3 for elution of Zn. As shown in Fig. S5, the elution curve indicates that those elements could be fully separated from interfering ions and collected in different fractions. In order to eliminate the interference from Cl ions during MC-ICP-MS analysis, the copper fraction was evaporated to dryness at 100 °C and redissolved in 0.7 M HNO_3, and this step was repeated twice. A copper isotopic standard sample in HNO_3 and 30% H_2O_2 was analyzed by the same procedures to ensure that no copper isotopic fractionation was caused by the sample preparation process. The recovery of copper during the whole sample preparation process was > 95%.

Measurement of Cu concentration and isotopic ratios

The copper concentration was measured by an Agilent 8800 inductively coupled plasma mass spectrometer (Santa Clara, CA, USA). For copper isotopic ratio measurement, a Nu Plasma II

MC-ICP-MS (Wrexham, UK) equipped with 16 Faraday cups was used. The sample was injected in wet mode with a peristaltic pump at a rate of 8 rpm. The optimized instrumental parameters and Faraday cup configuration are given in Table S10. The copper isotopic ratio ($^{65}Cu/^{63}Cu$, expressed as $\delta^{65}Cu$ value in permil) was expressed relative to a standard material (ERM-AE633):

$$\delta^{65}\mathrm{Cu} = \left(\frac{\left(^{65}\mathrm{Cu}/^{63}\mathrm{Cu}\right)_{\mathrm{sample}}}{\left(^{65}\mathrm{Cu}/^{63}\mathrm{Cu}\right)_{\mathrm{standard}}} - 1 \right) \times 1000‰ \quad (1)$$

Since samples have the equal concentration compared with standard (± 10% range) and the biological matrix effect was verified to be slight, the standard-sample-standard bracketing (SSB) method was adopted to correct the mass bias.[2-4] The $\delta^{65}Cu$ value of sample was calculated based on the results of two adjacent standards. The method was validated by two standard materials, ERM-AE633 and CAGS-Cu. The $\delta^{65}Cu$ value of CAGS-Cu relative to ERM-AE633 was 0.57 ± 0.1‰ (n = 3). This value was very close to that reported in the previous literature (0.57 ± 0.06‰ in Ref. 5 and 0.54 ± 0.07‰ in Ref. 4),[4, 5] proving that this method is highly accurate and precise. It is noteworthy that some reference materials of biological origins have been reported recently,[6] and inclusion of such materials in Cu isotopic analysis may further enhance the precision in future studies.

Evaluation of biological matrix effect in copper isotopic analysis

The experiments were carried out as follows: first, the biological samples were digested and purified by anion exchange column as described above. Then, all the eluted fractions were collected and combined again except for the copper fraction. In this way, we obtained a copper-excluded sample that had the same composition with the original one but with no copper. After that, an ERM-AE633 copper standard solution was added to the copper-excluded sample to obtain a final copper concentration of ~1 μg/mL. The mixture was purified and analyzed again as described above. The results are given in Fig. S2. It can be seen that no significant influence on the copper isotopic measurement caused by the sample matrix was found in the present study.

Machine learning model

Random forest (RF) is an ensemble supervised learning method that fits a number of

individual decision tree classifiers on sub-samples of the whole dataset. Each individual tree in the RF yields a class prediction and the class selected by most trees is the output of the random forest.[7] Such an ensemble strategy not only increases the performance but also reduces the risk of overfitting compared with single decision tree. Therefore, RF algorithm is particularly suitable for the problems with low data volumes. In this study, a RF classification model based on a total of 88 samples (41 BCa and 47 non-BCa participants) was established, where each sample was represented by a vector of four copper-related variables (i.e., plasma copper concentration, RBC copper concentration, plasma δ^{65}Cu value, and RBC δ^{65}Cu value). The leave one out cross-validation (LOOCV) was used to estimate the performance of RF classifier. During the training process, each sample was used once as a test set while the remaining 87 samples formed the training set. In this way, a total of 88 models have been constructed, and the overall precision, recall (also referred to as sensitivity or TPR (true positive rate)), TNR (true negative rate, also referred to as specificity), FPR (false positive rate, also referred to as 1-specificity), and accuracy were calculated by combing predicted label of the tested sample in each fold. The calculation of these metrics was defined as follows. Besides, the area under the receiver operator characteristic curve (AUC-ROC), an overall diagnostic accuracy, was adopted to summarize the trade-off between sensitivity and TNR for every possible cut-off for a combination of tests. Variable importance was computed as the total reduction of the criterion brought by the variables, known as the Gini importance. The t-SNE, RF model, and evaluation process were implemented by an open-source machine learning library scikit-learn in Python 2.7.

$$Precision = \frac{TP}{TP + FP} \tag{2}$$

$$Recall = \frac{TP}{TP + FN} \tag{3}$$

$$FPR = \frac{FP}{FP + TN} \tag{4}$$

$$Specificity = TNR = \frac{TN}{FP + TN} \tag{5}$$

$$Accuracy = \frac{TP + TN}{TP + TN + FP + FN} \tag{6}$$

where TP, FP, TN, and FN denote the number of true positives, the number of false positives, the number of true negatives, and the number of false negatives, respectively.

Statistical analysis

Before doing *P* value calculations, we carried out normal distribution test and homogeneity test of variance. We employed Mann Whitney test for *P* value calculation when the dataset did not conform to normal distribution. If the dataset conformed to normal distribution and passed the homogeneity test of variance, we employed the unpaired Student's two-tailed *t*-test for *P* value calculation. If the dataset did not pass the homogeneity test of variance, we employed Welch's *t*-test for *P* value calculation.[8-10]

Data and code availability

The data that support the findings of this study are given in this supporting material or available from the corresponding authors upon reasonable request. The codes that support the findings of this study are available from the corresponding author upon reasonable request. All analyses were conducted with built-in and freely available R packages.

2. Supplementary Discussion

Comprehensive correlation analysis of multiple factors.

To gain more information on copper metabolic imbalance of BCa patients, we performed a comprehensive correlation analysis of multiple factors (including blood metals and biochemical indexes) (Fig. S3). We mainly paid attention to the data with Pearson correlation coefficient (R) greater than 0.3 or less than -0.3 ($R > 0.3$ or $R < -0.3$) and the P value less than 0.05 ($P < 0.05$, marked with asterisks). When taking the three groups together (Fig. S3a), generally, there was no significant correlation between biochemical indexes and blood copper concentration or δ^{65}Cu value. The copper concentration and δ^{65}Cu value in plasma were positively correlated with those in RBC, indicating that the copper in plasma and RBC should have some common sources. The plasma and RBC δ^{65}Cu values were negatively correlated with age, but this correlation did not exist in BCa patients (Fig. S3d). This result accorded with that in Fig. 2I-2J, i.e., BCa might cover up the effect of age on the blood copper.

In the age-matched healthy group (Fig. S3b), the positive correlation between copper and zinc concentration in both plasma and RBC could be ascribed to the abundant presence of Cu-Zn binding protein SOD1 in blood.[11] In the BCa group (Fig. S3d), however, the Cu-Zn correlation was only present in RBC but absent in plasma. The plasma and RBC δ^{65}Cu values were negatively correlated with the plasma zinc but positively correlated with the RBC zinc (Fig. S3d). This meant that the negative shift in the blood δ^{65}Cu value of BCa patients should be accompanied with the down-regulation of SOD1 in RBC. As an important Cu-Zn storage protein, SOD1 was ^{65}Cu-depleted relative to blood.[12] Thus, the release of copper from SOD1 could cause a depletion of ^{65}Cu in plasma relative to RBC. This suggested that the storage processes of copper might also contribute to the BCa-induced copper isotopic fractionation, which needs further evidence in future studies.

3. Supplementary Tables

Table S1. Demographics, copper concentration, and δ^{65}Cu values in blood for study participants. [a]

	BCa patients	Benign patients	Age-matched healthy control [b]	Young healthy control [c]
Age (mean ± SD)	65.8 ± 8.7	59.1 ± 14.1	57.5 ± 6.7	21.3 ± 4.5
Gender (male/female)	28/13	11/6	15/15	9/9
Copper in plasma (μg/g)	0.93 ± 0.26	1.41 ± 0.58	1.50 ± 0.72	0.36 ± 0.22
δ^{65}Cu value in plasma (‰)	-0.57 ± 0.73	-0.06 ± 0.31	0.07 ± 0.23	-
Copper in RBC (μg/g)	0.69 ± 0.14	0.91 ± 0.36	0.76 ± 0.55	0.23 ± 0.11
δ^{65}Cu value in RBC (‰)	0.25 ± 0.74	0.76 ± 0.68	1.39 ± 0.38	-

[a] See Supplementary Table S2-S8 for detailed information.

[b] Age-matched healthy control means the healthy subjects who have a similar age range with BCa patients.

[c] Young healthy control means the healthy subjects with a lower age range.

Table S2. Elemental concentrations and δ^{65}Cu values in plasma and RBC for BCa patients.

ID	Plasma						RBC					
	[Cu] (μg/g)	δ^{65}Cu (‰)	SD (n = 1-6)	[Fe] (μg/g)	[Mg] (μg/g)	[Zn] (μg/g)	[Cu] (μg/g)	δ^{65}Cu (‰)	SD (n = 1-4)	[Fe] (μg/g)	[Mg] (μg/g)	[Zn] (μg/g)
1	1.01	-0.55	0.09	3.60	22.9	2.20	0.67	0.45	0.25	648	45.7	11.1
2	1.20	-0.66	0.07	2.80	25.0	3.55	0.74	-0.65	0.18	429	35.3	8.10
3	0.75	-0.56	0.13	3.14	17.5	2.75	0.70	0.10	0.06	613	56.8	8.58
4	0.74	-0.80	0.13	8.72	19.6	1.53	0.78	0.15	0.05	806	50.2	11.2
5	0.98	-0.75	0.30	5.01	21.4	2.68	0.67	-0.10	0.16	735	42.2	10.3
6	0.61	-0.67	0.19	3.48	19.1	1.53	0.65	-0.33	-	743	40.7	10.5
7	1.30	-1.63	0.25	6.34	21.6	3.41	0.80	-0.86	0.21	588	36.8	11.7
8	0.56	-1.39	0.15	21.7	15.9	1.97	0.58	-2.03	0.13	632	35.1	11.0
9	0.71	-1.49	0.01	7.75	18.7	1.55	0.89	1.09	0.25	396	33.8	7.9
10	0.69	-1.23	0.25	7.95	21.5	3.76	0.58	-0.42	0.06	338	32.2	6.14
11	0.66	-1.71	0.08	7.52	21.0	4.92	0.59	-0.71	-	89.3	23.8	2.94
12	0.55	-2.53	0.35	10.5	21.8	4.67	0.45	-	-	816	48.1	10.3
13	0.87	0.03	0.08	3.86	20.3	2.52	0.79	-0.50	0.01	69.8	23.9	3.82
14	0.64	0.79	0.23	15.7	20.8	2.04	0.53	-0.18	-	894	41.9	14.8
15	1.17	0.90	0.67	11.3	21.1	2.20	0.80	1.17	0.04	526	34.4	8.82
16	1.28	-0.62	0.15	3.35	15.4	0.94	0.68	2.23	0.01	885	51.2	10.8
17	0.97	0.59	0.01	3.11	21.0	1.99	0.45	0.65	-	874	43.2	11.5
18	0.59	0.51	0.11	2.90	19.8	1.88	0.69	1.40	0.09	859	44.3	12.5
19	0.78	0.10	0.15	6.12	21.5	1.29	0.64	0.47	-	790	52.0	10.7
20	0.63	0.08	0.23	2.15	18.8	1.32	0.63	-0.02	-	874	42.1	12.0
21	1.09	0.34	0.09	9.49	22.8	2.39	-	-0.33	0.18	-	-	-
22	0.93	-1.07	0.17	1.60	22.0	1.49	0.65	1.44	0.06	894	47.2	12.0

23	1.04	-1.22	0.13	4.13	24.8	2.68	0.25	0.87	0.01	329	15.5	3.85
24	1.26	-1.20	0.19	5.32	19.1	0.82	0.60	-0.82	-	1014	51.8	9.03
25	0.77	-0.38	0.03	1.45	22.1	0.76	0.68	0.50	0.06	753	46.6	11.4
26	0.81	-1.04	0.03	2.16	20.1	1.06	0.66	0.31	-	812	46.6	9.54
27	0.91	-0.62	-	1.69	24.2	1.85	0.64	0.53	-	867	51.4	12.2
28	0.80	-0.65	-	1.28	21.9	0.78	0.54	0.55	-	671	51.8	11.1
29	1.58	0.77	0.14	5.13	19.6	0.96	0.68	0.59	-	695	38.0	10.2
30	0.92	-0.40	0.01	10.1	23.3	1.00	0.60	0.25	-	788	42.5	9.05
31	0.80	-1.13	0.12	4.68	18.8	0.70	1.06	0.05	0.02	814	52.3	11.4
32	1.01	-0.38	0.15	1.87	24.1	0.99	0.66	0.63	0.16	864	49.7	9.04
33	0.83	-1.19	0.05	1.74	21.8	1.05	0.81	0.23	0.03	932	47.6	10.3
34	0.99	-0.48	0.03	3.05	22.9	2.24	0.87	0.41	0.09	984	51.0	14.5
35	1.48	-0.68	0.08	5.45	22.5	1.74	0.82	0.19	-	785	50.4	11.7
36	1.06	-0.18	0.09	5.11	22.5	3.31	1.00	0.76	0.14	1016	44.4	10.9
37	0.87	-0.08	0.12	5.87	23.8	2.38	0.75	0.51	0.16	914	50.9	12.5
38	1.09	-0.33	0.01	2.19	22.9	1.73	0.75	0.69	0.13	901	45.6	11.1
39	0.99	-0.78	0.14	18.6	26.0	1.57	0.74	-0.13	0.03	634	42.5	11.2
40	1.52	-0.82	0.04	2.11	19.8	1.14	0.83	0.36	0.01	929	56.6	13.8
41	0.81	-0.36	0.01	2.57	23.5	1.68	0.78	0.67	0.01	946	57.6	10.6

Table S3. Elemental concentrations and δ^{65}Cu values in plasma and RBC for benign patients.

ID	Plasma						RBC					
	[Cu] (μg/g)	δ^{65}Cu (‰)	SD (n = 1-2)	[Fe] (μg/g)	[Mg] (μg/g)	[Zn] (μg/g)	[Cu] (μg/g)	δ^{65}Cu (‰)	SD (n = 1-2)	[Fe] (μg/g)	[Mg] (μg/g)	[Zn] (μg/g)
1	1.44	-0.64	0.14	4.41	29.5	2.70	0.90	-0.62	0.05	722	46.1	14.6
2	2.19	-0.40	0.04	3.00	25.2	2.31	1.18	0.31	0.08	706	48.4	13.6
3	2.84	-0.15	0.09	4.70	28.1	2.05	1.90	0.33	0.00	591	50.0	12.9
4	1.56	0.39	0.07	3.45	28.0	3.98	0.95	1.07	0.02	696	51.3	14.2
5	2.17	-0.25	0.00	2.61	23.7	1.74	1.43	0.23	0.07	501	38.9	12.5
6	1.00	0.34	0.06	4.30	25.0	1.86	0.76	0.96	0.09	558	36.4	9.32
7	1.49	-0.15	0.16	9.49	25.2	2.77	1.19	0.38	0.03	737	53.2	12.6
8	1.43	0.25	0.02	9.08	23.3	2.84	1.08	1.18	0.17	701	47.5	12.3
9	1.82	-0.48	0.24	3.94	30.4	1.17	0.84	0.77	0.04	964	57.2	14.7
10	1.12	0.12	0.04	3.38	24.2	2.64	0.75	1.18	0.01	880	55.9	12.8
11	1.56	-0.14	0.01	3.42	24.3	2.53	0.47	1.26	0.05	676	38.1	10.3
12	0.77	0.44	0.11	2.13	25.8	2.74	0.67	1.68	0.02	770	48.5	8.59
13	1.23	0.22	0.12	3.82	23.3	1.18	0.51	2.22	0.25	753	42.9	9.57
14	0.64	-0.10	-	4.21	18.8	2.37	0.70	0.93	0.17	-	18.7	2.32
15	0.94	-0.11	-	13.5	23.7	1.43	0.79	0.39	0.09	334	30.6	4.93
16	0.92	-0.28	-	2.58	21.9	1.25	0.72	0.76	-	1007	52.1	11.6
17	0.89	-0.14	-	2.01	21.1	1.07	0.59	-0.17	0.07	820	58.9	9.62

Table S4. Elemental concentrations and δ^{65}Cu values in plasma and RBC for age-matched healthy subjects.

ID	Plasma						RBC					
	[Cu] (μg/g)	δ^{65}Cu (‰)	SD (n = 3)	[Fe] (μg/g)	[Mg] (μg/g)	[Zn] (μg/g)	[Cu] (μg/g)	δ^{65}Cu (‰)	SD (n = 3)	[Fe] (μg/g)	[Mg] (μg/g)	[Zn] (μg/g)
1	1.85	0.20	0.06	2.52	28.8	2.17	0.64	1.16	0.02	795	56.3	10.8
2	1.85	0.16	0.03	2.01	22.3	1.98	0.69	1.59	0.03	964	54.6	11.6
3	1.30	0.43	0.02	2.93	22.1	1.58	0.58	1.45	0.04	890	44.6	9.29
4	1.16	0.02	0.03	1.30	18.9	1.13	0.73	1.41	0.02	1002	61.7	11.4
5	1.63	0.29	0.09	2.45	23.2	1.74	0.71	1.68	0.03	1002	58.4	11.1
6	1.25	-0.16	0.05	2.25	22.5	1.34	0.59	1.20	0.03	906	49.6	12.8
7	1.31	-0.10	0.03	2.91	23.0	1.55	0.74	1.86	0.03	941	61.4	10.3
8	1.00	0.29	0.07	6.78	22.6	1.52	0.59	1.89	0.02	977	44.3	11.3
9	1.33	-0.12	0.02	1.69	19.4	1.52	0.73	1.16	0.02	948	51.3	11.5
10	0.91	0.24	0.04	1.63	19.7	1.41	0.63	1.34	0.03	929	49.7	10.8
11	1.10	0.17	0.04	1.96	23.4	1.63	0.63	1.22	0.03	977	58.9	13.3
12	1.28	0.02	0.01	2.07	26.1	1.42	0.68	1.31	0.02	941	47.5	10.6
13	1.39	0.08	0.05	2.57	22.6	1.58	0.64	1.76	0.01	930	47.5	10.3
14	1.74	-0.05	0.01	4.75	24.9	2.94	0.64	1.35	0.05	990	65.8	12.6
15	1.41	0.23	0.05	2.86	28.1	2.72	0.63	1.26	0.07	1021	56.3	12.5
16	1.18	0.38	0.02	1.62	22.6	1.45	0.79	1.46	0.00	1001	56.7	10.7
17	1.35	-0.42	0.10	2.12	21.9	1.94	0.79	2.18	0.03	1014	55.7	14.0
18	1.26	0.00	0.06	2.93	23.9	1.59	0.67	1.11	0.06	958	58.4	13.0
19	1.89	0.46	0.04	2.06	26.0	3.02	0.69	1.54	0.02	964	56.3	11.0
20	1.26	0.32	0.09	2.12	25.4	1.53	0.61	1.37	0.04	7667	44.8	10.2
21	1.40	0.05	0.04	3.28	22.8	2.01	0.57	1.41	0.05	954	49.7	9.45
22	1.26	0.06	0.01	1.30	26.2	1.46	0.57	1.20	0.02	953	56.5	11.8

23	5.01	0.16	0.02	1.64	25.5	4.19	0.65	1.17	0.04	1015	58.4	13.8
24	1.09	-0.09	0.08	2.53	20.0	1.48	0.67	0.79	0.04	926	41.5	12.1
25	1.06	-0.28	0.04	2.15	19.4	1.39	0.65	1.04	0.01	1028	56.0	12.5
26	2.03	-0.38	0.01	2.33	23.2	2.26	3.64	2.36	0.19	999	55.8	14.7
27	1.22	0.00	0.03	2.20	23.6	1.78	0.70	1.45	0.05	998	54.5	9.35
28	1.78	0.11	0.06	2.29	21.9	1.33	0.66	0.74	0.02	932	52.0	10.7
29	1.21	-0.26	0.02	2.08	27.4	1.44	0.64	0.59	0.02	937	50.3	11.3
30	1.35	0.16	0.04	2.22	21.3	1.77	0.64	1.51	0.03	939	54.1	11.1

Table S5. Information and elemental concentrations in plasma and RBC for young healthy subjects.

ID	Age	Gender	Plasma				RBC			
			[Cu] (μg/g)	[Fe] (μg/g)	[Mg] (μg/g)	[Zn] (μg/g)	[Cu] (μg/g)	[Fe] (μg/g)	[Mg] (μg/g)	[Zn] (μg/g)
1	33	Male	0.27	5.06	24.1	0.10	0.28	803	47.1	11.5
2	21	Female	0.39	6.49	25.7	0.19	0.19	703	45.0	8.42
3	20	Female	0.18	7.17	25.7	0.11	0.10	742	45.5	7.34
4	19	Female	0.29	7.35	30.8	0.21	0.14	701	49.4	8.88
5	16	Female	0.47	7.22	30.8	0.25	0.28	785	44.3	8.84
6	20	Male	0.60	10.6	29.4	0.12	0.26	809	41.5	8.44
7	14	Female	0.37	8.08	25.9	0.26	0.29	735	43.4	9.00
8	17	Female	0.34	12.6	25.6	0.54	0.40	770	42.3	9.14
9	19	Male	0.48	5.05	22.8	0.60	0.23	676	37.4	8.47
10	20	Female	0.15	6.92	25.3	0.18	0.17	635	32.9	6.42
11	19	Male	0.08	8.33	29.0	0.33	0.19	854	40.5	11.0
12	22	Female	0.77	9.68	29.4	0.39	0.19	790	39.7	7.95
13	23	Male	0.33	7.00	31.3	0.30	0.09	687	43.0	8.59
14	23	Female	0.18	6.13	27.3	0.29	0.44	796	47.8	10.5
15	23	Male	0.13	5.26	29.4	0.33	0.17	748	39.6	11.3
16	29	Male	0.19	4.05	32.6	0.09	0.16	888	43.4	11.5
17	25	Male	0.87	10.6	32.0	0.68	0.13	809	43.9	9.45
18	21	Male	0.36	5.25	29.7	0.06	0.47	742	42.1	11.3

Table S6. Information and blood biochemical indexes for BCa patients.

ID	Age	Gender	BMI	ABO	Grade of malignancy	IHC [a]	Hematuria	ALT	AST	NLR	Cre	BUN	LY (%)	RBC	WBC
1	80	Male	26.2	O	-	TFE3+, P504s, Vimentin+, CD10+	yes	29.2	20.2	62.6/28	99.2	6.1	28	5.47	5.5
2	63	Male	24.8	-	-	p53++, p21+++	yes	12.8	10	71.3/20.4	114	7.3	20.4	4.03	10.6
3	58	Male	25.4	O	high	CgA+, CK20+, SYN+, CK7+	yes	21.2	17.5	91.7/3.3	103	4.4	25.7	4.48	5.6
4	58	Male	24.2	B	high	p53+++, p21++	not	16.2	18.6	83.7/10.2	67.3	4.1	10.2	4.46	12.8
5	60	Male	21.5	AB	high	Ki-67++, p53++, p21+, p63+, CK7+	yes	23.7	17	66.3/25	55.6	2.5	25	5.51	8.2
6	52	Male	23.5	O	high	p53+++, p21+++	yes	24.4	17.7	45.1/38.9	85.8	6.8	38.9	5.23	6.8
7	67	Female	28.0	AB	-	-	yes	21.1	15.7	54.8/36	87.6	8.4	36	3.33	7.2
8	60	Male	24.0	-	high	Ki67++, p53++, p21++	yes	20.9	17.6	74.8/16.8	76.4	6.2	31.2	5.16	5.4
9	63	Male	21.1	-	high	p53+++, p21++	yes	5.9	11.5	86.6/9.5	51.6	2.5	9.5	3.2	9.7
10	54	Male	28.7	O	low	Ki67+, p53+, p21+	yes	19.8	13.2	48.4/41.3	80.5	3.5	41.3	4.82	6.1
11	59	Male	24.3	AB	high	Ki67+++, p53+, p21+	yes	32.5	29.8	77.1/45.4	66.9	4.4	45.4	6.2	7.2
12	68	Male	25.3	B	high	CK20+, Ki67++, Syn+, NSE+, CD56+	yes	48	36	55.4/34.7	90.4	4	34.7	5.11	5.2
13	67	Female	27.2	A	low	p53+, Ki67<1%TCP	yes	15	17	92.4/41.4	67.9	5.9	41.4	4.04	7.4
14	53	Male	23.5	A	low	p53++, Ki67≈15% TCP	yes	15.3	24.2	48.8/18.8	80.2	2.5	18.8	7.8	6.1
15	80	Male	21.0	O	low	p53++, p21++, Ki67≈10%TCP	yes	8.6	15	72.4/32.1	85.2	3.6	32.1	4.6	9.9

16	71	Male	19.1	B	high	-	yes	5.2	11.1	89.3/15.1	78.3	4.5	15.1	3.4	7.7
17	61	Male	23.4	O	high	CK27+, CgA+, p53+++	yes	15.7	14.1	56.4/12	91.2	7.9	12	6.8	3.7
18	59	Female	28.4	A	low	p21++, p53++ Ki67<5% TCP	yes	16.6	17.4	87.6/14.1	99.5	6.2	14.1	2.5	15.1
19	55	Female	63.9	AB	low	p53+, p21+, Ki67≈10%TCP	yes	27.9	22.9	48.3/7.4	66.2	3.5	7.4	3.2	9.3
20	78	Male	23.1	A	high	p53++, p21++ Ki67≈10%TCP	yes	7.9	18	55.1/22	102	3.8	22	4.37	7.2
21	62	Male	31.4	O	high	Ki67++, p53+, p21+	yes	32.1	38.1	87.1/41	87.4	2.6	41	5.92	5
22	73	Female	32.0	B	low	p53++, p21+, Ki67<10%TCP	yes	33.6	28.1	78.2/23	71.2	3.5	23	4.32	6.8
23	82	Male	25.1	B	high	p53+, p21+++, Ki67≈90%TCP	yes	33.6	26.5	63.1/26.7	67.7	4	26.7	5.15	6.6
24	75	Female	24.2	O	high	CK7+, CK20+, p53+, Ki67 index7%	yes	28.2	61.6	52.2/25	113	10.6	25	4.22	4.9
25	57	Male	27.1	B	low	p53++, p21+, Ki67<5% TCP	yes	30.6	15.9	52.4/33.5	64	2.6	33.5	4.6	10.3
26	62	Male	28.4	AB	low	p53++, p21+++, Ki67+++	yes	34	15	69.6/26.4	91	7	26.4	4.82	10
27	66	Female	21.4	-	low	p53+, p21++, Ki67+	yes	8.5	26.3	52.2/37.4	59.4	4.4	37.4	4.1	4.4
28	77	Male	24.6	B	high	CK7 Positive, Syn Positive, NSE Positive	yes	14.5	14.3	62.2/26.2	88.9	6.7	26.2	4.53	5.8
29	63	Female	22.8	AB	low	p53+, p21++, Ki67 individual cells+	yes	15	21	66.3/24.3	65	7	24.3	4.28	14.7

30	55	Female	29.3	-	high	p53+++, p21+, Ki67+++	yes	48.5	24.3	54.2/40.3	50.3	4	40.3	4.83	5.6
31	62	Female	30.4	-	low	p53+++, p21+++, Ki67<5%TCP	yes	32	15.7	43.3/46.5	71.8	3.9	46.5	4.49	6.4
32	77	Male	27.3	B	low	p53+, p21+, Ki67+	yes	28	18	70.1/24	74.6	4.5	5.3	5.13	10.9
33	-	Female	27.0	-	-	-	-	23.8	30.9	30.7/60.4	24.5	2.2	60.4	3.91	6.7
34	71	Male	29.4	B	high	p53+++, p21++, Ki67≈30%TCP	yes	26.8	20.8	55.4/34.7	67.9	6.3	36	4.53	7.5
35	61	Male	25.7	AB	high	p53+, Ki67 individual cells+	yes	9	15	68.4/21.3	111	8	32.3	5.3	8.4
36	67	Male	31.1	-	high	p53+++, p21++, Ki67≈30%TCP	yes	9.8	18.2	61.6/31.1	75.9	4.2	31.1	5.5	7.4
37	71	Male	27.0	O	low	p53++, p21++, Ki67≈5%TCP	yes	24.5	29.8	61.9/29.4	69.2	4.4	29.4	4.86	7.5
38	84	Female	21.0	O	low	p53+, p21+, Ki67++	yes	16.9	17.9	44.7/44.8	51.6	7.6	44.8	3.84	5
39	71	Male	20.6	A	high	CK7+, CK20+	yes	10.9	28.9	71.1/18.2	122	8	18.2	4.43	8.2
40	72	Female	30.0	O	high	CK7+, CK20+	yes	20	21	83.1/10.1	91.1	7	42.4	3.89	3.5
41	57	Male	22.3	AB	low	p53++, p21++, Ki67+	yes	13.9	36.4	60.8/30.3	80.7	3.8	30.3	4.34	4.8

[a] TCP in IHC means tumor cell positive.

Table S7. Information and blood biochemical indexes for benign patients.

ID	Age	Gender	BMI	ABO	IHC	Hematuria	ALT	AST	NLR	Cre	BUN	LY (%)	RBC	WBC
1	71	Male	26.3	B	-	no	14.3	18.8	62.5/27.8	73.9	4.5	27.8	4.80	5.9
2	79	Female	29.1	AB	-	no	7.40	19.5	75.4/13.8	245	15.9	13.8	4.01	12.9
3	61	Female	25.5	-	-	-	56.5	33.5	79.8/14	85.3	4.0	14	4.33	5.7
4	57	Female	28.6	A	-	no	26.9	29.0	71.8/22.3	63.0	5.5	22.3	4.26	7.3
5	75	Female	28.5	O	-	no	13.4	14.2	76.3/17.8	52.7	5.4	17.8	4.55	8.1
6	49	Male	26.4	B	-	no	33.6	24.4	53.5/38.9	72.2	4.3	38.9	4.48	5
7	68	Male	29.4	A	-	no	11.9	14.2	74.2/18.9	76.7	8.7	18.9	5.00	7.6
8	44	Female	22.8	O	-	no	16.8	18.9	53.6/40.6	57.6	2.1	40.6	4.47	6.9
9	66	Male	19.7	O	-	no	26.8	17.7	70.9/22.6	90.1	6.9	22.6	5.20	7.6
10	47	Female	25.4	-	-	no	31.3	24.8	83/11.8	50.3	4.2	11.6	4.04	11.5
11	71	Male	16.4	B	-	no	8.90	16.2	50.9/41.9	108	5.6	41.9	4.58	5
12	76	Male	23.5	-	-	no	13.8	15.1	87.5/6.1	74.5	3.5	6.1	5.16	11
13	33	Male	30.4	B	-	no	18.3	14.7	62.7/30.4	73.9	6.2	30.4	5.50	9.4
14	38	Male	22.2	O	CK20+, CK7+, p63+	no	13.9	12.3	91.3/15	67.2	3.3	15	3.70	7.9
15	56	Male	26.9	B	-	no	14.4	9.90	73.1/18.69	62.2	5.9	18.69	3.72	4.1
16	67	Male	29.4	B	-	no	90.7	32.1	62.2/27	65.2	5.2	27	5.07	10.4
17	47	Male	21.2	AB	-	no	32.6	24.3	62.7/22.1	75.0	5.9	22.1	4.87	5.9

Table S8. Information and blood biochemical indexes for age-matched healthy subjects.

ID	Age	Gender	BMI	ALT	AST	NLR	Cre	BUN	WBC
1	57	Female	25.2	14.6	18.2	59/31.2	61.4	8.47	6.08
2	58	Male	27.3	25.7	19.3	53.5/33.2	85.0	7.60	8.27
3	59	Male	29.1	80.7	49.3	53.3/39.0	85.2	4.72	8.96
4	56	Female	24.1	22.3	23.7	55.2/35.3	51.9	5.35	5.11
5	46	Male	31.0	54.4	35.7	52.7/37.5	65.8	4.73	6.91
6	90	Male	21.2	10.1	14.0	48.1/39.2	83.3	8.30	5.79
7	56	Male	36.8	22.4	17.5	57.4/36.3	89.1	6.30	8.97
8	54	Male	20.2	12.4	19.5	58.6/32.1	59.0	5.98	4.78
9	59	Female	27.4	14.6	14.4	54.2/38.6	67.6	7.54	5.96
10	56	Female	23.4	13.1	16.3	53.4/36.8	42.1	5.16	6.56
11	56	Female	31.3	30.0	19.5	62.1/30.9	60.0	5.74	9.09
12	59	Female	28.3	13.9	17.2	54.5/38.4	59.0	6.02	5.71
13	59	Male	26.9	14.0	18.2	55.3/32.4	73.8	5.15	5.03
14	56	Female	28.7	28.7	21.5	61.4/29.3	44.6	4.44	6.21
15	57	Male	27.8	39.5	18.3	62.5/29.1	75.1	3.96	6.30
16	59	Male	27.7	20.6	19.9	45.2/47.7	93.2	6.30	7.59
17	55	Female	23.3	14.8	17.2	56.9/35.6	47.8	4.08	5.27
18	58	Female	27.0	4.63	15.9	65.1/26.8	54.0	5.51	6.99
19	56	Male	23.5	16.8	13.8	61.0/31.1	67.3	6.53	6.09
20	56	Male	27.4	16.1	17.3	45.6/46.8	66.1	7.66	5.27
21	59	Male	29.1	20.4	15.8	63.3/29.5	59.1	4.63	9.49
22	57	Female	25.1	9.47	11.5	79.1/16.2	51.8	5.33	9.58
23	59	Female	21.0	13.2	18.6	41.8/48.9	58.8	6.00	4.49
24	58	Female	29.0	13.8	16.4	67.1/26.7	47.7	4.54	7.44

25	56	Female	25.2	20.6	14.2	58.8/33.6	44.4	3.93	6.54
26	55	Male	24.8	35.6	20.8	54.0/38.9	66.0	6.59	7.49
27	54	Male	23.5	12.2	23.3	36.9/53.7	65.0	3.97	4.85
28	52	Female	26.5	15.5	16.0	63.9/31.4	62.2	6.07	7.25
29	57	Female	28.6	30.3	20.8	52.2/38.8	63.0	3.33	7.28
30	56	Male	24.2	12.8	15.7	49.4/42.9	90.7	8.96	8.11

Table S9. Comparison of the performance of this method with established urinary biomarkers for BCa.[a]

Biomarker/test system	Overall sensitivity (%)	Overall specificity (TNR) (%)	Sensitivity for high-grade BCa (%)
UroVysion (FISH)*	30-86	63-95	66-70
Microsatellite analysis	58-92	73-100	90-92
Immunocyt/uCyt +*	52-100	63-79	62-92
NMP22*	47-100	55-98	75-92
BTA stat*	29-83	56-86	62-91
BTA TRAK*	53-91	28-83	74-77
Cytokeratins	12-88	73-95	33-100
2D Cu signatures (this work)	87.8	91.5	94.7

[a] The information is taken from the *EAU Guidelines on Non-muscle-invasive Bladder Cancer (TaT1 and CIS)*, European Association of Urology 2018.[13] The asterisk means FDA approved.

Table S10. Parameters for Cu isotope ratio measurement by MC-ICP-MS.

Sample preparation	
Digestion	1 mL or 0.5 mL with 7.5 mL of 14 M HNO_3 and 1 mL of 30% H_2O_2 in Teflon digestion tube
Column purification	Anion exchange resin (AG-MP-1M) 100-200 mesh
Solution for elution of Cu	12 mL of 5 M HCl + 0.001% H_2O_2 solution
Solution for elution of Fe	7 mL of 0.6 M HCl solution
Solution for elution of Zn	8 mL of 0.7 M HNO_3 solution
Elimination of chloride ion	Dryness at 100 °C and redissolution in 0.7M HNO_3
Instrument settings	
Sample introduction	Peristaltic pump at 8 rpm uptake rate
Sampler cone (nickel)	"experimental" WA cone (Nu Instruments)
Skimmer cone (nickel)	"experimental" WA cone (Nu Instruments)
Lens settings	Optimized for maximum analytical signal intensity
Torch	Glass
Collector	L2—^{63}Cu , H2—^{65}Cu
RF power	1300 W
Data acquisition parameters	
Scan type	Static measurement
Measurement mode	Low-resolution with standard-sample-standard bracketing
Measurement intensity (^{63}Cu)	10 V/ppm
Blank signal	20 to 40 mV
Magnet delay time	2 s
Number of blocks	1 block, 30 cycles

Table S11. Cu concentrations and δ^{65}Cu values in serum and RBC for HCC patients and controls reported in a previous paper. [a]

Group	ID	Serum		RBC	
		[Cu] (μg/g)	δ^{65}Cu (‰)	[Cu] (μg/g)	δ^{65}Cu (‰)
Non-HCC	3	3.14	-0.06	0.93	0.66
Non-HCC	12	2.44	0.38	1.02	1.05
Non-HCC	24	1.32	-0.20	0.97	0.58
Non-HCC	25	3.07	-0.08	1.22	1.19
Non-HCC	26	3.49	0.22	1.21	0.67
Non-HCC	41	1.58	-	0.94	0.79
Non-HCC	42	1.39	0.25	0.97	1.13
Non-HCC	44	1.89	0.33	1.09	0.88
Non-HCC	45	1.66	-0.39	0.93	0.89
Non-HCC	46	1.14	0.17	0.81	0.79
Non-HCC	47	1.24	0.33	0.93	1.24
Non-HCC	48	1.92	0.13	1.00	1.22
Non-HCC	49	2.63	0.07	0.98	0.86
Non-HCC	50	2.81	0.04	1.12	0.64
Non-HCC	51	2.86	0.30	1.10	0.97
Non-HCC	53	1.82	-0.21	1.22	0.62
Non-HCC	60	2.54	-0.09	0.92	0.75
Non-HCC	103	2.32	0.37	0.85	1.03
Non-HCC	111	1.21	0.33	1.11	0.99
Non-HCC	157	1.17	0.07	0.92	0.57
HCC	1	3.40	-0.01	1.04	0.35
HCC	10	3.41	-0.07	1.16	0.65
HCC	11	4.91	-	1.54	-0.07
HCC	16	4.96	0.47	1.61	0.90
HCC	20	3.39	-0.66	0.83	0.03
HCC	62	3.42	0.08	1.10	0.45
HCC	68	3.59	0.19	1.85	0.56
HCC	69	5.16	-	1.03	0.26
HCC	77	5.61	0.26	1.22	0.73
HCC	78	4.08	-0.30	0.99	0.25
HCC	86	3.42	-0.08	0.96	0.92
HCC	88	3.23	-0.19	1.05	0.62
HCC	90	3.68	-0.39	1.06	0.41
HCC	108	4.11	0.07	1.08	0.47
HCC	118	3.84	0.11	1.48	0.10
HCC	140	3.80	0.15	1.00	0.82
HCC	144	4.78	0.16	1.44	0.72
HCC	145	2.91	0.05	1.18	0.63
HCC	154	3.95	0.44	0.96	0.84

HCC	162	4.45	-0.22	1.77	0.21
HCC	174	3.97	-0.25	1.09	0.44
HCC	204	3.10	0.02	1.29	0.65
HCC	259	3.08	-0.19	0.92	0.76

[a] The Cu concentrations and δ^{65}Cu values for HCC patients and controls are from a previously published paper by Balter et al.[14]

4. Supplementary Figures

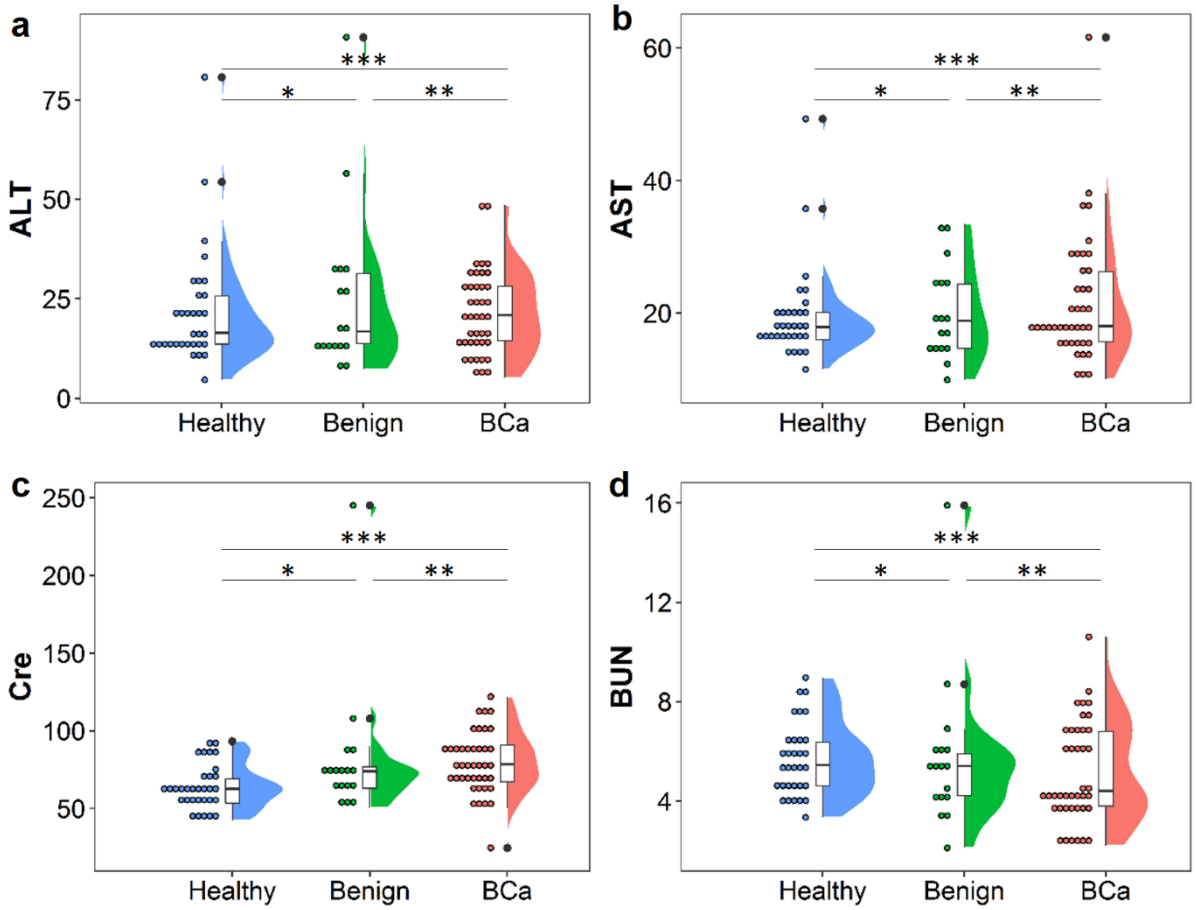


Figure S1. Blood biochemical indexes for the three groups of study participants. Each symbol presents an individual subject. (**a**) Glutamic-pyruvic transaminase (ALT) in blood. $^{*}P_a$ = 0.7950, $^{**}P_a$ = 0.9024, $^{***}P_a$ = 0.5231, Mann Whitney test. (**b**) Glutamic-oxalacetic transaminase (AST) in blood. $^{*}P_b$ = 0.8559, $^{**}P_b$ = 0.6815, $^{***}P_b$ = 0.4082, Mann Whitney test. (**c**) Creatinine (Cre) in blood. $^{*}P_c$ = 0.0436, $^{**}P_c$ = 0.2359, Mann Whitney test; $^{***}P_c$ = 0.0006, unpaired Student's two-tailed *t*-test. (**d**) Urea nitrogen (BUN) in blood. $^{*}P_d$ = 0.4440, $^{**}P_d$ = 0.7126, $^{***}P_d$ = 0.1090, Mann Whitney test. It can be seen that, except for Cre, no significant difference was observed in blood biochemical indexes among different groups.

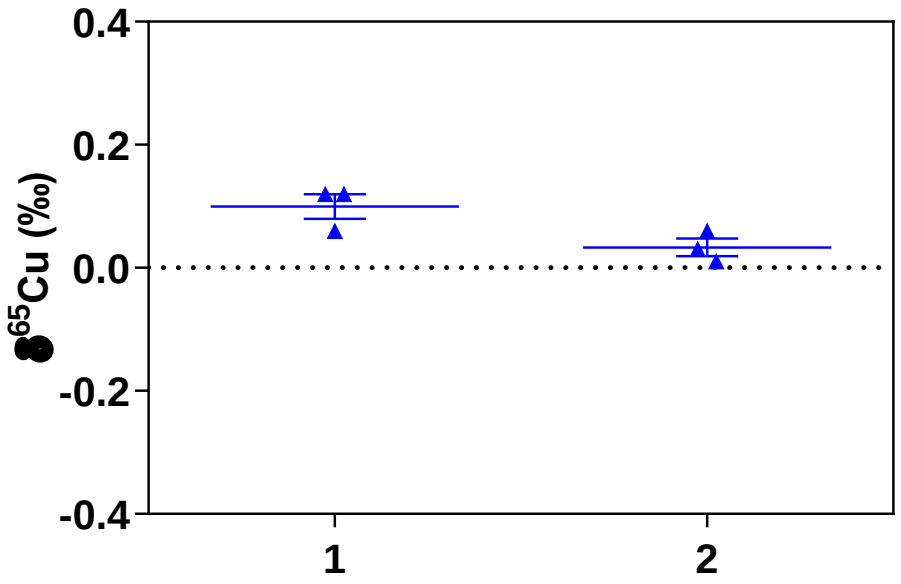


Figure S2. Evaluation of performance of sample purification procedure toward complex biological matrix. The experiments were carried out as follows: first, the biological samples were digested and purified by anion exchange column as described in the Experimental Section. Then, all the eluted fractions were collected and combined again except for the Cu fraction. In this way, we obtained a Cu-excluded sample that had the same composition with the original one but with no Cu. After that, an ERM-AE633 Cu standard solution was added to the Cu-excluded sample to obtain a final Cu concentration of ~1 μg/mL. The mixture was purified and analyzed again as described in the Experimental Section. Herein, **1** and **2** represent two batches of experiments and each batch consists of three parallel samples (n = 3). As shown in this figure, the average δ^{65}Cu values are 0.10 ± 0.03‰ and 0.03 ± 0.03‰ for batch **1** and **2**, respectively. These values are close to or less than the method uncertainty (0.1‰), indicating that the biological matrix in samples has no influence on the Cu isotopic measurement in the present study.

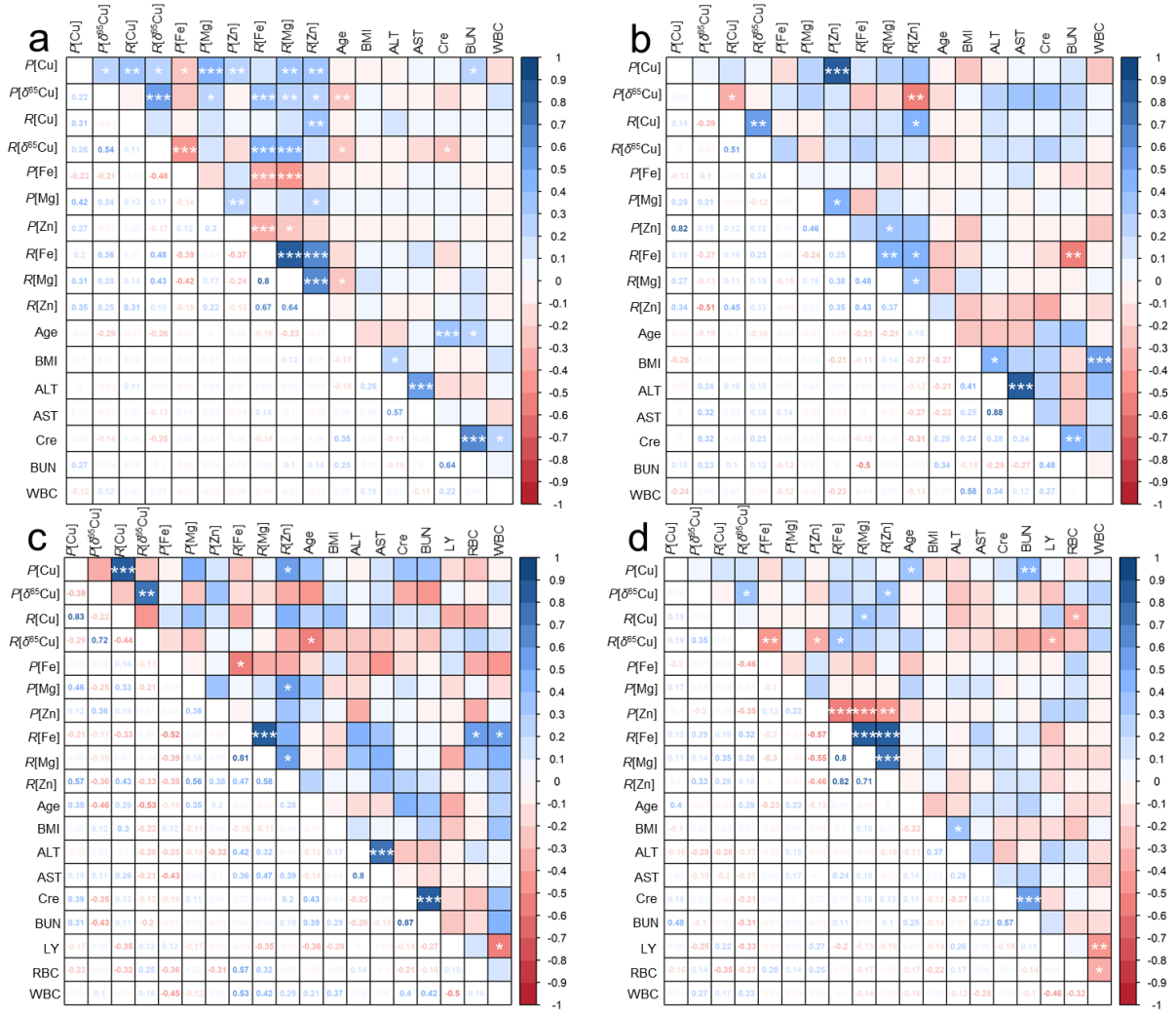
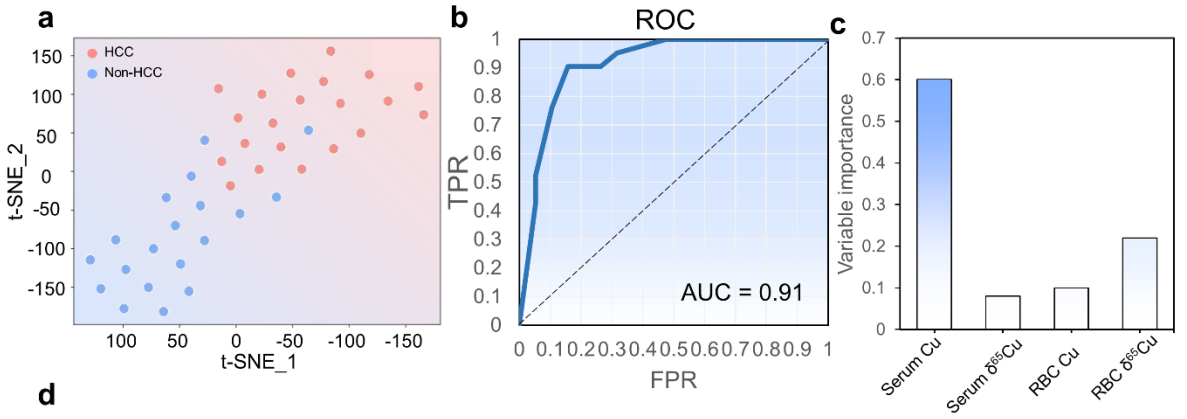


Figure S3. Comprehensive correlation analysis of multiple factors. (**a**) The correlation analysis for all groups together. (**b**) The correlation analysis for age-matched healthy group. (**c**) The correlation analysis for benign patient group. (**d**) The correlation analysis for BCa patient group. The Pearson correlation coefficient (*R*) is indicated by color along with the values given in the left bottom part of each figure. The "*", "**", and "***" represent *P* values < 0.05, < 0.01, and < 0.001, respectively, and data without asterisk means *P* > 0.05. The prefix "*P*" and "*R*" in axes represent plasma and RBC, respectively.



d

Study participant	Number	Classification result		
		HCC	Non-HCC	
HCC	21	19	2	**Recall: 90.5%**
Non-HCC	19	3	16	**TNR: 84.2%**
Total	40	Number of correct: 35		**Accuracy: 87.5%**
		Precision: 86.4%	**NPV: 88.9%**	**FPR: 15.8%**

Figure S4. Application of the machine learning model for classification of HCC and non-HCC subjects. To test the universality of the machine learning model, we also applied the model to the previously published data of hepatocellular carcinoma (HCC) patients (the raw data are also given in Table S11).[14] (**a**) The t-SNE dimensionality reduction results with the four copper-related variables (serum Cu, RBC Cu, serum δ^{65}Cu value, and RBC δ^{65}Cu value). (**b**) The receiver-operating characteristic curve (ROC) of the random forest model. The area under the receiver operator curve (AUC) reached 0.91. (**c**) The variable importance of four Cu-related variables in the RF model. (**d**) Classification results and model performance. HCC means hepatocellular carcinoma and non-HCC means controls. The "number of correct" means the number of subjects with correct classification result. NPV: negative predictive value. TPR: true positive rate. FPR: false positive rate. TNR: true negative rate. It can be seen that the machine learning model established in the present study also worked well for hepatocellular carcinoma (HCC) with accuracy, recall, TNR, and FPR of 87.5, 90.5, 84.2, and 15.8%, respectively.

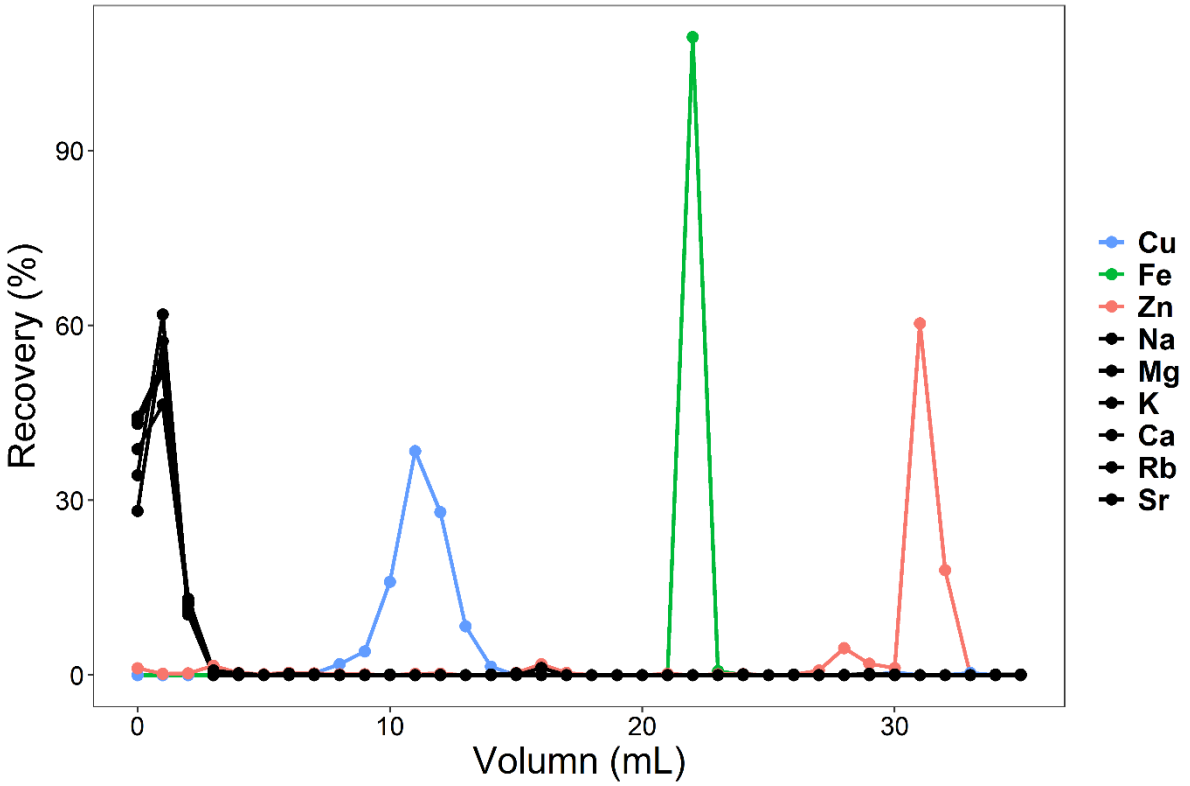


Figure S5. Elution curve of Fe, Cu, and Zn from interfering ions in anion exchange column chromatography. The elements were separated by an AG-MP-1M anion exchange resin column, and 8 mL of 8 M HCl + 0.001% H_2O_2 solution was used to elute the matrix, 12 mL of 5 M HCl + 0.001% H_2O_2 solution to elute Cu fraction, 7 mL of 0.6 M HCl solution to elute Fe fraction, and 8 mL of 0.7 M HNO_3 to elute Zn fraction. The elution curve indicates that Cu ions can be well separated from other interfering ions to ensure a high-precision MC-ICP-MS isotopic analysis.

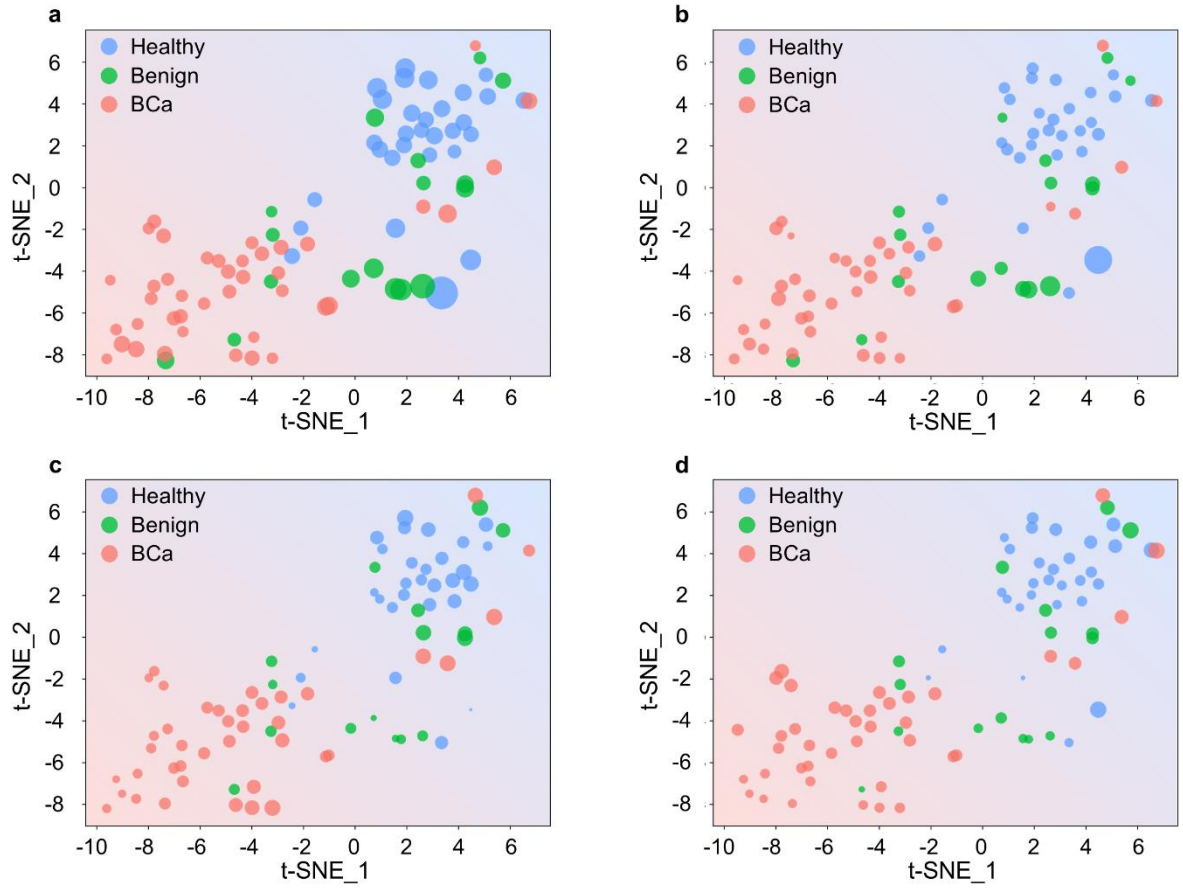


Figure S6. The t-SNE visualization with intensity distribution of plasma copper concentration, RBC copper concentration, plasma δ^{65}Cu, and RBC δ^{65}Cu. For all panels, the size of dots represents the intensity of variables in t-SNE. Because negative values of plasma δ^{65}Cu and RBC δ^{65}Cu cannot be represented by the dot size, the original data was rescaled to the range 0-1 (min-max normalization). (**a**) The intensity distribution of plasma copper concentration. (**b**) The intensity distribution of RBC copper concentration. (**c**) The intensity distribution of plasma δ^{65}Cu. (**d**) The intensity distribution of RBC δ^{65}Cu.

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