Natural variations of copper and sulfur stable isotopes in blood of hepatocellular carcinoma patients

Vincent Balter[a,1], Andre Nogueira da Costa[b], Victor Paky Bondanese[a], Klervia Jaouen[c], Aline Lamboux[a], Suleeporn Sangrajrang[d], Nicolas Vincent[a], François Fourel[a], Philippe Télouk[a], Michelle Gigou[e], Christophe Lécuyer[a,f], Petcharin Srivatanakul[d], Christian Bréchot[e,g], Francis Albarède[a], and Pierre Hainaut[h,i]

[a]UMR 5276, Laboratoire de Géologie de Lyon, École Normale Supérieure de Lyon, CNRS, Université de Lyon 1, BP 7000 Lyon, France; [b]Mechanistic Toxicology & Molecular Pathology Department of Non-Clinical Development, UCB BioPharma, SPRL Chemin du Foriest 1, B-1420 Braine L'Alleud, Belgium; [c]Department of Human Evolution, Max Planck Institute for Evolutionary Anthropology, 04103 Leipzig, Germany; [d]National Cancer Institute, Bangkok 10400, Thailand; [e]Unité 785, Pathogénèse et Traitement de l'Hépatite Fulminante et du Cancer du Foie, INSERM, Université Paris-Sud, 94800 Villejuif, France; [f]Institut Universitaire de France, 75005 Paris, France; [g]Institut Pasteur, 75015 Paris, France; [h]Unité 823, Ontogenèse et Oncogenèse Moléculaire, Institut Albert Bonniot, INSERM, Université Joseph Fourier, 38706 Grenoble, France; and [i]Strathclyde Institute of Global Public Health, International Prevention Research Institute, 69006 Lyon, France

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The widespread hypoxic conditions of the tumor microenvironment can impair the metabolism of bioessential elements such as copper and sulfur, notably by changing their redox state and, as a consequence, their ability to bind specific molecules. Because competing redox state is known to drive isotopic fractionation, we have used here the stable isotope compositions of copper ($^{65}Cu/^{63}Cu$) and sulfur ($^{34}S/^{32}S$) in the blood of patients with hepatocellular carcinoma (HCC) as a tool to explore the cancer-driven copper and sulfur imbalances. We report that copper is ^{63}Cu-enriched by ∼0.4‰ and sulfur is ^{32}S-enriched by ∼1.5‰ in the blood of patients compared with that of control subjects. As expected, HCC patients have more copper in red blood cells and serum compared with control subjects. However, the isotopic signature of this blood extra copper burden is not in favor of a dietary origin but rather suggests a reallocation in the body of copper bound to cysteine-rich proteins such as metallothioneins. The magnitude of the sulfur isotope effect is similar in red blood cells and serum of HCC patients, implying that sulfur fractionation is systemic. The ^{32}S-enrichment of sulfur in the blood of HCC patients is compatible with the notion that sulfur partly originates from tumor-derived sulfides. The measurement of natural variations of stable isotope compositions, using techniques developed in the field of Earth sciences, can provide new means to detect and quantify cancer metabolic changes and provide insights into underlying mechanisms.

stable isotopes | copper | sulfur | liver | cancer

Copper is an essential trace element (1), which has a pivotal role in the balance of oxidative stress: High levels are harmful because reduced copper promotes the generation of reactive oxygen species (2), and in the meantime, copper is involved in several enzymes (ceruloplasmin, hephaestin, Cu/Zn superoxide dismutase) that prevent the generation of reactive oxygen species (e.g., ref. 3). Three major types of ligand are associated with copper binding. Copper binds to nitrogen in histidine, and to sulfur in cysteine and methionine. The ratio of naturally occurring stable isotopes of copper, $^{65}Cu/^{63}Cu$, varies according to the nature of the donor ligand. Light isotopes favor soft bonds relative to hard bonds. Bonds with Cu^{+} are softer than those with Cu^{2+}, while bonds with sulfur are softer than those with nitrogen. These fairly general principles have been confirmed by ab initio calculations and density functional theory, and Cu isotope fractionations among various organic and inorganic compounds are now available (4). Heavy ^{65}Cu is preferentially oxidized compared with ^{63}Cu and prefers nitrogen donor ligands, such as histidine, or oxygen donor ligands, such as glutamate or aspartate (4). Sulfur possesses four stable isotopes, among which the $^{34}S/^{32}S$ ratio is the easiest to measure because ^{32}S and ^{34}S represent >99% of total sulfur (*Method*). The magnitude of variation of the $^{34}S/^{32}S$ ratio can reach one tenth permil during the incomplete reduction of sulfate and sulfide (5). Regardless of the element, differences in coordination and redox states are generally associated with isotopic effects known as isotopic fractionation. In animal models, the normalized $^{65}Cu/^{63}Cu$ ratio, expressed in delta units (*Method*), varies between organs from −1‰ in liver to +1‰ in kidney (6) (for comparison, this ratio ranges from −3‰ to +2.5‰ in terrestrial materials). In humans, the $^{65}Cu/^{63}Cu$ ratio in blood and bone differs between men and women (7, 8), partly because a sizeable proportion of the women's blood copper comes from the liver to balance menstrual losses (9, 10). The body $^{65}Cu/^{63}Cu$ ratio varies according to that of diet (11) and also seems to be age dependent (12). In life sciences, sulfur isotopic ratios are mainly used to trace animals' diet in wildlife (13) and in stockbreeding (14) contexts. To this date, one study reports on bodily sulfur isotopic systematics, which shows no significant variations relative to diet in a murine model (15).

The natural variations of the isotopic ratios of an element offers a new means to study the imbalances linked to pathological conditions (16–18). Here, we used the natural variations of the $^{65}Cu/^{63}Cu$ and $^{34}S/^{32}S$ ratios, expressed as $\delta^{65}Cu$ and $\delta^{34}S$ values (*Method*), to track the copper and sulfur imbalance in hepatocellular carcinoma (HCC). Liver cancer was chosen because the liver

Significance

In cancer, the metabolism of copper and sulfur are dysregulated, leading to deleterious side effects. These issues are commonly addressed by studying the variations of concentrations of the elements, but here we have used, for the first time to our knowledge, copper and sulfur stable isotope compositions variations, using methods widespread in Earth sciences. We show that in hepatocellular carcinomas patients, blood copper and sulfur are enriched in light isotopes compared with control subjects. These isotopic signatures are not compatible with a dietary origin, but rather reflect the massive reallocation in the body of copper immobilized within cysteine-rich proteins such as metallothioneins. We also propose that sulfur isotope compositions could serve to track sulfur originating from tumor-derived sulfides.

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[1]To whom correspondence should be addressed. Email: vincent.balter@ens-lyon.fr.

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is the main copper reservoir in the body and is known to be a pivotal organ for the metabolism of sulfur amino acids, and because the metabolism of copper and sulfur has been shown to be disrupted in cancer (e.g., refs. 19 and 20). To this end, we measured the δ^{65}Cu and δ^{34}S values in the serum and red blood cells of a series of Thai male HCC patients (n = 23, *Material*) and control subjects (n = 20, *Material*). We also analyzed biopsies of liver tumor and surrounding unaffected tissues from seven Caucasian patients (*Material*).

Results

Correlation of Copper and Sulfur Isotopes with Biological Parameters. Several parameters were measured in the framework of the International Liver Cancer Study (ILCS) initiative in Thailand, including alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT), which are classical tests for liver function and latent-transforming growth factor β binding-protein 2 (LTBP2), a new candidate biomarker of HCC (21). In general, tests for liver function fail to discriminate HCC patients from controls [analysis of variance (ANOVA); ALP, $P = 0.456$, AST, $P = 0.172$, ALT, $P^* = 0.010$], while LTBP2 values do (ANOVA; $P^{***} < 10^{-4}$). In serum, copper and sulfur isotope compositions are not correlated to any of the tests for liver function (copper; ALP, $R = 0.350$, $P^* = 0.027$, AST, $R = 0.231$; $P = 0.151$, ALT, $R = 0.144$, $P = 0.379$; sulfur; ALP, $R = 0.219$, $P = 0.271$, AST, $R = 0.245$; $P = 0.218$, ALT, $R = 0.186$, $P = 0.353$). Serum copper isotope compositions are not correlated to LTBP2 values ($R = -0.093$, $P = 0.569$). However, serum sulfur isotope compositions are correlated to LTBP2 values ($R = -0.509$, $P^{**} = 0.006$), but this mostly results from a group effect between HCC patients and controls.

In serum, copper and sulfur isotope compositions are not correlated to any anthropometrical parameter i.e., age, height, and body mass index (Tables S1 and S2). This holds for red blood cells (RBC) too (Tables S2 and S3), except that an inverse correlation ($R = -0.490$, $P^* = 0.028$) is observed between the age of control subjects and the copper isotope composition of RBC (Fig. S1), as previously observed in a remote Yakut population (12).

Copper and Sulfur Isotopes in Blood Fractions of HCC Patients and Control Subjects. Copper concentrations and isotope compositions were measured along with ceruloplasmin (Cp) concentrations and Cu/Zn superoxide dismutase (SOD1) activity in the blood fractions of HCC patients and control subjects. Results are given in Table S1 for serum and in Table S3 for RBC. As expected from a wealth of data (e.g., ref. 22), copper concentrations were higher in the serum and in RBC of HCC patients compared with controls (serum, $P < 10^{-4}$, Fig. 1*A*; RBC $P = 0.006$, Fig. 1*B*). In addition, we observed a small increase of Cp ($P = 0.012$, Fig. 1*C*) and a decrease in SOD1 activity ($P = 0.015$, Fig. 1*D*), two effects previously reported in a variety of cancers (23, 24).

The copper isotope composition of blood fractions showed a tendency toward enrichment in ^{63}Cu in HCC patients compared with control subjects (Fig. 1 *E* and *F*). This effect is significant in RBC only (RBC, $P < 10^{-4}$, serum, $P = 0.134$). However, there is a generally good correlation between serum and RBC copper isotope composition in HCC patients ($P < 10^{-4}$, $R = 0.579$), suggesting that the copper isotopic imbalance is reflected in both fractions (Fig. 2*A*). We hypothesize that the lack of significant isotopic difference in the serum of HCC patients is due to the short half-life of serum copper, which is determined by that of Cp ($t_{1/2}^{Cp} \approx 5$ d, ref. 25), whereas in RBC, the half-life of copper is about one order of magnitude higher ($t_{1/2}^{RBC} \approx 60$ d).

Measurements of sulfur isotope compositions indicate that sulfur is significantly ^{34}S-depleted in both serum and RBC in HCC patients compared with control subjects (serum, $P^{***} < 10^{-4}$, Fig. 1*G*; RBC, $P^{**} = 0.004$, Fig. 1*H*), and that the sulfur isotope compositions in serum are correlated to those in RBC ($P^{***} < 10^{-4}$, $R = 0.929$, Fig. 2*B*). HCC patients were characterized by lower δ^{34}S and δ^{65}Cu values compared with controls in RBC [multivariate analysis of variance (MANOVA); Wilks' $\lambda = 0.47$, $F(2,40) = 22.52$, $P^{***} < 10^{-4}$, Fig. 2*C*] and in serum [MANOVA; Wilks' $\lambda = 0.59$, $F(2,24) = 8.23$, $P^{**} = 0.002$, Fig. 2*D*].

Discussion

Otto Warburg and Adolf Krebs found in 1928 (26) that serum copper levels increased in various chronic diseases, including several types of cancers, resulting into a systemic and oncogenic copper accumulation (19, 27, 28). The present isotopic copper results suggest that this extra copper burden is unlikely of exogenous (dietary) origin. Enhanced exogenous copper uptake would be expected to attenuate the isotopic fractionation between dietary sources and blood, leading the δ^{65}Cu value of blood fractions to tend toward that of the diet. Such a mechanism has been proposed to explain the iron isotope composition of blood in patients with hemochromatosis (16). Assuming that the composition of a typical human diet may give typical δ^{65}Cu values of about +0.4‰ (6), enhanced dietary copper uptake would result in a ^{65}Cu enrichment in the blood of HCC patients, which is not compatible with our observations. If not exogenous, the extra copper burden may be caused by (*i*) reduced copper losses through bile and (*ii*) release of copper from endogenous stores.

Bile is the major normal pathway of copper excretion. The impairment of hepatic cells in HCC can lead to a reduced bile production, and consequently to an increase of systemic copper. Testing this hypothesis requires comparison of bile production and chemical composition for HCC patients and healthy controls.

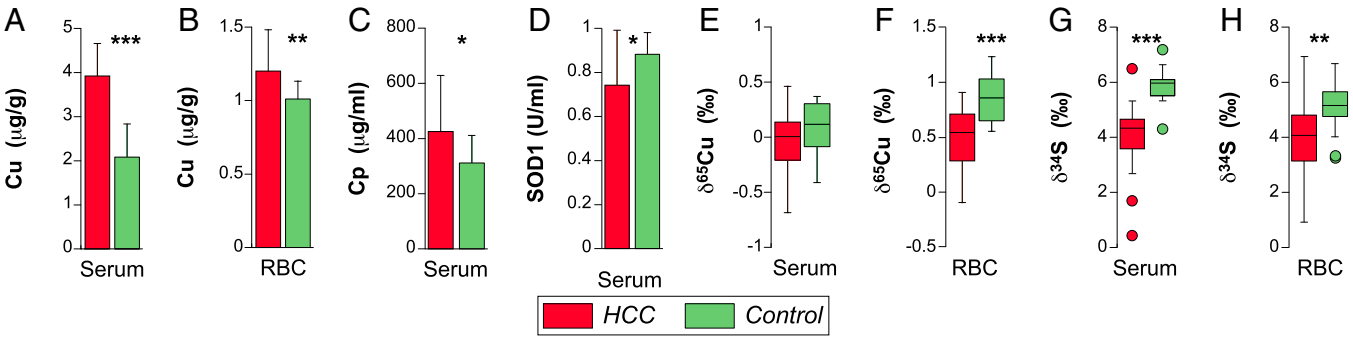

Fig. 1. Chemical and biochemical compositions of serum and RBC of HCC patients and controls. (*A*) Copper concentrations in serum. (*B*) Copper concentrations in RBC. (*C*) Cp concentrations in serum. (*D*) SOD1 concentrations in serum. (*E*) Copper isotope composition of serum. (*F*) Copper isotope composition of RBC. (*G*) Sulfur isotope composition of serum. (*H*) Sulfur isotope composition of RBC. For all panels, $^*P = 0.01$–0.05, $^{**}P = 0.001$–0.01, and $^{***}P < 0.001$.

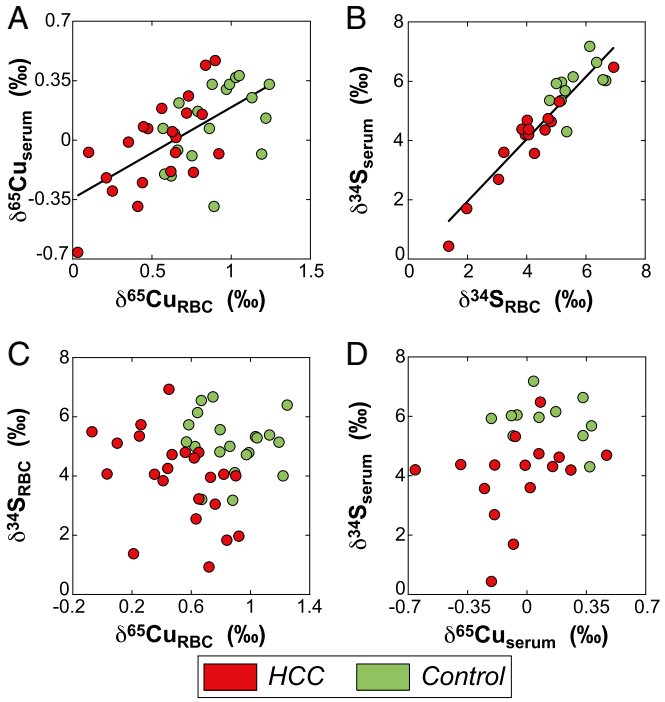

Fig. 2. Copper and sulfur isotope systematics in blood. (*A*) Copper isotope compositions in serum as a function of that of RBC. The black line stands for the least square correlation for all samples ($y = 0.504(\pm0.115)x - 0.316(\pm0.088)$, $R^2 = 0.335$, $P < 10^{-4}$). (*B*) Sulfur isotope compositions in serum as a function of that of RBC. The black line stands for the least square correlation for all samples ($y = 1.051(\pm0.084)x - 0.161(\pm0.406)$, $R^2 = 0.863$, $P*** < 10^{-4}$). (*C*) Sulfur isotope compositions as a function of that of copper in RBC. (*D*) Sulfur isotope compositions as a function of that of copper in serum.

This approach can be easily achieved in further experiments by using murine models. Such experiments should focus on HCC models but also on other cancer models where there is, in principle, no relationship with liver function impairment.

The release of copper from endogenous stores could be directly demonstrated by analyzing the copper concentration and isotope composition for various organs in cancer mice and comparing the results with control mice. Here, we use an indirect approach, which consists to predict the isotope composition of copper released from normal cells. In cells, copper is laden mainly to three metalloproteins, i.e., SOD1 and metallothionein (MT) in the cytoplasm and cytochrom c oxidase (CcO) in mitochondria (29–31). As heavier isotopes bind preferentially with ligands with stronger electronegativity (oxygen > nitrogen > sulfur, ref. 4), and taking the binding characteristics of the main three copper-laden proteins into account, one can predict that $\delta^{65}Cu_{SOD1} > \delta^{65}Cu_{CcO} >> \delta^{65}Cu_{MT}$. The low $\delta^{65}Cu$ value of the extra copper burden may thus be explained by the release of intracellular copper from cysteine clusters, with MT being the most likely source. Therefore, the links between copper imbalance in cancer and MT should be the focus of further attention.

A third mechanism, which is not exclusive from the previous ones, can explain the low $\delta^{65}Cu$ value of the blood extra copper burden. It involves mass conservation of copper isotopes between ^{65}Cu-depleted blood and ^{65}Cu-enriched tumors. In a series of liver biopsies of HCC patients sampled along with adjacent normal liver tissues, we found that tumors are systematically ^{65}Cu-enriched relative to normal tissues (Table S4 and Fig. S2). The accumulation of ^{65}Cu in tumors can therefore enhance the existing ^{65}Cu depletion observed in blood originally triggered by the release of intracellular copper from MT. This hypothesis implies that the blood $\delta^{65}Cu$ value would decrease as a function of the severity of the cancer, which would be of interest for the estimation of tumor burden.

The observation of lower $\delta^{34}S$ values in the serum and the RBC of HCC patients is unexpected because the two blood fractions have different proteomes, thereby suggesting that a common mechanism might be at work. The hypothesis of enhanced formation of sulfides, in which the $\delta^{34}S$ value is 20‰ less than in coexisting sulfate (32), may be involved. Tumor-derived hydrogen sulfide (33) and allosteric formation disulfide bonds in cancer-related proteins (20) are possible candidates, but it is still unclear how prevalent is the formation of these compounds. Considering that the sulfur content of proteins is stoechiometric and therefore constant, the blood $\delta^{34}S$ value of HCC patients ($\delta^{34}S_{HCC} = 4.0‰$) can be considered as a mixture of the normal sulfur isotope composition ($\delta^{34}S_N = 5.4‰$) and of total sulfides ($\delta^{34}S_{TS}$), with proportion x and $1 - x$, respectively:

$$\delta^{34}S_{HCC} = x * \delta^{34}S_N + (1-x) * \delta^{34}S_{TS}.$$

Such a simple mass balance indicates that about 6% of total sulfur in blood of HCC patients is coming from tumor produced sulfide with a $\delta^{34}S_{TS}$ of −20‰. This number decreases to 4% with a $\delta^{34}S_{TS}$ of −30‰. These numbers are very high and require further investigations, notably the direct determination of the $\delta^{34}S$ value of tumor produced H_2S. However, they already suggest that blood sulfur isotopic variations of HCC patients is a sizeable process that may provide new biomarkers for cancer detection and monitoring. However, the sulfur isotope compositions need to be measured on other types of cancer to know whether the origin of the low blood $\delta^{34}S$ values in HCC patients are linked to disorders of hepatic origin or sulfides production.

Material

Study Participants. Blood samples from patients and controls from Thailand were collected in the framework of a hospital-based case–control study conducted at the Cancer Control Unit of the National Cancer Institute of Thailand (Bangkok) from April 2008 to December 2009. All cases of primary liver cancer were recruited, and matched controls were obtained from outpatient clinics. Differential diagnosis of HCC versus cholangiocarcinoma was established by a combination of clinical examination, imaging using ultrasonography, computerized tomography or Magnetic Resonance Imaging, biochemistry (alpha-fetoprotein and liver function enzymes testing) and histological confirmation on a small subset of patients from whom needle biopsies were available. Individuals from the reference group presented no clinical evidence of liver disease. All study participants provided informed consent, and both Thailand and international institutional review boards approved the study protocol (21, 34).

Liver Biopsies Collection. Liver tumor and nontumor samples were collected in spring 2010 at the Paul Brousse Hospital, Centre Hépato-Biliaire, Villejuif, France, on seven patients further diagnosed for hepatocarcinoma. Copper concentrations and isotope compositions of tumor and nontumor samples are given in Table S1.

Method

Sample Preparation for Copper Isotope Analysis. RBC and serum samples were digested by a mixture of concentrated subboiled distilled HNO_3 and 30% H_2O_2. Further information concerning copper separation from the matrix is given in *SI Text*.

Copper Isotope Analysis. Copper isotope compositions were measured at the Laboratoire de Géologie de Lyon (LGLTPE). The ion exchange chromatography and mass spectrometry techniques have been described extensively elsewhere (35, 36), and are described in *SI Text*. The delta value is given by $\delta^{65}Cu = [(^{65}Cu/^{63}Cu)_{sample}/(^{65}Cu/^{63}Cu)_{standard} - 1] \times 10^3$. The NIST-SRM 976 solution was used as the copper isotopic standard. Copper isotope compositions are given in Tables S1 and S3.

Copper Concentration Analysis. Copper concentrations were measured by quadrupole inductively coupled plasma mass spectrometry (Q-ICPMS) at LGLTPE using a 7500 CX quadrupole mass spectrometer (Agilent Technologies). The

following operating parameters of Q-ICPMS were optimized: rf power (1,550 W), the plasma gas flow rate (15 $L \cdot min^{-1}$), the auxiliary gas flow rate (2 $L \cdot min^{-1}$), the carrier gas flow rate (0.9 $L \cdot min^{-1}$), and the makeup gas flow rate (0.15 $L \cdot min^{-1}$). The performance of the Q-ICPMS instrument was optimized and checked daily by testing a standard mixture containing 1 $ng \cdot mL^{-1}$ Li, Y, Co, Ce, and Tl in 3% HNO_3. Indium at 1 $ng \cdot mL^{-1}$ was used as an internal standard to correct for any long-term instrumental drift. Copper concentrations are given in Tables S1 and S3.

Sulfur Isotope Analysis. Sulfur isotope analysis was undertaken by Elemental Analysis Isotope Ratio Mass Spectrometry at IsoAnalytical and duplicated for some samples at LGLTPE. Further details on the analytical procedures are given in *SI Text*. In both laboratories, international standards NBS-127 (barium sulfate, $\delta^{34}S_{CDT} = +20.3‰$) and IAEA-SO5 (barium sulfate, $\delta^{34}S_{V\text{-}CDT} = +0.50‰$) were measured as quality control checks during batch analysis of the samples (Table S5 and Fig. S3). Sulfur isotope compositions are given in Tables S1 and S3.

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Supporting Information

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SI Text

Sample Preparation for Copper Isotope Analysis. Liver samples were dissolved by concentrated subboiled distilled HNO_3 in Teflon bombs using a microwave device. RBC and serum samples were digested by a mixture of concentrated subboiled distilled HNO_3 and 30% H_2O_2. Dissolved samples were then evaporated, redissolved in 1 mL of a mixture of subboiled distilled 7 M HCl and 0.001% H_2O_2, and evaporated again to remove any traces of remaining nitric acid. Finally, samples were taken up in 1 mL of a mixture of subboiled distilled 7 M HCl and 0.001% H_2O_2, and a 50-μL aliquot taken for elemental concentration measurements. The remaining solution was processed for metal separation according to the technique of Maréchal and Albarède (1). Briefly, before each elution, 1.6 mL of macroporous resin AGMP-1 (100–200 mesh) was cleaned three times with subboiled distilled 0.5 M HNO_3 alternating with H_2O, and conditioned by 6 mL of a mixture of subboiled distilled 7 M HCl and 0.001% H_2O_2. Matrix and Cu were removed using 30 mL of a mixture of subboiled distilled 7 M HCl and 0.001% H_2O_2. Iron and Zn were finally eluted with 10 mL of subboiled distilled 0.5 M HNO_3. The copper fraction was further purified using the same protocol.

Copper Isotope Analysis. Copper stable isotope compositions were determined by multicollector inductively coupled plasma mass spectrometry at Laboratoire de Géologie de Lyon (LGLTPE), using a Nu plasma 500 high-resolution double-focusing mass spectrometer (Nu Instrument) equipped with 12 Faraday detectors and with a mass resolution of 1,000. The operating conditions were: rf power (1,300 W), plasma gas flow rate (13 $L{\cdot}min^{-1}$), and auxiliary gas flow rate (0.9 $L{\cdot}min^{-1}$). The samples were introduced by free aspiration in 0.05 M subboiled distilled HNO_3. Instrumental mass fractionation on copper was corrected using Zn doping (Zn JMC3-0749L; Johnson Matthey) and standard-sample bracketing technique. Sample measurement solutions were diluted to match the concentration of the standard mixture (Zn 0.25 ppm to Cu 0.25 ppm). Samples were randomized during analysis, and duplicates were measured to check for systematic errors. The delta value is given by $\delta^{65}Cu = [(^{65}Cu/^{63}Cu)_{sample}/(^{65}Cu/^{63}Cu)_{standard} - 1] \times 10^3$. The NIST-SRM 976 solution was used as the copper isotope standard. The reproducibility of copper isotopic ratios was <0.05‰.

Sulfur Isotope Analysis. Sulfur isotope analysis was undertaken by Elemental Analysis Isotope Ratio Mass Spectrometry (EA-IRMS) at IsoAnalytical Ltd. and duplicated for some samples at LGLTPE.

At Isoanalytical, about 4 ± 0.1 mg amounts of blood samples were weighed into tin capsules (8 × 5 mm) and added to 10 mg of vanadium pentoxide catalyst before sealing the capsules into balls. The capsules were loaded into the 66-position autosampler on a Sercon CNS-EA elemental analyzer. The elemental analyzer was coupled to a Europa Scientific 20–20 istope ratio mass spectrometer on which the sulfur isotope analyses were performed. From the autosampler, samples dropped, in sequence, into a furnace held at 1,080 °C and combusted in the presence of oxygen. Tin capsules flash combust, raising the temperature in the region of the sample to ∼1,700 °C. The combusted gases are then swept in a helium stream over combustion catalysts (tungstic oxide/zirconium oxide) and through a reduction stage of high-purity copper wires to produce SO_2, N_2, CO_2, and water. Water is removed using a Nafion membrane. Sulfur dioxide is resolved from N_2 and CO_2 on a packed GC column at a temperature of 32 °C. The resultant SO_2 peak enters the ion source of the IRMS whereupon it is ionized and accelerated. Gas species of different mass are separated in a magnetic field and then simultaneously measured on a Faraday cup universal collector array. Analysis was based on monitoring of m/z 48, 49, and 50 of SO^+ produced from SO_2 in the ion source.

At LGLTPE, the EA system used is a VarioPYROcube (Elementar GmbH). As in most conventional EA-IRMS systems, the VarioPYROcube system used helium as a carrier gas and consisted of a combustion tube followed by a reduction tube. However, the chemical water trap is a SICAPENT dryer. Nitrogen gas is not trapped and goes straight through the EA system. CO_2 and SO_2 are trapped at room temperature and released at 110 °C and 220 °C, respectively, in separate traps. The EA system is connected online via an open split device to an IsoPrime IRMS system with a diluting system to allow high C/S ratio samples (2). The delta value is given by $\delta^{34}S = [(^{34}S/^{32}S)_{sample}/(^{34}S/^{32}S)_{standard} - 1] \times 10^3$. The "Canyon Diablo Troilite" was used as the sulfur isotope standard. The reproducibility of sulfur isotopic ratios was <0.4‰.

1. Maréchal CN, Albarède F (2002) Ion-exchange fractionation of copper and zinc isotopes. *Geochim Cosmochim Acta* 66:1499–1509.

2. Fourel F, Martineau F, Seris M, Lécuyer C (2015) Measurement of $^{34}S/^{32}S$ ratios of NBS 120c and BCR 32 phosphorites using purge and trap EA-IRMS technology. *Geostand Geoanal Res*, 10.1111/j.1751-908X.2014.00297.x.

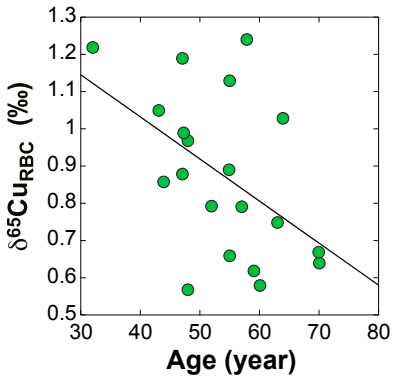

Fig. S1. Correlation of RBC δ^{65}Cu values with age for control individuals of the ILCS study. The black line stands for the least square correlation [$y = -0.011(\pm 0.005)x + 1.485(\pm 0.259)$, $R^2 = 0.240$, $P^* = 0.028$].

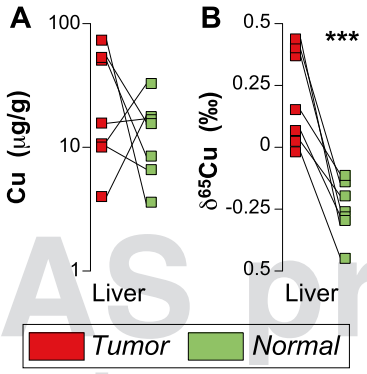

Fig. S2. Copper elemental and isotopic compositions of liver cancer tumor (HCC) and adjacent normal tissues (Normal). (*A*) Copper concentrations. (*B*) Copper isotope compositions. For all panels, $*P = 0.01$–0.05, $**P = 0.001$–0.01, and $***P < 0.001$.

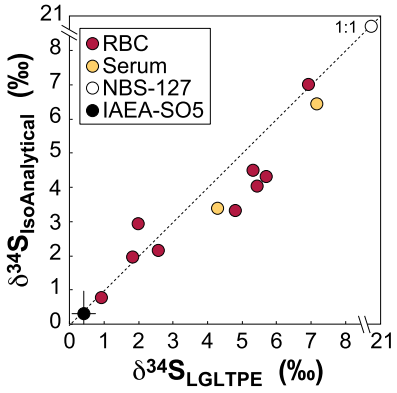

Fig. S3. Correlation of δ^{34}S values between ISoAnalytical Ltd and LGLTPE. The dashed line stands for the 1/1 line.

Table S1. Sample identification (ID), average δ^{65}Cu value, associated SD and number of replicates (n), average Cu concentration, average δ^{34}S value, Cu/Zn superoxide dismutase (SOD1) activity, ceruloplasmin (Cp) concentration, alkaline phosphatase (ALP) activity, aspartate aminotransferase (AST) activity, alanine aminotransferase (ALT) activity, and latent-transforming growth factor β binding-protein 2 (LTBP2) concentration in serum samples from the ILCS study

	Serum										
ID	δ^{65}Cu, ‰	sd	n	[Cu], ng/g	δ^{34}S, ‰	SOD1, U/mL	Cp, mg/mL	ALT, U/L	ALP, U/L	AST, U/L	LTBP2, ng/mL
Control											
3	−0.06	0.02	3	3140	6.05	0.770	570	132	88	47	13
12	0.38			2445	5.68	0.906	335	28	65	21	2
24	−0.20	0.02	2	1322		1.112	292	100	307	171	19
25	−0.08	0.10	2	3071	5.33	0.892	526	51	90	69	15
26	0.22			3494		0.817	309	49	122	116	11
41				1580		1.018	282	54	59	55	8
42	0.25	0.04	2	1387		0.929	290	42	148	61	14
44	0.33			1892		0.864	452	90	76	57	16
45	−0.39	0.03	2	1662		0.840	315	67	80	48	9
46	0.17	0.06	2	1141	6.15	0.756	336	29	89	27	3
47	0.33	0.02	2	1243	6.64	0.967	409	42	198	59	0
48	0.13			1920		0.915	256	39	140	39	18
49	0.07	0.04	2	2626		0.957	207	63	122	60	14
50	0.04	0.04	2	2811	7.18	0.911	237	98	197	140	7
51	0.30	0.05	2	2865		0.831	222	91	217	92	10
53	−0.21	0.06	2	1823	5.93	0.911	439	178	77	99	18
60	−0.09	0.06	2	2537	6.03	0.906	213	54	124	84	8
103	0.37	0.02	2	2319	4.30	0.653	264	221	365	306	10
111	0.33	0.06	2	1210	5.35	0.850	250	60	159	82	18
157	0.07	0.03	2	1173	5.97	0.808	235	88	120	141	12
HCC											
1	−0.01	0.10	3	3398	4.36	1.238	123	16	260	33	12
10	−0.07			3411		0.780	572	34	68	44	28
11				4911		0.803	1023	14	450	29	41
16	0.47	0.06	2	4961	4.69	0.798	224	55	584	184	41
20	−0.66	0.04	2	3390	4.20	1.285	91	30	87	27	23
62	0.08	0.00	2	3415	6.48	0.513	491	159	541	382	40
68	0.19	0.02	3	3588	4.63	0.934	417	37	186	83	36
69				5158		0.700	305	18	72	18	60
77	0.26			5607	4.19	0.677	357	30	101	16	34
78	−0.30	0.06	2	4079		0.339	496	14	42	27	27
86	−0.08	0.05	2	3418	1.70	0.723	568	168	101	146	44
88	−0.19	0.04	3	3231	4.36	0.723	478	30	59	24	60
90	−0.39	0.04	2	3677	4.38	0.639	574	21	45	24	37
108	0.07	0.01	2	4113	4.74	0.705	257	21	61	23	59
118	0.11	0.01	2	3837	5.32	0.620	803	16	56	21	35
140	0.15			3795	4.31	0.742	165	43	93	27	22
144	0.16			4775		0.471	449	32	78	28	44
145	0.05	0.02	3	2912		0.662	375	47	68	39	51
154	0.44			3946		0.152	313	74	64	40	58
162	−0.22	0.01	2	4452	0.44	0.831	459	16	67	21	37
174	−0.25	0.03	3	3972	3.57	1.079	240	13	68	20	27
204	0.02	0.02	3	3104	3.60	0.695	215	43	93	38	28
259	−0.19	0.01	3	3075	2.69	0.756	303	18	87	17	34

Table S2. Statistical results for correlation between anthropological parameters (age, height, and BMI) and copper and sulfur isotope compositions of serum and RBC

		Serum		RBC	
Isotopic ratio	Anthropological parameter	*R*	*P*	*R*	*P*
δ^{65}Cu	Age	−0.268	0.095	−0.156	0.317
	Height	0.191	0.239	0.168	0.282
	BMI	0.025	0.875	0.319	0.037
δ^{34}S	Age	−0.074	0.713	−0.035	0.821
	Height	0.121	0.549	0.116	0.459
	BMI	0.061	0.762	0.163	0.296

Table S3. Sample ID, age, BMI, average δ^{65}Cu value, associated SD and n, average Cu concentration, and average δ^{34}S value in RBC samples from the ILCS study

				RBC				
ID	Age, year	Height, cm	BMI	δ^{65}Cu, ‰	sd	n	[Cu], ng/g	δ^{34}S, ‰
Control								
3	55	163	19.6	0.66			929	6.58
12	43	163	25.6	1.05	0.05	3	1015	5.30
24	60	172	22.6	0.58			966	5.74
25	47	172	24.5	1.19	0.02	2	1215	5.14
26	70	155	20.2	0.67	0.08	2	1210	3.20
41	57	165	23.9	0.79			942	4.82
42	55	159	28.5	1.13			971	5.41
44	47	173	20.0	0.88	0.06	3	1094	3.18
45	55	159	26.9	0.89			926	4.14
46	52	168	22.3	0.79			807	5.57
47	58	169	20.3	1.24	0.05	2	926	6.36
48	32	176	30.7	1.22	0.14	2	997	4.02
49	44	172	28.1	0.86	0.10	3	979	5.01
50	70	180	30.8	0.64	0.12	2	1119	6.12
51	48	174	22.5	0.97	0.12	2	1102	4.74
53	59	165	25.0	0.62			1225	5.01
60	63	167	21.9	0.75			921	6.67
103	64	170	23.2	1.03			847	5.35
111	47	160	34.4	0.99	0.25	2	1109	4.77
157	48	163	27.9	0.57			923	5.17
HCC								
1	55	160	20.7	0.35			1045	4.07
10	57	156	23.7	0.65			1157	4.80
11	56	165	16.5	−0.07	0.07	4	1536	5.49
16	38	160	23.4	0.90			1607	4.01
20	55	161	19.7	0.03	0.03	2	828	4.07
62	47	169	22.4	0.45			1105	6.93
68	40	164	21.9	0.56			1854	4.81
69	46	178	24.9	0.26			1027	5.73
77	44	170	23.5	0.73			1221	3.96
78	45	169	30.1	0.25			987	5.35
86	58	170	29.4	0.92			961	1.97
88	68	167	20.1	0.62	0.11	2	1050	4.62
90	50	162	22.5	0.41			1057	3.85
108	55	164	20.1	0.47			1076	4.73
118	58	165	24.6	0.10			1480	5.11
140	68	173	25.0	0.82	0.14	2	1003	4.06
144	65	150	18.7	0.72			1435	0.92
145	56	168	17.7	0.63			1179	2.56
154	41	185	19.8	0.84			959	1.83
162	58	165	22.9	0.21			1774	1.37
174	52	160	23.0	0.44			1090	4.26
204	51	170	25.3	0.65			1287	3.23
259	64	170	22.1	0.76			915	3.05

Table S4. Copper concentrations and isotope compositions in tumor and nontumor normal adjacent tissues samples for seven patients with hepatocellular carcinomas from the Centre Hépato-Biliaire, Villejuif, France

		Tumor		Normal	
ID	Sex	δ^{65}Cu, ‰	[Cu], μg/g	δ^{65}Cu, ‰	[Cu], μg/g
1	F	0.15	73.2	−0.11	3.69
2	M	−0.02	10.5	−0.45	33.0
3	M	0.36	10.2	−0.26	6.65
4	M	0.07	4.02	−0.28	17.7
5	M	0.40	51.0	−0.14	8.40
6	M	0.02	15.8	−0.20	16.4
7	M	0.43	52.3	−0.30	15.5

Table S5. Average δ^{34}S value, associated SD, and n for IAEA-SO5 and NBS127 standards at LGLTPE and IsoAnalytical Ltd.

	LGLTPE			IsoAnalytical		
Standard	δ^{34}S, ‰	sd	n	δ^{34}S, ‰	sd	n
IAEA SO5	0.03	0.43	2	0.27	0.17	5
	0.30	0.26	4	0.14	0.35	3
	0.49	0.40	2	0.48	0.12	4
	0.82	0.09	3			
NBS127	20.31	0.12	4	20.37	0.13	11
	20.30	0.14	4	20.28	0.18	6
	20.30	0.25	4	20.38	0.14	8
	20.29	0.25	3			