Exploring the anticancer potential of hydrogen sulfide and BAY-876 on clear cell renal cell carcinoma cells: Uncovering novel mutations in VHL and KDR genes among ccRCC patients

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Abstract. The aim of the present study was to determine the cytotoxic effect of BAY-876 and NaSH alone or in combination with sunitinib against the 786-O cell line (renal adenocarcinoma). The IC_{50} of sunitinib, BAY-876 and NaSH were estimated. Cells were cultured in a 96-well plate and then different concentration of each drug alone was exposed for different incubation time; afterwards, cell cytotoxicity was measured using Cell Counting Kit-8 kit. The IC_{50} for each drug was used in next experiment to determine the influence of drug combinations. Furthermore, to observe the effect of mutations of few driver genes in development of clear cell renal cell carcinoma (ccRCC), direct sanger sequencing was used to find single nucleotide polymorphisms in exon 1 and exon 13 of tumor suppressor gene Von Hippel Lindau (VHL) and kinase insert domain receptor (KDR) genes respectively in ccRCC formalin fixed paraffin embedded block samples. The results revealed that the IC_{50} for sunitinib (after 72 h), BAY-876 (after 96 h) and NaSH (after 48 h) was 5.26, 53.56 and 692 μM respectively. The cytotoxic effect of sunitinib and BAY-876, sunitinib and NaSH combinations after 24- and 48-h incubation respectively was significantly higher ($P<0.05$) compared with the control group as well as to sunitinib group alone. These results proved that each of BAY-876 and NaSH have anticancer effect; thus, they could be used in future for ccRCC treatment purpose. Furthermore, direct sequencing results demonstrated unrecorded mutations of VHL and KDR genes is 43.7 and 31.5% of cases respectively. These findings confirmed the leading role of VHL gene in development of ccRCC and the crucial role of KDR gene in angiogenesis and drug resistance.

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Introduction

Renal cancer accounts for 5% of all cancer and ranks as the sixth most prevalent type of cancer in male patients. In female patients, it contributes to 3% of malignancies and stands as the tenth most popular form of tumor (1,2). Renal cell carcinoma (RCC) accounts for >90% of kidney tumors (2,3). Depending on population surveys, a 5-year survival rate was observed in 70% of patients diagnosed with regional cancer. However, for those with metastatic RCC, this rate drops down to 13% (4). Clear cell RCC (ccRCC) is the most common histological subtype of RCC, accounting for 75% of cases, which develops in the epithelium of kidney tubules and can metastasize to different organs (5).

The inactivation of the tumor suppressor gene Von Hippel Lindau (VHL) has a key role in the pathogenesis of clear cell carcinoma (6). It was demonstrated that >90% of patients with ccRCC experience a loss of VHL heterozygosity and inactivating mutations of VHL are detected in 50-65% of instances (7,8). Reducing the level of VHL proteins directly promotes an increase in the expression of both hypoxia-inducible factors (HIFs) including HIF-1A and HIF-2A (9). These factors activate various target genes in the tumor microenvironment which are stimulating tumor cell survival, cell proliferation, angiogenesis, metabolism of sugar and tumor metastasis (10-12). Targeting HIFs or downstream effector molecules in the VHL/HIF pathway (such as VEGFs) has been used for three decades in the treatment of disease outcomes in advanced patients with ccRCC (13).

Sunitinib is one of the earliest approved multi-targeted tyrosine kinase inhibitors (TKIs) that have been extensively used as first-line treatment for metastatic ccRCC (14). TKIs act by inhibiting the kinase activity of various receptors and are still used in therapy (15). Notably, despite its demonstrated efficacy in treating advanced RCC, a substantial proportion of patients develop primary resistance or acquired resistance to sunitinib within 6-15 months of treatment (16). BAY-876 was previously identified as a new-generation inhibitor of glucose transporter 1 (GLUT1) in ovarian cancer (17); however, since its discovery, there have been no studies investigating the effect of BAY-876 as GLUTI in ccRCC.

Hydrogen sulfide (H_2S) is a gaseous transmitter found in mammalian tissues; along with nitric oxide (NO) and

carbon monoxide (CO), it has a variety of biological functions (18,19). Other studies demonstrated that H_2S gas has a dual effect on cancerous diseases, acting as both a pro- and antitumor agent (20,21). Sodium hydrosulfide (NaSH) salt is a solid analog of H_2S gas that provides immediate and direct access to biologically significant forms of sulfide. NaSH has been used extensively as an exogenous delivery method for H_2S to evaluate its therapeutic potential (22). To the best of the authors' knowledge, the effects of NaSH and BAY-876 on RCC remain unclear and a significant number of patients with ccRCC have demonstrated a lack of response, recurrence, or resistance to sunitinib. For these reasons, the present study aimed to investigate the effects of NaSH and BAY-876 alone and in combination with sunitinib on ccRCC cells. The evaluation of genotypic variations and polymorphisms in the VHL and kinase insert domain receptor (KDR) genes in patients with ccRCC was another aim of the present study.

Materials and methods

Cell line. The human metastatic ccRCC cell line (786-O) was obtained from the American Type Culture Collection (cat. no. CRL-1932). The cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc.; cat. no. 31800089) which was supplemented with 10% fetal bovine serum (FBS) and 1% of antibiotic (consisting of 10,000-unit penicillin, 10 mg streptomycin and 25 μg amphotericin; Sigma Aldrich; Merck KGaA; cat. no. A5955). Cells were split every 2-3 days to maintain their density and maintained at 37˚C in a humidified incubator with 5% CO_2 and 95% air (RS Biotech Galaxy Model R+). Following an adaptation step of three passages, the experiments were conducted within passages 11 to 16 of 786-O cells (23).

Determining IC_{50}. To determine the IC_{50} of sunitinib (MedChemExpress; cat. no. HY-10255A), BAY-876 (MedChemExpress; cat. no. HY-100017) and NaSH on 786-O viability, cells were seeded in a 96-well culture plate ($4x10^3$ cell/well) and incubated at 37˚C in a humidified incubator with 5% CO_2. After 24 h, the cells were treated with various concentrations of sunitinib (range, 1-100 μM), BAY-876 (range, 10-1,000 μM) and NaSH (range, 10-10^3 μM) in quadruplicate for each concentration. The plates were incubated for 24, 48 and 72 h at 37˚C with 5% CO_2. For BAY-876 another plate was incubated for 96 h. After each incubation interval, the culture media of the plate wells were changed with new media and then the cell cytotoxicity was measured by using Cell Counting Kit-8 (CCK-8; MedChemExpress; cat. no. HY-K0301) (24) according to the manufacturer's instruction. Briefly, 10 μM of the CCK-8 reagent was added to each well, incubated for 2-4 h at 37˚C and then the absorbance was measured at 540 nm by using an ELIZA reader (BioTek Instruments, Inc.). Each experiment was carried out three times.

Drug combinations. The IC_{50} of sunitinib after 72 h ($5.26x10^{-6}$ M), BAY-876 after 96 h ($53.56x10^{-6}$ M) and NaSH after 72 h ($692x10^{-6}$ M) were used to determine the cytotoxicity of the combination of drugs against 786-O cells. Briefly, $4x10^3$ cells/well were seeded in 96 well plates and incubated at 37˚C with 5% CO_2. After 24 h incubation, the cells were exposed to the IC_{50} of each drug alone and a combination of sunitinib with BAY-876, sunitinib with NaSH and BAY-876 with NaSH. Following incubation for 24 and 48 h at 37˚C and 5% CO_2, cell cytotoxicity was measured by using the CCK-8 kit.

Statistical analysis. Data analysis was conducted via GraphPad Prism 9 Software (Dotmatics). Non-linear regression was employed to establish the IC_{50} of medications, while ordinary one-way ANOVA with Tukey's multiple comparisons test was performed to assess the statistically significant differences in the anticancer activity between various agents and untreated cells. The data were reported as the mean value ± standard error (SE) of the difference. $P<0.05$ was considered to indicate a statistically significant difference.

Formalin-fixed paraffin-embedded tissues of patients with ccRCC. A total of 30 formalin-fixed paraffin-embedded tissues (FFPE) of patients with ccRCC were collected form PAR and Rzgary teaching hospitals (Erbil, Iraq) between July 2022 and May 2023. After histological confirmation of ccRCC through hematoxylin and eosin (H&E) staining, the blocks were collected for extraction of DNA. The approval (approval no. 45/90) of the present study was granted by the Human Ethics Committee of the College of Education of Salahaddin University-Erbil (Erbil, Iraq). The study was conducted according to the criteria set by The Declaration of Helsinki (October 2013) and signed informed consent forms were obtained from the participants approving the use of their tissues for investigation. DNA samples were extracted from the FFPE tissues. Clinicopathological characteristics of patients (including sex and age distribution) are presented in Table I.

Stage assessment of ccRCC. The TNM cancer staging system provides significant predictors of prognosis in RCC. The present study used the eighth edition (TNM8) of the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) for ccRCC tumor staging (25,26). In TNM, the T refers to the size and regional extension of the primary tumor, the N refers to the tumor involvement in the regional lymph nodes and the M refers to metastasis or spread of the tumor to other parts of the body away from the original location (27).

DNA extraction from FFPE tissues. Genomic DNA extraction from FFPE was performed using the QIAamp DNA FFPE Tissue kit (Qiagen GmbH; cat. no. 56404) according to the manufacturer's protocol. Briefly, 5-μm thick ribbon sections were created from the paraffin blocks, followed by deparaffination by xylene. The cells were lysed with lysis buffer and proteinase k. The DNA bound to the membrane and the contaminants were flowing through the membrane. Finally, pure DNA was eluted from the membrane with an elution buffer. The eluted DNA was quantified based on the absorbance (A) measured at 260 nm (A260) and purity was estimated based on the A260/A280 ratio using a Nanodrop (Thermo Fisher Scientific, Inc.). An elution buffer was used as a blank for the Nanodrop instrument. The assessment of DNA purity relied on an ideal A260/A280 ratio of 1.8 (28).

Genotype determination. The current study examined variants of each VHL and KDR gene. Based on the results of a

Table I. Clinicopathological characteristics of patients with clear cell renal cell carcinoma.

Clinicopathological characteristics	Percentage (%)
Sex	
Male	44.40
Female	56.60
Age, years	
>50	50
51-60	20
61-70	20
<71	10
Degree of differentiation	
Well-differentiated tumor	43.33
Moderately differentiated tumor	50
Poorly differentiated	6.66
TNM staging	
Stage I	
pT1a N0 Mx	50
pT1b N0 Mx	26.60
Stage II	
pT2 N0 Mx	3.33
Stage III	
pT1b N1 Mx	6.33
pT3a Nx Mx	3.66
pT3a N0 Mx	6.33
pT3 N1 Mx	3.33

previous study, exon 1 of VHL (29) and exon 13 of KDR genes were chosen for the current study. Initially, the purified DNA underwent an independent process of amplification. For this purpose, a pre-prepared master mix (including dNTP, $MgCl_2$, Taq DNA polymerase and PCR reaction buffer; Promega Corporation) and the following primer pairs were used: VHL forward, 5'-GTCTGGATCGCGGAGGGAAT-3' and reverse, 5'-GGACTGCGATTGCAGAAGAT-3'; KDR forward, 5'-CGT GTCTTTGTGGTGCACTG-3' and reverse, 5'-CTAGGACTG TGAGCTGCCTG-3'. The thermocycling conditions used for PCR were: Initial denaturation at 95˚C for 5 min; 35 cycles of denaturation at 95˚C for 30 sec, annealing at 61˚C for 30 sec and extension at 72˚C for 1 min; final elongation at 72˚C for 6 min and the samples were maintained at 4˚C.

The PCR products were separated by 1.5% agarose gel electrophoresis. DNA ladder of 100 base pairs (GeneDireX, Inc.; cat. no. DM003-R500) was used for band composition as shown in Fig. S1. Before pouring it into the tray, the gel was mixed with Safe gel stain Dye (ADD BIO) and then it was visualized using the UV Transilluminator UST-20M-8K (Biostep GmbH). Following the PCR protocol, the products were delivered to the cytogenetic laboratory-Zheen International Hospital-Erbil-Iraq for sequencing performed on the Automatic 3130 Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the same forward primers for each exon in the genes. The sequencing data were deposited into the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/; accession no. OR294044 and OR339875). Analysis and interpretation of the Sanger sequencing data were performed using the Mutation Surveyor software package 5.1 (SoftGenetics, LLC) by comparing the sequencing results with reference genes in public databases, including ClinVar, dbSNP, gnomAD and COSMIC (30).

Database's gene mutation retrieval and gene interactions. Genome aggregation database (gnomAD) version 3.1.2 (https://gnomad.broadinstitute.org) was used for mutation retrieval of selected genes. This population referencing database currently is one of the most powerful tools that provides information about genetic variation and gene interpretation found in the human population. This resource is developed by worldwide investigators who collect and integrate both genome and exome data from massive sequencing programs. The database nowadays is widely used in clinical genetics and genomic research (31).

All recorded gene mutations of chosen genes (VHL and KDR) in RCC were retrieved from both the Mutagene database (https://www.ncbi.nlm.nih.gov/research/mutagene) and Catalog of Somatic Mutation of Cancer (COSMIC v98, released 23-MAY-23; https://cancer.sanger.ac.uk/cosmic). The Mutagene database contains 441 RRC samples with 33,063 gene mutations. Based on B_{score} thresholds, Mutagene is used to determine the cancer driver, potential cancer driver and passenger mutations of selected genes. COSMIC, which is readily available to researchers globally, is a thorough database encompassing somatic mutations in cancer. It provides extensive information about mutation location, frequency and impact, along with clinical and pathological data. By utilizing this database, researchers can identify prevalent mutations and other genetic changes that potentially play a role in the development or advancement of cancer. Moreover, these findings may offer potential targets for therapeutic interventions (32).

In addition, the interaction of VHL and KDR genes in RCC is predicted through the GeneMANIA anticipation tool (https://genemania.org/). Based on genetic connection, physical protein-protein interaction and co-expression pattern, this public online database can provide gene interaction. By examining the anticipated associations among these two genes, valuable insights into their participation in cellular processes and disease pathways can be gained. This, in turn, could pave the way for the identification of fresh therapeutic targets or diagnostic biomarkers. GeneMANIA through anticipation of gene interaction will lead to determining the function of genes, uncovering gene interaction in specific cellular assays (33) and identifying potential gene candidates for further study.

Results

Anticancer activity of sunitinib, BAY-876 and NaSH. To determine the *in vitro* cytotoxic effect of sunitinib, BAY-876 and NaSH, dose-dependent viability studies were performed to find the appropriate concentration of each drug that can eliminate 50% (IC_{50}) of 786-O cells by using the CCK-8 kit. The IC_{50}s for sunitinib were found to be $9.85x10^6$, $9.1x10^{-6}$ and $5.26x10^{-6}$ M at 24, 48 and 72 h, respectively (Fig. 1A), with no significant difference among the measured timepoints. The IC_{50} for BAY-876 was reached only after 4 days of treatment

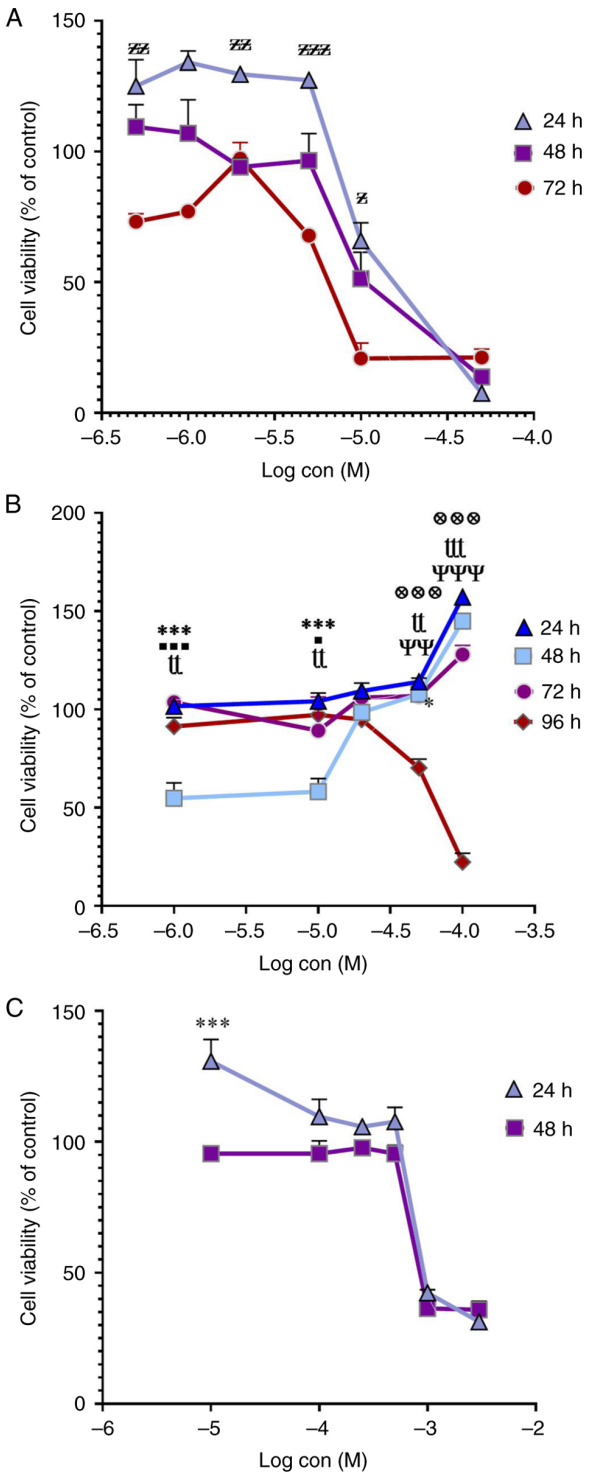

Figure 1. Time- and dose-dependent effect of (A) sunitinib, (B) BAY-876 and (C) NaSH on viability of 786-O cells. Cell viability (%) was expressed as the percentage of treated cells versus log of concentration. Data are expressed as the mean values ± standard error. The *, ■, ŧ, ⊗, Ψ and ≠ represent significant difference in the cytotoxicity effect of specific concentration between 24 and 48, 48 and 72, 48 and 96, 24 and 96, 72 and 96 and 24-72 h of incubation respectively. ■,≠P<0.05, ŧŧ, ΨΨ,≠≠P<0.01 and ***,■■■,ŧŧŧ,⊗⊗⊗,ΨΨΨ,≠≠≠P<0.001.

and it was 53.56x10^{-6} M (Fig. 1B). Furthermore, the present study also revealed that the IC_{50} for NaSH as an exogenous donor of H_2S after 24 and 48 h of treatment were equal to 726x10^{-6} and 692x10^{-6} M, respectively (Fig. 1C), with no significant difference between the two timepoints.

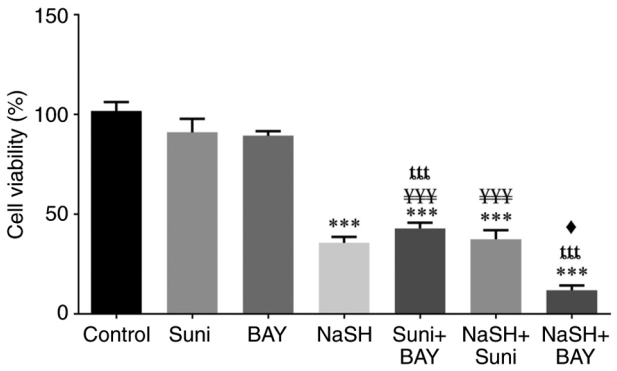

Figure 2. Cytotoxicity effect of sunitinib, BAY-876 and NaSH alone and in combination against 786-O cells after 24 h of treatment. The *, ¥, ŧ and ♦ represent statistical differences from control, sunitinib, BAY-876, and NaSH respectively. Each column represents the mean of four data; three independent experiments were performed. ♦P<0.05 and ***,¥¥¥,ŧŧŧP<0.001.

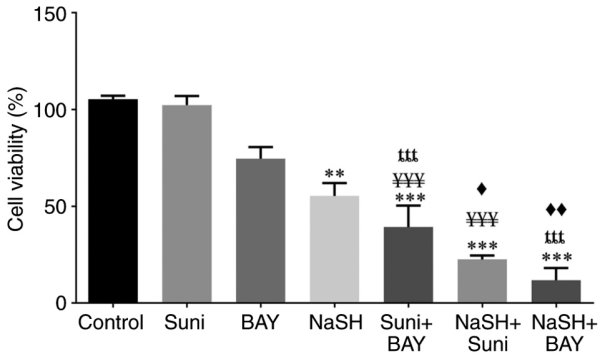

Figure 3. Cytotoxicity effect of sunitinib, BAY-876 and NaSH alone and in combination against 786-O cells after 48 h of treatment. The *, ¥, ŧ and ♦ represent statistical differences from control, sunitinib, BAY-876, and NaSH respectively. Each column represents the mean of four data; three independent experiments were performed. ♦P<0.05, **,♦♦P<0.01 and ***,¥¥¥,ŧŧŧP<0.001.

In addition, the cytotoxic effect against 786-O cells of several drug combinations was assessed. Sunitinib IC_{50} after 3 days, BAY-IC_{50} after 4 days and NaSH after 2 days were used alone and in combination for 24 (Fig. 2) and 48 h (Fig. 3). The results after 24 h demonstrated that there were no significant differences in cytotoxicity between sunitinib and BAY-876 treatments alone compared with the control group; however, their combination demonstrated statistically different cytotoxicity (P<0.001) compared with the control group and each drug used individually.

Regarding the cytotoxic effect of NaSH, NaSH alone and in combination with either sunitinib or BAY-876 revealed a statistically significant increase (P<0.001) in cytotoxicity compared with the control group. The cytotoxicity of NaSH in combination with BAY-876 was significantly higher (P<0.05) compared with NaSH alone. However, there was no significant difference between NaSH and sunitinib co-treatment and NaSH.

The outcomes of the drug combinations at 24 and 48 h were comparable and the only exception was that there was a statistically significant difference (P<0.05) between the NaSH and sunitinib co-treatment and NaSH alone in reducing cell viability.

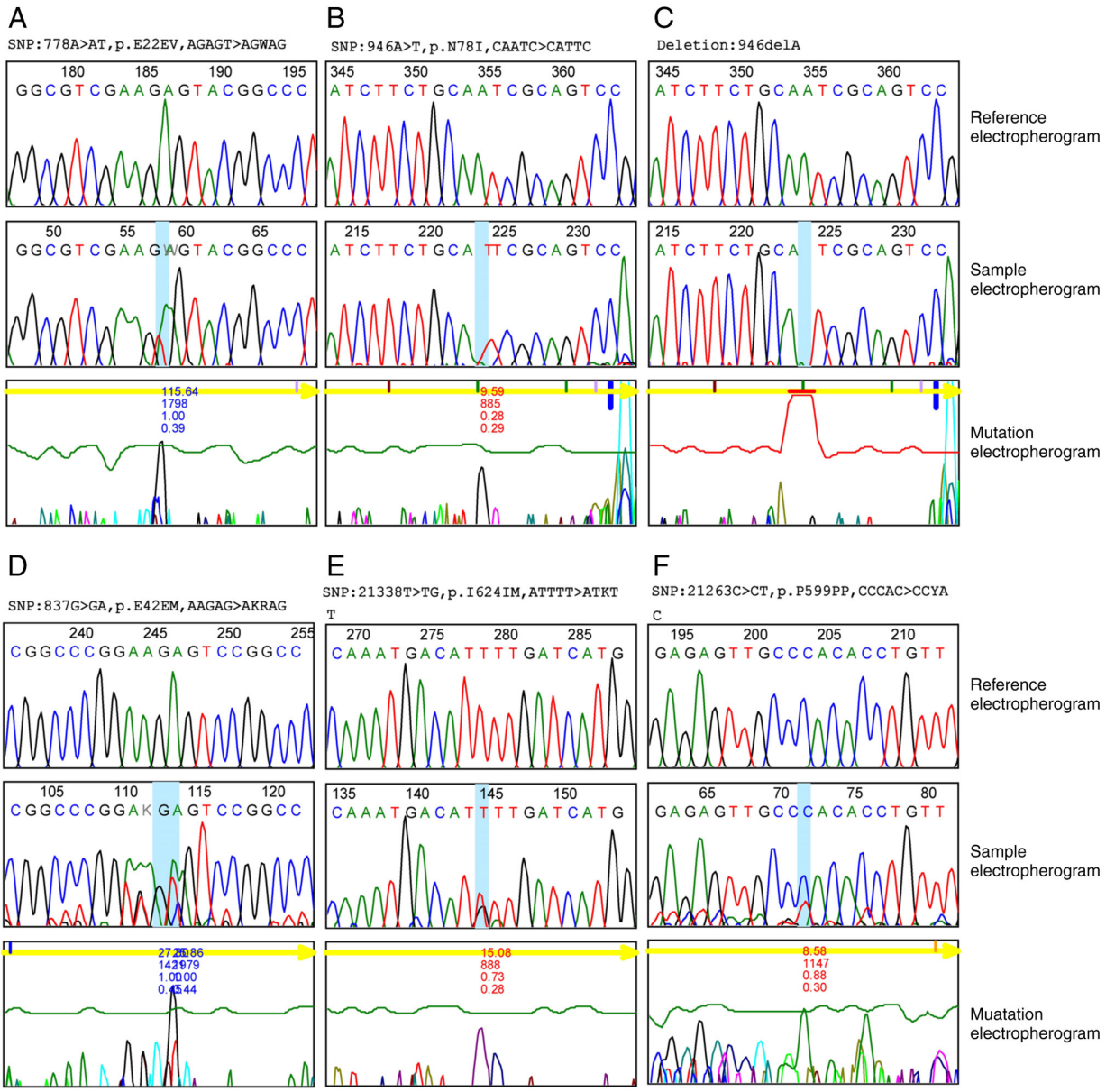

Figure 4. Graphical representation of recognized SNPs in exon 1 of VHL and exon 13 of KDR genes in patients with ccRCC. (A) and (B) Heterozygous mutation from A to AT and homozygous mutation from A to T respectively in exon 1 of VHL genes. (C) and (D) Homozygous deletion (946del) and heterozygous substitution from G to GA respectively in VHL gene that lead to change in amino acid sequences. (E) and (F) Heterozygous substitution from T to TG and C to CT respectively in exon 13 of KDR gene, the first one led to a change in amino acid sequence, while the second one did not. SNPs, single nucleotide polymorphisms; VHL, tumor suppressor gene Von Hippel Lindau; KDR, kinase insert domain receptor.

Patient characteristics. In the current study, out of 30 patients with ccRCC, 17 were male (56.6%) and 13 were female (44.40%). Regarding the patient age, 50 (50%) were under 50 years, 6 (20%) were between 51 and 60 years, 6 (20%) were between 61 and 70 years, and 3 (10%) were >71 years old. The degree of cell differentiation varied among patients with ccRCC. A large number of patients were grouped as medium-differentiated (50%) and well-differentiated carcinomas (43.33%), while poorly-differentiated carcinomas accounted only for 6.66% (Table I).

Mutation analysis. Single nucleotide polymorphism (SNP) was recorded in 47.7% of the VHL gene and 31.5% of the KDR gene (data not shown). In exon 1 of the VHL gene of ccRCC, 36 SNP mutations were identified, including 35 substitution mutations (A>AT, C>CT, A>AG, G>GA, T>TC, C>CA, A>T) and one deletion mutation (delA). Both heterozygous (n=35) and homozygous (n=1) variant mutations were identified as demonstrated in Fig. 4. In total, 35 variant mutations were previously not recorded in external databases and 30 of them had their sequences of amino acids changed (Table II). The single homozygous substitution variant (946A>T) accounts for the greatest percentage (40%) among all other variants; however, among heterozygous variants, two heterozygous variants (778A>AT and 793A>AT) constitute the second greatest percentage (33.30%) among other mutation of VHL gene.

Regarding to KDR gene, 16 heterozygous substitution variant mutations (C>CG, C>CT, G>GT, T>TC, A>AC, G>GC, C>CA, G>GA) were recorded in exon 13; all of them were not recorded previously in external databases. A

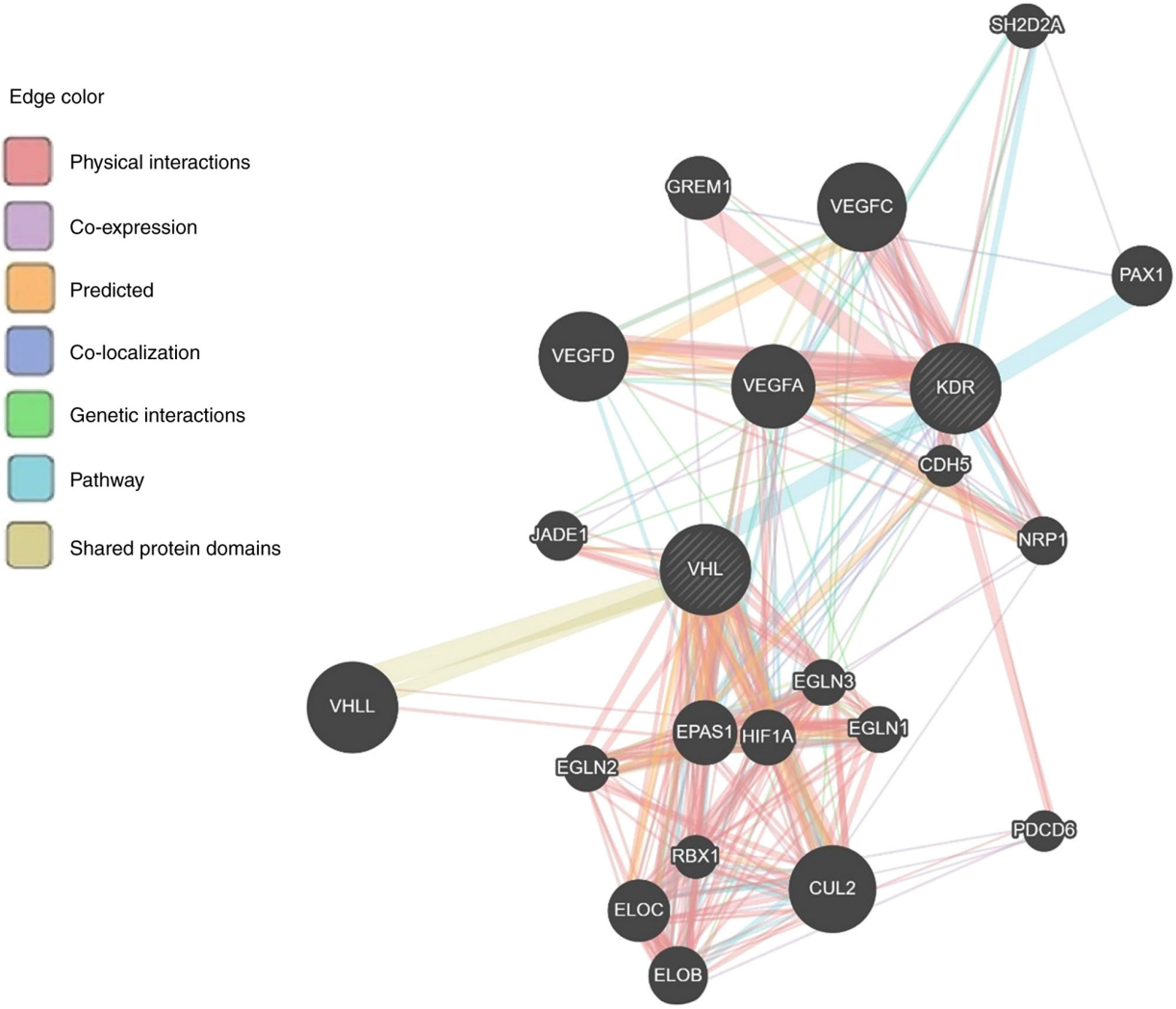

Figure 5. The relation network of VHL and KDR genes with other genes by GeneMANIA. A total of 13 genes have relationship with VHL and 12 with KDR genes. Some genes such as VHLL and VEGFC are surrounded by big circles indicating their crucial roles in signaling pathways VHL and KDR genes. Physical interaction is the most and shared protein domains is the least common type of relation between genes. VHL, tumor suppressor gene Von Hippel Lindau; KDR, kinase insert domain receptor.

total of 11 of these mutations are associated with changes in the sequence of amino acids. Furthermore, the heterozygous variant (21234C>CG) constitutes the largest percentage (28%) among other variants (Table II).

Database mutations and gene interactions. The GnomAD tool was used to retrieve the entire recorded mutations of VHL and KDR genes in all types of cancer. VHL had 386 mutations, while KDR had 1,595 mutations as presented in Table SI.

Additional analysis of retrieved VHL and KDR gene mutations in RCC was performed using Metagene (all genome-wide studies in ICGC) and COSMIC databases as demonstrated in Tables SII and SIII. The number of mutations in VHL was 125 and 1,569 in the Metagene and COSMIC databases, respectively; however, the number of mutations of the KDR gene was 229 and 27 in the same two databases, respectively. In the Mutagen database the most common type of mutation in the VHL gene of patients with RCC was missense mutation followed by nonsense mutation. Out of 125 mutations, 66 were passengers followed by 34 drivers and 25 potential driver mutations. Regarding the KDR gene, the most common type of mutation was missense followed by silent mutation, and their status in cancer participation was mostly passenger (n=179) followed by potential driver (n=34) and driver (n=16) mutations. In the COSMIC database, the frameshift deletion (n=530) was the most common type of mutation of VHL in patients with ccRCC followed by missense-substitution (n=339), frameshift insertion (n=219) and nonsense substitution (n=62). However, missense substitutions (n=20) were common types of mutations of the KDR gene in patients with ccRCC followed by frameshift deletions (n=3), nonsense substitution (n=2) and substitution-coding silent (n=2).

Moreover, data from the GeneMANIA predicting tool revealed that 13 genes (RBX1, VHLL, CUL2, VEGFA, EPAS1, ELOC, PAX1, ELOB, HIF1, JADE1, EGLN1, EGLN2 and EGLN3) and 12 genes (CDH5, PDCD6, VEGFA, VEGFC, VEGFD, EPAS1, GREM1, HIF1, JADE1, NRP1, EGLN3 and SH2D2A) are associated with VHL and KDR genes, respectively, mainly through physical interaction then co-expression, prediction, colocalization, genetic interaction, pathway and minimum level by sharing protein domain (Fig. 5 and Table SIV). A total of five genes (VEGFA, EPAS1, HIF1,

Table II. Variants identified in VHL and KDR genes in ccRCC patients analyzed with mutation DNA variant analysis.

	Chromosome position	Mutation	Mutation enotype	Heterozygous/ Homozygous	Variants	Variant percentage (%)	Amino acid change	External database
VHL gene								
1	3:10183764	Substitution	A>T	Homozygous	946A>T	40.00	Asparagine/Isoleucine	Not found
2	3:10183623	Substitution	A>AT	Heterozygous	805A>AT	30.00	Glutamic acid/Valine	Not found
3	3:10183626	Substitution	A>AT	Heterozygous	808A>AT	30.00	Glutamic acid/Valine	Not found
4	3:10183638	Substitution	A>AT	Heterozygous	820A>AT	30.00	Glutamic acid/Valine	Not found
5	3:10183641	Substitution	A>AT	Heterozygous	823A>AT	30.00	Glutamic acid/Valine	Not found
6	3:10183656	Substitution	A>AT	Heterozygous	838A>AT	30.00	Glutamic acid/Methionine	Not found
7	3:10183671	Substitution	A>AT	Heterozygous	853A>AT	30.00	Glutamic acid/Valine	Not found
8	3:10183673	Substitution	C>CA	Heterozygous	855C>CA	10.00	Lucine/Methionine	Not found
9	3:10183686	Substitution	A>AT	Heterozygous	868A>AT	30.00	Glutamic acid/Valine	Not found
10	3:10183730	Substitution	A>AT	Heterozygous	912A>AT	18.20	Asparagine/Tyrosine	Not found
11	3:10183570	Substitution	A>AT	Heterozygous	752A>AT	25	none	Not found
12	3:10183578	Substitution	A>AT	Heterozygous	760A>AT	25	Glutamic acid/Valine	Not found
13	3:10183581	Substitution	A>AT	Heterozygous	763A>AT	25	Glutamic acid/Valine	Not found
14	3:10183582	Substitution	G>GA	Heterozygous	764G>GA	25	Glutamic acid/Valine	Not found
15	3:10183596	Substitution	A>AT	Heterozygous	778A>AT	33.30	Glutamic acid/Valine	Not found
16	3:10183597	Substitution	A>AT	Heterozygous	793A>AT	33.30	Glutamic acid/Valine	Not found
17	3:10183612	Substitution	A>AG	Heterozygous	794A>AG	22.20	Glutamic acid/Valine	Not found
18	3:10183635	Substitution	C>CT	Heterozygous	817C>CT	30.00	Alanine/Valine	Not found
19	3:10183655	Substitution	G>GA	Heterozygous	837G>GA	10	Glutamic acid/Methionine	Not found
20	3:10183611	Substitution	A>AT	Heterozygous	793A>AT	33.30	Glutamic acid/Valine	Not found
21	3:10183614	Substitution	A>AT	Heterozygous	796A>AT	11.10	Aspartic acid/Valine	Not found
22	3:10183764	Deletion	A		946delA	25	none	Not found
23	3:10183635	Substitution	C>CT	Heterozygous	817C>CT	30.00	Aspartic acid/Valine	Not found
24	3:10183579	Substitution	G>GA	Heterozygous	761G>GA	12.50	Glutamic acid/Valine	Not found
25	3:10183608	Substitution	A>AT	Heterozygous	790A>AT	11.10	Glutamic acid/Valine	Not found
26	3:10183624	Substitution	G>GA	Heterozygous	806G>GA	10.00	Glutamic acid/Valine	Not found
27	3:10183740	Substitution	A>AT	Heterozygous	922A>AT	16.70	Glutamic acid/Valine	Not found
28	3:10183672	Substitution	A>AG	Heterozygous	854A>AG	9.10	Glutamic acid/Valine	Not found
29	3:10183762	Substitution	C>CT	Heterozygous	944C>CT	10	none	Not found
30	3:10183763	Substitution	A>AG	Heterozygous	945A>AG	12.50	Isoleucine/Valine	Not found
31	3:10183691	Substitution	A>AT	Heterozygous	873A>AT	9.10	Methionine/Leucine	Not found
32	3:10183753	Substitution	C>CT	Heterozygous	935C>CT	8.30	none	Not found
33	3:10183759	Substitution	C>CT	Heterozygous	941C>CT	9.10	none	Not found

Table II. Continued.

	Chromosome position	Mutation	Mutation enotype	Heterozygous/ Homozygous	Variants	Variant percentage (%)	Amino acid change	External database
VHL gene								
34	3:10183756	Substitution	C>CT	Heterozygous	938C>CT	8.30	none	Not found
35	3:10183719	Substitution	T>TC	Heterozygous	901T>TC	9.10	Leucine/Proline	Not found
36	3:10183764	Substitution	A>AG	Heterozygous	946A>AG	12.50	Asparagine/serine	found
KDR gene								
1	4:55971029	Substitution	C>CG	Heterozygous	21234C>CG	28.00	Proline/Alanine	Not found
2	4:55970820	Substitution	C>CT	Heterozygous	21443C>CT	8.30	None	Not found
3	4:55970985	Substitution	G>GT	Heterozygous	21278G>GT	16.70	Lysin/Asparagine	Not found
4	4:55970981	Substitution	T>TC	Heterozygous	21282T>TC	12.50	Leucine/Proline	Not found
5	4:55970980	Substitution	T>TC	Heterozygous	21283T>TC	12.50	Leucine/Proline	Not found
6	4:55970991	Substitution	T>TC	Heterozygous	21272T>TC	16.70	None	Not found
7	4:55970969	Substitution	T>TG	Heterozygous	21294T>TG	20	Tryptophane/Glycine	Not found
8	4:55970819	Substitution	A>AC	Heterozygous	21444A>AC	8.30	Threonine/Proline	Not found
9	4:55971034	Substitution	C>CT	Heterozygous	21229C>CT	25.30	Proline/Leucine	Not found
10	4:55971000	Substitution	C>CT	Heterozygous	21263C>CT	25.10	None	Not found
11	4:55970978	Substitution	G>GC	Heterozygous	21285G>GC	12.50	Aspartic acid/Histidine	Not found
12	4:55970970	Substitution	T>TC	Heterozygous	21293T>TC	10.00	None	Not found
13	4:55970996	Substitution	C>CG	Heterozygous	21267C>CG	25.00	Proline/Aspartic acid	Not found
14	4:55970995	Substitution	C>CA	Heterozygous	21268C>CA	25.00	Proline/Aspartic acid	Not found
15	4:55970892	Substitution	G>GA	Heterozygous	21371G>GA	7.10	None	Not found
16	4:55970925	Substitution	T>TG	Heterozygous	21338T>TG	7.10	Aspartic acid/Histidine	Not found

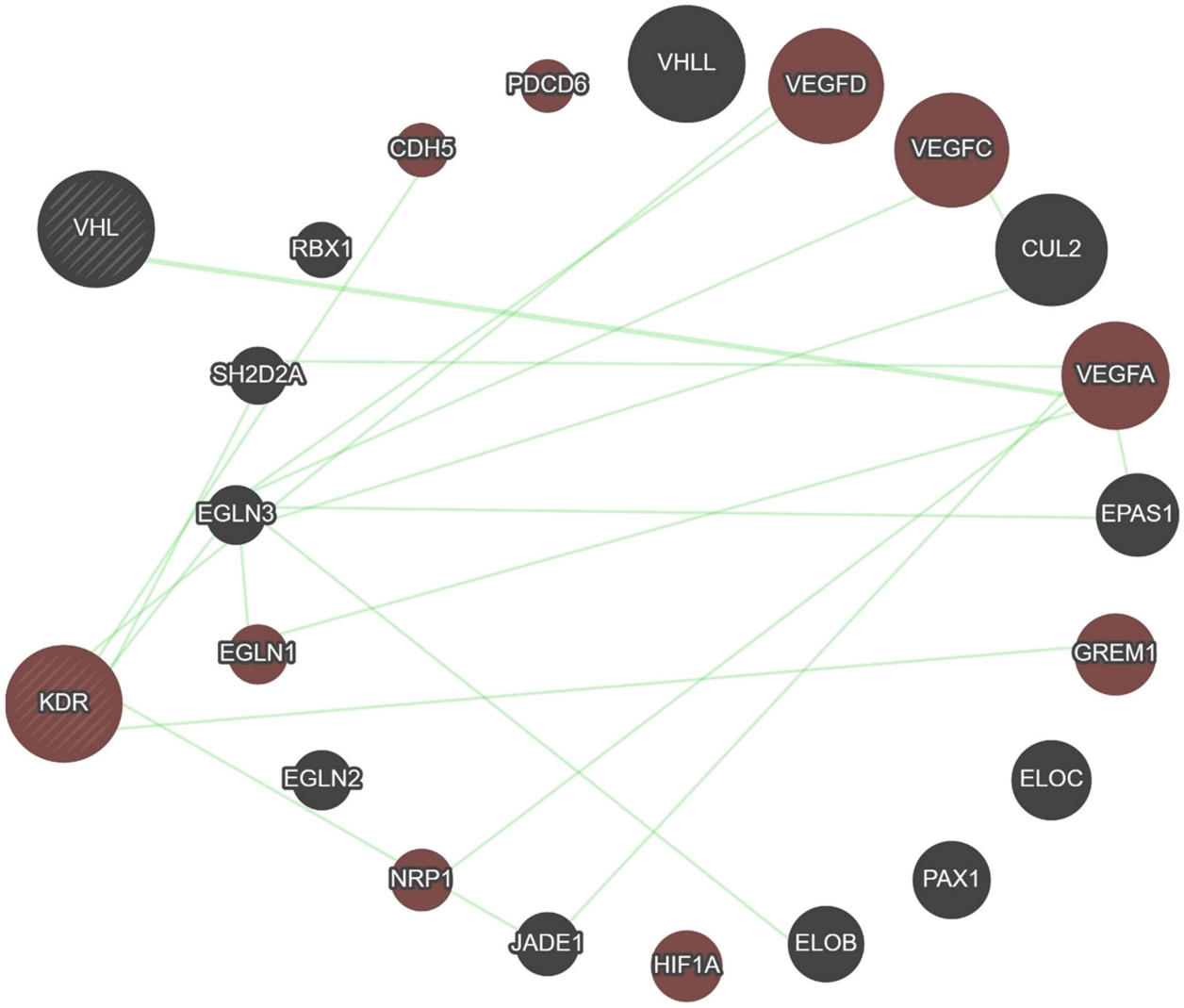

Figure 6. The genetic interaction of VHL and KDR genes with other genes which have a role in cancer angiogenic (highlighted with brown) by GeneMANIA. KDR gene interacts with most of these angiogenesis genes while VHL interaction with these genes is lower. VHL, tumor suppressor gene Von Hippel Lindau; KDR, kinase insert domain receptor.

JADE1 and EGLN3) are shared between VHL and KDR genes, and this is considered a close relation between these two genes. Furthermore, the KDR gene showed genetic interaction with most of the genes having a role in angiogenesis (Fig. 6).

Discussion

In the present study, the sunitinib IC_{50} after 24 and 48 h was ~$9x10^{-6}$ M, whereas after 72 h it dropped to $5.26x10^{-6}$M. These results are very similar to previous results demonstrating the cytotoxicity of sunitinib against RCC cell lines. Sato *et al* (34) identified that the IC_{50} value of sunitinib against 786-O cells (3,000/well) was 5 μM after a 48-h incubation. This disparity in the results might be attributed to the initial cell count used in the experiment as the current study involved plating 4,000 cells in each well of a 96-well plate, which could have affected the observed IC_{50} value due to variations in cell density. Another study reported that sunitinib IC_{50} was 3.99 μM against Caki-1 cells after 48 h (35). In this case, the discrepancy with the present results might be attributed to the differences in the cell line.

In the current study, ccRCC 786-O cells were exposed for 24, 48, 72, and 96 h to several concentrations of BAY-876. The results demonstrated that BAY-876 can reduce 786-O cells in a dose-dependent manner and the IC_{50} was $53.56x10^{-6}$ M. As RCC specimens demonstrated a remarkably significant rise in the expression levels of GLUT1 compared with the corresponding normal kidney tissue (36,37), the present finding proves that reducing cell glucose intake by inhibiting GLUT1 through BAY-876 treatment, could represent a new strategy to suppress the growth of ccRCC. Moreover, the present result is consistent with a recent study (38) showing that BAY-876 has an inhibitory effect against bladder urothelial carcinoma (BLCA) cell lines and tumor inhibition effect in xenograft models. It was proposed that CLUTI inhibition through BAY-876 could be an effective approach to inhibiting the growth of BLCA tumors. Furthermore, another study (17) revealed that BAY-876 is a strong blocker of GLUT1 activity, mechanisms of glycolysis and ovarian cancer cell growth in ovarian cancer cell lines, as well as cell line-derived xerographs and ovarian cancer patient-derived xenografts.

The present results identified that the combination of BAY-876 and sunitinib has a significant cytotoxic effect on 786-O cells at shorter incubation times (after 24 and 48 h) compared with each drug alone, which had no cybotactic effect at these intervals. This combination may eliminate the cell by reducing the sugar intake by blocking GLUT1 transporters as well as VEGF, PDGF and c-Kit receptors (39). The multi-shunt elimination of 786-O cells during drug combination exposure may be attributable to a reduction in the duration of cell death when compared with each drug administered separately. This could be a promising advancement in the treatment of RCC.

RCC presents a contradictory aspect of the action of endogenous H_2S. Numerous studies demonstrated an increase in H_2S levels or H_2S-producing enzymes in RCC cell lines, xenograft models and ccRCC tissues. These findings suggested a correlation between H_2S and RCC growth and progression (40,41). On the contrary, another study argued that the expression of H_2S-producing enzymes in ccRCC was reduced (42). The findings of the present study indicated that the use of NaSH alone resulted in a significant increase in cell death compared with the cells that were not treated. This finding aligns with the results of a previous study which demonstrated that NaSH has a significant impact on reducing cell viability in three different breast cancer cell lines (MCF-10, MCF-7 and MDA-MB-23) (43). The aforementioned study also demonstrated that exogenous H_2S can suppress the progression of RCC by inhibiting the P13K/AKT pathway as well as by inducing apoptosis.

The cytotoxic activity of both sunitinib and BAY-876 increased when combined with NaSH. Overall, the combination of NaSH and BAY-876 could represent a novel approach to treating resistant metastatic ccRCC. If they are used in treatment, they may reduce the serious health impact and adverse reaction of sunitinib and other TKIs.

The clinicopathological characteristics of the patients in the present study revealed a slightly higher incidence rate of ccRCC in male patients (56.6%) compared with female patients (44.4%). However, a recent study demonstrated a significantly higher incidence rate of ccRCC in male patients (80%) compared with female patients (20%) (44). In addition, the TNM staging revealed that the incidence rates for stages I, II and III were 76.66, 3.33 and 20%, respectively.

Inactivation of the gene encoding the tumor suppressor VHL mutations is the most prevalent in ccRCC (45,46). This present study revealed that the incidence of intragenic VHL mutation was 43.7%, which is marginally lower than the percentage observed in a study conducted in Japan, which was 51% (47). This may be because only exon 1 was sequenced in the present research, whereas the entire gene was sequenced in the study by Kondo *et al* (47). In addition, the present study identified 36 missense SNP mutations in exon 1 of the VHL gene. This funding is consistent with the findings of a recent study which demonstrated that all mutations in VHL are missense mutations in ccRCC (48), and with COSMIC databases, which demonstrated that missense mutation is superior to other types of mutations in RCC. Based on this number of point mutations in VHL, mostly with an amino acid change (30 out of 36), the present study suggested that mutations in this gene could be the primary cause of ccRCC and confirmed that TKIs such as sunitinib may be one of the best options for inhibiting the growth of this type of kidney cancer, as they inhibit angiogenesis by inhibiting the downstream pathways of VHL/HIF.

VEGFR-2 serves a crucial role as a primary mediator in tumor angiogenesis and is regarded as a significant target for therapeutic interventions to counteract angiogenesis. Several anti-angiogenic medications, including ramucirumab, sunitinib, axitinib and sorafenib, have been developed to target this pathway (49). A previous study revealed that the rs1870377 A>T genetic polymorphism of VEGFR-2 is associated with the prognosis of gastric cancer (50). The present study demonstrated that there were 16 heterozygous missense variants in exon 13 of KDR, which is consistent with data from both the COSMIC and mutagen databases. This may explain the function of KDR gene mutation in the development of ccRCC and resistance to numerous anti-angiogenic drugs. KDR mutation testing is crucial for guiding the choice of therapy and improving patient outcomes.

Moreover, the present study found that BAY-876 and NaSH could improve sunitinib's anticancer efficacy in ccRCC cell lines, suggesting a novel treatment approach. It also found that VHL mutation is the main cause of ccRCC development and KDR mutation is necessary for angiogenesis. Testing these mutations might help choose the right therapy and understand the illness process. To the best of the authors' knowledge, the present study is the first to identify a significant number of VHL and KDR gene mutations in patients with ccRCC. By interpreting these gene mutations and relating their detection with relevant mutation-related datasets, the molecular mechanisms that initiate and progress ccRCC were elucidated. The present investigation is important in the context of RCC research and lays the groundwork for future studies. However, additional research is needed to confirm the current outcomes on other cell lines, also to determine the cellular and physiological effects of these agents. Functional studies could investigate the molecular effects of these mutations, their effects on clinical outcomes in larger groups, their predictive value for treatment response and genomic analysis to identify more mutations and variations. Notably, the significance of tracking response rate and cancer regression rate over time is recognized as a crucial avenue for future research and it is intended by the authors to contemplate integrating this data into subsequent research endeavors or offering a more comprehensive analysis in subsequent studies.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

PSH conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article. KAM conceived and designed the experiments, performed the experiments, authored, or reviewed drafts of the article. PSH and KAM confirm the authenticity of all the raw data. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved (approval no. 45/90) by the Human Research Ethics Committee of Affiliated Salahaddin University (Erbil, Iraq). Written informed consent forms were obtained from the participants approving the use of their tissues for investigation.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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