The Evaluation of the Relationship between Bladder Cancer and Oxidative Stress Using NRF-2/KEAP-1 Pathway, Zinc and Copper Levels

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Purpose: It has been shown that Copper and Zinc contribute to the structure of the antioxidant enzymes. In addition, NRF-2 and KEAP-1 complex have a powerful effect on the intracellular organization of the antioxidants. We evaluated the relation of Copper, Zinc, NRF-2, and KEAP-1 complex regarding the oxidative stress with tumor stage - grade in patients with bladder cancer.

Materials and Methods: A total of 52 patients (32 bladder cancer and 20 control group) were included in the study. The demographic properties of groups were identical. Serum NRF-2, KEAP-1, Cu, and Zn levels were compared by ELISA method between the groups, and tissue NRF-2, KEAP-1, Cu and Zn levels were evaluated also by ELISA method in cancer patients.
Results: Serum levels of NRF-2 and KEAP-1 of the bladder cancer patients were found to be higher than the control group ($p = 0.004$ and $p = 0.001$, respectively). On the other hand serum levels of Copper and Zinc were found to be lower than the control group ($p = 0.008$ and $p = 0.001$, respectively). However, the subgroup analysis according to the stages and grades of the tumour showed no difference. The Copper level obtained from the tissue analysis was detected to be considerably decreased with tumour stage and grade.

Conclusion: Bladder cancer patients had higher serum NRF-2 and KEAP-1 levels and lower serum Copper and Zinc levels. In addition, the Copper levels decreased with the tumour stage and grade. Studies with larger number of patients are needed to demonstrate the efficacy of these markers.

Keywords: bladder cancer; NRF-2; KEAP-1; urinary markers

INTRODUCTION

Bladder cancer (BC) is the 9th most frequently detected and 13th most-lethal common malignancy worldwide. In addition, non-muscle invasive bladder cancer (NMIBC) accounts approximately for 75% of the patients at admittance[1]. The important risk factors known for the aetiology include smoking, exposure to aromatic amines and polycyclic hydrocarbons, genetic predisposition, and chronic irritation[2]. The gold standard method for the diagnosis and treatment of BC is cystoscopy. Although several diagnostic markers have also been identified to minimize this invasive procedure, none of them could get ahead of cystoscopy[3-4].
Exposure to toxic metabolites such as aromatic amines and oxidative stress appear to be important factors in carcinogenesis. The changes caused by these factors at the cellular level lead to the initiation of the oncological process and chronicity. Reactive oxygen radicals (ROR) are the most important radical type formed by living systems originated from the reduction of the molecular oxygen[5]. ROR play a significant role in the different complex course of carcinogenesis. A higher value of ROR concentration is associated with the presence of tumours. There are a lot of epidemiologic evidences for ROR in cancer pathogenesis. ROR have also close relationship with BC. It has been demonstrated that ROR is one of the important reasons for the presence and recurrence of BC[6,7].
When the cell is exposed to oxidative stress, it activates its own antioxidant systems in the first stage. These systems might be mediated via enzymatic or non-enzymatic pathways, and exert activity to limit the catabolic effects of the ROR[8]. Trace elements in the structure of the enzymes responsible for the antioxidant system as co-factors are as functionally important as the enzymes themselves against oxidative stress. The imbalance of these elements may lead to lack of limitation of the oxidative stress[9]. Copper and Zinc are the essential molecules which contribute to the structure of the antioxidant enzymes as co-factors and play an important role in the metabolism of oxidative stress[10]. The abnormal changes in the levels of the trace elements may cause mutation and cancer by affecting the structural characteristics of the antioxidant enzymes[11]. The presence of an association between trace elements and BC is also observed in the previous studies. The imbalance of these important trace elements might play an important role in BC induction[12].
Total antioxidant capacities have close relationship in the presence of carcinogenesis. Novel studies reported that antioxidants could prevent the initiation and promotion of carcinogenesis[7]. The physiological role of

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Table 1. The Demographics of Patients and Control Group.

	Control group	Bladder group tumour
Number	20	32
Median age (± SD)	60.2 ±11.2	67.7 ± 10.95
Gender (female / male)	5/15	4/28
Comorbidity (DM/HT)	2/2	6/5

antioxidants is to prevent damage to cellular components that may arise as a result of chemical reactions involving ROR. Nuclear transcription factor erythroid 2p45 related factor 2 (NRF-2) and Ketch-like ECH related protein 1 (KEAP-1) complex have an important effect on the intracellular organization of the antioxidant system[8]. When the cell is exposed to oxidative stress, NRF-2 is separated from KEAP-1 and translocated to the nucleus, and provides the transcriptional activation of the detoxification enzymes and antioxidant molecule genes[13]. Schematic diagram of the 'NRF2 - KEAP1-Antioxidant Response Element' signaling pathway is shown in **Figure 1**[14]. In addition to protecting the normal cells, this complex may also protect the cancer cells exposed to stress and prolong the survival of the cancer cells[15]. While its relation with several types of cancer has been evaluated in the literature, there is no study evaluating the relationship between the BC and NRF-2/KEAP-1 complex.

In this trial, we aimed to assess the level of NRF-2, KEAP-1 molecules and Copper and Zinc levels associated with oxidative stress. Furthermore, we evaluated the place of NRF-2, KEAP-1, Zinc, and Copper molecules in the diagnosis of BC, which may be possible BC markers.

PATIENTS AND METHODS

All procedures in this cross-sectional study were performed in accordance with the ethical standards of the institutional / national research committee with the standards of Helsinki declaration. This study was approved by the local / national ethics committee (no: 2015/12/01/12) and all patients gave written informed consent prior to surgery and the study.

The sample size was calculated based on the formula according to the previously published works[6,7,12]. The sample size estimation was 35 cases. For this reason we included 35 patients to the study, however, 3 patients were excluded from the study because of unacceptable values.

While patients with < 2 cm of mass in their bladder were included in the trial, patients with a history of non-bladder cancer, chronic inflammatory disease, and rheumatic disease were excluded. Transurethral resection of bladder tumour (TUR-B) was performed under general anaesthesia. Patients diagnosed with a BC type other than transitional cell carcinoma were excluded from the trial. Approximately 0.5cm3 tumour tissue pieces were collected from the bladder mass, and stored

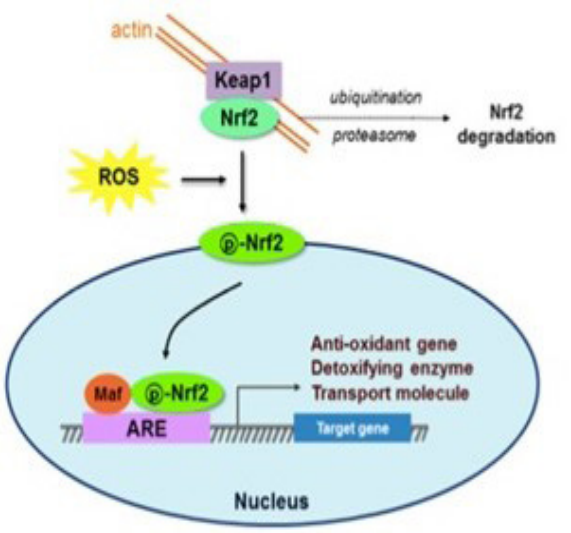

Figure 1. Schematic diagram of the Nrf2-Keap1-ARE signaling pathway. Nrf2 is constantly ubiquitinated through Keap1 and degraded in the proteasome. After exposure to oxidative stress, Keap1 is inactivated and Nrf2 becomes phosphorylated. Phosphorylated Nrf2 (p-Nrf2) accumulates in the nucleus and binds to Antioxidant Response Element sites. It subsequently activates other genes including antioxidants, detoxifying enzymes, and transport molecules.

for analysis at -86°C in freezer condition. Moreover, 8 mL of blood samples were collected to the serum separating gel tubes and centrifuged at 3000 rpm for 10 minutes. The resultant sera, then, were taken into Eppendorf tube and stored in a -86°C freezer to analyse the levels of NRF-2, KEAP-1, Copper, and Zinc. No tissue sample was collected from the patients in this group due to ethical reasons.

The control group was composed of totally healthy individuals. Both the patient and the control group were similar patterns for age, sex, smoking status and region of living area. We performed cystoscopy to the control group as a gold standard to exclude BC. The individuals to whom cystoscopy was performed were included as a control group for different evaluations such as benign prostate hypertrophy (BPH) etc. without ethical restriction. The cystoscopy of these individuals was also normal for BPH and other diseases. The exclusion criteria in the study group were also applied to the control group.

Bladder tumour tissue samples were homogenized in saline buffer solution. Following the homogenization, NRF-2 levels (Cusabio brand kit with the lot number of CSB-EL 015752HU) and KEAP-1 levels (Cusabio brand kit with the lot number of CSB-EL 012147HU) in the tissue were measured by ELISA method. Tissue samples for measuring Copper and Zinc levels were boiled with 2 mL nitric acid at 100°C for 1 hour and

Table 2. The Correlation Between Parameters of Serum Levels in Bladder Tumour Group and Control Group.

	Control group average values (Min.-Max.)	Bladder tumour group average values (Min.-Max.)	*P* value
KEAP-1 (serum) pg/mL	577 (193-1843)	1133 (150-10897)	0.004
NRF-2 (serum) pg/mL	3227 (2340-5150)	5708 (1463-20645)	0.001
Copper serum pg/mL	126 (80-247)	101 (38-219)	0.008
Zinc serum pg/mL	180 (135-326)	146 (61-279)	0.001

Table 3. The Comparison of Copper Levels in Bladder Cancer Tissue for Significant Subgroups.

Bladder tumour staging/grading	N (%)	Cu tissue values (pg/mL)	*P* value
Ta low grade versus	7 (21%)	9.96 (3.7-119.8)	0.001
T2 high grade	6 (28%)	2.8 (1.1-3.7)	
CIS versus	2 (6%)	11.1 (10.8-11.3)	0.04
T2 high grade	6 (28%)	2.8 (1.1-3.7)	
Ta high grade versus	3 (9%)	4.6 (4.3-4.9)	0.02
T2 high grade	6 (28%)	2.8 (1.1-3.7)	

digested, and then 2 mL of perchloric acid (60%) was added. The digested material was diluted with deionized water and the levels of Copper and Zinc were measured using flame atomic absorption spectrophotometry (TM Shimadzu AA-6800).

For the analysis of the data, SPSS version 20.0 software was used. The normality of the data was checked using Shapiro-Wilk test. As the variables do not distribute normally, non-parametric statistical tests were preferred. Mann-Whitney U test was used for the comparison of two independent groups. For the comparisons of more than two independent groups, Kruskal-Wallis test was used. Mann-Whitney U test was used for the comparison of subgroups. The Receiver Operating Curve analysis (ROC) was performed to detect the predictive accuracy of the studied markers (NRF-2 and KEAP-1). The results were expressed as median and minimum-maximum values using marginal or cross tables. $P < 0.05$ value was accepted to be statistically significant.

RESULTS

A total of 32 patients with the diagnosis of BC were included in the trial. Twenty-eight (87.5%) of the patients were male, 4 (12.5%) of them were female, and the mean age of the patients was 67.7 ± 10.9 years. Six (18%) of the patients had diabetes mellitus (DM) and 5 (15%) had hypertension (HT) (**Table** 1). After the resection, 2 (6%) of the patients were detected to have carcinoma in situ (CIS), 7 (21%) of them were found to have Ta/low grade, 3 (9%) of them had Ta/high grade, 9 (28%) of them had T1/low grade, 5 (15%) of them had T1/high grade, and 6 (18%) of the patients were found to have T2/high grade tumour.

A total of 20 healthy individuals were included in the control group for the trial. Fifteen (75%) of the patients in this group were male and 5 (25%) of them were female. The mean age was 60.2 ± 11.2 years. Two (10%) of the patients in the control group had diabetes mellitus and 2 (%10) patients were detected to have hypertension (**Table 1**).

A serum median levels of NRF-2 and KEAP-1 in the study group were 5708 pg/mL (min:1463-max:20645) and 1133 pg/mL (min:150-max:10897), respectively. On the other hand, serum median level of NRF-2 was 3227 pg/mL (min:2340-max:5150) and KEAP-1 was 577 pg/mL (min:193-max:1843) at the control group. The study group values of NRF-2 and KEAP-1 were found to be significantly higher than the control group ($p = 0.001$ and $p = 0.004$). The ROC analysis for the serum NRF-2 and KEAP-1 in BC patients was shown in **Figure 2**. The cut-off value for serum NRF-2 was 4291 pg/mL (sensitivity: 75%- specificity: 55%) (AUC=0.800, $p < 0.0001$, %95 CI (0.679-0.931)). In addition the cut-off value for serum KEAP-1 was 913 pg/mL (sensitivity: 65%- specificity: 60%) (AUC = 0.741, $p < 0.004$, %95 CI (0.606-0.875)).

The normal value of serum total Copper level in healthy population was 63.7-140.12 pg/mL. A serum median level of total Copper value in BC patients was 101 (min:38-max:219) pg/mL (min:1463-max:20645) and in control group was 126 pg/mL (min:80-max:247). A serum median level of Zinc in BC patients was 146 pg/mL (min:61-max:279) and in control group was 180 pg/mL (min:135-max:326). Serum levels of Copper and Zinc were found to be statistically significantly lower than the control group (p=0.008 and p=0.001, respectively) (**Table 2**). Subgroup analysis performed based on the stages of the BC showed no difference in the staging and grading of the tumours caused by serum levels of Zinc and Copper ($p = 0.26$ and $p = 0.89$, respectively). In addition, tissue levels of NRF-2 and KEAP-1 were detected to cause no significant difference in the staging and grading of the tumours. The only significant difference between BC stages/grades was found in the tissue Copper level (p : 0.038). When we analysed the subgroups for tissue Copper level, this significance only occurred between Ta low grade versus T2 (p : 0.001), Ta high grade versus T2 (p : 0.02), and CIS versus T2 (p : 0.04) stages (**Table 3**). Values and statistical analyses of the trial data for serum and tissue Copper, Zinc, NRF-2, and KEAP-1 according to

Table 4. The Correlation Between Bladder Tumour Stage and KEAP-1 Levels.

	Bladder tumour staging	N (%)	Average values (Min-Max)	*P* value
KEAP-1 (serum) pg/mL	Ta low grade	7 (21%)	1025 (150-10897)	0.67
	CIS	2 (6%)	3452 (1154-5750)	
	Ta high grade	3 (9%)	975 (788-1960)	
	T1 low grade	9 (28%)	1088 (383-3050)	
	T1 high grade	5 (15%)	2175 (375-4160)	
	T2 high grade	6 (28%)	1038 (420-4160)	
KEAP-1 (tissue) pg/mL/mgPrt	Ta low grade	7 (21%)	130 (8-600)	0.62
	CIS	2 (6%)	166 (68-265)	
	Ta high grade	3 (9%)	37 (25-106)	
	T1 low grade	9 (28%)	50 (19-185)	
	T1 high grade	5 (15%)	111 (22-219)	
	T2 high grade	6 (28%)	70 (22-219)	

Table 5. The Correlation Between Bladder Tumour Stage and NRF-2 Levels.

	Bladder tumour staging	Average values (Min-Max)	*P* value
NRF-2 (serum) pg/mL	Ta low grade	7500 (1462-206445)	0.73
	CIS	17261 (5771-28750)	
	Ta high grade	4875 (3938-9800)	
	T1 low grade	5438 (1916-15250)	
	T1 high grade	10875 (1875-20800)	
	T2 high grade	5188 (2100-20800)	
NRF-2 (tissue) pg/mL/mgPrt	Ta low grade	6598 (1056-74967)	0.65
	CIS	20785 (8457-33113)	
	Ta high grade	4685 (3094-13282)	
	T1 low grade	6287 (2358-23114)	
	T1 high grade	13902 (2748-27366)	
	T2 high grade	8801 (2723-27366)	

the stages and grades are shown in **Tables 4 to 7**.

DISCUSSION

The dysregulation of the ROR mechanism may play a role in the pathology of tumorigenesis. It activates the abnormal introduction of the signalling pathway that triggers the tumor formation. The uncontrolled growth of the cells leads to the development of the cancer mass with the help of the reactive oxygen-nitrogen species, signal transduction, transcription factors, and kinases/phosphatases cascades[16]. Copper and Zinc are some of the fundamental molecules involved in oxidative stress. They contribute to the structure of antioxidant enzymes as co-factors[10]. Previous studies have shown that oxidative stress may be an important factor in the development of BC[7,16-19]. It has known that the intake of vitamins and antioxidants decreases BC recurrence [20]. Mazdak et al. also stated that the distribution of total antioxidant activity did not show normal pattern in patients with BC. They revealed that the patients with BC had a lower level of total antioxidant activity[7].

NFR-2/KEAP-1 complex has also an important role in antioxidant activity. NFR-2 is a transcription factor belonging to Cap'n'Collar family (CNC) in this complex and has a close interaction with KEAP-1. KEAP-1 is another molecule of this complex. It acts as a sensor to identify the oxidant and electrophilic compound[8]. When the cell is exposed to oxidative stress, it separates from KEAP-1. The free NRF-2 quantity increases in the cytoplasm. When a certain concentration is achieved, NRF-2 molecules translocate to the nucleus and bind to Antioxidant Response Element (ARE) region. ARE is present in the promotor region of many antioxidant enzymes. This region provides the transcriptional activation of the detoxifying enzymes and antioxidant molecules in the cells exposed to oxidative stress[15].

While its relation with several types of cancer has been evaluated in the literature, there is no study evaluating the relationship between the BC and NRF-2/KEAP-1 complex. The somatic mutation of the NRF-2 prevents it from being identified by KEAP-1, and leads to NRF-2 up-regulation. This mechanism has been demonstrated to play a role in pulmonary, head/neck, and oesophagus cancer[21-23]. Similarly, KEAP-1 mutations had been detected in pulmonary and gallbladder carcinoma tissues[24-25]. KEAP-1 mutations cause over-expression of NRF-2 in the cancer cells and activation of the cytoprotective proteins in response. Similar findings are also thought to be in association for BC.

In our trial, when the BC and the control groups are compared, serum NRF-2, KEAP-1, Copper and Zinc levels were observed to have statistically significant differences. Being an important part of the oxidative stress, trace elements in the serum were detected to be significantly reduced in patients with BC. We believe that this reduction may be associated with the fact that trace elements such as Zinc and Copper are used as co-factors of the enzymes acting to prevent oxidative stress or used for system activation. Furthermore, NRF-2 and KEAP-1 increased in BC patients that show the presence of oxidative stress indirectly. The increase of NRF-2 and KEAP-1 was statistically considerable in patients with BC compared to the control group. It suggests that oxidative stress is an important factor in the development of BC. Similar trials are showing the relationship between BC and Copper and Zinc levels in the serum[10,12,26]. When the results of these studies are examined, different data are obtained. In the previous studies, the serum levels of Zinc have been found to be generally low in cases with BC and in the meta-analysis by Song et al. this reduction was found to be significant [10]. Consistent with the literature findings, in the pres-

Table 6. The Correlation Between Bladder Tumour Stage and Copper Levels.

	Bladder tumour staging	Average values (Min-Max)	*P* value
Copper (serum) pg/mL	Ta low grade	101 (38-146)	0.89
	CIS	106 (97-114)	
	Ta high grade	88 (86-1467)	
	T1 low grade	100 (63-219)	
	T1 high grade	115 (98-146)	
	T2 high grade	105 (84-155)	
Copper (tissue) pg/mL/mgPrt	Ta low grade	10 (4-120)	0.03
	CIS	11.07 (10.84-11.31)	
	Ta high grade	4.59 (4.29-4.87)	
	T1 low grade	4.32 (1.68-11.50)	
	T1 high grade	3.99 (2.97-13.59)	
	T2 high grade	2.75 (1.06-3.74)	

Table 7. The Correlation Between Bladder Tumour Stage and Zinc Levels.

	Bladder tumour staging	Median values (Min-Max)	*P* value
Zinc (serum) pg/mL	Ta low grade	140 (102-179)	0.26
	CIS	202 (193-211)	
	Ta high grade	130 (112-147)	
	T1 low grade	159 (61-204)	
	T1 high grade	143 (129-164)	
	T2 high grade	150 (116-279)	
Zinc (tissue) pg/mL/mgPrt	Ta low grade	36 (13-148)	0.06
	CIS	67 (48-87)	
	Ta high grade	16 (6-36)	
	T1 low grade	21 (4-127)	
	T1 high grade	20 (9-76)	
	T2 high grade	11 (2-25)	

ent trial, the serum levels of Zinc of the cases with BC were found to be significantly lower. The relationship between BC and the serum level of Copper has been reported in varying degrees in the literature. The general opinion is that the serum levels of Copper in cases with BC are increased compared to the control group. In the meta-analysis by Song et al., serum level of Copper was detected to be increased in patients with BC.[10] In our study, the serum level of Copper was detected to be considerably lower than the control group which is not consistent with the literature data[12]. On the other hand, there were some studies that state no relationship between BC and control groups[26,27]. The subgroup heterogeneity of BC for stage and grade might be one reason for this inconstancy. We choose most of our cases from high-risk NMIBC and muscle invasive BC. The status of stage and grade might affect the Copper levels. In addition, the Copper level may also be involved in different mechanisms in this patient group. We believe that this inconsistency is associated with the fact that there are not enough studies to conduct a meta-analysis on this subject. In our study, we found that only the tissue level of Copper was significant for BC and this significance only occurred between Ta low versus T2, Ta high versus T2, and CIS versus T2 stages. We could not find any correlation between T1 and other stages. It might be due to the invasion of lamina propria (T1) is the transition layer for muscle invasion. For this reason, the results might show only significant results with distinct stages.

The serum levels of NFR-2 and KEAP-1 in cases with BC were found to be considerably higher than those of the control group. While there are no data regarding the BC, the serum levels of NRF-2 and KEAP-1 were found to be increased in patients with prostate, breast, and pulmonary cancers[28]. The blood levels of these molecules that are the active components of the antioxidant system were also found to be increased in our study. We also evaluated the position of serum and tissue Zinc, Copper, NRF-2 and KEAP-1 molecules as BC markers. No relationship was detected between these molecules and the pathological stages except the Copper tissue levels of the BC. While there is no study regarding the BC, there are some studies showing the relationship between NRF-2 and KEAP-1 molecules, and other urological cancers[29]. NRF-2 levels differ in benign and malignant prostate tissue with being significantly more expressed in tissues with prostate cancer compared to benign tissue. Furthermore, a correlation was detected between the stage of the prostate cancer and NRF-2 levels[29].

Limited number of participants and the non-homogeneous distribution of the BC stages were the negative aspects of this trial. The highly broad reference ranges of NRF-2 and KEAP-1 were also the other negative aspects of the study. In addition, the patient and control

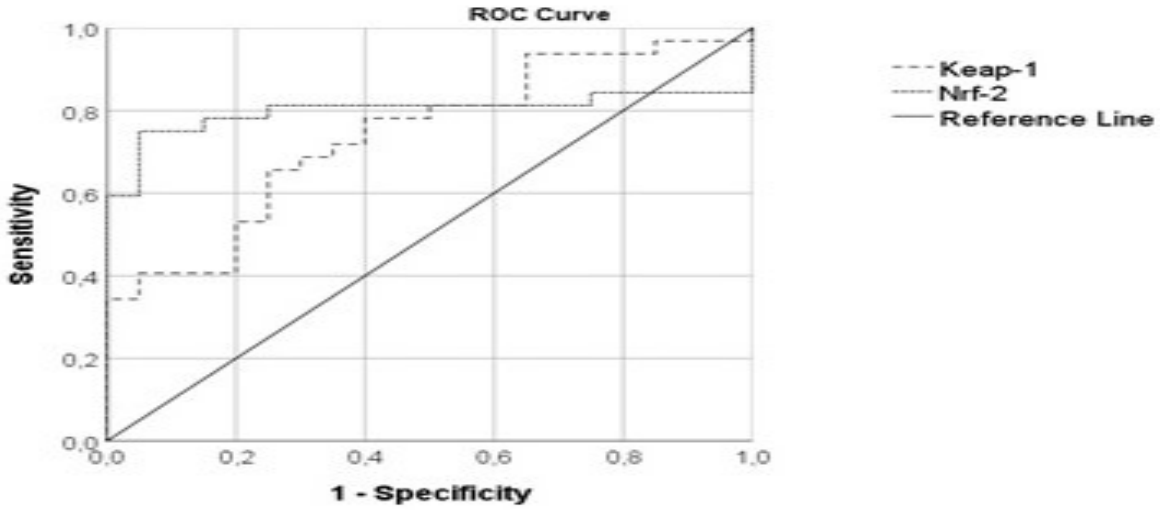

Figure 2. The ROC curve for serum NRF-2 and KEAP-1 in bladder cancer patients.

group could not have exactly similar patterns for the type of ROR stress, the region of inhabitance of participants and the duration of exposure to agents. We know that Copper and Zinc had previously been studied in BC, however, as NRF-2 and KEAP-1 had not been studied before, we hope that this trial paves the way for future trials. We think that conducting much larger, comprehensive, new trials on this timely subject will give way to make the follow-up protocol of the patients with BC less invasive and more cost effective.

CONCLUSIONS

The serum levels of Zinc and Copper were found to be significantly decreased and the serum levels of NRF-2 and KEAP-1 were found to be increased in patients with BC. For the prediction of the stage of the BC, it was detected that only the tissue level of Copper was significant and this significance only occurred between Ta low grade versus T2, Ta high grade versus T2, and CIS versus T2 stages. Other variables were observed to be non-significant for the prediction of the tumour stage. In this regard, we believe that the tissue level of Copper may aid other markers and cystoscopy in the diagnosis and follow-up of the patients.

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CONFLICT OF INTEREST

We have no conflict of interest to declare.

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