Studies on Association Between Copper Excess, Zinc Deficiency and *TP53* Mutations in Esophageal Squamous Cell Carcinoma From Kashmir Valley, India-A High Risk Area

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Abstract:
Trace element deficiency or excess is implicated in the development or progression in some cancers. Here we report the elevated levels of copper and low level of zinc in the plasma of esophageal cancer patients in Kashmir India- a high incidence area. The average level of copper was significantly higher for patients than for controls ($p<0.0001$) with a mean concentration of 169 µg/dl and 149 µg/dl for patients and controls, respectively. In contrast, the average level of zinc in patients was significantly lower than in controls ($p<0.0001$) with a mean concentration of 86.8 µg/dl and 96.1 µg/dl for patients and controls, respectively. No significant difference in copper and zinc levels was observed for different age groups in controls or patients. For controls, the level of copper was not significantly different in males and females (median: 155 µg/dl for males and 144 µg/dl for females, $p=0.10$), but we observed a higher level of zinc in females (median: 90.5 µg/dl for males and 101 µg/dl for females, $p=0.03$). Copper or zinc concentrations were not significantly associated with gender, tumor site, green tea with salt (*nun chai)* consumption, smoking habits or snuff in cases. Patients with poorly differentiated tumors had a higher copper concentration than those with moderately or well-differentiated tumors ($p<0.0001$). No association was found between copper concentration and *TP53* mutation status but patients with *TP53* mutant tumor had lower zinc levels than those with no mutation. Our results point towards a role of the trace element imbalance in the esophageal tumorigenesis in high risk Kashmiri population exposed to a range of nitroso compounds or their precursors. Further prospective cohort studies are warranted to determine whether change in the plasma zinc and copper homeostasis may represent an independent risk factor for this malignancy as well as possible target for preventive intervention.

Abbreviations: ESCC-esophgeal squamous cell carcinoma,TE-trace elements, PCR- polymerase chain reaction

Key words: Esophgeal squamous cell carcinoma, Trace elements, copper excess, Zinc deficiency, TP53 mutations, Kashmir

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Introduction:

The contribution of diet and nutrition status to cancer risk and conversely to its prevention and treatment has been a major focus of research, as well as public health policy.[1] Trace elements (TE) are essential nutrients but are also frequent environmental dietary contaminants. Deficiency or excess in TEs like zinc has multiple carcinogenic consequences, including effects on mutagenesis, DNA repair, DNA synthesis, cell proliferation, apoptosis and differentiation, and on the global cellular anti-oxidant balance.[2] The most abundant intracellular TE, zinc, exerts preventive effects on mutagenesis and cell transformation by at least three mechanisms. First, zinc is a potent anti-oxidant agent, in particular through its capacity to bind and protect sulfhydryls in proteins from radical attack. Second, zinc is a co-factor, which regulates the catalytic activity of over 300 enzymes belonging to all major classes of mammalian enzymes. Third, zinc binds to specific residues in protein and stabilizes protein tertiary and quaternary structures.[2-7] The anti-oxidant roles of zinc include the removal of superoxide ions by superoxide dismutase impairing hydroxyl ion formation by redox-active transition metals like copper and iron [8] and the regulation of expression and activity of metallothioneins, a major class of metal and radical buffer proteins involved in many stress-responses and detoxification reactions.[2,9] Severe zinc deficiency may occur as a result of genetic defects in zinc absorption (e.g. acrodermatitis enteropathica) or as the conesquence of sub-optimal zinc intake. Low zinc status has been proposed as a risk factor that may enhance the carcinogenicity of certain nitrosamines (e.g. N -nitrosomethylbenzylamine) that act as esophageal carcinogens in rodents.[10-11] This effect has been well character-rrized in rodents (rats fed on a low-zinc diet and exposed to nitrosamines) with regards to cell proliferation[12], P450-dependent metab-olism of nitrosamines[13], alkyl guanine DNA methyltransferase activity[14] and the anticarcinogenic impact of zinc replenish-ment.[15] In contrast with zinc, copper is a redox-active TE that generates hydroxyl radicals via a Fenton-type reaction.[16-17] Excess of copper may not only promote radical damage, it may also substitute for zinc in many proteins and enzymes, thus altering their activities. It has been shown that excess copper causes cellular injury via an oxidative pathway, giving rise to enhanced lipid peroxidation, thiol oxidation and ultimately DNA damage and helps in angiogenesis of newly formed tumors.[18] In rodents (LEC rats), excess copper accumulation in the liver has been shown to result into the formation of liver tumors.[19]

Squamous cell carcinoma of the esophagus is the 6th most common cancer worldwide, with considerable geographical variations in incidence. The risk factors respo-nsible for the high incidence rates are still a matter of conjectures. Comparative studies of TEs between areas of high and low incidence for esophageal carcinoma have revealed an inverse association between mortality due to esophageal cancer and the levels of zinc, selenium, molybdenum and other TEs in crops, soil and foodstuffs.[20-22]Early studies have reported lower levels of zinc in the serum of newly diagnosed esophageal carcinoma patients than normal controls and elevated level of copper in cases.[23] In Lixian County, China, a region that has one of the highest rates of esophageal cancer, nutrition intervention trials have shown that supplementation of the diet with minerals in combination with vitamins, may cause a reduction in cancer risk.[24]

Reports and observations in Kashmir valley, in Northern India, indicate that this area has a high rate of esophageal cancer, similar to reports from several other regions of Central Asia. This high rate is associated with the exposure to dietary amines, nitrite and nitrate through unique dietary habits such as dried and smoked fish, dried and pickled vegetables, salt tea and *hukka* smoke.[25-27] Until now, there is no report on the bioavailability of TE like copper and zinc in esophageal cancer patients and normal controls. The aim of the study was to assess the plasma levels of copper and zinc from the population exposed to a range of N-nitroso moieties, and in esophageal cancer patients, in relation with the presence in their tumors of *TP53* mutations.

Methods

Blood specimens

Patients (n=55) registered in the Departments of Gastroenterology and the Cardiovascular Thoracic Surgery of Sher-i-Kashmir Institute of Medical Sciences Srinagar,

Jammu and Kashmir, India, between July, 2002 and July, 2003 for treatment of primary oesophageal cancer were recruited after informed consent. Information regarding dietary habits, lifestyle and family history of disease was collected using questionnaire. Heparinized blood samples were collected from all the patients as well as from healthy controls matched for place of residence, age, sex and dietary habits. Plasma was separated and stored at –80^{o}C before analysis of TEs. The demographic details and tumor characteristics of the patients have been omitted for brevity.

Trace element estimation

Copper and zinc were analysed by double beam atomic absorption spectrophotometry (GBC 902, Brooklyn, Australia), perfo-rmed essentially according to Mc Master *et al.*[28] Reagents used were trichloroacetic acid 20% (Merck), copper atomic absorption standard solution (1000 µg copper per ml in 1.0 % HNO_3) (Sigma), zinc atomic absorption standard solution (1000 µg zinc per ml in 1.9% HCl) (Sigma). The instrument was calibrated with zinc or copper standards of 50, 100, 150, 200 and 300 µg/dl and acetylene-air flame was used for excitation. The cylinder and line pressure (kpa) were maintained at 100 and 95 respectively. The slit width was 0.5 nm and sample uptake was optimized to 5ml/min. The λ_{max}, lamp current, working range and the sensitivity were 324.7nm, 3.0mA, 1-5 µg/ml and 0.025 µg/dl for copper and 218.5 nm, 5.0 mA 0.4-1.5 µg/ml and 0.008 µg/dl for zinc respectively . Two ml of plasma were treated with equal volume of 20% Trichloroacetic acid, vortexed and stored at 4^{o}C overnight in parafilm sealed, metal-free glass tube. The reaction mixture was then centrifuged at 1500 g for 30 min and the supernatant was used for estimation of TEs. Each sample was run in duplicate and results were expressed as the mean of the two analyses.

DNA extraction, PCR, Sequencing

Fragments of biopsies and pieces of resected tumor were used for isolation of genomic DNA, PCR amplification of *TP53* exons 5 to 8 and sequencing as described previously.[29]

Statistical analysis

Plasma levels of copper and zinc were compared between patients and controls by using *t test*. To asses potential modification of plasma levels of copper and zinc, we tested correlation with age and local tea consumption. The differences of concentration of copper and zinc in relation to gender, smoking habits, snuff, clinical characteristics and *TP53* status of the tumor were analyzed by a non-parametric Kruskall-Wallis test.

Results

This study comprised of 55 patients and equal number of controls with a distribution of 36 males and 19 females. Sixteen (29%) were smokers and 15 (27%) consumed snuff. In most of the patients (56%) the lower third and middle of the esophagus was involved whereas the upper third was involved in only 2 cases (3.6%).The tumors were poorly differentiated in 7 (12%), moderately differentiated in 36(65%) and well differentiated in 12(21%) patients respectively. Copper and zinc levels in the plasma of patients and controls matched on age and sex were estimated spectrophotometrically. The average level of copper in patients was significantly higher for patients than for controls ($p<0.0001$) with a mean concentration of 169 µg/dl and 149 µg/dl for patients and controls, respectively. In contrast, the average level of zinc in patients was significantly lower than in controls ($p<0.0001$) with a mean concentration of 86.8 µg/dl and 96.1 µg/dl for patients and controls, respectively. No significant correlation of copper and zinc levels with age was observed in patients as well as controls. For controls, the level of copper was not significantly different in males and females (median: 155 µg/dl for males and 144 µg/dl for females ($p=0.10$), but we observed a higher level of zinc in females (median: 90.5 µg/dl for males and 101 µg/dl for females, $p=0.03$). No significant difference was found in copper or zinc levels between male and female patients. Similarly copper or zinc concentrations were not significantly associated with local tea consumption, smoking habits or snuff in patient group (Table 1).

Patients with poorly differentiated tumors had a higher copper concentration than those with moderately or well-differentiated tumors ($p<0.0001$, Table 2).

Table (1). The comparison of median values of Copper and Zinc for cases by demographic and habit characteristics. The copper and zinc concentrations are presented as median with Interquartile range in brackets. P values are from t-test between zinc or copper concentration and respective variables like age, sex, smoking habits, snuff and salt tea consumption. *Age and salt tea consumption were available as continuous variable.

Variable		Number of cases in each group (Total cases = 55	Copper(µg/dl)	*p* value	Zinc(µg/dl)	*p* value
Age	<60 years	27	163 (147-195)	0.69*	86 (80-94)	0.24*
	>60 years	28	165 (147-194)		84 (80-92)	
Sex	Males	36	166 (147-194)	0.91	86 (80-93)	0.42
	Females	19	162 (146-195)		84 (78-92)	
Smoking habits	Non Smokers	16	152 (140-184)	0.18	85 (79-94)	0.73
	1-5 cigarettes/ day	3	179 (148-195)		80 (70-90)	
	6-10 cigarettes/day	12	160 (148-174)		88 (79-95)	
	11-15 cigarettes/day	24	175 (149-205)		86 (81-91)	
Snuff consumption	No	40	164 (147-197)	0.47	86 (82-93)	0.12
	Yes	15	162 (146-179)		81 (79-90)	
Salt tea consumption	Details not available	7	149 (139-168)	0.28*	90 (84-107)	0.36*
	Less than 1000 ml	23	162 (146-188)		85 (80-91)	
	Between 1000 ml and 1499ml	12	185 (164-207)		87 (82-94)	
	1500 ml and more	13	162 (148-180)		86 (78-94)	

Table (2). The association of copper and zinc concentrations with tumor grade, site and TP53 mutant status in 55 esophageal squamous cell carcinoma patients. The copper and zinc concentrations are presented as median with Interquartile range in brackets.

Variable		Number of cases in each group	Copper(µg/dl)	*p* value	Copper(µg/dl)	*p* value
Tumor Grade	Poorly differentiated	7	206 (202-209)	<0.0001	86 (79-91)	0.92
	Moderately differentiated	36	154 (146-170)		85 (81-92)	
	Well differentiated	12	165 (145-179)		84 (78-104)	
Site of the tumor	Lower third	31	165 (146-188)	0.23	86 (81-96)	0.51
	Middle third	22	165 (148-195)		86 (80-91)	
	Upper third	2	133 (119-148)		88 (76-100)	
TP53 status						
	Wild type	35	163 (146-200)	0.69	88 (93-94)	0.03
	Mutant	20	161 (147-179)		81 (77-88)	

observed according to tumor grade (p=0.92). The levels of serum copper were not significantly different in patients with wild type and mutant tumors while as patients with *TP53* mutant tumor had lower zinc levels than those with no mutation (p=0.03, Table 2). Copper and zinc concentrations were not found to be associated with site of tumor as well.

TP53 *mutations*

All tumors were analysed for *TP53* mutation in exons 5-8; which contain over 90% of all mutations reported in oesophageal cancers. These results have originally been reported in Mir et al., 2005.[29] Mutations were detected in 20 of the 55 ESCC patients (36.4%), including 3 (15%) deletions, 5 (25%) transitions or transversions at A: T base pair, 6 (30%) G: C>A: T transitions (2 (10%) of which at CpG sites), 3 (15%) G: C>C: G transversions and 3 (15%) G: C>T: A transversions. The majority of these mutations were missense (12/20, 60%). Eleven of the 20 patients harbouring a mutation were females (p=0.016), suggesting a gender bias since they represent only 34 % of all the patients. Moreover, mutations were more prevalent in females categorized as heavy smokers (who smoked over 275-375g of tobacco a day ; 3/4, 75%) than in male heavy smokers (3/20, 15%).[29]

Discussion

Kashmir valley, located in the extreme north of India, is a high-risk area for esophageal cancer with an incidence of 27 and 42 in females and males, respectively, per 10^5 individuals per year.[30] Suspected sources of environmental risk factors include various dietary carcinogens and their *in vitro* effects.[31-34] Our results show significantly elevated levels of plasma copper in patients as compared to controls (p<0.0001). In contrast, the average level of zinc in patients was significantly lower than in controls (p<0.0001). The fact that the levels of these metals vary according to different patterns suggests that tumor development is associated with an imbalance in TE metabolism. Low levels of zinc may decrease the overall anti-oxidant defences, whereas increased levels of copper, a redox-active metal, may enhance the production of reactive oxygen species and the generation of radical-induced tissue, cellular and genetic damage. Whether this imbalance pre-exists and affects tumor development (and may thereby play a causal role) or is the reflect of metabolic changes in cancer patients, remains to be assessed. On one hand, an increase in the mean copper and copper: zinc ratio in serum has been reported in most malignancies, as well as decreased zinc level in patients with advanced metastasis.[35] On the other hand, the lifestyle of the subjects recruited in the present study may have a profound influence on their TE status.

Most of the patients and controls are from rural areas and have low socio-economic status, two factors that have been described as being strongly associated with the risk of esophageal cancer in other high-risk areas in Asia.[36] It is interesting to note that their lifestyle involves frequent and repeated use of artefacts made from elemental copper. Most of the daily foodstuffs, including the local salty tea, are commonly prepared and served using the copper utensils. Water and milk samples stored in copper vessels, as well as salt tea prepared in copper vessels, were found to contain markedly high levels of copper, which in turn may explain the high plasma copper levels.[37] On the other hand, plasma TE levels may also be influenced by the dietary patterns of the subjects recruited, which is characteristic of their low-socio-economic status. It has been shown that subjects who eat relatively little meat and large quantities of whole grains are more likely to be zinc deficient and copper sufficient than those who eat more meat and more refined grains.[38-39] Thus low meat and high whole cereals consumption, as well as frequent use of copper utensils, may contribute to the high copper, low zinc status of cases and controls. In areas of China with high incidence of esophageal cancer, the average intake of zinc has been reported as being low (72% of RDA)[40] and to be positively associated with a higher risk of esophageal squamous cell carcinoma[41-42], while individuals who take sufficient zinc were less likely to develop esophageal cancer.[43] Intervention studies have shown that dietary supplementation with zinc and selenium reduces the incidence and mortality of esophageal cancer.[44] And this may be the reason that females are at lower risk in valley as they show average high value of zinc in control groups. Our results are in agreement with earlier data on zinc deficiency in cancer patients in high-risk populations.[45-47] Low levels of zinc in blood plasma have been reported in some other parts of Indian subcontinent as well.[48]

Poorly differentiated esophageal squamous cell carcinomas are most aggressive and have a worse prognosis than well differentiated ones and our results point to a possible association between high copper status and poorly differentiated tumor status ($p<0.0001$). This observation suggests accumulation of copper may influence tumor phenotype. A recent study has suggested that copper accumulation may enhance angiogenesis.[49] Interestingly, we observed a probable association between low zinc status and *TP53* mutant tumors ($p=0.03$). This observation is compatible with the notion that imbalance in zinc and copper levels may either increase mutagenic DNA damage (e.g. G: C > C: G; through radical damage to DNA) or decrease DNA repair mechanisms, both phenomena resulting in a higher prevalence of *TP53* muta-tions. Current data are too limited to assess whether these mechanisms may account for the pattern and distribution of TP53 mutations in this case series.

In summary, the results presented here indicate that an imbalance in plasma levels of zinc and copper may have an association with oesophageal tumorigenesis in the population of the Kashmir Valley. Further, prospective cohort studies are now required to determine whether changes in plasma zinc and copper homeostasis may represent a risk factor for this cancer, as well as a possible target for preventive intervention.

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