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**Molecular imaging and therapy targeting copper metabolism in hepatocellular carcinoma**

Wachsmann J *et al*. Copper Metabolism and hepatocellular carcinoma

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**Abstract**

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide. Tremendous efforts were devoted to search for new biomarkers for molecular imaging and targeted therapy of HCC. Copper is a nutritional metal required for the function of numerous enzymatic molecules in metabolic pathways of human cells. Emerging evidence suggests that copper plays a role in cell proliferation and angiogenesis. Increased accumulation of copper ions was detected in tissue samples of HCC and many of other cancers in humans. Altered copper metabolism is a new biomarker for molecular cancer imaging with position emission tomography (PET) using radioactive copper as a tracer. It has been reported that extrahepatic mouse hepatoma or HCC xenografts could be localized with PET using copper-64 chloride as a tracer, suggesting copper metabolism as a new biomarker for detection of metastasis of HCC in the areas of low physiological copper uptake. In addition to copper modulation therapy with copper chelators, short-interference RNA specific for human copper transporter 1 (hCtr1) may be used to suppress growth of HCC by blocking increased copper uptake mediated by hCtr1. Furthermore, altered copper metabolism is a promising target for radionuclide therapy of HCC using therapeutic copper radionuclides. Copper metabolism holds potential as a new theranostic biomarker for molecular imaging as well as targeted therapy of HCC.

**Key words:** Hepatocellular carcinoma; Copper metabolism; Positron emission tomography; Radionuclide therapy; RNA interference; Gene therapy

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**Core tip:** Copper is required for cell proliferation and tumor angiogenesis. This article provided an up-to-date review of copper metabolism as a novel theranostic biomarker in hetatocellular carcinoma. Altered copper metabolism is not only a novel biomarker for molecular imaging of extrahepatic metastasis of hepatocellular carcinoma using radioactive copper, but also a promising target for copper modulation and radionuclide therapy of hepatocellular carcinoma.

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**INTRODUCTION**

Copper is a trace metal that is required for numerous metabolically important enzymes involved in variousmetabolic pathways of human physiology[1,2]. These include ceruloplasmin, superoxide dismutase, dopamine monooxygenase, lysyl oxidase, cytochrome c oxidase, factor V, and tyrosinase. These enzymes are used for a variety of purposes such as melatonin production, bone production, thrombosis and neurotransmitter synthesis. The amount of daily dietary copper content for an average adult is 1.0 to 1.6 mg according to the third National Health and Nutrition Survey[3]. zero point nine mg per day of copper is the recommended daily allowance, and less than 10 mg per day is recommended by the National Academy of Sciences[4]. The adult human body contains about 75 mg of copper[5]. The liver and brain have about one third of the overall quantity present, but copper is distributed throughout the human body and found in many organ systems. This includes the heart, kidneys, pancreas, spleen, bone and muscle[5].

A majority of the daily intake of copper is from vegetables and legumes, with other sources such as various meats. On average, vegetable sources of dietary copper require a more complex enzymatic process to be absorbed, compared to non-vegetable sources such as meat or milk. The variable amount of copper in food sources is dependent on the various amounts of copper in the soil as well as different food processing techniques[1,6]. After copper is ingested from food sources, dietary absorption of copper predominately occurs in the stomach and small bowel, with only approximately 30%-40% of ingested copper being absorbed by those living in industrialized countries. However, depending on one’s dietary intake of copper, the human body can theoretically absorb as much as 63%-67% in the copper deficient diet, or as little as 12% in those whose copper intake is very high. The high acidic environment in the stomach is believed to cause release of copper from natural organic complexes. Subsequently, absorption in the small bowel is influenced by a change in the pH as well as pancreatic enzymes[6–8].

Metallthionein within the absorptive cells of the bowel are able to bind copper via mercaptide bonds and then released to the plasma cell membrane on the serosal side. After being released from the intestinal mucosa, copper is bound to amino acids and albumin in the portal venous system. A small portion of the copper in the portal venous system is able to pass through to systemic circulation, while the remainder is transferred into the cytosol of hepatocytes *via* cell membrane receptors. Within the liver, copper is bound to various proteins, but preferentially metallothionein[5,9].

The liver is a critical organ for systemic regulation of copper metabolism and themaintenance of copper homeostasis. Wilson’s disease (WD) is an inherited copper metabolism disorder caused by mutation of ATP7B gene located on chromosome 13, for which numerous specific mutations have been identified[10-12]. Long-Evans Cinnamon rat, an animal model for WD, has a deletion in the copper transporting ATPase gene and develops hereditary hepatitis followed by spontaneous HCC[13]. When these rats are treated with the copper chelating agent D-pencillamine, as commonly used in humans with Wilson’s disease, there was not only the prevention of the onset of hepatitis, but also the inhibition of elevated serum transaminases[14]. Togashi *et al*[14] therefore concluded that abnormal copper accumulation in the liver of Long-Evans Cinnamon was associated with the pathogenesis of hereditary hepatitis and subsequent development of HCC. Both low and high molecular weight copper binding species have been described. The high molecular weight species is predominated in gallbladder bile, while low molecular weight species is more prevalent in hepatic bile. The low molecular weight species is thought to assist in the membrane transport of copper across the biliary canaliculus. The high molecular weight portion of copper binding species is principally related to copper homeostasis[9,15]. This is supported by the inability to adequately remove hepatic copper in the absence of the higher molecular weight copper binding species, in the setting of protein synthesis inhibitors[16]. Copper that is tightly bound to bile salts is predominately unable to be absorbed in gastrointestinal tract, and is lost in feces, which is the predominate route of excretion[5,6,9,17].

The plasma concentration of copper has been shown to increase throughout life, peaking around the age of 60, and then having a minimal decline afterwards[18]. This process thought to be related to progressive reduction in biliary clearance later in life, rather than an increase in gastrointestinal absorption[6,19]. Differences in the plasma concentration of copper have also been demonstrated in sexes, with females on average having higher concentrations than men. Women between the ages of 20 and 59 were shown to absorb more and have a quicker turnover of radiolabeled copper in a meal, when compared to men. Higher levels of ceruloplasmin are also present in females[18].

**COPPER AND HCC**

HCC is the fifth most common cancer worldwide. It is the third leading cause of cancer related death worldwide. Overall, about 75%-80% of HCC occurs in patients with hepatitis B or C, with many other known risk factors including aflatoxin B1, obesity, alcohol usage, diabetes, and tobacco[20,21]. It has been demonstrated that the copper content in hepatic parenchyma of patients with HCC is significantly higher in those without HCC, with no significant difference in hepatic iron levels. In fact, the copper level in the liver was the only significant factor associated with the presenceof HCC in the cohort of patients with hepatitis C and chronic liver disease[22]. There were reports of increased incidence of HCC in the patients diagnosed with WD[23,24]. The copper content and amount of copper binding proteins of HCC has been shown to be higher than the amounts seen in other liver malignancies such as cholangiocellular carcinoma and metastatic tumors[25,26]. Additionally, the serum copper to zinc ratios are significantly higher in patients with HCC than matched controls[27].

On the other hand, there were also reports of decreased incidence of HCC in patients with copper metabolism disorders[28]. It has been proposed that WD patients treated with D-penicillimine have an elevated risk of developing HCC[29]. This may be secondary to the associated decrease of copper content of the liver, when on chelation therapy. This discrepancy could reflect either a carcinogenic or a protective role of copper in pathogenesis of HCC, which remains to be elucidated and requires further investigation.

**COPPER METABOLISM AS BIOMARKER FOR METABOLIC IMAGING OF HCC**

Currently, the detection of liver masses is predominately evaluated with the use of anatomic imaging modalities[30], such as ultrasound, computed tomography (CT) and magnetic resonance imaging (MRI). The topic of molecular imaging is gaining momentum and is being applied to various disease states[31]. Positron emitting fluorine-18-2-deoxy-gluocose (F-18 FDG) is a radioactive tracer for assessment of glucose metabolism in both benign and malignant tissues. After being delivered to the cells via blood flow, F-18 FDG is transported by GLUT transporters and then phosphorylated once intracellular. Typically, the FDG-6-phosphatase is trapped within the cells, unless there is a high level of phosphatase activity, as seen in the liver[32]. Secondary to the high level of phosphatase in the liver, the sensitivity for detecting well differentiated HCC is poor. However, there is usually high F-18 FDG uptake in moderately and poorly differentiated HCC. Positron emission tomography/computed tomography using F-18 FDG (F-18 FDG PET/CT) is also useful for the detection of recurrence and extrahepatic metastasis of HCC[33,34].

The sensitivity of FDG PET/CT in detection of HCC was about 50%, compared to the sensitivity of 75% by CT[35]. However, Wang *et al*[36] were able to show improved performance in detection of HCC when an early dynamic F-18 FDG PET/CT was performed for the 240 s after tracer injection. Even better detection rates were able to be obtained when early dynamic and conventional delayed whole body information was used in combination. The detection rates improved from 56.7% to 91.9% when using whole body delay versus the combination early dynamic and whole body scans, respectively. In patients who were to undergo liver transplantation, F-18 FDG PET/CT was found to be useful for prediction of microvascular invasion by HCC. Presence of microvascular invasion by HCC was predicted when the ratio of maximum standardized uptake value (SUV) of HCC to mean SUV of normal liver parenchyma was 1.2 or greater[37].

C-11 acetate, a tracer that evaluates free fatty acid synthesis, may have better sensitivity than that of F-18 FDG[38]. According to a study performed by Ho *et al*[39], the sensitivity in detection of HCC in patients with less than 3 lesions was 87% for C-11 acetate and 47% for F-18 FDG. When this was correlated with histologic findings, it appears that well differentiated tumors were better detected by C-11 acetate, while the poorly differentiated tumors are better detected by F-18 FDG. None of the non-HCC tumors were seen to demonstrate abnormal C-11 acetate uptake. The use of dual phase C-11 acetate, using the change in uptake values in early and conventional imaging, was able to correctly differentiate between small, 1-3 cm, well differentiated HCC from FNH’s and hemangiomas[40].

The tracer C-11 choline is used to evaluate the metabolism of phospholipids subsequently used as constituents for the cell membrane. F-18 FDG negative HCC showed elevated uptake of C-11 choline, which predominately was seen in the moderately differentiated group[41]. F-18 fluorocholine has also been shown to perform better than F-18 FDG for well differentiated HCC, with a combination of both tracers appearing to be the best option[42]. Compared to a single modality, a combination of imaging modalities, including F-18 FDG PET, CT, MRI and ultrasound, currently has higher sensitivity, with minimal effect on specificity[38].

There are continuous efforts to develop new tracers for molecular imaging of HCC. Radioactive copper has been used for assessment of copper metabolism disorders in patients diagnosed with WD using nuclear imaging for at least 45 years[43-46]. Exploring copper metabolism as a biomarker for molecular imaging of HCC, Peng *et al*[47], for the first time, demonstrated that mouse extrahepatic hepatoma could be visualized by PET using copper-64 chloride (64CuCl2) as a tracer, based on increased copper uptake mediated by mouse copper transporter 1. There was relatively less 64Cu uptake in the hepatoma compared to the liver, which was thought to be related to several factors: less mCtr1 in the tumor relative to the liver, the possibility that endogenous mCtr1 may be less active on the tumor, other copper transporter in the normal hepatocytes not expressed on the tumor, and efflux of copper more rapidly in tumor cells than normal hepatocytes[48]. More recently, human HCC xenografts in athymic mice were also visualized by PET after intravenous injection of 64CuCl2 as a tracer[49]. There was abundant physiologic distribution of 64Cu in the liver, which would cause a limited evaluation of primary HCC in the liver. Given the normal intense uptake of FDG by the cortical brain tissue and low physiological cerebral uptake of 64Cu[47-53], 64CuCl2-PET is a promising technique for non-invasive assessment of intracranial and other extrahepatic metastasis of HCC located in the areas with low physiological copper uptake (Figure 1). Prognosis for the patients with intracranial HCC metastasis is poor as they are often resistant to systemic chemotherapy. Use of 64CuCl2-PET/CT for early detection of HCC intracranial metastasis is significant for improving prognosis of the patients with metastatic HCC. On the other hand, 64CuCl2-PET is expected to be useful to exclude extra-hepatic metastases in pre-transplant work up of the patients who are considered as candidates for liver transplant. Positron emitting 64Cu radionuclide has a half-life of 12.7 h, making it possible to ship it to an imaging facility distant from production site of this radiotracer. Preclinical radiation dosimetry of 64CuCl2 using *Atp7b*-/- knockout mouse model of WD was comparable to that of F-18 FDG[50], supporting the use of 64CuCl2 as a radiotracer for PET of HCC metastasis, except the metastatic lesions in the abdomen due to excreted 64Cu in the intestinal tract.

**TARGETING COPPER METABOLISM FOR TREATMENT OF HCC**

Early detection and treatment are most critical for reduction of mortality in HCC[54,55]. The use of conventional transarterial chemoembolization (TACE) for the treatment of unresectable HCC has been found to improve the overall survival of patients compared to available supportive care[56]. The use of cisplatin or doxorubicin in a large review comparing chemoembolization showed a significant benefit compared to embolization alone[57]. A major limitation in the literature regarding TACE is the lack of consistent methods between various investigators[56]. The use of TACE with drug eluting beads (DEB-TACE), primarily using doxorubicin allows for slow drug release and lower levels of systemic of the chemotherapeutic agent when compared to TACE using lipiodol[58]. Though no survival benefit was shown, Malagari *et al*[59] were able to also show that there were longer times to progression, less recurrence, and a better local response when using doxorubicin-eluting beads compared to bland embolization. Despite additional studies that have not shown a difference in radiographic response, survival or adverse events[60], Sieghart *et al*[56], still recommends that any future trials should include drug eluting bead TACE secondary to the lower systemic levels of doxorubicin and then possible reduction in drug-drug interactions.

The ability to bridge a patient to liver transplant has been performed using several types of neo-adjuvant therapies including TACE, radiofrequency ablation, trans-arterialradioembolization (TARE) external beam radiotherapy and surgical resection. The idea of bridging has been shown to decrease waiting list dropout, reducing HCC recurrence, and improved post-transplant survival with the goal to have similar post transplant outcomes as non-HCC patients[61].

Palliation for patients with end stage or terminal HCC includes various options, with the primary goal of improving patient symptoms rather than definitive treatment[62]. Average survival for patients with end stage or terminal HCC is 3-4 mo, and includes about 15%-20% of all HCC patients at presentation. Some of the various treatment options with end stage disease are opiates, acetaminophen and corticosteroids[62]. HCC can be difficult to treat despite tremendous efforts devoted to development of effective therapies for treatment of this devastating disease[55]. There are continuous efforts to search new targets for treatment of HCC. Angiogenesis is an important pathway for tumor growth and copper is an important angiogenic factor for tumor growth[63]. Copper has been shown to be a cofactor in several mediators of angiogenesis including angiogenin, matrix metalloproteinase and fibroblast growth factor[64-66]. Moriguchi *et al*[67] have demonstrated antiangiogenic effects of copper chelator, trientinedihydrochloride, on hepatocellular carcinoma in a rat model. Other groups have also show that the copper chelatorpencillamine along with diet modification can lower copper levels and microvascular density in cerebral rabbit models. Brem *et al*[68] also therefore came to the conclusion that using pharmacologic withdrawal and dietary depletion of copper that there was suppression of intracerebral tumor angiogenesis. However, prolonged anti-copper cancer therapy with copper chelators or long-term use of D-pencillamine for anti-inflammatory treatment in rheumatoid arthritis has been shown to cause toxicity such as bone marrow suppression, rash and neurologic symptoms[69,70]. Significant advancement has been made in understanding molecular biology of copper transporters and chaperons regulating cellular copper homeostasis[71]. Recent advances in understanding of the role of copper in signal transduction pathway of cellular proliferation[53,72-76] support further study of copper metabolism as a target for molecular therapy of HCC. Selection of patients with copper hypermetabolic, metastatic HCC by 64CuCl2-PET/CT may be helpful to improve efficacy of anti-copper therapy of HCC. Human copper transporter 1(hCtr1) is a high affinity copper transporter mediated cellular copper uptake in humans[77]. To overcome side effects of anti-copper therapy with long-term or high-dose use of copper chelators, RNAi-mediated knockdown of hCtr1[53] may be a promising approach fortargeted anti-copper therapy of HCC.

The use of external beam radiation for HCC has been limited secondary to the liver being considered a radiosensitive organ, which may have led to early under-dosing of patients[78]. This limitation can be compounded when HCC occurs in the setting of an already diseased burdened liver as seen with hepatitis C. Radiation-induced liver disease in the patients subjected to external beam radiation can cause endothelial damage, platelet activation, fibrin thrombus and venous occlusion. These changes can lead to subsequent hepatic fibrosis. However, there may be a role for radiotherapy in patients with tumors that are in challenging locations, palliative purposes, bridge to transplant, or in combination with other treatment options[79]. External beam radiation as well as percutaneous cementoplasty has been used for palliative purposes with successful management of symptoms[80,81].

The targeted deliver of radionuclide therapy has been done with intrarterial delivery of various conjugates radiolabeled with therapeutic radioisotopes which include Yttrium-90, Iodine-131, Holmium-166 and Rhenium-188[82,83]. Yttrium-90 labeled microspheres are used for interventional radionuclide therapy of HCC[84]. Currently, there are both glass and resin based particles available for radioembolization of HCC. The glass based form has a smaller size with a lesser embolic effect and lower incidence of post-embolization syndrome. One limitation of TACE is the possible decompensation of the liver after their use in patients with hepatic artery and portal thrombus. The use of Y-90 glass microspheres in patients with HCC and branch or lobar portal vein thrombosis both showed favorable tumor response rates and was safe in a trial that included 108 patients[85]. Y-90 does not emit gamma rays and therefore is not optimal for imaging. In contrast, rhenium-188 is a therapeutic radionuclide with a physical half-life of 16.9 h and emits both beta and gamma rays. The use of intra-arterial Rhenium-188-conjugates for radioembolization of HCC has been shown to inhibit tumor growth[86]. Attempts were also made to develop I-131 radiogene therapy of HCC based on tumor-specific expression of human sodium/iodide symporter (hNIS) under control of alpha fetoprotein promoter and enhancer[87-89]. Tumor-specific expression of the hNIS in HCC cells was achieved by transfection of HCC cells with a vector encoding hNIS gene driven by an alpha fetoprotein promoter/enhancer.Increased uptake of I-131 by the cells expressing hNIS was detected by gamma counting in vitro and by imaging with gamma camera in vivo. Growth of extrahepatic tumor xenografts derived from the cells expressing hNIS was inhibited, secondary to radiation effects of 131I accumulated in the transfected HCC cells expressing hNIS[89]. Development of technologies to allow safe and efficient delivery of the vector encodinghuman sodium/iodide symporter gene is critical for clinical application of I-131 radiogene therapy of HCC, based on the finding of preclinical studies.

Multiple copper isotopes are available for cancer imaging and therapy[90-93]. Copper-64 emits both β+ and β –particles and holds potential as a theranostic copper radionuclide for both cancer imaging and therapy.Apelgot *et al* demonstrated that 64Cu had a lethal effect in mammalian cells similar to that of 67Cu radionuclide[94]. Tremendous efforts were made to develop 64Cu-radiolabeled conjugates for cancer imaging and therapy[95-99]. Based on its simplicity andincreased tumor uptake of 64Cu demonstrated by PET[47-49,52,53,100,101], ionic 64CuCl2 holds potential as a therapeutic radiopharmaceutical for treatment of tumors expressing high level of hCtr1. Recently, it was reported that growth of malignant melanoma overexpressing hCtr1 was suppressed in mice treated with 64CuCl2[102]. In addition to its potential as a reporter gene for tracking gene delivery with PET, targeted overexpression of hCtr1 may be used for copper radiogene therapy of the tumors expressing low level of endogenous hCtr1[103]. The findings from preclinical studies support further investigation of ionic 64CuCl2 as a radiopharmaceutical for targeted radionuclide therapy of HCC, in addition to copper modulation therapy with copper chelators (Figure 2).

**CONCLUSION**

Copper is a transitional metal required for regulation of cell proliferation and angiogenesis. Copper’s exact role in the development of HCC is still poorly understood, as demonstrated by the paradoxical suppression or increase of HCC in patients with copper metabolic disorders such as WD. The findings of increased uptake of radioactive copper by the extrahepatic HCC xenografts in mice invite clinical exploration of altered copper metabolism as a new imaging biomarker for metabolic imaging of HCC metastasis with PET using 64CuCl2 as a radioactive tracer. Additionally, copper metabolism holds potential as a target for copper modulation gene therapy of HCC based on RNAi-mediated knockdown of hCtr1 followed by administration of copper chelators. Furthermore, 64CuCl2 or 67CuCl2 may be used as radiopharmaceuticals for radionuclide therapy of HCC and ablation of extrahepatic HCC metastasis.

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**Figure 1 Metabolic imaging of metastasis of hepatocellular carcinoma with 64CuCl2-position emission tomography and computed tomography.** A: Preclinical metabolic imaging of HCC xenografts in mice injected with 64CuCl2 as a tracer. 64Cu bound to copper binding molecules in the blood immediately after intravenous injection of 64CuCl. PET/CT Images were then obtained that show the expected biodistribution of 64CuCl2 in the liver and intestinal tracts, with low uptake in the brain and muscular tissues. The HCC xenografts implanted on the shoulder showed increased 64Cu uptake on the PET/CT images; B: Schematic presenting clinical perspective of metabolic imaging of HCC in humans. The human patients may be injected with 64CuCl2 and subjected to PET/CT for detection of HCC metastasis in the areas of low physiological 64Cu uptake, such as brain and skeletomuscular tissues. HCC: hepatocellular carcinoma; PET/CT: hybrid positron emission tomography and computed tomography.



A B

**Figure 2 Perspective on targeting copper metabolism for treatment of hepatocellular carcinoma**. A: Copper metabolism in hepatocytes. Copper metabolism in hepatocytes is regulated by a network of copper transporters and chaperons. After copper uptake mediated by influx copper transporter, hCtr1, copper is transported intracellularly by copper chaperons and copper homeostasis is maintained by outflow of copper mediated by efflux copper transporter, ATP7B; B: Targeting copper metabolism for treatment of HCC. Copper is required for cell proliferation and may play a role in signaling transduction pathway regulating proliferation of hepatocellular carcinoma (HCC) cells. Targeting copper metabolism, copper chelators have been tested for anti-copper therapy of HCC, with variable response. RNAi-mediated knockdown of hCtr1 and/or other copper chaperons are potential new approaches for targeted anti-copper gene therapy of HCC. Furthermore, ionic 64CuCl2 or 67CuCl2 hold potential as new radiopharmaceuticals for systemic radionuclide therapy of HCC, based on increased 64Cu uptake of HCC visualized on preclinical PET/CT images. hCtr1: Human copper transporter 1; ATOX-1: Antioxidant 1; Cox 17: Cytochrome c oxidase 17; CCS: Copper chaperone for superoxide dismutase; SOD1: Superoxide dismutase 1; GSH: Glutathione; MT: Metallothionein; ATP7A: Copper-transporting ATPase 1; ATP7B: Copper-transporting ATPase 2; PET/CT: Hybrid position emission tomography and computed tomography.