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# Cyclin D1 as a therapeutic target of renal cell carcinoma- a combined transcriptomics, tissue microarray and molecular docking study from the Kingdom of Saudi Arabia

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From 3rd International Genomic Medicine Conference  
Jeddah, Saudi Arabia. 30 November - 3 December 2015

## Abstract

**Background:** Renal cell carcinoma (RCC) is a seventh ranked malignancy with poor prognosis. RCC is lethal at metastatic stage as it does not respond to conventional systemic treatments, and there is an urgent need to find out promising novel biomarkers for effective treatment. The goal of this study was to evaluate the biomarkers that can be potential therapeutic target and predict effective inhibitors to treat the metastatic stage of RCC.

**Methods:** We conducted transcriptomic profiling to identify differentially expressed genes associated with RCC. Molecular pathway analysis was done to identify the canonical pathways and their role in RCC. Tissue microarrays (TMA) based immunohistochemical stains were used to validate the protein expression of cyclinD1 (CCND1) and were scored semi-quantitatively from 0 to 3+ on the basis of absence or presence of staining intensity in the tumor cell. Statistical analysis determined the association of CCND1 expression with RCC. Molecular docking analyses were performed to check the potential of two natural inhibitors, rutin and curcumin to bind CCND1.

**Results:** We detected 1490 significantly expressed genes (1034, upregulated and 456, downregulated) in RCC using cutoff fold change 2 and  $p$  value  $< 0.05$ . Hes-related family bHLH transcription factor with YRPW motif 1 (HEY1), neuropilin 2 (NRP2), lymphoid enhancer-binding factor 1 (LEF1), and histone cluster 1 H3h (HIST1H3H) were most upregulated while aldolase B, fructose-bisphosphate (ALDOB), solute carrier family 12 (SLC12A1), calbindin 1 (CALB1) were the most down regulated genes in our dataset. Functional analysis revealed Wnt/ $\beta$ -catenin signaling as the significantly activated canonical pathway ( $z$  score = 2.53) involving cyclin D1 (CCND1). CCND1 was overexpressed in transcriptomic studies (FC = 2.26,  $p$  value = 0.0047) and TMA results also showed the positive expression of CCND1 in 53 % (73/139) of RCC cases. The ligands – rutin and curcumin bounded with CCND1 with good affinity.

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**Conclusion:** CCND1 was one of the important upregulated gene identified in microarray and validated by TMA. Docking study showed that CCND1 may act as a potential therapeutic target and its inhibition could focus on the migratory, invasive, and metastatic potential of RCC. Further in vivo and in vitro molecular studies are needed to investigate the therapeutic target potential of CCND1 for RCC treatment.

**Keywords:** Renal cell carcinoma, Cyclin D1, Gene expression profiling, Tissue microarray, Molecular docking, Therapeutic target, Saudi Arabia

## Background

Renal Cell carcinoma (RCC) is a major health problem and accounts for approximately 1.5 percent of all cancer deaths [1, 2]. It accounts for about 3 % of all cancers and 2-3 % per year increase in global incidence [1, 3]. For RCC treatment, surgery is the best option at advance stage, however, one third of patients develop metastases even after surgery [4]. At metastatic stage, prognosis is very poor because RCC patients hardly respond to conventional existing systemic treatments and leads to death [5]. RCC treatment is a big challenge without identification of new drug targets and effective remedies. Although previous studies have reported role of gene alterations, their expression and deregulation of molecular signals to be linked with cancer initiation and progression, there still lack of curative therapy for RCC [6–8]. Therefore, identification of a potential drug target and prediction of suitable ligand is crucial for the patients with RCC.

The cyclin D members (D1, D2 and D3) bind to CDKs and are required for the hematopoietic cells proliferation and survival and perform a rate-limiting antiapoptotic function in vivo [9]. Cyclin D1 (CCND1) overexpression is predominantly correlated with early cancer onset, tumor progression, shorter cancer patient survival and increased metastases [10–12]. Induction of vascular endothelial growth factor (VEGF) production by CCND1 promotes oncogenesis by increasing growth and angiogenesis, while downregulation of death receptor, Fas by CCND1 causes chemotherapeutic and apoptosis resistance [13]. Overexpression of CCND1 has been previously reported in many cancers including lung cancers [14], esophageal squamous cell carcinoma [15], head and neck cancer [16], pancreatic cancer [17], pituitary cancer [18], and breast cancer [19].

CCND1 is a proto-oncogene and a good biomarker for tumor progression, found to be deregulated in several cancers, including RCC. CCND1 along with associated cyclins activates cyclin-dependent kinases (CDKs) - CDK4 and CDK6. G<sub>1</sub>-S phase transition during cell cycle, requires phosphorylation of retinoblastoma (Rb) by CDK4 and CDK6. Hyperphosphorylation of Rb allows expression of genes involved in DNA replication and cell division [20–23]. The ability of CCND1 to exhibit oncogenic property and to regulate a critical G<sub>1</sub>-S transition checkpoint

by activating CDK4/CDK6, makes it a potential therapeutic target of RCC [24–28].

Alternative or synergistic anticancer therapies using natural compounds and their derivatives (polyphenols, flavonoids, alkaloids, saponins, etc.) have been extensively studied [29]. Rutin is a flavonol glycoside found in many plants, including buckwheat; tobacco; asparagus, green tea etc. and contributes to the antibacterial [30], hepatoprotective [31], neuroprotective [32] and antioxidant [33] properties of the plant. It is structurally very similar to quercitrin and has been used therapeutically to decrease capillary fragility, to protect blood capillaries, and as ingredients of multivitamin nutritional supplements and alternative herbal remedies. It can attach to iron ion, thereby averting its binding to H<sub>2</sub>O<sub>2</sub> and free radical generation. In addition, rutin acts as an angiogenesis inhibitor and can stall the VEGF in vitro; also has potential anticancerous and antiproliferative property [34, 35].

Curcumin commonly known as turmeric is a phytopolyphenol pigment isolated from the plant *Curcuma longa*, and possesses a variety of pharmacologic properties like anti-inflammatory, antineoplastic, antiproliferative, anticancer, apoptosis inducer, chemopreventive [36, 37]. It can inhibit the reactive-oxygen species formation, cyclooxygenases (COX) and other metabolic enzymes involved in inflammation; and can disrupt cell signal transduction via inhibition of protein kinase C. It can interact with myriad of biomolecules by covalent and non-covalent binding. The H-bonding and hydrophobic interactions, arising from the aromatic and tautomeric structures in addition to the flexible linker group owe for the non-covalent interactions [38]. Curcumin reportedly suppress cyclin D1 expression by promoting proteolysis and down-regulating its expression and causes inhibition of CDK4-mediated phosphorylation of retinoblastoma protein [39]. It has been reported that curcumin-treated cells show decreased expression of CCND1, resulting in low cell growth rate. This curcumin-induced CCND1 mRNA down-regulation is perhaps mediated by induction of BTG2 as well as inhibition of nuclear translocation of NF-kappaB [40].

In this study, expression profiling of RCC (CEGMR data) identified 1490 significantly differentially expressed genes and molecular pathway analysis predicted alteration in many important cancer related pathways. However, the

major finding of this study was identification and tissue microarray based validation of CCND1 as important over-expressed gene/proteins of RCC. Overexpression of CCND1 can trigger cancer by activating many pathways, including Wnt/ $\beta$ -catenin signaling pathway and has been shown to exhibit oncogenic property, making it a potential therapeutic target. We, therefore, attempted docking study to show the therapeutic potential of anticancerous natural ligands (rutin and curcumin) against the identified potential drug target (CCND1).

## Methods

### Patients and samples

The study was executed on RCC patients from Saudi Arabia and resected tissue samples were collected from collaborating hospitals of Jeddah during the period 2010–2014. For gene expression analysis, fresh surgically resected tumor and normal tissue were collected and stored in RNALater (Invitrogen/Life Technologies, NY, USA) till RNA extraction. All patients included in the present study were Saudi in origin and diagnosed with clear cell or chromophobe renal cell carcinoma without any prior chemotherapy or radiotherapy exposure.

### Ethical approval

Local ethical committee has approved this study (08-CEGMR-02-ETH). Patients were included in the present study only after their prior consent.

### RNA extraction and array processing

Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) was used to extract total RNA from fresh kidney tissue, Nano Drop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used for concentration determination and RNA quality was checked with Bioanalyzer (Agilent Technologies, CA, USA). Out of 20 specimen, only 7 tumor and 5 control samples passed the selection criteria of RNA integrity number (RIN)  $>5$  and were judged fit to be used for array expression analysis. We used Human Gene 1.0 ST GeneChip arrays (Affymetrix, Santa Clara, CA, USA) for transcriptomics studies (Life Technology, Grand Island, NY), interrogating 764,885 probes and 36,079 annotated reference sequences (NCBI build 36). We processed 250 ng RNA of 12 samples using the Ambion WT Expression Kit (Life Technologies, Austin, TX), GeneChip Hybridization, Wash and Stain Kit (Affymetrix, Santa Clara, CA) and GeneChip WT Terminal Labeling and Controls Kit (Affymetrix, Santa Clara, CA). The hybridization of 5500 ng of cDNA was done in a hybridization oven at 45 °C under rotation (60 rpm) for 17 h. After complete processing, the arrays were scanned in the GeneChip Scanner 3000 7G and GeneChip Command Console Software (AGCC) were used to generate probe cell intensity data (CEL files).

### Gene expression analysis

We carried transcriptomic profiling of 12 samples, seven RCC and five normal kidney tissues. To gain confidence with our limited number samples, we performed a comparative analysis with independent expression datasets from NCBI's GEO database (GSE781,  $n = 34$ ; GSE7023,  $n = 47$ ; and GSE6344,  $n = 40$ ) for confirmation. Affymetrix. CEL files were imported and analyzed using Partek Genomics Suite version 6.6 (Partek Inc., MO, USA). Default settings robust multi-chip averaged (RMA) was used to log-transform data set and for normalization. Analysis of Variance (ANOVA) was applied, and differentially expressed genes (DEGs) were identified with cut off fold change  $> 2$  and  $p$  value  $< 0.05$ . Principal component analysis (PCA) was performed to assess overall expression pattern among sample groups, similar samples were grouped together.

### Tissue microarray and immunohistochemistry

Tissue microarrays (TMA) were designed and constructed for 139 primary RCC and 34 normal kidney tissue as previously described [41]. Experienced pathologist reviewed hematoxylin and eosin (HE) slides of RCC and normal kidney tissue. 1.5 mm tissue cores from areas of interest were chosen from donor block(s) and transferred to recipient paraffin block of TMA Master 1.14 SP3 (3D Histech Ltd, Budapest, Hungary). HE staining of TMA slides was repeated to assess basic morphology of slide construction.

Immunohistochemical studies were performed on positive-charged leica plus slides (Leica Microsystems, Wetzlar, Germany) mounted with 4  $\mu$ m of TMA paraffin blocks. Deparaffinisation of sections was done using xylene, followed by rehydration in an automated BenchMark XT immunostainer (Ventana® Medical systems Inc., Tucson, AZ, USA) and pretreatment in prediluted cell conditioning 1 (CC1) solution for an hour. Immunostaining of TMA slides was done by incubating anti-CCND1 antibody at 37 °C for 16 min, followed by washing, counterstaining (with Mayer's hematoxylin) and mounting using Ventana® Ultraview Universal DAB detection kit. For analysis and interpretation both negative (with tris-buffered saline only) and positive (with primary antibody) control slides were used. Sections were evaluated independently by the pathologist without knowing the clinicopathological characteristics of RCC patients. Immunostainings were scored semiquantitatively from 0 to 4 + .

### Functional and pathway analysis

We performed pathway analyses and Gene ontology (GO) studies for differentially regulated genes in RCC to find associated biological networks and molecular processes, using Ingenuity Pathways Analysis (IPA) software



(Ingenuity Systems, Redwood City, CA). Significantly expressed genes with Affymetrix ID, expression level and *p*-value were uploaded into IPA software to identify the most significant altered biological functions and networks. Fisher's exact test was used to calculate the significance of association between transcriptomic data and canonical pathways of RCC.

### Molecular docking studies

The 3-D crystal structure of cyclin D1 was retrieved from RCSB's Protein Data Bank (PDB) – PDB id: 2w96: Chain A. Structure visualization and illustration was done using PyMol (DeLano Scientific) (Fig. 1). The molecular structure of rutin and curcumin were retrieved from NCBI's PubChem compound database with CID 5280805 and 969516 respectively (Fig. 2).

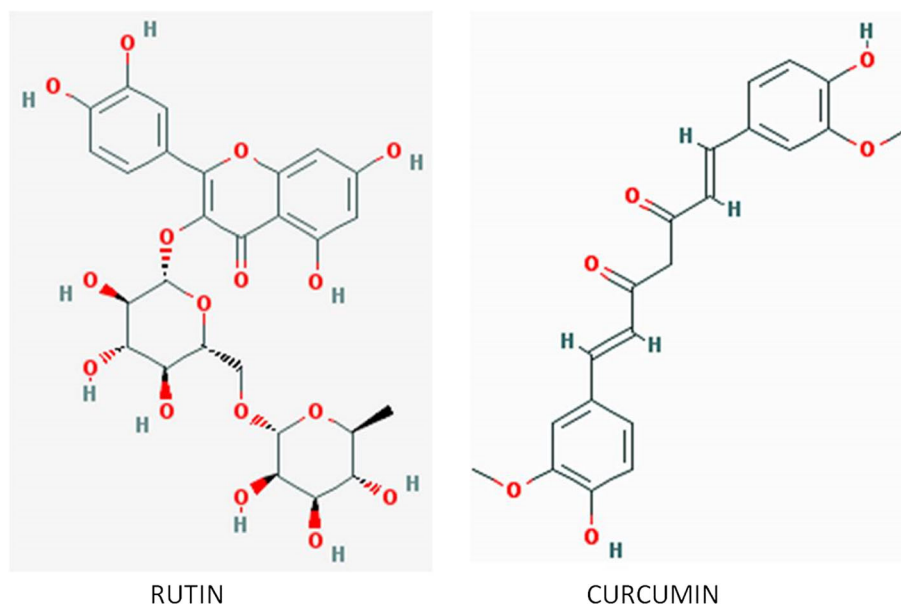
Molecular docking was performed using Molecular Docking Server on [42]. The MMFF94 force field geometry optimization method was used for energy minimization of ligand molecule: rutin and curcumin using DockingServer. Gasteiger partial charges were added to the ligand atoms at pH 7.0. Non-polar hydrogen atoms were merged, and rotatable bonds were defined. Rest methodology was followed in sequential manner as previously described [2, 6, 43].

### Supporting data availability

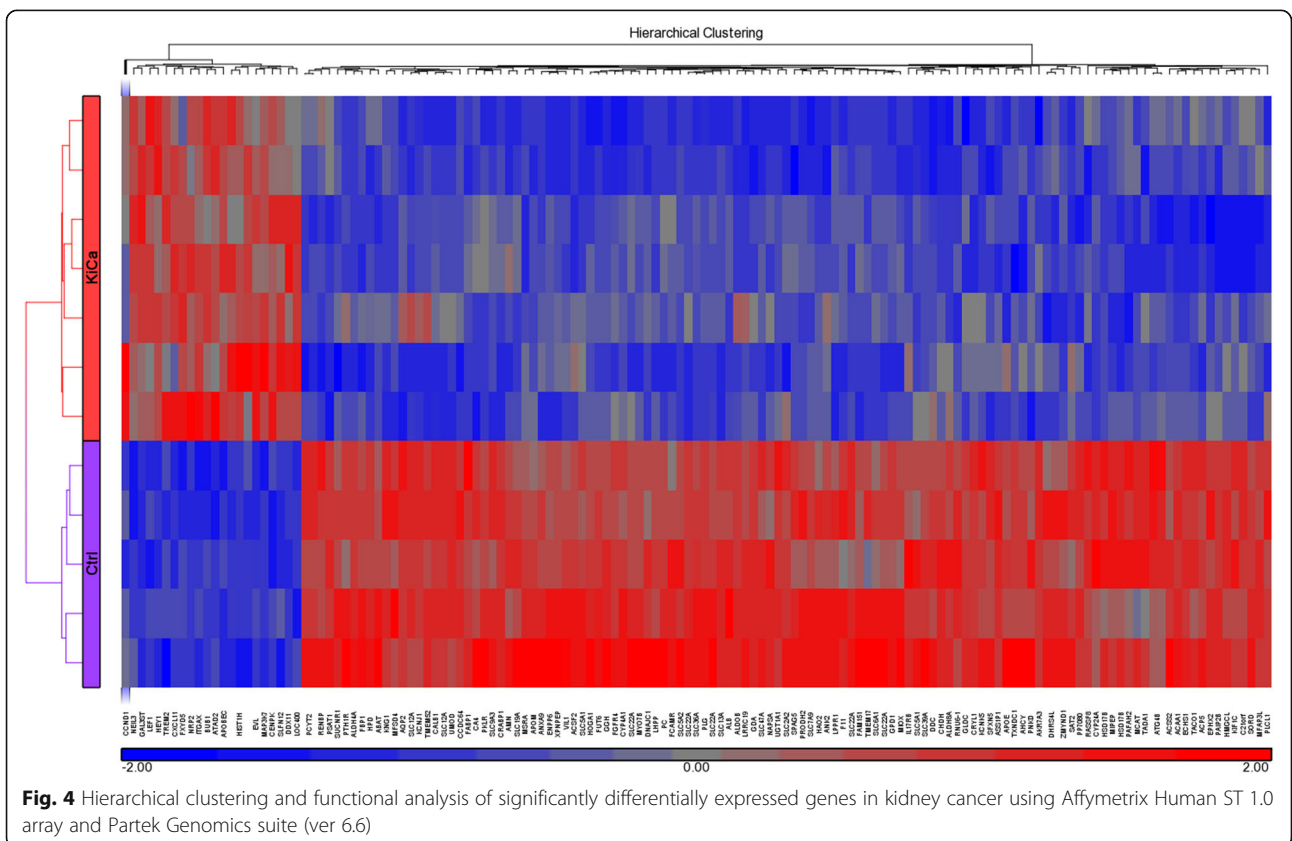
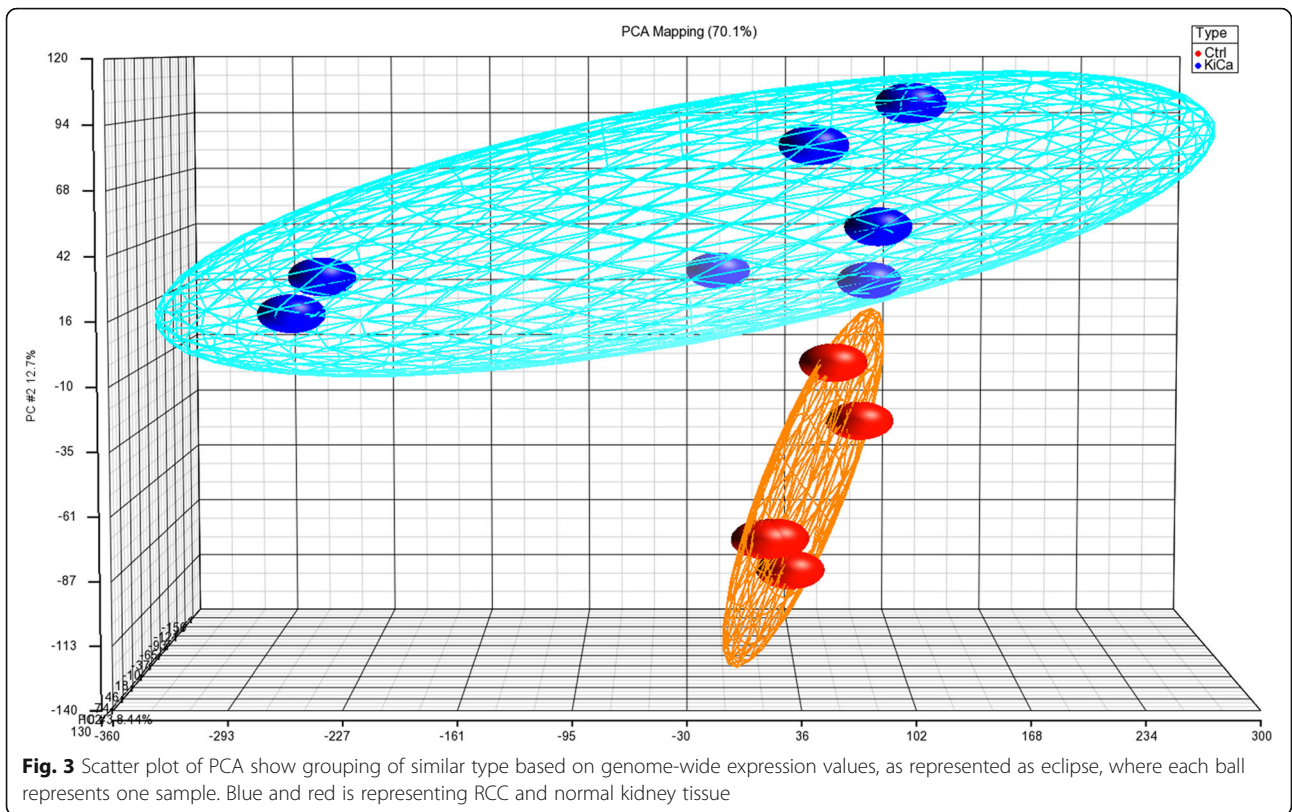
Data series (Accession No. GSE781, GSE7023, GSE6344) used in present study are available at NCBI's Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo/>).

### Results

This study focused on utilizing transcriptomic profiling to identify biomarkers associated with RCC and conducting molecular docking analysis to assess the interactions between potential target and drugs. We identified CCND1 as important overexpressed gene/proteins of RCC and demonstrated its potential as possible anticancer drug target.



**Fig. 2** Overall cyclin D1 structure depicted as ribbon diagram (PDB: 2 W96)



**Table 1** Differentially expressed significant genes in RCC

| Gene symbol | Gene name  | RefSeq           | p-value         | Fold-change    |
|-------------|--|------------------|-----------------|----------------|
| HEY1        | hes-related family bHLH transcription factor with YRPW motif 1 | NM_001040708     | 7.88E-06        | 3.64128        |
| NRP2        | neuropilin 2   | ENST00000272849  | 0.00017         | 3.63215        |
| LEF1        | lymphoid enhancer-binding factor 1                             | NM_001130713     | 4.04E-05        | 3.54448        |
| HIST1H3H    | histone cluster 1, H3h   | NM_003536        | 3.29E-05        | 2.87948        |
| ITGAX       | integrin, alpha X (complement component 3 receptor 4 subunit)  | NM_000887        | 3.23E-05        | 2.69367        |
| BUB1        | BUB1 mitotic checkpoint serine                                 | NM_001278616     | 3.35E-05        | 2.62163        |
| MAP3K7CL    | MAP3K7 C-terminal like   | NM_001286620     | 2.81E-05        | 2.54523        |
| FXYD5       | FXYD domain containing ion transport regulator 5               | NM_001164605     | 0.000158        | 2.45618        |
| HIST1H2AI   | histone cluster 1, H2ai  | NM_003509        | 0.000108        | 2.27918        |
| CENPK       | centromere protein K   | NM_001267038     | 6.01E-05        | 2.27502        |
| CCND1       | cyclin D1  | <b>NM_053056</b> | <b>0.004789</b> | <b>2.25898</b> |
| DDX11       | DEAD   | NM_001257144     | 0.000178        | 2.24119        |
| APOBEC3D    | apolipoprotein B mRNA editing enzyme, catalytic polypeptide-li | NM_152426        | 2.61E-06        | 2.23905        |
| ATAD2       | ATPase family, AAA domain containing 2                         | NM_014109        | 0.000129        | 2.18905        |
| HIST1H3F    | histone cluster 1, H3f   | NM_021018        | 0.00016         | 2.14196        |
| TREM2       | triggering receptor expressed on myeloid cells 2               | NM_018965        | 0.00021         | 2.13092        |
| GAL3ST4     | galactose-3-O-sulfotransferase 4                               | NM_024637        | 1.50E-05        | 2.11736        |
| LOC400464   | uncharacterized LOC400464                                      | AK127420         | 9.33E-05        | 2.11281        |
| CXCL11      | chemokine (C-X-C motif) ligand 11                              | NM_005409        | 0.0002          | 2.07422        |
| NEIL3       | nei endonuclease VIII-like 3 (E. coli)                         | NM_018248        | 2.73E-05        | 2.06874        |
| EVL         | Enah   | ENST00000553771  | 5.57E-05        | 2.06506        |
| SLFN12L     | schlafen family member 12-like                                 | ENST00000361112  | 9.07E-05        | 2.02832        |
| ALDH4A1     | aldehyde dehydrogenase 4 family, member A1                     | NM_001161504     | 4.94E-07        | -16.1809       |
| SLC22A12    | solute carrier family 22 (organic anion                        | NM_001276326     | 9.50E-06        | -16.4997       |
| SLC47A2     | solute carrier family 47 (multidrug and toxin extrusion), me   | NM_001099646     | 5.60E-06        | -17.2214       |
| HAO2        | hydroxyacid oxidase 2 (long chain)                             | NM_001005783     | 0.000192        | -17.3322       |
| SLC6A19     | solute carrier family 6 (neutral amino acid transporter), me   | NM_001003841     | 0.000195        | -18.4585       |
| XPNPEP2     | X-prolyl aminopeptidase (aminopeptidase P) 2, membrane-bound   | NM_003399        | 0.000131        | -18.9386       |
| CYP4A11     | cytochrome P450, family 4, subfamily A, polypeptide 11         | XR_246241        | 7.59E-05        | -19.2237       |
| SLC22A6     | solute carrier family 22 (organic anion transporter), member 6 | NM_004790        | 5.33E-06        | -20.6522       |
| KCNJ1       | potassium inwardly-rectifying channel, subfamily J, member 1   | NM_000220        | 8.79E-05        | -22.4359       |
| TMEM52B     | transmembrane protein 52B                                      | NM_001079815     | 0.000112        | -23.1374       |
| SLC12A3     | solute carrier family 12 (sodium                               | NM_000339        | 1.14E-06        | -27.7638       |
| HPD         | 4-hydroxyphenylpyruvate dioxygenase                            | NM_001171993     | 1.88E-05        | -29.7949       |
| SLC5A12     | solute carrier family 5 (sodium                                | XM_006718157     | 0.000155        | -30.9613       |
| KNG1        | kininogen 1  | NM_000893        | 4.28E-06        | -34.7144       |
| SLC13A3     | solute carrier family 13 (sodium-dependent dicarboxylate tra   | NM_001011554     | 1.93E-06        | -37.6483       |
| SLC36A2     | solute carrier family 36 (proton                               | NM_181776        | 4.06E-07        | -37.8957       |
| PLG         | plasminogen  | NM_000301        | 4.87E-07        | -43.0535       |
| SLC22A8     | solute carrier family 22 (organic anion transporter), member   | NM_001184732     | 4.50E-06        | -45.8474       |
| UMOD        | uromodulin   | NM_001008389     | 2.44E-06        | -68.9599       |
| CALB1       | calbindin 1, 28 kDa  | NM_004929        | 2.29E-06        | -78.3947       |
| SLC12A1     | solute carrier family 12                                       | ENST00000330289  | 0.000135        | -79.6698       |
| ALDOB       | aldolase B, fructose-bisphosphate                              | NM_000035        | 2.56E-05        | -87.9122       |

Negative fold change value indicates the downregulation

\*bold data shows CCND1 (Cyclin D1) was overexpressed (fold change = 2.258) and statistically significant (p-value = 0.00478)

### Identification of differentially expressed genes

Three-dimensional scatter plot of PCA demonstrated that RCC and control tissues are distinctly clustered (Fig. 3). We did genome-wide transcription profiling of fresh RCC specimens and identified 1490 differentially expressed genes; 1034 up-regulated and 456 down-regulated using unadjusted  $p$  value  $< 0.05$  (Additional file 1). Number of differentially expressed genes reduced to 141 (22 up-regulated and 119 down regulated) on applying the stringent condition of false discovery rate with  $p$  value  $< 0.05$  while keeping all other above parameter same (Fig. 4, Table 1). Hes-related family bHLH transcription factor with YRPW motif 1 (HEY1), neuropilin 2 (NRP2), lymphoid enhancer-binding factor 1 (LEF1), and histone cluster 1 H3h (HIST1H3H) were the most upregulated ones while aldolase B, fructose-bisphosphate (ALDOB), solute carrier family 12 (SLC12A1), calbindin 1 (CALB1) were the most down regulated genes in our dataset. We compared our identified differentially expressed genes list with re-analyzed GEO data series (GSE781, GSE6344 and GSE7023) and identified over-expression of CCND1 in all dataset, thus supporting our result (Table 2).

### Validation of CCND1

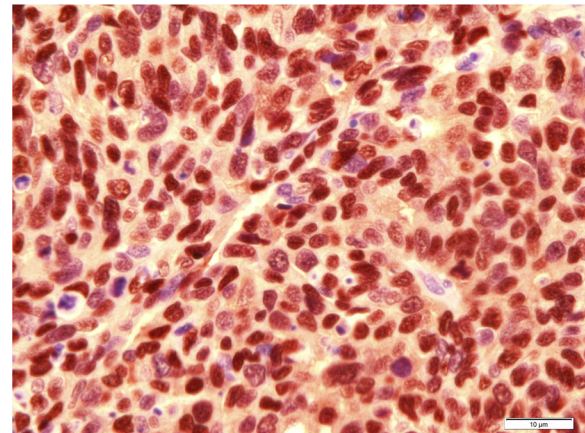
Transcriptomic profiling revealed distinct CCND1 over-expression (FC = 2.26,  $p$  value = 0.0047). Validation study based on TMA-immunohistochemistry staining showed the positive expression of CCND1 in 53 % (73/139) of RCC cases (Fig. 5).

### Pathways and networks underlying RCC

Pathway analysis of identified DEGs revealed the bio-functions, molecular network and canonical pathways association with RCC (Table 3). Most significantly inhibited pathways were synaptic long term potentiation (z-score = -2.33), NRF2-mediated oxidative stress response (z-score = -2.33), production of nitric oxide and reactive oxygen species in macrophages (z-score = -2.324), and renin-angiotensin signaling (z-score = -2.121). Wnt/ $\beta$ -catenin signaling was significantly activated pathway (z-score = 2.53) involving following genes; cyclin D1 (CCND1, FC = 2.26), CD44 molecule (CD44, FC = 2.31), v-myc avian myelocytomatosis viral oncogene homolog (c-Myc, FC = 2.31), HNF1 homeobox A (TCF1, FC = -2.26), secreted frizzled-related protein 1 (SFRP1, FC = -4.45)

**Table 2** Expression of CCND1 in Saudi RCC patients (CEGMR dataset) and GEO dataset

| Dataset          | Sample size | P-value               | Fold change |
|------------------|-------------|-----------------------|-------------|
| CEGMR (own data) | 12          | 0.0047                | 2.26        |
| GSE781           | 34          | 0.0030                | 2.41        |
| GSE6344          | 40          | $1.04 \times 10^{-9}$ | 4.82        |
| GSE7023          | 47          | $6.55 \times 10^{-5}$ | 3.33        |



**Fig. 5** Immunohistochemistry stain for CCND1 show positive staining in RCC (original magnification  $\times 60$ )

(Fig. 6). We found over expression of CCND1 playing important role in regulation of Wnt/ $\beta$ -catenin signaling along with other cancer related pathways like Acute Myeloid Leukemia Signaling, Non-Small Cell Lung Cancer Signaling, PTEN Signaling, Regulation of Cellular Mechanics by Calpain Protease, ErbB2-ErbB3 Signaling, HER-2 Signaling in Breast Cancer, HER-2 Signaling in Breast Cancer, Thyroid Cancer Signaling, Endometrial Cancer Signaling etc. Further extensive molecular pathway analysis may help to better understand the mechanism of RCC initiation, invasion and metastasis.

### Docking studies

We made a structural attempt to study possible binding of two natural famed ligands with the potential therapeutic drug target, Cyclin D1 for cancer therapeutics. CCND1 protein has a classical double cyclin box domain fold, comprising of 11 alpha-helices [44].

Molecular docking studies predicted good interactions between three dimensional structure of drug target (CCND1, PDBID: 2w96) and selected ligands; rutin and curcumin. Molecular docking revealed that both the compounds are able to bind in the ligand binding domain. *In silico* docking studies revealed interaction of two active compounds with the common vital ligand binding site residues (Leu91, Lys149, Asn151) of cyclin D1. Both rutin and curcumin docked at a common ligand binding site of CCND1 slightly varied intensity as estimated by their size, structure, stereochemistry (Figs. 7 and 8; Table 4). We also examined their complete interaction profile including hydrogen bonds, HB plot, polar, hydrophobic, pi-pi and cation-pi interactions. The estimated free energy of binding with Cyclin D1 for rutin was -4.26 kcal/mol and for curcumin was -4.67 kcal/mol which is very similar, however, the estimated inhibition constant ( $K_i$ ) was 757.57  $\mu$ M and 380.02  $\mu$ M respectively.

**Table 3** Canonical pathways predicted by Ingenuity Pathway Analysis for significant genes differentially expressed in kidney cancer

| Ingenuity canonical pathways   | -log (p-value) | z-score | Molecules   |
|--|----------------|---------|---|
| Wnt/ $\beta$ -catenin Signaling  | 0.271          | 2.530   | CSNK1E,MYC,PPP2R4,TGFBR3,CD44,LEF1,SFRP1,UBC, <b>CCND1</b> ,HNF1A,ACVR2A,LRP1   |
| Synaptic Long Term Potentiation  | 0.481          | -2.333  | PLCB4,PPP1R1A,PPP1R3C,PPP3R1,PRKAR2A,CACNA1C,PLCL1,PLCD4,PRKCZ,PRKCA  |
| NRF2-mediated Oxidative Stress Response  | 1.5            | -2.333  | GSTA3,AKR7A2,AKR7A3,GSTM1,GSTM3,NQO2,ABCC2,NQO1,DNAJC19,SOD1,PRKCZ,DNAJC11,AKR1A1,SCARB1,FMO1,GSTA1,AOX1,TXN,PRKCA,EPHX1  |
| Production of Nitric Oxide and Reactive Oxygen Species in Macrophages          | 0.512          | -2.324  | PPARA,MAP3K15,APOE,APOM,PPP1R3C,PRKCZ,APOL1,ALB,PPP2R4,CYBA,APOC1,CHUK,APOD,RBP4,PRKCA  |
| Sperm Motility   | 1.19           | -2.309  | PLA2G16,SLC16A10,PLCB4,PLA2R1,PRKAR2A,PNPLA3,PLCL1,PLA2G12B,PDE1A,PLCD4,PLA2G7,PRKCZ,PRKCA  |
| Renin-Angiotensin Signaling  | 0.279          | -2.121  | ADCY9,GRB2,REN,PRKAR2A,CCL5,PRKCZ,AGT,PRKCA   |
| Nitric Oxide Signaling in the Cardiovascular System                            | 0.27           | -1.890  | KNG1,CAV1,PRKAR2A,CACNA1C,PDE1A,PRKCZ,PRKCA   |
| Antioxidant Action of Vitamin C  | 3.01           | 1.897   | PLA2G16,NAPEPLD,PLA2R1,SLC23A3,PLA2G7,GLRX,SLC2A3,PLCB4,SLC23A1,SLC2A2,PNPLA3,CHUK,TXN,PLA2G12B,PLCL1,PLCD4   |
| Aldosterone Signaling in Epithelial Cells                                      | 1.34           | -1.897  | DNAJC12,DNAJC19,PDPK1,HSPD1,HSPA2,PRKCZ,HSPA12A,DNAJC11,PLCB4,SCNN1G,SLC12A1,CRYAA/LOC102724652,SCNN1B,PLCL1,PLCD4,PRKCA,AHCY   |
| Valine Degradation I   | 10.11          | NaN     | ECHS1,ABAT,ACADSB,BCKDHB,BCAT1,HIBCH,HIBADH,AUH,DLD,DBT,EHHADH,HADHA,ALDH6A1  |
| Ethanol Degradation II   | 9.4            | NaN     | HSD17B10,ADH6,ALDH1B1,ALDH4A1,ACSS1,ALDH9A1,ADH5,ALDH2,AKR1A1,ALDH3A2,ACSS2,ADHFE1,ACSL1,ALDH7A1,DHRS4  |
| Fatty Acid $\beta$ -oxidation I  | 9.4            | NaN     | HSD17B10,ECHS1,SLC27A2,ACAA1,ACAA2,SCP2,ECI2,AUH,ACSL4,IVD,EHHADH,ACADM,HADHA,ACSL1,HADH  |
| FXR/RXR Activation   | 9.17           | NaN     | PPARA,KNG1,APOE,PKLR,APOH,ABCC2,SLC22A7,HNF1A,CYP8B1,MTTP,PCK2,SCARB1,SLC10A2,FGFR4,LPL,GC,AGT,APOM,SDC1,UGT2B4,CYP27A1,SERPINF2,APOL1,ALB,FABP6,APOC1,FBP1,G6PC,SLC51B,RBP4,APOD |
| Serotonin Degradation  | 8.46           | NaN     | ADH6,HSD17B10,ALDH4A1,ALDH1B1,UGT3A1,UGT2B4,UGT2B7,UGT1A1,ALDH9A1,ADH5,ALDH2,AKR1A1,SMOX,ALDH3A2,ADHFE1,DHRS4,ALDH7A1,MAOA  |
| Noradrenaline and Adrenaline Degradation                                       | 7.86           | NaN     | ADH6,HSD17B10,ALDH4A1,ALDH1B1,ALDH9A1,ADH5,ALDH2,AKR1A1,SMOX,ALDH3A2,ADHFE1,DHRS4,ALDH7A1,MAOA  |
| Tryptophan Degradation   | 7.34           | NaN     | ALDH4A1,ALDH1B1,ALDH2,AKR1A1,SMOX,ALDH3A2,DDC,ALDH9A1,ALDH7A1,MAOA  |
| PXR/RXR Activation   | 4.04           | NaN     | PPARA,SCD,GSTM1,ABCB1,ABCC2,PRKAR2A,CES2,HMGCS2,UGT1A1,PCK2,ALDH3A2,GSTA1,G6PC,CYP2B6   |
| Acute Myeloid Leukemia Signaling   | 0.491          | 0.816   | RUNX1,MYC,GRB2,LEF1, <b>CCND1</b> ,HNF1A,IDH1   |
| Non-Small Cell Lung Cancer Signaling   | 0.481          | -0.816  | GRB2,PDPK1,EGF,ERBB2, <b>CCND1</b> ,PRKCA   |
| PTEN Signaling   | 0.662          | 0.905   | FGFR3,GRB2,FGFR4,TGFBR3,PREX2,ITGA5,FGFR2,PDPK1,CHUK, <b>CCND1</b> ,PRKCZ   |
| Regulation of Cellular Mechanics by Calpain Protease                           | 0.453          | -1.000  | GRB2,ITGA5,EGF, <b>CCND1</b> ,ACTN1   |
| ErbB2-ErbB3 Signaling  | 0.967          | -1.890  | MYC,GRB2,NRG3,PDPK1,ERBB3,ERBB2, <b>CCND1</b>   |
| Aryl Hydrocarbon Receptor Signaling  | 2.07           | NaN     | GSTA3,GSTM1,ALDH4A1,ALDH1B1,GSTM3,NQO2,NQO1,ALDH8A1, <b>CCND1</b> ,ALDH9A1,MYC,ALDH1L1,ALDH1L2,ALDH3A2,GSTA1,ALDH5A1,ALDH6A1,ALDH7A1  |
| HER-2 Signaling in Breast Cancer   | 1.01           | NaN     | GRB2,PARD6B,EGF,ERBB3,ITGB8,ERBB2, <b>CCND1</b> ,PRKCZ,PRKCA  |
| Thyroid Cancer Signaling   | 0.828          | NaN     | CXCL10,MYC,LEF1, <b>CCND1</b> ,HNF1A  |
| Endometrial Cancer Signaling   | 0.765          | NaN     | MYC,GRB2,PDPK1,LEF1,ERBB2, <b>CCND1</b>   |
| Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis | 0.615          | NaN     | CXCL8,FN1,IL1RL1,CXCL12,CCL5,HNF1A, <b>CCND1</b> ,FCGR1A,PRKCZ,C5,MYC,PLCB4,F2RL1,PPP3R1,LEF1,CHUK,SFRP1,PLCL1,PLCD4,FCGR3A/FCGR3B,TNFSF13B,LRP1,PRKCA,ADAMTS4                    |
| Estrogen-mediated S-phase Entry  | 0.278          | NaN     | MYC, <b>CCND1</b>   |

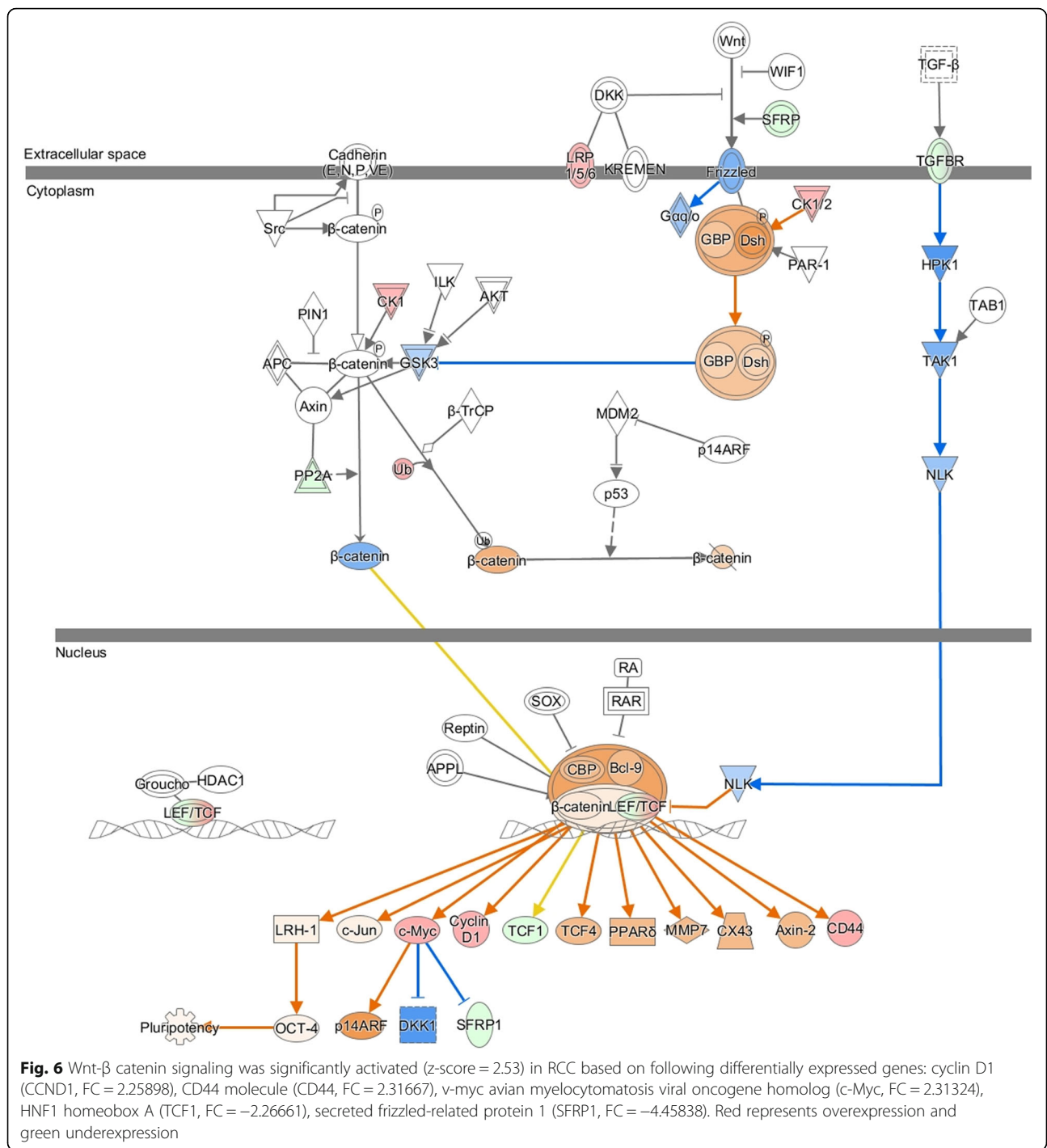
\*bold data shows presence and importance of CCND1 among identified canonical pathways

## Discussion

RCC is a complex heterogeneous tumors involving altered genes and proteins. We performed a transcriptional profiling

and functional analysis of RCC to understand the role of identified significant genes in regulation of physiological processes through biological pathways/networks. We



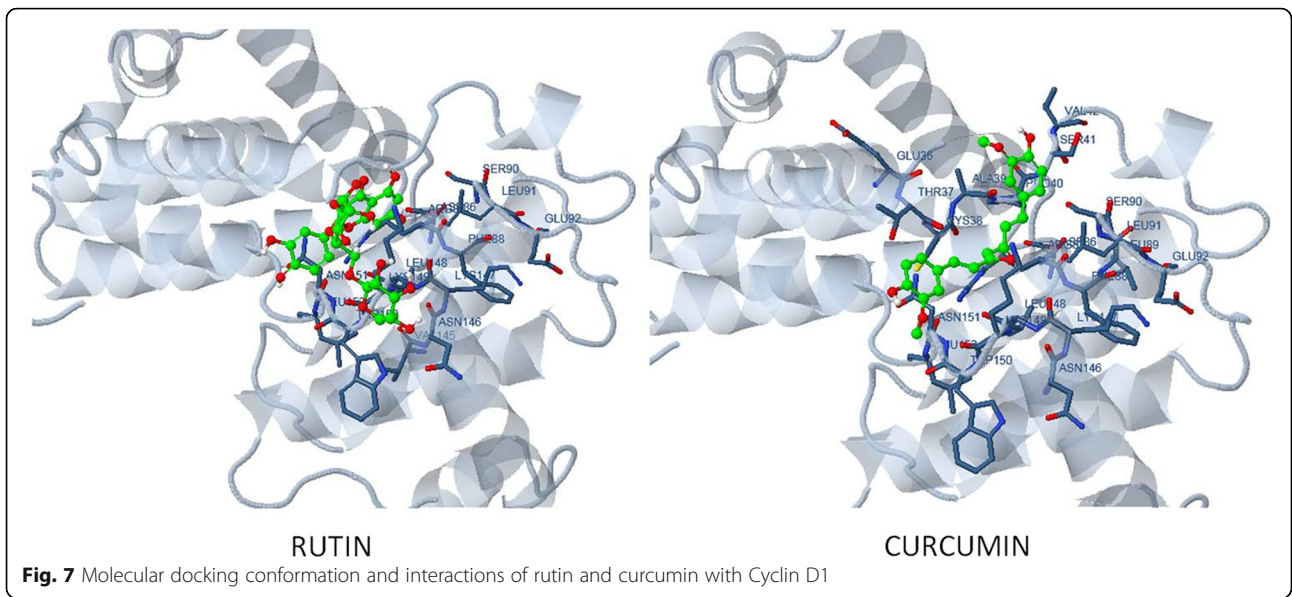


**Fig. 6** Wnt-β catenin signaling was significantly activated (z-score = 2.53) in RCC based on following differentially expressed genes: cyclin D1 (CCND1, FC = 2.25898), CD44 molecule (CD44, FC = 2.31667), v-myc avian myelocytomatosis viral oncogene homolog (c-Myc, FC = 2.31324), HNF1 homeobox A (TCF1, FC = -2.26661), secreted frizzled-related protein 1 (SFRP1, FC = -4.45838). Red represents overexpression and green underexpression

found CCND1 as one of the significantly expressed gene and potential biomarker RCC.

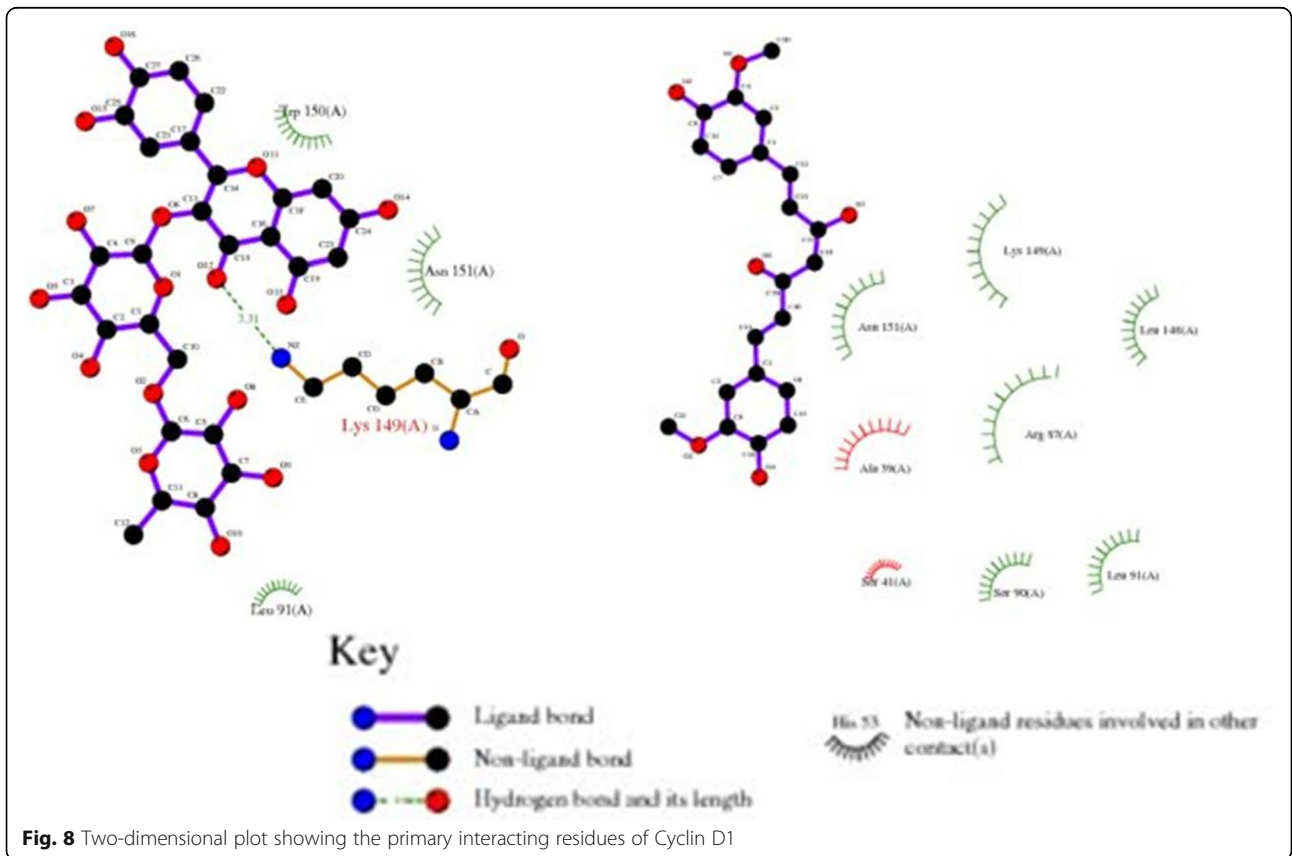
HEY1, an upregulated gene, has been reported to be mediator of notch signaling, showing pro-oncogenic function and promotes cancer progression [45, 46]. Neuropilin-2 (Nrp2) is a well known receptor for the vascular endothelial growth factor-C (VEGF-C) and activates lymph nodes as well as promotes tumor metastasis

by lymphangiogenesis [47, 48]. Lef1 interacts with β-catenin and plays critical role in proliferation of RCC by activating downstream target genes [49, 50]. Wnt/β-catenin signaling, found activated, regulates embryonic development and is involved in many diseases including cancer, polycystic kidney disease [51–54]. WNT signal and its paracrine mode to growth of cancer cells makes it clinically important to understand the metastasis of tumor



cells [53, 55, 56]. HIST1H3H is frequently altered chromatin factors in many cancers [57, 58]. Aldolase, a family member of glycolysis enzymes, was found to be significantly affecting RCC. Aldolase-A was reportedly upregulated while aldolase-B was downregulated in RCC and human primary hepatocellular carcinoma

[59–62]. SLC12 family members are involved in regulation of cell volume, blood pressure and chloride concentration, and play a critical role in diseases like cancer, epilepsy and osteoporosis [63]. In the present study, SLC12 was down regulated that is in accordance to other findings [64]. CALB1 is reported to be



**Table 4** Docking features and values for rutin and curcumin

| Features                     | RUTIN          | CURCUMIN       |
|------------------------------|----------------|----------------|
| Est. Free energy of binding  | -4.26 kcal/mol | -4.67 kcal/mol |
| Est. Inhibition Constant, Ki | 757.57 $\mu$ M | 380.02 $\mu$ M |
| vdW + Hbond + desolv Energy  | -5.43 kcal/mol | -6.37 kcal/mol |
| Electrostatic energy         | -0.01 kcal/mol | -0.12 kcal/mol |
| Total intermolecular energy  | -5.44 kcal/mol | -6.49 kcal/mol |
| Interaction surface          | 653.668        | 684.416        |

altered in RCC and found to be negatively stained compared to normal tissue [61, 65].

CCND1 was overexpressed in our as well as other transcriptomics studies [66–69]. We validated CCND1 overexpression by using tissue microarray platform and *in silico* docking analysis was done to check its therapeutic potential as it plays a key role in G1-S phase transition of cell cycle. There are reports of anti-proliferative, apoptosis inducing and chemopreventive effects of natural bioactive flavonoids like baicalein, catechin, genistein, quercetin, and rutin. Docking analysis showed that rutin and curcumin binds to CCND1 and can potentially inhibit downstream CCND1/CDK4/CDK6 complex formation, required for G1-S phase transition. Our finding demonstrate the anticancer drug targets potential of CCND1 and rutin and curcumin as potential inhibitors, however, this *in silico* docking study has to be validated further.

## Conclusion

Our microarray and immunohistochemistry results suggest significantly high levels of cyclin D1 expression in RCC. Distinct transcriptomic signatures identified for RCC needs verification at larger dataset and additional significant genes need to be further validated for identification of novel biomarkers. The critical role of CCND1 in RCC metastasis by activating G1-S transition of cell cycle has drawn our attention to examine its potential as anticancer drug target. Our *in silico* docking study shown CCND1 protein as an attractive anticancer target and natural flavanoids rutin and curcumin as potential anticancer drug of RCC and they may be promising in the prevention of kidney cancer too. Quantitative structure-activity relationship studies, ligand binding, efficacy and toxicity should be further investigated before clinical trials. Clinical and therapeutic applications of these natural ligands were initially limited by their low solubility and bioavailability but combination with adjuvant and nano-technology based delivery vehicles can immensely improve their potential. Moreover, these are reported to act in synergism with several other natural compounds or synthetic agents routinely used in chemotherapy and can assist in cancer prevention and treatment

when used alone or in combination with other conventional treatments.

## Additional file

**Additional file 1:** Differentially expressed genes of RCC from Saudi patients on comparing RCC with normal kidney tissue. Transcriptomics profiling revealed 1490 differentially expressed genes, 1034 up-regulated and 456 down-regulated, with fold change  $\geq 2$  and  $p$ -value  $< 0.05$ . (XLSX 209 kb)

## Acknowledgment

This project and publication was supported by the NSTIP strategic technologies program in the Kingdom of Saudi Arabia – Project No (10-BIO1258-03, 09-BIO1073-03, 08-MED120-03). The authors also acknowledge with thanks Science and Technology Unit, King Abdulaziz University for technical support. Authors would like to acknowledge the Deanship of Scientific Studies, King Abdulaziz University, Jeddah, Saudi Arabia for funding the research (HiCi-1434-117-2). We also thank the patients, physicians, nurses, and pathologists of the King Abdulaziz University Hospital, and King Faisal Specialist Hospital and Research Center, Jeddah, Saudi Arabia.

## Declaration

This article has been published as part of *BMC Cancer* Volume 16 Supplement 2, 2016: Proceedings of the 3rd International Genomic Medicine Conference: cancer. The full contents of the supplement are available online at <http://bmccancer.biomedcentral.com/articles/supplements/volume-16-supplement-2>.

## Availability of data and material

Data series (Accession No. GSE781, GSE7023, GSE6344) used in present study are available atNCBI's Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo/>).

## Authors' contributions

SK, JM and HF participated in the study design. HJS, AAA, FA and MS performed data collection, DNA extraction and microarray studies. AB and JM did tissue microarray, immunohistochemistry and pathological studies. SK, ZM and HN analyzed data, interpreted the results and drafted the manuscript. KA, AMA, AC, AS and MHQ participated in critical review, editing and finalization of manuscript. All authors have read and approved the final manuscript.

## Competing interests

The authors declare that they have no competing interests.

## Ethical approval

Local ethical committee has approved this study (08-CEGMR-02-ETH). Patients were included in the present study only after their prior consent. Ethical approval information is included in the materials and methods section. Local ethical committee has approved this study under the approval number 08-CEGMR-02-ETH.

## Consent for publication

Patients' consent information are included in the material and methods section. Patients were only included in the present study after their prior consent.

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Published: 5 October 2016

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