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Von Hippel-Lindau Gene Mutations and Vascular Endothelial Growth Factor Immunoexpression in Clear Cell Renal Cell Carcinoma

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Renal cell carcinoma (RCC) is the most common type of kidney cancer in adults representing about 3% of all newly diagnosed cancers in the United States. Clear cell RCC is the most common subtype (70-80%) of RCC. Clear cell RCC can be familial, but 95% of cases are sporadic resulting from the germline or acquired mutation of Von Hippel-Lindau (VHL) gene. VHL tumor suppressor gene functions as a down regulator of vascular endothelial growth factor (VEGF). Mutations of VHL gene result in overexpression of VEGF, neoangiogenesis and tumor metastasis. Nowadays, anti-VEGF targeted therapy is used for treating metastasis clear cell RCC. However, drug resistance occurs over time. VHL gene targeted therapy combined with anti-VEGF therapy should be considered and detection of VHL gene mutations status becomes essential in these cases. The present study was aimed to detect the VHL gene mutations status and VEGF immunoexpression in 62 clear cell RCC patients by conventional polymerase chain reaction and immunohistochemistry. Three primer pairs were used to detect the mutations of 3 exons in VHL gene. The positive cases for VHL exon 1 mutation, exon 2 mutation and exon 3 mutation were checked by 2% agarose gel electrophoresis. Tumor grading was done by Fuhrman nuclear grading system and staging was done by pathologic TNM staging system. Fifty cases (80.65%) were VHL gene mutation positive and 12 cases (19.35%) were negative. VHL gene mutations were significantly associated with histological grades (p=0.005). Out of 62 cases, 24 cases were weakly positive (1+) and 38 cases were strongly positive (2+) VEGF immuno-reactivity. There was statistically significant association between VEGF immunoexpression and histological grades of clear cell renal cell carcinoma (p=0.00) as well as tumor stage (p=0.01). It was also found that VEGF immunoexpression of clear cell RCC was significantly associated with VHL gene mutation positive tumours (p=0.00). These results can be helpful in further invention of molecular targeted therapy for drug-resistant clear cell RCC patients.

Keywords: Clear cell renal cell carcinoma, VHL gene, VEGF immunoexpression, Polymerase chain reaction, Immunohistochemistry

INTRODUCTION

Kidney cancer is the 14th most common cancer in the world. Renal cell carcinoma (RCC) is the most common type of kidney cancer in adults. In 2014, a total of 63,990 new cases of renal cancer were diagnosed and 14,400 deaths were due to renal cancer. According to the Hospital Statistics of New Yangon General Hospital and Yangon Specialty Hospital, the total number of RCC patients attending the Urosurgical Ward were 62 cases in 2013, 41 cases in 2014, 46 cases in 2015 and 47 cases in 2016.[1, 2]

Renal cell carcinoma comprises a broad spectrum of histopathological entities. Among them, clear cell RCC is the most common subtype accounting for 70-80% of RCC. Clear cell RCC can be familial or sporadic due to germline or acquired mutation of VHL gene. The incidence of somatic VHL mutations in sporadic clear cell RCC increases up to 91%. The VHL gene is located on the short arm of

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the chromosome 3 (3p25-26). It has 3 exons and 2 introns. VHL exon 1 mutation is seen in 26-48% of cases, exon 2 mutation in 22-68% of cases and exon 3 mutation in 26-32% of cases.[3, 4, 5] Normally, VHL tumor suppressor gene degrades hypoxia inducible factor and down-regulates vascular endothelial growth factor. VHL gene mutations results in over-expression of VEGF which promotes the neo-angiogenesis, tumor metastasis and progression of clear cell RCC. Therefore, clear cell RCC is associated with advanced disease at the time of diagnosis.

VEGF is a 45 kDa heparin binding polypeptide of platelet derived growth factor family and is secreted by a variety of malignant cells. Upregulation of VEGF is associated with high nuclear grade and stage. At present, there are several targeting drugs approved for treating metastatic clear cell RCC. These include anti-VEGF targeted therapy and tyrosine kinase inhibitors. However, drug resistance occurs over time. These drug resistances are encouraging factors to consider VHL gene targeted therapy combined with anti-VEGF. Detection of VHL gene mutation status becomes essential for the treatment of these drug-resistant patients.

This study was carried out to detect VHL gene mutations and VEGF immunoexpression in correlation to histological grade and stage of clear cell RCC. The findings from this study will give information for the potential use of drugs designed to stabilize or enhance the VHL gene in combination with anti-VEGF for the treatment of clear cell RCC.

MATERIALS AND METHODS

Gross examination of nephrectomy specimens of clinically diagnosed RCC and careful sampling were done according to guidelines for handling of nephrectomy specimen. After adequate fixation, tissue processing was done by automatic tissue processor and haematoxylin and eosin stained tissue sections were carefully studied to confirm the clear cell RCC, Fuhrman nuclear grade and pathologic TNM staging were done. Then the representative paraffin wax-blocks were further processed for immuno-histochemical staining with polyclonal antibody to VEGF by using peroxidase-antiperoxidase method. According to the degree of distribution of immunoreactive clear cell RCC cells, VEGF immunoexpression were graded into 3 categories: negative (0) - no staining of tumor cells, weakly positive (1+) - membranous stain with no cytoplasmic immunostaining or with light cytoplasmic staining of some tumor cells (<50%), strongly positive (2+) - diffuse and strong membranous and cytoplasmic staining of some tumor cells (<50%), and most tumor cells (>50%).

Detection of VHL gene mutations was done by conventional PCR. DNA extraction from formalin-fixed paraffin-embedded (FFPE) tissue blocks was done by using QIAamp DNA FFPE tissue kit. Three primer pairs were used to detect the mutations of 3 exons in VHL gene.

Three primer pairs for detection of VHL gene mutations[5]

Name of the primers	Sequence (5'-3')
• PCR primers for Exon 1 (Forward)	= 5'-GGTCTGGATCGCGGAGGGA-3'
• PCR primers for Exon 1 (Reverse)	= 5'-GCCCGGCCTCCATCTCCT-3'
• PCR primers for Exon 2 (Forward)	= 5'-AGTCGGGCGCCGAGGAGT-3'
• PCR primers for Exon 2 (Reverse)	= 5'-CCGTCGAAGTTGAGCCATAC-3'
• PCR primers for Exon 3 (Forward)	= 5'-CCCAGGTCATCTTCTGCAAT-3'
• PCR primers for Exon 3 (Reverse)	= 5'-CTGCTGGGTCGGGCCTAAG-3'

PCR reaction mixture was prepared in a sterile micro centrifuge tube by adding 0.5 microgram of DNA template, 20 picomole of each of the primers, 2.5 units Taq DNA polymerase, 1x Qiagen PCR buffer, 200 µM of each dNTP and RNAase-free water. The tubes were transferred to G-storm thermal cycler for amplification. PCR cycling condition consists of 1 cycle of initial incubation 95°C for 5 minutes, a total of 40 cycles of denaturation for 94°C for 45 seconds, annealing at 53°C for 45 seconds and elongation at 72°C for 90 seconds and 1 cycle of final elongation at 72°C for 7 minutes.[5]

After amplification, PCR amplicons were mixed with DNA loading dye and the mixture was analyzed by electrophoresis on 2% agarose gel at 135V for 30 minutes. The products were evaluated under UV transilluminator. The positive cases for VHL exon 1 mutation were seen at 110 bp, exon 2 mutations were seen at 150-159 bp and exon 3 mutations were seen at 194 bp. The patients were regarded as VHL gene mutation positive if one of the three exons showed positive result. The negative cases

showed no DNA band at the above bp of molecular weight marker. The patient was regarded as VHL gene mutation negative if all of the three exons showed negative results on agarose gel electrophoresis.

Data management

The VHL gene mutations and VEGF immuno-reactivity of each case were recorded in the proforma. Data were entered in Microsoft Excel Spread Sheet. Data were summarized in frequency tables; outliers, missing data and pattern were checked to obtain reliable data. Any mismatch data were checked again with primary record and validated if necessary.

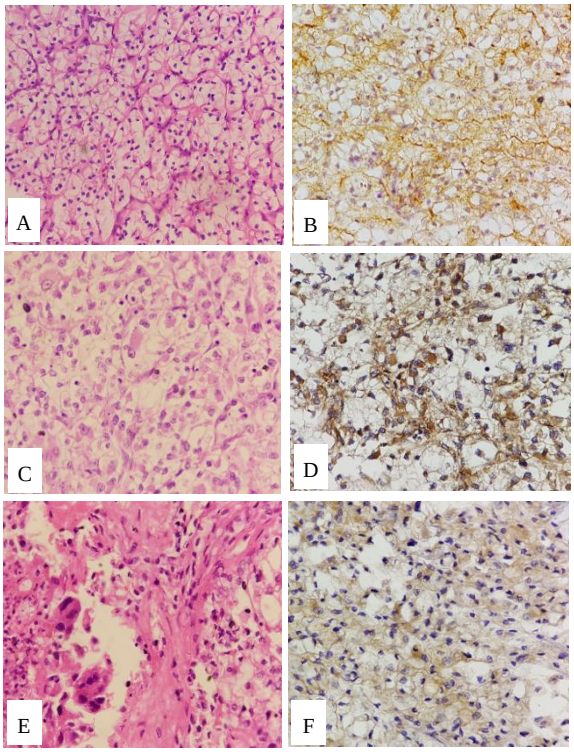
The final data file in Microsoft Excel was reported as data-based file and it was analyzed in Statistical Software STATA version 15.0. Simple descriptive analysis for each single variable was done. General characteristics such as age was summarized by mean, standard deviation, minimum and maximum ranges. The VHL gene mutation status was also categorized by exon 1 mutation, exon 2 mutation and exon 3 mutation. The categorical variables such as Fuhrman nuclear grades, stages, VHL gene mutations and VEGF immunoexpression were summarized as frequency and percentage. Association between categorical variables were calculated by chi-square test and independent sample 't' test. The association between VHL gene mutation and VEGF immunoexpression were calculated by chi-square test. The level of significance was set at $p<0.05$ (95% confidence level).

Ethical consideration

The study received human subject approval from Ethical and Research Committee, University of Medicine 1, Yangon on 21. 12. 2015. Participants provided written informed consent to participate.

RESULTS

In this study, the mean age of the patients was 58.41±12.10 years and the median age of the patients was 60 (50-68) years. Forty-two patients (67.74%) were male and 20 patients (32.26%) were female. Male to female ratio was 2.1:1 which showed that males were more affected than female.

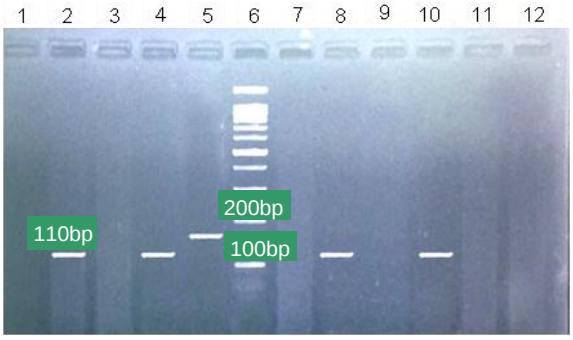


A = Fuhrman nuclear grade (2), clear cell renal cell carcinoma (H&E X400) (Case No. 22)
B = Fuhrman nuclear grade (2), clear cell renal cell carcinoma, VEGF immunoexpression (score 1+) X400 (Case No. 22)
C = Fuhrman nuclear grade (3), clear cell renal cell carcinoma (H&E X400) (Case No. 13)
D = Fuhrman nuclear grade (3), clear cell renal cell carcinoma, VEGF immunoexpression (score 2+) X400 (Case No. 13)
E = Fuhrman nuclear grade (4), clear cell renal cell carcinoma (H&E X400) (Case No. 49)
F = Fuhrman nuclear grade (4), clear cell renal cell carcinoma, VEGF immunoexpression (score 2+) X 400 (Case No. 49)

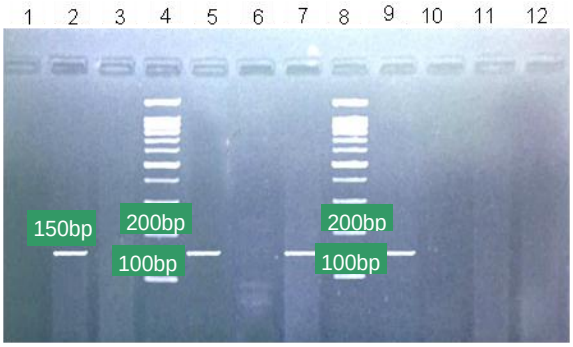
Fig. 1. Fuhrman nuclear grade 2, 3 & 4 clear cell renal cell carcinoma

In the present study, among 62 cases of clear cell renal cell carcinoma, 26 cases (41.94%) were grade 2, 32 cases (51.61%) were grade 3, 4 cases (6.45%) were grade 4 according to Fuhrman nuclear grading system (Fig. 1).

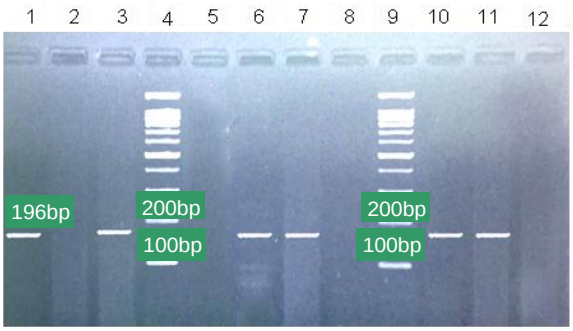
Among 62 cases of clear cell renal cell carcinoma, 13 cases (20.97%) were stage I, 10 cases (16.13%) were stage II, 25 cases (40.32%) were stage III and 14 cases (22.58%) were stage IV according to TNM staging system. Out of 62 patients, 50 patients (80.65%) were VHL gene mutations positive. Eighteen patients (29.03%) were VHL gene exon 1 mutation positive. Thirty-two patients (51.61%) were exon 2 mutation positive. Forty-five patients (72.58%) were exon 3 mutation positive (Fig. 2).



(a) Lane 6 - Molecular size marker of different 100 base pairs ladder, Lane 5 - Distinct band of DNA as positive control, Lane 2, 4, 8 and 10 - Distinct band of DNA at 110 bp (Case No. 21, 23, 25 and 49), Lane 1, 3, 7, 9, 11 and 12 - No distinct band of DNA (Case No. 19, 22, 24 and 26)



(b) Lane 4 and 8 - Molecular size marker of different 100 base pairs ladder, Lane 5 - Distinct band of DNA as positive control, Lane 2, 7 and 9 - Distinct band of DNA at 150 bp for exon 2 mutation (Case No. 13, 33 and 34), Lane 1, 3, 6, 9, 10, 11 and 12 - No distinct band of DNA at 150 bp for exon 2 mutation (Case No. 28, 30, 31, 35, 36 and 37)



(c) Lane 4 and 9 - Molecular size marker of different 100 base pairs ladder, Lane 3 - Distinct band of DNA as positive control, Lane 1, 6, 7, 10 and 11 - Distinct band of DNA at 196 bp (Case No. 49, 31, 33, 35 and 36), Lane 2, 5, 8 and 12 - No distinct band of DNA (Case No. 29, 30, 34 and 37)

Fig. 2. Amplified DNA from FFPE tissue with specific primer for VHL gene exon 1 mutation (a), exon 2 mutation (b), and exon 3 mutation (c)

Table 1. Association between VHL gene mutation and histological grades and stages of clear cell renal cell carcinoma

	VHL gene mutations		Total
	Positive(%)	Negative(%)	
*Histological grade**			
Grade 2	16(25.81)	10(16.13)	26(41.94)
Grade 3	30(48.39)	2(3.23)	32(51.61)
Grade 4	4(6.45)	0(0.00)	4(6.45)
Total	50(80.65)	12(19.35)	62(100.00)
*Stages***			
Stage I	8(12.90)	5(8.06)	13(20.96)
Stage II	7(11.29)	3(4.84)	10(16.13)
Stage III	21(33.87)	4(6.45)	25(40.32)
Stage IV	14(22.58)	0(0.00)	14(22.58)
Total	50(80.65)	12(19.35)	62(100.00)

*Pearson chi^2 =10.56, p=0.005 (significant)
**Pearson chi^2 =7.31, p = 0.063

Out of 50 VHL gene mutation positive cases, 16 cases (25.81%) were grade 2, 30 cases (48.39%) were grade 3 and 4 cases (6.45%) were grade 4. According to Pearson chi^2 test, VHL gene mutations were statistically significantly associated with histological grades of clear cell RCC (p =0.005) (Table 1).

Out of 50 VHL gene mutation positive cases, 8 cases (12.90%) were stage I, 7 cases (11.29%) were stage II, 21 cases (33.87%) were stage III and 14 cases (22.58%) were stage IV. According to Pearson chi^2 test, there was no significant association between VHL gene mutations and stages of clear cell RCC (p=0.063) (Table 1).

Table 2. Association between VEGF immunoexpression and histological grades and stages of clear cell renal cell carcinoma

	VEGF Immunoexpression		Total
	Weakly positive (1+) (%)	Strongly positive (2+) (%)	
*Histological grade**			
Grade 2	22(35.48)	4(6.45)	26(41.94)
Grade 3	2(3.23)	30(48.39)	32(51.61)
Grade 4	0(0.00)	4(6.45)	4(6.45)
Total	24(38.71)	38(61.29)	62(100.00)
*Stages***			
Stage I	10(16.13)	3(4.84)	13(20.97)
Stage II	4(6.45)	6(9.68)	10(16.13)
Stage III	7(11.29)	18(29.03)	25(40.32)
Stage IV	3(4.84)	11(17.74)	14(22.58)
Total	24(38.71)	38(61.29)	62(100.00)

*Pearson chi^2=39.83, p=<0.01 (significant)
**Pearson chi^2=10.98, p=0.01

All the 62 cases of clear cell RCC showed positive immunoreactivity and no case showed negative immunoreactivity. Weakly positive (1+) immunoreactivity was detected in 24 cases (38.71%) and strongly positive (2+) immunoreactivity was detected in 38 cases (61.29%).

In this study, 35.48% of cases of weakly positive (1+) immunoreactivity were grade 2 and 3.23% were grade 3. Out of 38 cases of strongly positive (2+) immunoreactivity of VEGF, 6.45% of cases were grade 2, 48.39% were grade 3 and 6.45% were grade 4. According to Pearson chi^2 test, there was statistically significant association between VEGF immunoexpression and histological grades of clear cell RCC (p=<0.01) (Table 2).

In this study, 16.13% of cases of weakly positive (1+) immunoreactivity were stage I, 6.45% were stage II, 11.29% were stage III and 4.84% were stage IV. Out of 38 cases of strongly positive (2+) immunoreactivity of VEGF, 4.84% of cases were stage I, 9.68% were stage II, 29.03% were stage III and 17.74% were stage IV. According to Pearson chi^2 test, there was statistically significant association between immunoexpression and stages of clear cell RCC (p=0.01) (Table 2).

Table 3. Association between VHL gene mutation and VEGF immunoexpression in clear cell renal cell carcinoma

Clear cell renal cell carcinoma		VEGF immunoexpression (%)		Total (%)
		1+	2+	
VHL mutations	+	14(22.58)	36(58.06)	50(80.65)
	–	10(16.13)	2(3.23)	12(19.35)
Total		24(38.71)	38(61.29)	62(100.00)

Pearson chi^2=12.49, p=<0.01

Out of 50 VHL gene mutation positive cases, 14(22.5%) cases showed weakly positive (1+) immunoreactivity and 36 (58.06%) cases showed strongly positive (2+) immunoreactivity. According to Pearson chi^2 test, VEGF immunoexpression of clear cell RCC was significantly associated with VHL gene mutation positive tumour compared to that of VHL gene mutation negative tumour (p=< 0.01) (Table 3).

DISCUSSION

Renal cell carcinoma is the most common primary malignant tumor of the kidney and its incidence and new cases are increasing worldwide. In Myanmar, the number of RCC patients is also increasing annually. Among three major histological subtypes of RCC, clear cell RCC is the most common subtype and it also has the poor prognosis with a 5-year survival rate of nearly 10%.

Most of the clear cell RCC have germline or somatic mutations in VHL gene resulting in increased production of vascular endothelial growth factor, tumor metastasis and a poor treatment outcome. For the treatment of clear cell RCC, anti-VEGF and several tyrosine kinase inhibitors are widely used. But some patients show resistance to these treatments. This drug resistance triggers the consideration of VHL gene targeted therapy in combination with anti VEGF. Detection of VHL gene mutation status will become essential for treatment of clear cell RCC.

VHL gene mutations were studied in 62 cases of clear cell RCC by conventional PCR method in this study. Fifty cases (80.65%) were VHL gene mutation positive and 12 cases (19.35%) were VHL gene mutation negative. The present study revealed that VHL gene mutations were more commonly seen in higher nuclear grades and significant association was present between VHL gene mutation and nuclear grade (p=0.005) favouring the VHL gene mutations as one of the prognostic factors. But there was no significant association between stages of clear cell RCC and VHL gene mutation (p=0.063).

Most of the studies were done on nephrectomy specimen. All the patients in the present study also underwent nephrectomy. It is known that not all the patients undergo surgery for treatment of RCC. Most of the patients with stage IV cancers do not receive surgical treatment. These patients were not included in our study. In the present study, only nephrectomy specimen of clear cell RCC patients from Urosurgical Unit, Yangon Specialty Hospital were included. Tru-cut biopsy were excluded from the study because greater amount of tumor tissue was required for genetic study and immunohistological study. Further large-scale study is necessary to assess the true prevalence of different stages of renal cancer.

Among 62 cases of positive VEGF immunoreactivity, 24 cases (38.71%) revealed weakly positive (1+) immunoreactivity and 38 cases (61.29%) showed strongly positive (2+) immunoreactivity. VEGF immunoexpression was significantly associated with histological grade and stage of clear cell RCC, p=<0.01 and p=0.01, respectively. Immunoexpression of the VEGF is proportional to the formation and progression of RCC, which may allow VEGF

to be used as a prognostic marker for renal cell carcinoma.

VEGF immunoexpression of clear cell RCC was significantly associated with VHL gene mutation positive tumour compared to that of VHL gene mutation negative tumour. Cells with an inherited VHL mutation and VHL loss of heterozygosity might express high levels of VEGF which cause endothelial cells proliferation and tumor progression. The results presented in this study also suggest novel therapeutic targets for cancer treatment.

Nowadays, anti-VEGF targeted therapies are widely used in the treatment of RCC. Although it has many beneficial effects, drug resistance to that targeted therapy occurs with time. As the VHL gene is the master regulator of these angiogenic agents, drugs that can stabilize or maintain the VHL function or increase the VHL expression should be considered in that situation. By knowing the association between VHL gene mutations and immunoexpression of VEGF in this study, it can be helpful in further invention of molecular targeted therapy for drug resistant clear cell RCC patients.

Competing interests

The authors declare that they have no competing interests.

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