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High Expression of Nicotinamide Adenine Dinucleotide Phosphate Oxidase 4 Correlates with High Grade of Clear Cell Renal Cell Carcinoma

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ABSTRACT

Background
Clear cell renal cell carcinoma (ccRCC) is the most common histological subtype in renal cell tumors (RCT). This is a very aggressive cancer, often diagnosed at advanced stage leading to increase morbidity and mortality rates. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 (NOX-4) expression plays role in the regulation of several cancer types. The expression of NOX-4 is associated with a poorer degree of differentiation and a shorter survival in RCT. Therefore, NOX-4 expression is predictor of prognosis and potential therapeutic target. Research on the correlation of NOX-4 expression in RCTs is still very limited, while Correlation of NOX-4 expression in ccRCC does not exist yet.

Objective
To assess the relationship between NOX-4 expression and ISUP grading in ccRCC.

Method
In this study, 30 slide samples and ccRCC paraffin blocks were diagnosed histopathologically at Department of Anatomic Pathology of H. Adam Malik General Hospital from January 2012 to April 2022. All clinicopathological data were taken from medical records/pathology archives. The expression of NOX-4 immunohistochemical was assessed in nucleus and cytoplasm of tumor cells. The assessment was carried out semiquantitatively based on intensity and percentage of expressed tumor cells. The relationship between NOX-4 expression and ISUP grading was assessed using Fisher's exact test.

Results
Of the 30 ccRCC samples, high grade ccRCC was found in 21 cases (70%) and strong expression of NOX-4 in cytoplasm and nucleus in 20 cases (66.7%). Strong expression of NOX-4 in high grade ccRCC was found in 17 cases (81.0%). A significant relationship was observed between NOX-4 expression and ISUP grading on ccRCC with a p-value of 0.030 ($p<0.05$) using Fisher's exact test.

Conclusion
There is a significant relationship between NOX-4 expression and ISUP grading in ccRCC. Therefore, NOX-4 expression can be used for the prognosis of ccRCC and therapy development.

Keywords: clear cell renal cell carcinoma, ISUP grading, nicotinamide adenine dinucleotide phosphate oxidase 4

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INTRODUCTION

Kidney cancer is the most common cause of death of malignancies of the urinary tract and is included in the top 15 most common cancers based on GLOBOCAN data for 2020. Statistically, the incidence of kidney cancer has increased in the United States and Europe over the last three decades. The incidence of death from kidney cancer was reported to have increased from 68,140 cases in 1990 to 138,530 cases in 2017 with a total number of deaths of 89,620 cases in men and 48,910 cases in women. The incidence of death is lower in high-income countries than in low-income countries with a ratio of 0.4:0.5. Based on GLOBOCAN data for 2018, the incidence of kidney cancer in Indonesia was reported as many as 2,112 cases, then increased to 2,394 cases in 2020. Meanwhile, the incidence of death from 1,225 cases became 1,358 cases in 2020.[1-3]

Clear cell renal cell carcinoma (ccRCC) is the most common subtype of renal cell tumors (RCT) originating from proximal tubular epithelial cells of the kidney which have heterogeneous cell morphology with clear cytoplasm, sometimes eosinophilic with many blood vessels.[4,5] Sinaga *et al* at the H. Adam Malik General Hospital, Medan, observed that 80% of RCT cases were ccRCC.[6] The incidence of ccRCC is estimated at 3.59 cases per 100,000 persons per year. Most ccRCC are sporadic and rarely hereditary, this malignancy is associated with germline mutations in Von Hippel-Lindau syndrome (VHL) which trigger the growth of cancer cells.[4]

Determination of histopathological grading is a simple method to assess the prognosis of ccRCC. The International Society of Urological Pathology (ISUP) Grading System was introduced at the ISUP conference in Vancouver in 2012 and was designated as the newest grading system. This grading system has been shown to significantly predict cancer-specific survival. In the ISUP grading system, a microscopic image was assessed in one of the visual fields showing the highest level of cell pleomorphism. The ISUP grading system can only be used on the ccRCC and papillary RCC subtypes.[1,4,7]

Free radicals have a close relationship in various pathological processes of cancer. Reactive oxygen species (ROS) are free radical compounds resulting from the oxidation of several oxidative enzymes.[8] Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) is the main ROS-producing enzyme that participates in various cellular functions of the body such as cell proliferation, cell differentiation, gene regulation, protein expression, apoptosis, and immunological responses to host defense. Dysregulation of NOX activation is associated with various pathophysiologies of diseases such as diabetes, cardiovascular disease, neurodegenerative disease, aging, atherosclerosis, and cancer.[9]

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 (NOX-4) is an isoform of the NOX family that produces constitutive hydrogen peroxide (H_2O_2). This protein is negatively regulated upon adenine triphosphate (ATP) binding and is mainly expressed in the kidney and blood vessels. Beside plays a role in oxygen redox which is involved in kidney disease NOX-4 is also regulate several other types of cancer. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 (NOX-4) through ROS activates oncogenic signaling pathways, promotes DNA damage, genetic instability, reprogramming metabolic phenotypes and mediating immune responses resulting in drug resistance, cancer cell metastasis, cancer cell proliferation and various other cellular responses.[10,11]

The expression of NOX-4 plays a role in the regulation of several types of cancer. NOX-4 expression is associated with a poorer degree of differentiation and shorter survival rates in RCTs, thereby increasing attention to NOX-4 inhibitors as agents targeting therapy to prevent disease progression.[10-12] Research on the relationship of NOX-4 expression in RCTs are still very limited. As far as our knowledge this is the first reserach on the relationship between NOX-4 expression of ccRCC. The purpose of this study was to determine the relationship between NOX-4 expression and ISUP grading of ccRCC.

METHOD

A cross-sectional analytic study design was conducted at the Department of Anatomic Pathology, Faculty of Medicine, University of North Sumatra and the Anatomic Pathology Unit of H. Adam Malik General Hospital, Medan using slides and paraffin blocks which had been diagnosed histopathologically as ccRCC. This study used all paraffin slides and blocks that were histopathologically diagnosed as ccRCC from January 2012 to April 2022 and met the inclusion and exclusion criteria. The inclusion criteria in this study were complete clinical data including age, sex and tumor size from kidney CT-scan readings and slide preparations as well

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as paraffin blocks from nephrectomy/kidney biopsy surgery which were diagnosed histopathologically as ccRCC. While the exclusion criteria were paraffin blocks that were damaged or could not be serially re-cut because the tissue was inadequate.

The ages in this study were grouped into ≤10 years old, 11-20 years old, 21-30 years old, 31-40 years old, 41-50 years old, 51-60 years old, 61-70 years old, and ≥70 years old.[4,13] Gender was grouped into male and female. Tumor size was categorized as ≤4 cm, >4 - ≤7cm, >7 - ≤10 cm, and 4 ≥ 10 cm.[4,14] Lymphovascular invasion (LVI) and perineural invasion (PNI) are positive if tumor cells are found in the lymph vessels, blood vessels and nerves in ccRCC histopathological preparations. LVI and PNI are negative if no tumor cells are found in lymph vessels, blood vessels and nerves on ccRCC histopathological preparations.[15-17]

Histopathological grading in ccRCC refers to the ISUP consensus divided into grade 1 if the nucleus is not visible or not clear and basophilic in color at 400 times magnification, grade 2 if the nucleus is conspicuous, eosinophilic in color at 400times magnification and can be seen although not prominent at 100 times magnification, grade 3 if the nuclei are clearly visible and eosinophilic at 100 times magnification and grade 4 if the nuclei are heavy pleomorphic, multinucleated giant cells, sarcomatoid and rhabdoid differentiation.[4] ISUP grading in this study was grouped into low grade in grades 1-2 and high grade in grade 3-4.[14]

The expression of NOX-4 was indicated by the presence of brownish colored granules in the nucleus and cytoplasm of tumor cells using anti-NOX-4 rabbit antibody at 1:400 dilution (Cat No.E-AB-70215: Elabscience, Inc). The assessment was carried out semi-quantitatively based on the intensity and percentage of expressed tumor cells assessed in 5 microscopic preparation fields with 400 times magnification. The intensity of NOX-4 expression was categorized as follows: 0 (negative, no brown pigment), +1 (weak, yellowish brown pigment), +2 (moderate, light brown pigment), and +3 (strong, brown pigment old). The percentages of NOX-4 expression were categorized as follows: 0 (≤5% stained positively), 1 (6-25% stained positively), 2 (26-50% stained positively), 3 (51-75% stained positively), and 4 (76-100% positive smear). The expression NOX-4 was calculated by multiplying the intensity score by the percentage which was categorized as follows: weak expression (score ≤4) and strong expression (score >4).[12,18] The relationship between NOX-4 expression and ISUP grading was analyzed by Fisher's Exact test. The statistical test is significant if the p value <0.05.

RESULTS

In this study, 30 samples of ccRCC patients were diagnosed histopathologically and met the inclusion and exclusion criteria at H. Adam Malik General Hospital Medan from 2012 to 2022.

Table 1. Characteristics of the patients' sample.

Characteristics	Amount (n)	Percentage (%)
Gender		
Man	20	66.7
Female	10	33.3
Age group, average ± SD	48.5±16.5	
<10 years old	1	3.3
11-20 years old	1	3.3
21-30 years old	1	3.3
31-40 years old	7	23.4
41-50 years old	4	13.4
51-60 years old	10	33.3
61-70 years old	3	10.0
>70 years old	3	10.0
Tumor size, average ± SD	8.1± 3.5	
≤4 cm	6	20.0
>4 - ≤7 cm	5	16.7
>7 - ≤ 10 cm	6	20.0
>10 cm	13	43.3
Lymphovascular invasion (LVI)		
Negative	25	83.3
Positive	5	16.7
Perineural invasion (PNI)		
Negative	27	90.0
Positive	3	10.0
Grading ISUP		
Grading I	1	3.3
Grading II	8	26.7
Grading III	8	26.7
Grading IV	13	43.3
NOX-4 expression		
Weak expression		
Nuclear	0	0
Cytoplasm	8	26.7
Nuclear+cytoplasm	2	6.7
Strong expression		
Nuclear	0	0
Cytoplasm	0	0
Nuclear+cytoplasm	20	66.7

SD: Standard Deviation

Based on clinical data from medical records/pathology archives, the distribution of ccRCC samples was more in men as many as 20 cases (66.7%) and women as many as 10 cases (33.3%). The mean age of the patients in this study ranged from 48.5 ± 16.5 years old, where the youngest one was 10 years old and the oldest was 81 years old. The most cases were found in the 51-60 years old age group of 10 cases (33.5%), followed by the 31-40 years

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old age group consisted of 7 cases (23.4%), the 41-50 years old age group was 4 cases (13.4%), age group 61-70 years old and >70 years old each 3 cases (10.0%). The fewest cases of ccRCC were in the age group <10 years old, 11-20 years old and 21-30 years old, 1 case each (3.3%) (Table 1).

Tumor size was assessed based on the AJCC TNM system obtained from clinical data and referred to the T component. In this study, samples were taken from patients who underwent nephrectomy and kidney biopsy. The mean tumor size ranged from 8.1 ± SB 3.5 with the largest tumor size being 17 cm and the smallest tumor size being 2 cm. The most tumors had a size of >10 cm in 13 cases (43.3%), followed by tumors measuring ≤ 4 and >7-≤10 cm respectively in 6 cases (20.0%) and the least tumors were >4 - ≤7 cm in 5 cases (16.7%). Assessment of LVI and PNI is positive if tumor cells were found in lymph vessels, blood vessels and nerves. In this study, LVI was found to be negative in 25 cases (83.3%) and positive LVI in 5 cases (16.7%). Meanwhile, PNI was found to be negative in 27 cases (90.0%) and positive PNI in 3 cases (10.0%) (Table 1).

The histopathological grading of ccRCC was assessed based on the ISUP grading system which was graded according to the assessment of the nucleus and cell pleomorphism. In this study, the majority of ccRCC had grade IV in 13 cases (43.3%), followed by grade III and grade II each in 8 cases (26.7%) and at least 1 case in grade I (3.3%). Evaluation of NOX-4 immunohistochemical staining was carried out semiquantitatively based on the intensity and percentage of expressed tumor cells. The expression NOX-4 was indicated by brownish chromogen granules in the nucleus and cytoplasm of tumor cells, which were assessed in 5 microscopic preparation fields at 400 times magnification. In this study, the weak expression of NOX-4 in the cytoplasm was observed in 8 cases (26.7%) followed by the weak expression of NOX-4 in the cytoplasm and nucleus in 2 cases (6.7%). Most of the NOX-4 expression was strong in the cytoplasm and nucleus which were detected in 20 cases (66.7%) and there was no strong expression of NOX-4 in the cytoplasm or nucleus alone (Table 1).

Table 2. Relationship between NOX-4 expression and ISUP grading in ccRCC.

Characteristics	Grading ISUP		p-value
	Low grade N (%)	High grade N (%)	
NOX-4			*0.030
Weak expression	6 (66.7)	4 (19.0)	
Strong expression	3 (33.3)	17 (81.0)	

*Fisher exact test

Of the 30 ccRCC samples, weak expression of NOX-4 was found in 6 cases (66.7%) of low grade followed by weak expression of NOX-4 in 4 cases of high grade (19.0%). Strong expression of NOX-4 was found in high grade of 17 cases (81.0%) and strong expression of NOX-4 in low grade was detected in 3 cases (33.3%). The data were analyzed statistically using the fisher's exact test. Based on statistical test analysis, there was a significant relationship between strong expression of NOX-4 and high grade ISUP on ccRCC with p-value of 0.030 (p<0.05) (Table 2).

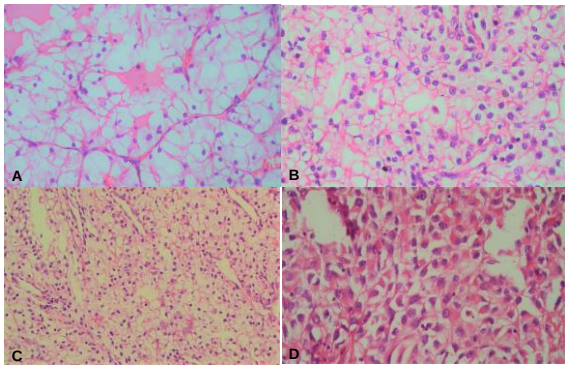

Figure 1. ISUP grading on ccRCC. A. Grade I (H&E, 400 times). B. Grade II (H&E, 400 times). C. Grade III (H&E, 100 times). D. Grade IV, rhabdoid differentiation (H&E, 400 times)

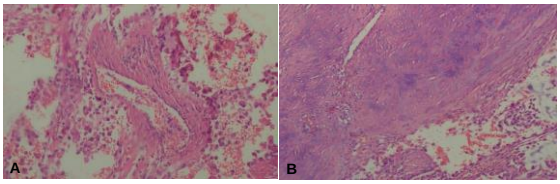

Figure 2. LVI and PNI on ccRCC. A. LVI positive (H&E, 100 times). B. PNI positive (H&E, 100 times).

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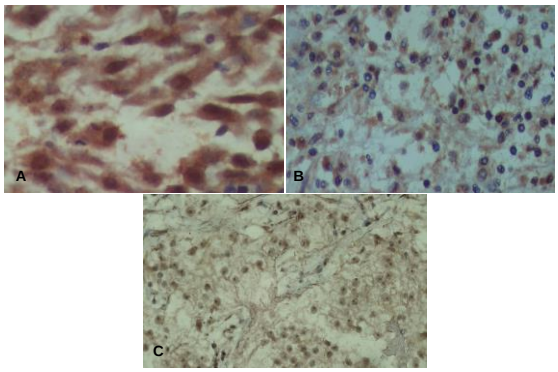

Figure 3. NOX-4 expression on ccRCC. A. Strong expression of NOX-4 in the nucleus+cytoplasm, 400 times. B. Weak expression of NOX-4 in the cytoplasm, 400 times. C. Weak expression of NOX-4 in the nucleus+cytoplasm, 400 times.

DISCUSSION

Clear cell renal cell carcinoma is a kidney tumor originating from the proximal tubular epithelial cells of the kidney, having heterogeneous cell morphology with clear cytoplasm, sometimes eosinophilic cytoplasm with many blood vessels. The incidence of ccRCC was significantly higher in men than in women with a ratio of 1.94:1.[4] In this study there were 30 ccRCC samples, which were found more in men as many as 20 cases (66.7%) than women as many as 10 cases (33.3%) (Table 1). The results of this study are in line with the research of Siegel et al which states that the ratio of incidence of ccRCC in men compared to women is 2:1.[19] Thaib et al in a study stated that the incidence of ccRCC was more in men than women with a ratio of 2.5:1.[20] In line with previous research, Roldan et al stated that the incidence of ccRCC was more in men as many as 18 cases (69.2%).[21] Men have a greater risk of getting ccRCC than women because they are associated with ccRCC risk factors such as smoking, exposure to environmental pollution (such as asbestos, benzene and tricholoroethylene) which men often inhale at work, poor diet and lifestyle.[4]

Most ccRCC is developed at the age 60 years old (average age is 62 years old).[4] In this study, most cases were found in the age group of 51-60 years old (mean age 48.5 ± 16.5 years old) with the youngest age of 10 years old and the oldest age of 81 years old (Table 1). The results of this study are in line with the research of Kharismawaty et al which stated that the most cases of ccRCC were found in the age group of 51-60 years old with 7 cases (30.4%).[22] The same thing was also found in the study of Thaib et al which stated that the most cases of ccRCC were found in the age group of 51-60 years old with 12 cases (31.6%).[20] The results of this study were not in line with those of Lopez et al who found that there were more cases of ccRCC at an older age, namely the average age of 66 years.[23] Aging is a normal process that will be experienced by every human being. In the aging process, there is a decrease in the natural function of tissues or organs, which is manifested in different degrees and forms in each tissue and organ. In the aging process, there are complex changes in body tissues. In this process, the tissue's ability to repair itself and maintain its normal structure and function will slowly disappear, making it very vulnerable to infections and other diseases.[24]

In old age, there is an accumulation of free radicals in the body. Normally free radicals will be destroyed by antioxidants, but some free radicals manage to escape and accumulate in the organs of the body. Free radicals are very reactive substances that can damage cell membranes and integrity. If free radicals accumulate in large quantities in the body, the body cannot neutralize it through the body's defense mechanisms, so it will damage several cell components such as proteins, lipids, carbohydrates and nucleotides and other macromolecules. Unsaturated fatty acids are the cell components most sensitive to free radicals and will form lipid peroxide chain reactions. The main target of free radicals is to damage mitochondrial DNA (mtDNA), which is found in the cell nucleus, which serves as the "command center" of the cell, and in the mitochondria. Free radical damage in cells is associated with various diseases including cancer, arthritis, atherosclerosis, alzheimer's disease, and diabetes. In old age, histological changes occur in the stroma. The stroma will form a protumorigenic environment caused by senescence-associated secretory phenotype (SASP). SASP has a role in the initiation and progression of age-related cancer.[25]

Tumor size in ccRCC varies from 12 mm to 140 mm (mean tumor size 57 mm). Recent studies reported an average tumor size of 23 mm to 170 mm (average tumor size 63 mm).[4] In this study the majority of patients had tumors >10 cm in 13 cases (43.3%) (mean 8.1 ± SB 3.5 cm) with the largest tumor size being 17 cm and the smallest being 2 cm (Table 1). This findings were in line with the results of Kharismawaty et al who found that the majority of ccRCC sufferers had tumors measuring >10 cm in 30.4% (mean 8.513 cm).[22] Thaib et al in their study found that ccRCC patients had tumors >10

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cm in size in 28 cases (73.7%).[20] However, the results of this study were different from those of Ali et al who examined 19 people with ccRCC in Canada showed that most tumors have small size, ranging from 25-67 mm (mean: 38.6 mm).[26] Whereas in our study, most patients had larger tumor size, most likely this was due to early detection/screening in developed countries such as Canada. While, cases of ccRCC in developing countries is still lacking and patients search for help are often late compared to those in developed countries, consequently the tumors are already large and have metastasized to other organs at the first time of diagnosis. In this study, tumor size could only be assessed up to T2b stage because tissue samples were also taken from kidney biopsies. In this study, tumor invasion of the great veins, ipsilateral adrenal gland and Gerota's fascia could not be assessed.

In this study, most of the LVI were negative in 25 cases (83.3%) and positive LVI in 5 cases (16.7%) (Table 1). This results are in line with the results of Belsante et al where LVI was found in 14.3% of all non-metastatic ccRCC patients.[27] The results of this study are different from those of Bedke et al where LVI was found in 32 cases of ccRCC (5.5%) from 573 samples.[28] Study by Ha et al found LVI in 121 cases of ccRCC (3.4%) from 3585 study samples.[29] LVI is suspected to be associated with a tendency for localized recurrence or metastases. Metastases started with tumor cells invading the circulating blood and lymphatic vessels.[27-29] In this study, the majority of PNI were negative in 27 cases (90.0%) and PNI positive in 3 cases (10.0%) (Table 1). The results of this study are in line with those of Simsek et al who reported a PNI of 2–7% in ccRCC nephrectomy specimens.[30] Capek et al reported one case of ccRCC with PNI in a spinal lesion located between T12 and L4.[16] In the study Muppa et al PNI was found in 44 cases (4.6%) of 964 bladder cancer samples.[31] PNI assessment varies widely because it requires expertise and accuracy from the assessor. The spread of ccRCC may reach the lumbosacral plexus along the pelvic autonomic nerves and along the intradural spinal nerves. Infiltration of the sympathetic nerves produces symptoms of dry foot syndrome and abdominal complaints. Involvement of the spinal cord in the lower thorax only causes pain. Intradural lesions can cause cauda equine syndrome, sexual dysfunction, bowel symptoms, lower extremity weakness, pain, and sensory disturbances.[32]

Assessment of tumor grading is one method to predict the prognosis in ccRCC. This study used the ISUP grading system to assess the grade of the nuclear child and cell plemorphism.[4] In this study, the majority of ccRCC had grade IV in 13 cases (43.3%), followed by grade III and grade II each in 8 cases (26.7%) and the fewest cases in grade I were 1 case (3.3%) (Table 1). This results are in line with the results of Seker et al where they found that the majority of ccRCC patients had grade IV which was dominated by males and an average tumor size of >7 cm.[33] The results of this study were different from those of Mohamed et al where the most ccRCC was obtained in grade II as many as 37 cases (44%).[34] The study by Galtung et al observed the most ccRCC was in grade II as many as 86 cases (47%).[35] In this study most of the ccRCC patients had grade IV. This may be due to hereditary VHL gene mutations. For this reason, further genetic examination is needed to prove it.[4] Ribosomepathies that are owned by people with hereditary diseases cause changes in the biogenesis and function of ribosomes which will translate proteins to produce a certain phenotype.[36] In addition, early detection/screening of ccRCC in developing countries still lacking and often too late so that the tumor when diagnosed has a higher grade.

The protein of NOX-4 is a member of the NOX enzyme family which has an important role in cellular physiological and pathological responses. The expression of NOX-4 plays a role in the regulation of several types of cancer.[12] In this study NOX-4 expression was assessed by microscopic examination of in the nucleus and cytoplasm of tumor cells. Weak expression of NOX-4 was mostly in the cytoplasm in 8 cases (26.7%) followed by expression in the cytoplasm and nucleus in 2 cases (6.7%). Moreover, NOX-4 was strongly expressed mostly in the cytoplasm and nucleus in 20 cases (66.7%) and no expression was found either in the cytoplasm or nucleus alone (Table 1). The results of this study are in line with the study by Du et al, which found strong expression of NOX-4 in 57 cases of 90 samples of gastric cancer.[18] Research by Soo et al in hepatocellular carcinoma, NOX-4 generally showed higher expression in the nucleus and cytoplasm of tumor cells with $p<0.001$.[37] The results of this study were different from that of Kaushik et al where strong NOX-4 expression was found in 50 ccRCC cases in the nucleus of tumor cells (54.35%).[12] Most of NOX-4 is expressed in the nucleus of tumor cells but NOX-4 can also be expressed in the cytoplasm

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of tumor cells. The variations in the subcellular locations of different NOX-4 expression are not well understood. The expresion of NOX-4 has been shown to transit from one intracellular compartment to the others and to have variations between different cell types.[38]

The expression of NOX-4 is associated with a worse degree of differentiation and shorter survival rates in RCTs.[12] Out of 30 ccRCC samples, 17 cases (81.0%) had strong NOX-4 expression in high grade and NOX-4 strong expression in low grade as many as 3 cases (33.3%). Weak expression of NOX-4 in low grade was 6 cases (66.7%) and weak expression of NOX-4 in high grade was 4 cases (19.0%). The data were analyzed statistically using the fisher's exact test. Based on statistical test analysis, it was shown that there was a significant relationship between strong expression of NOX-4 and high grade of ISUP grading on ccRCC with a p-value of 0.030 (p <0.05) (Table 2). The results of this study are in line with the results of Pan et al which stated that the increase in NOX-4 expression was in line with the increase in the grade of gallbladder cancer. In poorly differentiated tumors, high expression of NOX-4 was found in 32 cases (80%), in moderately differentiated NOX-4 expression was found in 18 cases (53%) and in well differentiated NOX-4 expression was found in 4 cases (36.4%)).[39] In the study of Kaushik et al showed that increased NOX-4 expression in high-grade ccRCC showed an important prognostic factor and had an influence on drug resistance.[12] The results of this study were different from those of Lin et al who found that poorly differentiated human colorectal cancer showed high expression of NOX-4 which was lower than well/moderately differentiated human colorectal cancer. Poorly differentiated human colorectal cancer showed high expression of NOX-4 in 11 cases (21%), while well/moderately differentiated human colorectal cancer showed high expression of NOX-4 in 35 cases (58.3%).[40] Increased NOX-4 expression in line with the grade increase in ccRCC. The expression of NOX-4 can be used as a prognostic factor in patients and future therapy development using NOX-4 inhibitors.

CONCLUSION

This study showed NOX-4 expression increased in high grade ISUP. There was a significant relationship between NOX-4 expression and ISUP grading on ccRCC with a p-value of 0.030 (p<0.05).

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