RESEARCH ARTICLE

Combination Therapy with Low Copper Diet, Penicillamine and Gamma Knife Radiosurgery Reduces VEGF and IL-8 In Patients with Recurrent Glioblastoma

Alireza Feli[1], Shima Jazayeri[2], Mohammad Ali Bitaraf[3], Masoud Solaymani Dodaran[4], Karim Parastouei[5], Mohammad Javad Hosseinzadeh-Attar[1]*

Abstract

Purpose: Vascular Endothelial Growth Factor (VEGF) and interleukin-8 (IL-8) appear important in tumor growth. In this study, we have investigated the effect of copper reduction along with gamma knife radiosurgery on IL-8 and VEGF in patients with recurrent glioblastoma multiforme (GBM). **Materials and Methods:** In a 3-month randomized clinical trial, patients with recurrent GBM were allocated randomly between intervention and placebo groups. Radiosurgery was performed for both groups (Reference dose: 16-18 Gray, in one fraction). The intervention group received low copper diet and penicillamine while the patients in the placebo group continued with their usual diet. VEGF and IL-8 were measured at baseline and the end of intervention. **Results:** VEGF in intervention group significantly reduced compared to placebo group (Mean ± SD, 4.5±1.91 vs. 7.8±3.21; P<0.001). IL-8 in intervention group decreased compared to placebo group but not significant (2.7±1.91 vs. 3.2±3.20; P=0.49). We also detected a significant positive correlation between serum copper and VEGF (r=0.57; P<0.05) and a negative correlation between KPS and serum copper. **Discussion:** Our results could reflect that low copper diet and penicillamine may decrease serum VEGF in patients who underwent gamma knife radiosurgery for recurrent glioblastoma multiforme.

Keywords: Gamma knife radiosurgery- Glioblastoma- IL-8- VEGF- Penicillamine

Introduction

Glioblastoma multiforme (GBM) classified as a World Health Organization Grade 4 glioma (Chamberlain, 2011), is the most common and most malignant brain tumor in humans with an incidence rate of 3-4/100,000/year (Polivka et al., 2017). GBM is characterized by extreme and abnormal vascularization and is highly resistance to radiation and cytotoxic chemotherapy. There are only very limited choices for the treatment of subsequent recurrences, with minimal clinical efficacy (Polivka et al., 2017). Angiogenesis, defined as the development of new blood vessels from pre-existing vessels (Gupte and Mumper, 2009). In physiological situations, angiogenesis takes place rarely in adults and is limited to few organs such as placenta, ovaries, uterus and fallopian tubes (Nasulewicz et al., 2004). A variety of signals that are able to initiate angiogenesis have been mentioned, and include: inflammatory response, mechanical trauma and genetic mutations, but the most important factor is metabolic stress, particularly the lack of oxygen within

the tumor cells (Nasulewicz et al., 2004). Tumors cannot grow more than 1–2 mm in diameter without angiogenesis (Gupte and Mumper, 2009). Malignant cells have different ways to either produce their own angiogenic stimulators or to employ endothelial cells to synthesize them (Gupte and Mumper, 2009). These angiogenesis stimulators include Vascular Endothelial Growth Factor (VEGF), basic Fibroblast Growth Factor a (b-FGF), Angiogenin, Epidermal Growth Factor (EGF), Tumor Necrosis Factor a (TNF-a), cytokines such as Interleukin (IL) 1, 6 and 8, and also trace elements such as copper (Gupte and Mumper, 2009). The major role in tumor angiogenesis is played by VEGF, the key regulator of angiogenesis (Chamberlain, 2011). VEGFs are a group of angiogenic proteins which are necessary for vasculogenesis and hypoxia-induced angiogenesis. Copper may be an essential cofactor of VEGF mediated angiogenesis. VEGF is highly expressed in brain tumors (Chamberlain, 2011). In GBM the highest levels of VEGF are seen in regions of hypoxia, necrosis and endothelial proliferation (Chamberlain, 2011). Therefore the study of anti-VEGF agents in GBM is highly desired

[1]Department of Clinical Nutrition, School of Nutritional Sciences and Dietetics, [3]Department of Neurosurgery, Tehran University of Medical Sciences, [2]Department of Nutrition, School of Public Health, [4]Minimally Invasive Surgery Research Centre, Iran University of Medical Sciences, [5]Department of Nutrition, School of Public Health, Baqiyatallah University of Medical Sciences, Tehran, Iran. *For Correspondence: mhosseinzadeh@tums.ac.ir

(Polivka et al., 2017). On the other hand increased levels of IL-8 have been detected in several types of tumors such as breast, melanoma and glioblastoma where its actions have been linked to tumor invasion, proliferation, survival and angiogenesis (Dwyer et al., 2012). Copper plays a significant role in promoting physiological and malignant angiogenesis (Goodman et al., 2004). The role of copper in both the etiology and expansion of tumors has been widely studied (Gupte and Mumper, 2009). Copper reduction as an anti-cancer approach is now under intense investigation (Gupte and Mumper, 2009). Copper-reducing agents suggest advantages to cancer therapy for multiple reasons, including effectiveness in various types of tumors, low toxicity hazard, low cost and feasibility of combination with almost every other treatment strategy (Nasulewicz et al., 2004). A significant limitation of using copper-reducing strategies in antitumor treatment is the long period of administration required (Nasulewicz et al., 2004). In aggressive tumors such as GBM, vascularization facilitates tumor progression (G Linkous and M Yazlovitskaya, 2011). Combining antiangiogenic agents with radiation therapy, radiosurgery and cytotoxic chemotherapy can be considered as possible treatment approaches for these tumors (Norden et al., 2009). The present standard treatment for primary GBM includes micro-surgical resection, followed by radiotherapy and adjuvant temozolomide (Frischer et al., 2016). Despite progress in surgical techniques, radical resection is nearly impossible because of the infiltrative nature of GBM. Therefore, glioblastoma inevitably recurs (Frischer et al., 2016). Treatment options for recurrent glioblastoma stay controversial and depend on tumor size, location, and the patient's performance status (Frischer et al., 2016). Gamma knife radiosurgery (GKRS) is one of the most precise and powerful treatments for some brain disorders and uses beams of highly focused gamma rays (Lunsford et al., 1990; Redmond and Mehta, 2015). Gamma knife could be considered as a relatively safe treatment option for recurrent GBM for well-selected patients but should be seen as part of a multimodal treatment protocol (Frischer et al., 2016). However, most studies have considered patients with limited tumor measuring < 4-5 cm in diameter (Crowley et al., 2006). The technique is most suitable in patients with focally recurrent tumor (Crowley et al., 2006). Penicillamine was introduced as a copper chelating agent for the treatment of Wilson's disease in 1956 (Gupte and Mumper, 2007; Gupte and Mumper, 2009). Laboratory studies have shown that penicillamine has antineoplastic (Wadhwa and Mumper, 2013) and antiangiogenic (Antoniades et al., 2013) properties. Other than copper chelation, it also inhibits some of growth factors and cytokines such as VEGF and IL-8 (Gupte and Mumper, 2009). According to mentioned issues, this study aimed to assess the efficacy of low copper diet and penicillamine associated with gamma knife radiosurgery on VEGF and IL-8 suppression in patients with recurrent glioblastoma multiforme.

Materials and Methods

Subjects and recruitment procedure

In a randomized single blind clinical trial, fifty patients with pathologically confirmed GBM were selected. Prior to the start of the study, ethical approval was received from the research ethics committee of Tehran University of Medical Sciences (Approval No. 116770). All the participants signed written informed consent forms and agreed to provide 10-ml blood samples. Inclusion criteria: tumor recurrence after operation, radiotherapy or chemotherapy; age ≥18; Karnofsky performance score (KPS) ≥60; proper hematological, renal and hepatic function (WBC ≥4000/mm3, Hb ≥10g/dl, platelets ≥100000/mm3, BUN ≤40 mg, creatinine ≤1.5 mg, AST and ALT ≤4 folds normal, PT <1.5 folds normal). Patients with tumors up to 3 cm in diameter, in time of admission that present with focal recurrence were considered for study (Crowley et al., 2006; Thumma et al., 2012; Frischer et al., 2016; Imber et al., 2016). KPS is a gold standard for the measurement of physical performance in clinical oncology (Schaafsma and Osoba, 1994). Exclusion criteria: hepatic, renal or hematological insufficiency; sensitivity to penicillin; concomitant use of Bevacizumab; pregnancy and breast feeding. At the next step, based on random block statistical method, we divided the patients into intervention and placebo groups (25 patients in each group). Using a block size of four, there were 6 possible ways to equally assign participants to a block. Allocation proceeded by randomly selecting one of the orderings and assigning the next block of participants to study groups according to the specified sequence.

Data collected and tools used

KPS and anthropometric values such as weight, height and BMI of all patients were measured. Serum samples were obtained from patients at the start and at the end of the study to measure biomarkers such as VEGF and IL-8 and copper. Gamma knife radiosurgery (The Leksell Gamma Knife unit Model C, Sweden) was performed for two groups at a reference dose of 16-18 Gray and 50-60% isodose, in a single fraction. Initial dose of penicillamine (Cuprimine, Rubio, Spain) or placebo was 250mg daily with dose escalation every other day for one week to reach final dose of 1gr per day. Concurrently with penicillamine, a low-copper diet was used in intervention group. Foods rich in copper (organ meat such as heart; liver; brain, shellfish, bran bread, mushrooms and chocolate) were replaced with foods low in copper to supply sufficient micronutrients and calories (Brem et al., 2005). Placebo group were requested not to change their diet. A 3-days food diary questionnaire was used to collect food consumption data. Patients were followed for 1-2 years.

Data analysis

Data were analyzed statistically by applying SPSS 23 version for windows (SPSS Inc., Chicago, IL). One-sample Kolmogorov-Smirnov test was used for testing normality. For each group, we compared baseline values with final measurement by paired t-test. To compare two groups at the beginning and end of the study, Student's t-test was

Table 1. Anthropometric and Demographic Characteristics of Patients

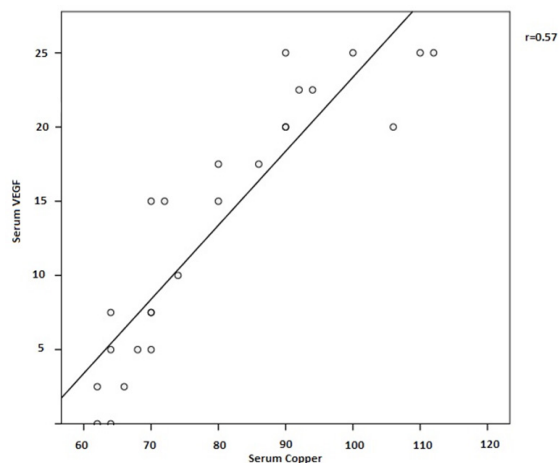| Variable*	Value	Value	*p*
	(Intervention group)	(Placebo group)	
Total patients	23	21	-
Weight (Kg)	74.39 ± 9.8	75.14 ± 15.85	0.85
Height (m)	1.69 ± 0.09	1.70 ± 0.04	0.93
BMI (kg/m²)	25.79 ± 3.6	25.73 ± 4.45	0.96
Age (years)	44.82 ± 12.03	42.47 ± 11.6	0.51
Males/Females	14/9	12/9	0.8

* Data are shown as Mean ± Standard Deviation (SD)

Figure 1. Correlation between Serum Copper and VEGF

used. A value of p<0.05 was considered as significant.

Results

Patient data

Forty-four of 50 (88%) patients completed the 3-month intervention. Before the end of intervention, two patients in the intervention group and four patients in the placebo group, died due to tumor-related complications. Demographic and anthropometric characteristics of

Table 2. Results of Food Analysis for Energy, Macronutrients and Copper

Variable*		Value	Value	*P*
		(Intervention group)	(Placebo group)	
Energy (kcal)	Before	2005.82 ± 260.74	2039.52 ±197.9	0.69
	After	1976 ±239.8±	2038 ±206.21	0.98
Carbohydrate (gr)	Before	258.47 ±21.82	264.90 ±22.04	0.33
	After	264.42 ±21.67	257.85 ±23.41	0.34
Protein (gr)	Before	72.34 ±13.19	75.71 ±11.73	0.37
	After	73.13 ±9.30	71.90 ±16.68	0.76
Fat (gr)	Before	55.21 ±4.36	54.47 ±3.66	0.55
	After	55.30 ±6.61	56.0 ±5.41	0.7
Copper (mgr)	Before	1.05 ±0.14	1.09 ±0.18	0.44
	After	0.40 ±0.09	1.05±13.93	0.001

* Data are shown as Mean ± Standard Deviation (SD)

Table 3. Serum Levels of VEGF and IL-8

Variable		Intervention	Placebo	P
		(Mean ± SD)	(Mean ± SD)	
VEGF (pg/ml)	Before	7.4 ± 2.87	7.2 ± 4.12	0.85
	After	4.5 ± 1.91	7.8± 3.21	< 0.001
IL-8 (pg/ml)	Before	3.3 ± 2.39	2.4 ± 2.74	0.21
	After	2.7± 1.91	3.2 ± 3.20	0.49

Table 4. Some Correlations between VEGF, IL-8, Copper and KPS

Parameters	Intervention		Placebo	
	r	p	r	p
Serum IL-8-Copper	0.37	0.07	0.14	0.51
Serum VEGF-Copper	0.57	0.04	0.34	0.12
Serum copper-KPS	-0.14	0.52	-0.03	0.88

patients at entry are listed in Table 1. Quantification of food diaries showed the amount of copper used in the intervention group has been reduced (Table 2). One patient underwent brain tumor surgery in the course of intervention. After 1.5 months (middle of the study) both intervention and placebo groups were examined clinically and paraclinically. Diarrhea was observed in two patients and a decrease in hemoglobin concentration was detected in one patient. Other side effects such as skin, liver and bone complications did not happen to any of the patients.

Biomarker data

In intervention group compared to placebo group, VEGF was significantly decreased (Mean ± SD, 4.5±1.91 vs. 7.8±3.21, P<0.001). The level of IL-8 was decreased nonsignificantly in intervention group (2.7±1.91 vs. 3.2±3.20, P=0.49) (Table 3). Correlations between VEGF, IL-8, copper and KPS in intervention and placebo groups have been shown in table 4. A significant positive correlation between serum copper and VEGF (r=0.57, P<0.05) and also a negative correlation between KPS and serum copper was seen (Figure 1).

Discussion

Our results have shown that penicillamine and low copper diet, reduced serum copper and serum VEGF in patients who underwent stereotactic radiosurgery for recurrent GBM. Since there is no definite treatment for GBM, the use of non-invasive methods, for instance, dietary interventions and/or pharmacological agents seems to be a logical approach. Angiogenesis has a key role in tumor expansion therefore decrease of VEGF as an important angiogenic factor by this approach may decelerate tumor growth. Glioblastoma multiforme (GBM) is the most common and lethal type of brain cancer (Ahluwalia and Chang, 2014). Tumors expand in a complex, stepwise method and several factors can affect this process. Copper plays a central role in tumor angiogenesis, particularly at its early stages (Nasulewicz et al., 2004). Copper chelators such as penicillamine and trientine have been shown to inhibit angiogenesis both in

vitro and in vivo (Brem et al., 1990; Pan et al., 2002; Ding et al., 2005; Hayashi et al., 2007). Some animal studies have supported the theory of using copper chelators as anti-angiogenic agent. Moriguchi et al., (2002) published a decreased tumor growth and IL-8 production with trientine in hepatocellular carcinoma (HCC). Yoshida et al., (1995) described a reduction in the tumor weight and vascular density after low copper diet plus penicillamine treatment of gliosarcoma xenografts. Brem et al., (1990) observed a reduced tumor growth and vascularization after low copper diet and penicillamine in glioma implanted intracerebrally in rabbits.

Low oxygen pressure in tumor cells induce the synthesis of several proteins, among which VEGF plays the most important role in angiogenesis (Nasulewicz et al., 2004). The expression of VEGF has also been shown to be upregulated via hypoxia-independent mechanisms (Schmidt et al., 1999; Brat et al., 2005; Reiss et al., 2005; Wang et al., 2008). In this study we investigated the possible role of copper reduction in suppression of VEGF in patients with recurrent glioblastoma. Intra- and inter-assay coefficient of variation for VEGF kit in our study was <10% and <12% respectively. Patients were exposed to a dietary intervention and penicillamine together with gamma knife radiosurgery. In agreement with our study, Brem has shown that copper reduction by low copper diet and penicillamine blocks angiogenesis by down regulating the angiogenic activity of VEGF, bFGF, TNF-a, IL-1, IL-6 and IL-8 (Brem and Wotoczek-Obadia, 1998). Interleukin-8 is known to possess proangiogenic and tumorigenic properties. In human glial tumors, IL-8 is secreted at high levels and it is essential for tumor neovascularity and progression (Brat et al., 2005). The essential role of angiogenic factors including VEGF and IL-8 are impaired by copper reduction (Hanahan and Folkman, 1996; Fukumura and Jain, 2007). To yield therapeutic effects, copper level in the patient's serum needs to be reduced to 20% of the physiological concentration, which takes from several weeks to 6 months (Nasulewicz et al., 2004). Unfortunately, long-term administration of penicillamine brings several adverse effects, including reduction of the antioxidative activity of Cu/Zn enzymes and, as a result, increased sensitivity to oxidative stress, neurological symptoms, fever, rash and joint pains (Yoshii et al., 2001). Also, persuasive data proposes that copper reduction causes a considerable inhibition of activity of cupric enzymes without any effect on their levels. This may result in overproduction of free radicals, irregularity in the connecting tissue and also immune system dysfunction (Cordano, 1998). There are also data showing a relationship between copper deficiency and coronary artery disease. It has been shown that experimental copper deficiency notably increases the susceptibility of lipoproteins and cardiovascular tissues to lipid peroxidation, therefore increasing the risk of cardiovascular disease (Rayssiguier et al., 1993). One may also suppose that tumor growth and angiogenesis need a copper optimum, so we cannot rule out the possibility that supplementation with high doses of copper may cause, similarly to copper deficiency, an inhibitory outcome on tumor growth (Nasulewicz et al., 2004).

However, there are certain limitations of using copper-reducing compounds in antitumor treatment. One of them is the long time of drug administration necessary to develop therapeutic effects. Thus, patients with rapidly progressing tumors such as GBM may be relatively poor candidates for 'anti-copper' therapy as a single modality. In addition, more work has to be done to explore the precise mechanisms of action of copper-reducing agents and, to describe the effects of a long-term copper deficiency.

The results in this study were strengthened by the use of specific inclusion and exclusion criteria and prospective data collection. Due to the loss of cases in either intervention or placebo group the duration of intervention was three months. It was a limitation of our study.

Results of this study showed that nutritional interventions such as low copper diet, along with or after other treatments like surgery, radiotherapy and gamma knife radiosurgery may decrease some important angiogenic factors such as VEGF.

Conflict-of-Interest

The authors report no declarations of interest.

Acknowledgments

References

Ahluwalia MS, Chang SM (2014). Medical therapy of gliomas. *J Neurooncol*, **119**, 503-12.

Antoniades V, Sioga A, Dietrich E, et al (2013). Is copper chelation an effective anti-angiogenic strategy for cancer treatment?. *Med Hypotheses*, **81**, 1159-63.

Brat DJ, Bellail AC, Van Meir EG (2005). The role of interleukin-8 and its receptors in gliomagenesis and tumoral angiogenesis. *Neuro Oncol*, **7**, 122-33.

Brem S, Grossman SA, Carson KA, et al (2005). Phase 2 trial of copper depletion and penicillamine as antiangiogenesis therapy of glioblastoma. *Neuro Oncol*, **7**, 246-53.

Brem S, Wotoczek-Obadia M (1998). Regulation of angiogenesis by copper reduction and penicillamine: antagonism of cytokine and growth factor activity. AACR special conference: Angiogenesis and cancer research. Orlando, Fla, 1998. pp 24-8.

Brem SS, Zagzag D, Tsanaclis A, et al (1990). Inhibition of angiogenesis and tumor growth in the brain. Suppression of endothelial cell turnover by penicillamine and the depletion of copper, an angiogenic cofactor. *Am J Pathol*, **137**, 1121.

Chamberlain MC (2011). Bevacizumab for the treatment of recurrent glioblastoma. *Clin Med Insights Oncol*, **5**, 117.

Cordano A (1998). Clinical manifestations of nutritional copper deficiency in infants and children. *Am J Clin Nutr*, **67**, 1012-6.

Crowley RW, Pouratian N, Sheehan JP (2006). Gamma knife surgery for glioblastoma multiforme. *Neurosurg Focus*, **20**, 17.

Ding W-Q, Liu B, Vaught JL, et al (2005). Anticancer activity of the antibiotic clioquinol. *Cancer Res*, **65**, 3389-95.

Dwyer J, Hebda JK, Le Guelte A, et al (2012). Glioblastoma cell-secreted interleukin-8 induces brain endothelial cell

permeability via CXCR2. *PLoS One*, **7**, e45562.

Frischer JM, Marosi C, Woehrer A, et al (2016). Gamma knife radiosurgery in recurrent glioblastoma. *Stereotact Funct Neurosurg*, **94**, 265-72.

Fukumura D, Jain RK (2007). Tumor microvasculature and microenvironment: targets for anti-angiogenesis and normalization. *Microvasc Res*, **74**, 72-84.

G Linkous A, M Yazlovitskaya E (2011). Angiogenesis in glioblastoma multiforme: navigating the maze. *Anticancer Agents Med Chem*, **11**, 712-8.

Goodman V, Brewer G, Merajver S (2004). Copper deficiency as an anti-cancer strategy. *Endocr Relat Cancer*, **11**, 255-63.

Gupte A, Mumper RJ (2007). Copper chelation by D-penicillamine generates reactive oxygen species that are cytotoxic to human leukemia and breast cancer cells. *Free Radic Biol Med*, **43**, 1271-8.

Gupte A, Mumper RJ (2009). Elevated copper and oxidative stress in cancer cells as a target for cancer treatment. *Cancer Treat Rev*, **35**, 32-46.

Hanahan D, Folkman J (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell*, **86**, 353-64.

Hayashi M, Nishiya H, Chiba T, et al (2007). Trientine, a copper-chelating agent, induced apoptosis in murine fibrosarcoma cells in vivo and in vitro. *J Vet Med Sci*, **69**, 137-42.

Imber BS, Kanungo I, Braunstein S, et al (2017). Indications and efficacy of gamma knife stereotactic radiosurgery for recurrent glioblastoma: 2 decades of institutional experience. *Neurosurgery*, **80**, 129-39.

Lunsford LD, Flickinger J, Coffey RJ (1990). Stereotactic gamma knife radiosurgery: Initial North American experience in 207 patients. *Arch Neurol*, **47**, 169-75.

Moriguchi M, Nakajima T, Kimura H, et al (2002). The copper chelator trientine has an antiangiogenic effect against hepatocellular carcinoma, possibly through inhibition of interleukin-8 production. *Int J Cancer*, **102**, 445-52.

Nasulewicz A, Mazur A, Opolski A (2004). Role of copper in tumour angiogenesis-clinical implications. *J Trace Elem Med Biol*, **18**, 1-8.

Norden AD, Drappatz J, Wen PY (2009). Antiangiogenic therapies for high-grade glioma. *Nat Rev Neurol*, **5**, 610-20.

Pan Q, Kleer CG, Van Golen KL, et al (2002). Copper deficiency induced by tetrathiomolybdate suppresses tumor growth and angiogenesis. *Cancer Res*, **62**, 4854-9.

Polivka J Jr, Polivka J, Holubec L, et al (2017). Advances in experimental targeted therapy and immunotherapy for patients with glioblastoma multiforme. *Anticancer Res*, **37**, 21-33.

Rayssiguier Y, Gueux E, Bussiere L, et al (1993). Copper deficiency increases the susceptibility of lipoproteins and tissues to peroxidation in rats. *J Nutr*, **123**, 1343.

Redmond KJ, Mehta M (2015). Stereotactic radiosurgery for glioblastoma. *Cureus*, **7**, e413.

Reiss Y, Machein MR, Plate KH (2005). The role of angiopoietins during angiogenesis in gliomas. *Brain Pathol*, **15**, 311-7.

Schaafsma J, Osoba D (1994). The karnofsky performance status scale re-examined: a cross-validation with the EORTC-C30. *Qual Life Res*, **3**, 413-24.

Schmidt NO, Westphal M, Hagel C, et al (1999). Levels of vascular endothelial growth factor, hepatocyte growth factor/scatter factor and basic fibroblast growth factor in human gliomas and their relation to angiogenesis. *Int J Cancer*, **84**, 10-8.

Thumma SR, Elaimy AL, Daines N, et al (2012). Long-term survival after gamma knife radiosurgery in a case of recurrent glioblastoma multiforme: a case report and review of the literature. *Case Rep Med*, **2012**, 545492

Wadhwa S, Mumper RJ (2013). D-penicillamine and other low molecular weight thiols: Review of anticancer effects and related mechanisms. *Cancer Lett*, **337**, 8-21.

Wang L-F, Fokas E, Juricko J, et al (2008). Increased expression of EphA7 correlates with adverse outcome in primary and recurrent glioblastoma multiforme patients. *BMC Cancer*, **8,** 79.

Yoshida D, Ikeda Y, Nakazawa S (1995). Suppression of tumor growth in experimental 9L gliosarcoma model by copper depletion. *Neurol Med Chir*, **35**, 133-5.

Yoshii J, Yoshiji H, Kuriyama S, et al (2001). The copper-chelating agent, trientine, suppresses tumor development and angiogenesis in the murine hepatocellular carcinoma cells. *Int J Cancer*, **94**, 768-73.