

Full Length Research Paper

Evaluation of serum level of some angiogenic factors in non hodgkin's lymphoma

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Accepted 24 September, 2012

Background: Angiogenesis is a prerequisite for growth of tumors whether solid or liquid tumors. The process of angiogenesis is very complex and tightly regulated by positive and negative regulatory factors, the precise role of these processes in lymphoma pathogenesis is under active investigation. This work aimed at assessing serum levels of certain angiogenic factors in cases of non Hodgkin's lymphoma (NHL) whether newly diagnosed or relapsed and there correlations to disease progression and staging. **Methods:** Serum levels of certain positive regulatory factors for angiogenesis namely "Angiogenin, Nitric oxide, Copper", and Zinc as a negative regulatory factor were assayed, and Copper / zinc ratio were determined in 57 patients of NHL classified into four groups according to the disease stage investigated before the start of chemotherapy and 20 healthy controls. **Result:** Patients in 1st group (stage I and II) showed significant elevation in serum levels of angiogenin, copper, and insignificant changes occurred in serum levels of zinc, nitric oxide and in copper zinc ratio in comparison with the control group. On the other hand; patients in 2nd group (stage III) and 3rd group (stage IV) showed highly significant increase in serum levels of copper and copper zinc ratio; while insignificant changes occurred in serum levels of angiogenin and nitric oxide. Conversely, highly significant decrease occurred in serum levels of zinc in 2nd group only. Patients in 4th group (relapsed cases) showed highly significant increase in serum levels of copper, significant increase in serum levels of angiogenin and in copper/inc ratio, while insignificant changes occurred in serum levels of zinc, and nitric oxide. The comparison between different patients groups revealed no significant differences in all special investigations except for zinc where there was a significant lower level of zinc in 2nd group than 1st group, and for copper and copper/zinc ratio; there were significant rise of each in 4thgroup in comparison to 1st group. **Conclusion:** The serum angiogenin and copper levels may play an important role in early detection of NHL as it increased significantly in early stages, the highest levels were found in advanced cases together with low zinc level suggesting their role in follow up of NHL together with consideration of copper /zinc ratio while limited role of nitric oxid had been observed.

Keywords: non hodgkin's lymphoma, angiogenin, copper

INTRODUCTION

Angiogenesis, the sprouting of new capillaries from preexisting ones, is an important component in many physiological and pathological processes which requires dynamic expansion, assembly and stabilization of vascular endothelial cells in response to proangiogenic

stimuli. In hypoxia; tissues reacts in two ways : they switch into a protective way by producing a specific set of hypoxia sensing proteins called hypoxia-inducible factors (HIFs); the other way is by producing angiogenesis proteins that will induce blood vessels

formation hence restoration of local blood flow (Ruan et al., 2009).

In cancer, active angiogenesis is a prerequisite for tumor growth beyond a few cubic millimeters in size. In addition, antiangiogenic strategies have become an important therapeutic modality for solid tumors. Angiogenesis is regulated by a balance of various positive (e.g., angiogenin) and negative regulatory factors (e.g., Zinc). Increased levels of positive regulatory molecules have been correlated with poor prognosis in patients with solid tumors (Giles et al., 2004).

While many aspects of postnatal pathological angiogenesis have been extensively studied in the context of nonhematopoietic neoplasms, the precise role of these processes in lymphoma pathogenesis is under active investigation (Fang et al., 2011).

Lymphoma growth and progression is potentiated by at least two distinct angiogenic mechanisms: autocrine stimulation of tumor cells via expression of vascular endothelial growth factor (VEGF) and VEGF receptors by lymphoma cells, as well as paracrine influences of proangiogenic tumor microenvironment on both local neovascular transformation and recruitment of circulating bone marrow-derived progenitors (Jørgensen et al., 2007). These distinct angiogenic mechanisms appear to be important therapeutic targets in selected non-Hodgkin's lymphoma (NHL) subtypes (Ruan et al., 2009).

Angiogenin (Ang), a potent inducer of neovascularization, normally circulates in human plasma in a concentration of 250-360 ng/ml. Its relatively high concentration in plasma may provide prompt repair of blood vessel damage caused by a variety of physical, chemical and pathological mechanisms as trauma, oxidative stress and ischemia (Suzumori et al., 2004).

High level of Ang correlate with a poor prognosis in patients affected with acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS). Also elevated blood levels of Ang feature chronic myeloid leukemia (CML) and essential thrombocytosis suggesting the role of Ang in the pathogenesis of the disease (Musolino et al., 2004).

Copper is one of the trace elements found in the serum in a concentration of 70-140 µg/dl in adult men and 80-155 µg/dl in women. It acts as an obligatory cofactor and is permissive to the angiogenic activator; copper is an integral component of many metalloenzymes which involved in oxidation-reduction reactions, including ceruloplasmin, cytochrome C oxidase, superoxide dismutase, etc (Brewer, 2001).

Moreover; it was noticed that angiogenin binding capacity increased in the presence of Copper in vitro. Serum copper level can be used as prognostic indicator for monitoring disease activity and response to treatment in malignant lymphoma. They also can be used as predictors of successful treatment in pediatric patients

with lymphoma (Juarez et al., 2006).

Nitric oxide (NO) is an important mediator of both physiological and patho-physiological process. NO is produced by Nitric oxide synthase (NOS) an enzyme that exists in three isoform (Neuronal, Inducible, and Endothelial) encoded by distinct genes (Roberts et al., 2007).

Nitric oxide lays a central role in angiogenesis as a mediator of signaling by VEGF and other angiogenic factors as well as acting as a downstream effector of VEGF action. There is evidence that NO is involved in both tumor angiogenesis and in immune cell killing of tumor cells and, therefore, can have positive and negative effects on tumor progression. At a relatively low concentration, NO can protect tumor cells from apoptosis, stimulate tumor angiogenesis and increase tumor blood flow thus promoting tumor growth. At higher levels, it can induce apoptosis and arrest tumor growth (Uneda et al., 2003).

Zinc is not only an important nutrient, cofactor of numerous enzymes and transcription factors, but also it acts as an intracellular mediator, similarly to calcium (Chimienti et al., 2003).

Zinc intracellular concentration is correlated to cell fate, ie, proliferation, differentiation or apoptosis. Red blood cells contain 6 to 8 times the amount of zinc as blood plasma, which is around 100 µg/dl. White blood cells contain up to 25 times the amount of zinc in the serum. Mast cells and basophils contain extremely high amounts of zinc, being found in granules with histamine (Guntupalli et al., 2007).

Leukemic cells contain only about 10% of the zinc contained in normal lymphocytes. Plasma zinc concentrations are lower and plasma copper concentrations are higher in children with untreated acute lymphoblastic leukaemia (ALL) than in the same children after successful treatment or healthy children in NHL (Rosas et al., 1995) and Hodgkin's disease (HD) (Cunzhi et al., 2001).

Copper/zinc ratio has a diagnostic and prognostic value in solid tumors as well as hematological tumors. Copper/Zinc ratio is higher in patients with lymphoma or acute and chronic leukemias compared to gender and age-matched control subjects. Both the serum copper level and copper/zinc ratio were correlated to histopathological changes, clinical stage, and prognosis of HD (Cunzhi et al., 2001).

In the current study we determine the serum levels of some known angiogenic stimulators as angiogenin, copper and nitric oxide and serum level of zinc as one of the angiogenic inhibitors and determine copper/zinc ratio in newly diagnosed and relapsed patients with NHL to study the significance of the previous factors in relation to disease progression and staging.

Table 1. Data of patients and controls

	Patients	controls
Total number	57	20
New n(%)	44 (77)	
Relapsed n(%)	13 (23)	
Age range(yrs)	18- 78	20- 45
Mean \pm SD	45.7 \pm 18.7	32.3 \pm 8.4
Sex n(%)		
Males	47(82)	12 (60)
Females	10 (18)	8 (40)
Newly diagnosed n(%):	44(77)	
*Stage I or stage II	9(16)	
*Stage III	17(30)	
*Stage IV	18(31)	
Relapsed cases n(%)	13 (23)	

* Stage of malignancy (Ann Arbor staging), *SD*= standard deviation

MATERIALS AND METHODS

The present study was performed on fifty seven patients with NHL attending both of South Egypt Cancer institute and Mabara health insurance hospital in Assiut and 20 apparently healthy controls (Table 1).

Careful clinical history, routine investigation [Complete blood picture, Liver function tests, Renal function tests, uric acid and lactate dehydrogenase (LDH)] were carried out on all patients and controls. Diagnostic and staging investigations were done in the form of: Chest X-ray, abdominal ultrasound, Computerized tomography (CT), Bone marrow aspiration and/or biopsy were done for all the cases, lymph node biopsy or splenic aspirate if needed.

Special investigations were done included assay of serum level of : Angiogenin [by enzyme linked immunosorbent assay (ELISA) technique using Quantikine Human angiogenin ELISA kit–UK], Copper and Zinc[by Colorimetric technique using (Spinreact kits-Spain) Prime semi automated colorimeter], and Nitric oxide [by colorimetric technique micro method using Stat Fax ELISA reader].

Statistics

Data was entered and analyzed using SPSS version 13. Numeric data was expressed as mean \pm Standard deviation (SD). Statistical significance was calculated using t-test for numeric data. P value less than 0.05 was considered significant while p value less than 0.001 was considered highly significant.

RESULTS

This study comprised (57) patients with NHL [44 (77%) were newly diagnosed cases and 13 (23%) were relapsed cases(after 6 months interval of complete remission)] classified into four groups: group 1 consisted of 9 (16%) patients in stage I or II, group 2 consisted of 17 (30%) patients in stage III, group 3 consisted of 18 (31%) patients in stage IV, and 13 (23%) relapsed cases as group 4.

Assay of the serum level of angiogenin

Only group 1 and 4 showed significant elevation in comparison to control group ($p < 0.05$) (Table 2), (Figure 1). Comparison between the different groups of patients showed no significant differences between them as regard Ang level.

Assay of the serum level of copper

Showed significant elevation in group 1 and highly significant elevation of the serum copper in groups (2, 3, and 4) of patients in comparison with control group; and maximal elevations observed in group 4 ($p < 0.001$) (Table 2) , (Figure 2). Comparison between the different groups of patients showed only a significant difference between group1 and 4 in the serum copper level.

Assay of the serum level of zinc

Showed highly significant decrease only in group 2

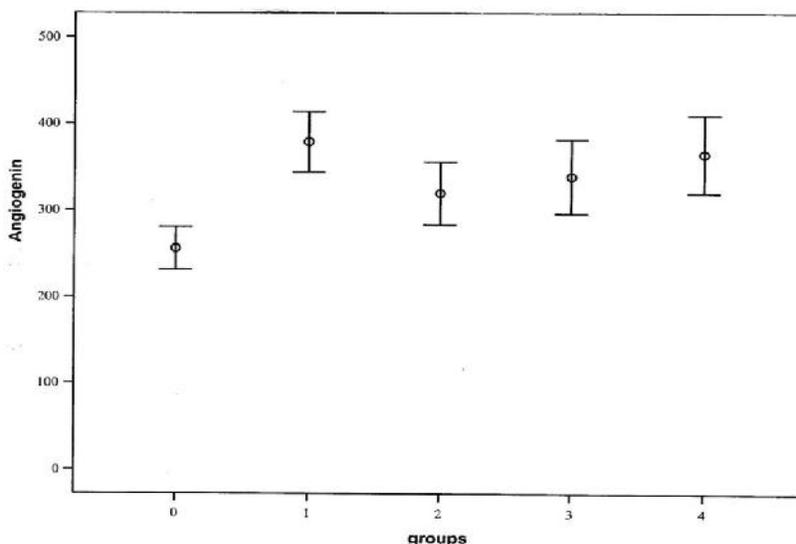


Figure 1. Mean serum level of Angiogenin (ng/ml)±SD in patients(group 1-4) and controls (group 0)

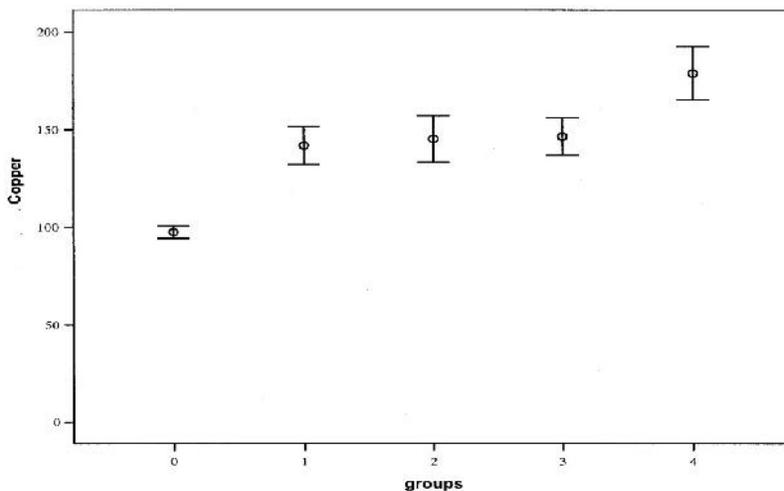


Figure 2. Mean serum level of Copper (µg/dl)±SD in patients(group 1-4) and controls (group 0)

compared to control group ($p < 0.001$) while no significant difference between each of 1st, 3rd and 4th groups with the control group ($p > 0.05$) (Table 2), (Figure 3). Comparison between the different groups of patients showed only a significant difference between group 1 and 2 in the serum zinc level.

Assay of the copper/zinc ratio

These values highly significant elevation in group 2, and 3 compared with control group ($p < 0.001$). Significant

elevation of copper/zinc ratio in group 1 and 4 ($p < 0.05$) compared to control group (Table 2), (Figure 4). Comparison between the different groups of patients showed a significant difference between group 1 and 4 in the copper zinc ratio.

Assay of the serum level of nitric oxide

Showed no significant difference between control group and patients' groups (Table 2), (Figure 5) and between the different groups of patients as regard NO level.

Table 2. Serum levels of studied angiogenic factors in patients groups and control groups.

	Group1 (n= 9)	Group 2 (n= 17)	Group 3 (n=18)	Group 4 (n=13)	Controls (n=20)
Angiogenin (ng/ml) mean±SD	378.6±103.9*	319.5 ± 149.2	338.7±180.6	346.7±161.6*	256.2 ± 89
Copper (µg/dl) mean±SD	141.8 ± 29.5*	145.2 ± 48.8**	146.4± 41.1**	179 ± 49**	97.5 ± 14.2
Zinc (µg/dl) mean±SD	85.7 ± 32.4	67± 32.6**	95.8 ± 59.5	96 ± 47.3	107.2±39.2
Copper /Zinc mean±SD	1.8 ± 0.6*	2.8 ± 1.9**	1.8 ± 0.8**	2.6 ± 2.7*	1 ± 0.5
NO (µM/L) mean±SD	21.1 ± 5.1	22.6 ± 5.4	19.4 ± 6.8	20.6 ± 9.6	21.2 ± 6.5

* Significant, ** highly Significant, SD= standard deviation

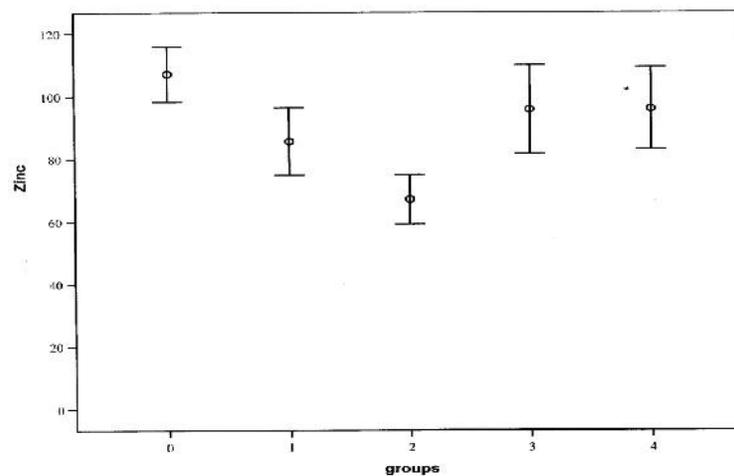


Figure 3. Mean serum level of Zinc (µg /dl)± SD in patients (group1-4) and controls (group 0)

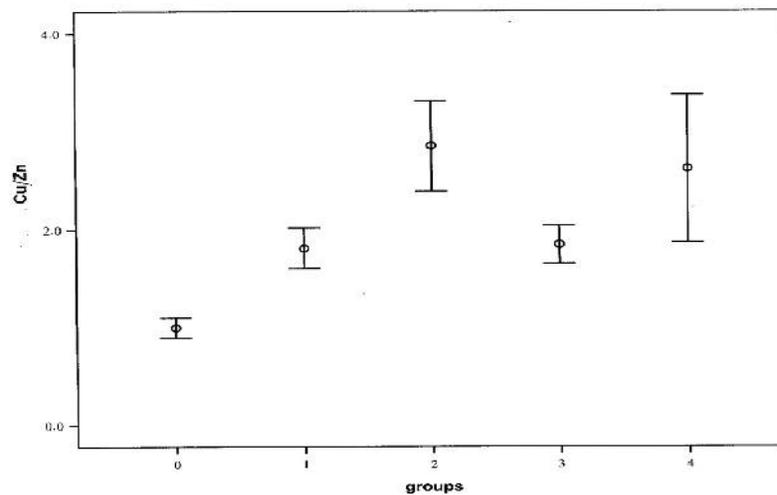


Figure 4. Mean serum level of cu/zn ratio±SD in patients (group 1-4) and controls (group 0)

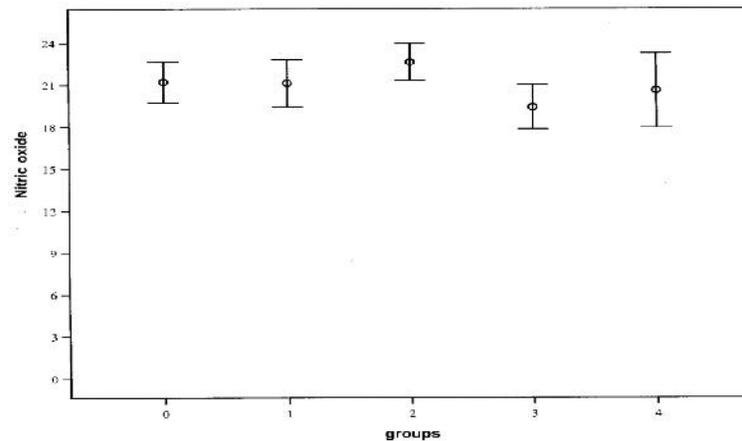


Figure 5. Mean serum level of Nitric oxide ($\mu\text{M} /\text{L}$) $\pm\text{SD}$ in patients (group 1-4) and controls (group 0)

DISCUSSION

Normal tissues receive a constant blood supply of oxygen carried by continuous flow of blood. Tissues can survive a hypoxic state for longer than others depending on their individual metabolic requirements. Growing evidence has demonstrated that control and modulation of angiogenic activity is important for the development, repair and growth of normal and abnormal tissues including cancer tissues. Antiangiogenic therapy could therefore play an important role in the clinical treatment of number of diseases including cancer, arthritis, psoriasis and diabetic retinopathy (Passam et al., 2008).

Angiogenesis, is an angiogenic stimulator, its serum level had been widely estimated in solid tumors as well as hematological malignancies. The serum angiogenesis was significantly higher in advanced MDS and AML than healthy individual but it was higher in advanced MDS than AML. Higher level of angiogenesis correlated with prolonged survival periods in both AML and advanced MDS (Verstovsek et al., 2001). Molica et al (2004) found that there were no significant differences between controls (median 264 ng/ml) and patients with B-cell chronic lymphoid leukemia (CLL) (median 295 ng/ml) but noticed that increased angiogenesis level was associated with high LDH and 2-microglobulin concentrations. However, it did not reflect the extent of bone marrow (BM) angiogenesis as evaluated by microvessel area and circulating VEGF and FGF-2. No correlation was found between serum angiogenesis and cytogenetic aberrations. Conversely, in CML, serum angiogenesis level was significantly higher than normal controls (Musolino et al., 2004).

Although the study of serum angiogenesis levels in both hematological and non hematological malignancies attracted so many authors, few studies were done on

cases of NHL.

Giles et al in 2004 measured levels of VEGF & FGF and angiogenesis in patients with NHL and patients with HD before and after therapy. Angiogenesis levels were significantly lower in both NHL and HD patients than in healthy individuals fell significantly after treatment in all patients.

Recent study revealed that elevated serum angiogenesis surfaced as an independent predictor for failure in long-term treatment response and for poor overall survival in NHL patients (Fang et al., 2011).

In this study, the mean level of angiogenesis in total patients' group was insignificantly higher than normal controls (345.2 ± 154.5 ng/ml; 256.2 ± 89.1 ng/ml respectively; $p > 0.05$).

Bertolini et al (1999), evaluated the circulating levels of some angiogenic growth factors including angiogenesis in NHL patients in complete remission and relapsed patients. They found there was no significant difference in patients in complete remission and relapsed cases in serum level of angiogenesis.

Angiogenesis seems to have limited value in angiogenesis in NHL in contrast to non hematological malignancies, the explanation of that is due to the differences in angiogenesis pathways and in the characteristics of blood vessels between lymphomas of various histologic subtypes and non hematological malignancies. Several authors reported that blood vessels in lymph nodes in follicular lymphoma are predominantly in the interfollicular areas with relative low vascularity in the neoplastic follicles (Arias and Soares, 2000; Ridel and Norrby, 2001). This pattern of vascular distribution is similar to that found in reactive lymph nodes and is lost in aggressive lymphoma (Stewart et al., 2002). The second reason is the phenotype of blood vessels in different subtypes of lymphoma seems to vary.

Overrepresentation of disorganized immature blood vessels characterized by a lack of pericytes has been found in aggressive lymphomas and HD compared with reactive lymph nodes and indolent lymphoma (Hyjek et al., 1999). Using different markers to assess blood vessel maturity showed a higher number of immature blood vessels in diffuse large B-cell lymphoma than in reactive nodes or follicular lymphoma (Passalidou et al., 2003).

In this study, significant elevation was found in group 1 (stage I and II) and group 4 (relapsed cases; $p < 0.05$) but insignificant changes occurred in group 2 and 3 in comparison with the control group.

Serum angiogenin levels in this study are negatively correlated with the disease stage as they were higher in stage I and II than both III and IV. This agreed with what was done by Molica et al. (2004) who found that serum angiogenin levels did not reflect the extent of BM angiogenesis evaluated by microvessels density.

The controversy between the different authors and the current study is due to the heterogeneity of the subtypes of NHL in the studied groups with different histologic subtypes and different grades of malignancies and subsequently had different angiogenic profile (Jørgensen et al., 2007).

Another contributing factor in angiogenesis is that angiogenin expression is correlated with macrophage infiltration as it is induced by inflammatory cytokines derived from infiltrated macrophages (Etoh et al., 2000). Mazur et al. (2004) studied the macrophage antigen CD-68 expression in neoplastic NHL and reactive lymph nodes by immunohistochemical. They reported that macrophage infiltration was statistically significantly higher in aggressive NHL than in indolent NHL as indicated by an increased number of CD-68 positive macrophage.

Copper ions stimulate proliferation and migration of endothelial cells. It has been shown that serum copper concentration increases as the cancer progresses and correlates with tumor incidence and burden. Copper ions also activate several proangiogenic factors as VEGF, tumor necrosis factor (TNF)- and interleukin (IL)-1. It also enhances binding of angiogenin to endothelial cells (Nasulewicz et al., 2004).

Numerous studies since the 1970s established excess copper and high copper /zinc ratio as prognostic indicators for lymphoma where higher levels correspond to more aggressive disease as the most aggressive tumors are the highly vascular (Rak et al., 2006) but now it is known that the elevation of serum copper in patients is due to tumor growth and associated angiogenesis which needs copper as an important element in stimulation of endothelial growth, proliferation and activation of other angiogenic factor. The increase of serum copper in late stages is due to increased tumor burden.

In this study, we found significant elevation in all groups of patients but maximal elevation was found in 4th group (relapsed cases), also the increase in serum copper is correlated with the disease stage, higher level was observed among patients of the 4th group (mean value $179 \pm 49 \mu\text{g/dl}$; $p < 0.001$). These results agreed with the results of Gozdazoglu et al., 1982 who performed their study on patients of untreated pediatric NHL the mean copper level was significantly higher than controls. Many researchers studied the copper level in patients with untreated malignant lymphoma; they found that serum copper level was significantly higher before treatment and patients in stage III and IV had higher serum copper level (Wu, 1988; Gupta et al., 1994; and Rosas et al., 1995).

Zinc helps block the absorption of copper and acts to remove accumulated copper from the body as well as prevent its accumulation (Rosas et al., 1995).

In this study, there was a decrease in the serum zinc levels in patients groups compared to controls but this was highly significant only in stage III (group 2) (67.1 ± 32.6 $p < 0.001$), and this agreed with previous works (Gozadazoglu et al., 1982; Gupta et al., 1994; Rosas et al., 1995) While Wu in 1988, found no significant difference between patients and controls.

In this study, the mean copper /zinc ratio showed highly significant elevations group 2, and 3 (mean 2.8 ± 1.9 , 1.8 ± 0.8 ; $p < 0.001$) and significant elevation in group 1 and group 4 (mean 1.8 ± 0.6 , 2.6 ± 2.7 ; $p < 0.05$) compared to controls. This result agreed with Gozdozoglu et al., 1982; Gupta et al., 1994; and Rosas et al., 1995: they found significant higher levels of copper/zinc ratio in patients than controls. On the other hand; Wu, 1988 noticed that copper/zinc ratio in patients in stage III and IV was higher than that measured in patients in stage I and II.

Nitric oxide (NO) mediates a diverse array of biological activities, including vasodilatation, neurotransmission and immune defense. Insignificant differences between controls and patients in serum NO levels were found in renal cell carcinoma and even lower than normal but still insignificant in stage 3 and 4 (Sozen et al., 2004). This support the theory that lower concentration of NO promotes angiogenesis and hence tumor growth (Carmeliet and Jain, 2000). Conversely, higher levels of NO were observed to be correlated with tumor burden in gastric cancer (Eroglu et al., 1999). In metastatic cancer breast, mean plasma NO level was 20 folds increased in comparison to mean control level (Gaballa et al., 2001).

In the current study, the mean NO level was high in patients in stage III but not significant. Also in the other groups there were no significant differences between controls and patients. This may be due to the lower expression of NOS 3 in cases of NHL as stated by Mondes et al (2001); so the NO levels are not suspected to be high in cases of NHL.

CONCLUSION

The serum angiogenin and copper levels have a diagnostic role in detection of conditions with increased angiogenesis as malignancy. Also serum copper, can be used to detect progression of NHL as marked increase occurred with advanced stages. Moreover, they have a value in follow up cases of NHL after complete remission as the highest levels were found in relapsed cases. Conversely, decrease in serum zinc level has bad prognosis. Monitoring copper/zinc ratio have prognostic role than copper only as increased copper/zinc ratio occurred in advanced stages.

The serum level of nitric oxide seems to have limited role in diagnosis and follow up of cases of NHL, however, larger sample size together with the estimation of tissue levels in lymph nodes or bone marrow are still required.

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