Characterization of tumor suppressor CYLD expression in clear cell renal cell carcinoma

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Abstract

Cyld is a tumor suppressor gene that has attracted particular interest recently, due to its involvement in many types of neoplasia, including head and neck squamous cell carcinoma, multiple myeloma, melanoma, hepatocellular carcinoma and colon cancer. Cyld encodes a predominantly cytoplasmic protein (CYLD), which is a deubiquitinating enzyme that regulates primarily cell survival and cell division pathways. Studies on the molecular function of CYLD have shown that it can modulate NF-κB, JNK, p38, TGF-beta, Wnt and Notch signaling. The present study aimed to investigate whether CYLD expression can be correlated with the development of clear cell renal cell carcinoma (ccRCC). Towards this goal, immunohistochemistry and Real Time PCR experiments were performed in order to analyze CYLD expression in clear cell renal cell carcinoma and matched normal tissue specimens. In addition, a clonogenic assay was performed to analyze the effect of CYLD wt and a catalytically inactive mutant CYLD on the growth of human embryonic kidney cells. The results of the present study show that CYLD is downregulated at protein and mRNA level in patients with ccRCC. This is further corroborated by the results of a clonogenic assay, which showed a deubiquitinating activity-dependent growth inhibitory role of CYLD in human embryonic kidney cells. Our results support the notion that CYLD can have a tumor suppressing role, at least in a subset of clear cell renal cell carcinoma, suggesting that it can be incorporated in the future in the development of targeted therapeutic approaches.

Keywords: clear cell renal cell carcinoma; CYLD; tumor suppressor; deubiquitinating enzyme

Introduction

Renal Cell Carcinoma (RCC) is the ninth most common malignancy affecting both genders and it accounts for 5% of new cancer cases [1]. RCC as an entity is a rather heterogenous collection of different types of tumors, with clear cell RCC (ccRCC) being the most common variant, making up about 70% of all tumors. Unfortunately, up to 17% of patients are diagnosed with metastatic disease, which seriously affects the prognosis of these patients and results in RCC being the most lethal cancer among the rest of the genitourinary tumors [2]. Therapeutic management of these patients...
has been altered dramatically during the last decade, as the understanding of the molecular mechanisms of this disease is enhanced. As a result, we have witnessed the induction of immune checkpoint inhibitors in the therapeutic algorithm, in parallel with the use of targeted therapies [3].

Information on ccRCC is constantly increasing. Despite the novel drugs that are available, the mortality in the group of metastatic patients is still very high. Therefore, there is need for new therapeutic targets.

Ubiquitination acts as a posttranslational modification that is of outmost importance for a variety of eukaryotic cellular processes. It may mark a protein for degradation or act as a scaffold for interactions between proteins, thus interfering with cell proliferation and death [4]. On the other hand, deubiquitinating enzymes (DUBs) are proteases that reverse the protein modifications or remodel the polyubiquitin chains. Almost 100 DUBs are encoded in the human genome, and likewise they have a key role in various cellular signaling pathways, such as the NF-κB, PI3-mTORC and PI3/AKT [5, 6]. Therefore, DUBs have been implicated in several types of cancer and can be targeted in the future in order to develop new therapeutic protocols [7]. Cylindromatosis gene cyld encodes CYLD protein, which is a tumor suppressor functioning as a DUB [8, 9]. It was firstly discovered by Biggs et al., who investigated its role in skin cylindromatosis tumors [10]. Further evaluation of the role of CYLD deubiquitinase has shown that it prevents the NF-κB pathway activation, by promoting the deubiquitination of specific tumor necrosis factor receptor signaling molecules, such as NEMO/IKKγ, TRAF2 and TRAF6 [11, 12]. NF-κB is a transcription factor that is involved in inflammation and promotes cells survival and oncogenesis [13]. Other key signaling pathways that were found to be regulated by CYLD are the c-Jun NH2-terminal kinase (JNK), p38, Wnt-1, Notch and TGF-beta pathways [14, 15, 16].

Further studies have revealed a role for CYLD in diverse cell processes, such as immune responses and inflammation, probably by regulating B cell homeostasis and T cell development [17]. In addition, it is involved in germ cell apoptosis and spermatogenesis and most importantly in regulating cell mitosis, proliferation and cell death, thus proving its role as a tumor suppressor [14, 17, 18, 19, 20, 21].

One important aspect of CYLD is the regulation of inflammation, as CYLD deregulation is accountable for many diseases that result from inflammation-induced tissue damage [15]. This may be associated to its function as a tumor suppressor and its involvement to various types of neoplasias, besides familial Cylindromatosis [10]. Complete or even partial suppression of CYLD has been implicated in the development of human malignancies, such as multiple myeloma, melanoma, colon adenocarcinoma, hepatocellular carcinoma, carcinomas of breast, pancreas and lung [16, 22, 23, 24, 25, 26, 27, 28, 29]. However, the role of CYLD in kidney neoplasias is unknown.

The aim of this study is to investigate CYLD expression in patients with clear cell renal cell carcinoma and explore whether CYLD expression is correlated with the tumorogenesis of ccRCC. Better understanding of CYLD function could lead to new therapeutic approaches.

Methods

Immunohistochemistry (IHC)

Fifteen ccRCC cases were selected from the archives of the Department of Pathology of “G. Gennimatas” General Hospital of Thessaloniki. All Hematoxylin and Eosin (H&E) stained sections from the tissue blocks of the selected cases were reviewed by an experienced in renal pathology surgical pathologist for confirmation of the diagnosis and adequacy of the material. After the evaluation, formalin fixed paraffin embedded (FFPE) tissue blocks were selected for further analyses using IHC. The present translational research protocol was approved by the Bioethics Committee of the Aristotle University of Thessaloniki School of Medicine (7.389/20.04.2021).

IHC staining was performed on 2.5 mm thick sections which were cut from the FFPE blocks. After deparaffinization and rehydration, the analytical phase of IHC was performed in an automated immunostaining platform (Bond Max II, Leica, Germany). The rabbit polyclonal anti-CYLD antibody (dilution: 1/300, Sigma-Aldrich, USA, SAB4200060) was used for the detection of CYLD protein and DAB used as a chromogen. The evaluation of all IHC sections was done by an experienced pathologist (blinded to the patients’ clinical characteristics and survival data).

For the interpretation of the CYLD IHC results, a semiquantitative assessment of CYLD expression, as the product of the scores of staining intensity and quantity of immunoreactive tumor cells was performed, as previously described [30]. Specifically, the staining intensity was categorized as 0 = negative, 1 = low, 2 = medium, and 3 = high, whereas the staining quantity was categorized as 0 = no expression, 1 = positivity in less than 1% of cells, 2 = positivity in 1–9%, 3 = positivity in 10–50%, and 4 = positivity in more than 50% of cells. The final immunoreactive score
(IRS), ranging from 0 to 12, was obtained by multiplication of the intensity of positive staining and number of stained cells (quantity).

CYLD expression in every cell compartment (cell membrane, cytoplasm and nucleus) was noted and calculated for each tumor case (renal tumor tissue and, if existed, adjacent normal renal tissue).

**Real time PCR (RT-PCR) from FFPE samples**

Our tissue samples were fixed by formalin, embedded into paraffin and then cut to thin slices of 2.5 mm of thickness. Two such slices from each ccRCC patient’s sample were used for total cellular RNA extraction, using the “Total RNA isolation from FFPE samples” kit of Macherey-Nagel (MN, Duren, Germany). The total quantity of the isolated RNA was determined spectrophotometrically. The synthesis of cDNA was conducted using the RevertAid Reverse Transcriptase system (Thermo Fisher Scientific, Waltham, MA, USA). For the synthesis of cDNA, random hexamers were used, provided by the supplier.

The cDNA samples were analyzed with RT-PCR, using the Applied Biosystems StepOne system and SYBR-green (Sigma-Aldrich, St Louis, MI, USA) according to the manufacturer’s instructions.

The PCR program included 1 cycle at 95°C for 3 min and 40 cycles at 95°C for 3 sec and 60°C for 1 min followed by melting curve analysis up to 95°C.

For RT-PCR, specific primers were used for the amplification of CYLD mRNA (forward: 5’-GATTCTGCCTGGCTTCTTTT-3’; reverse: 5’-CAGGTCCTCCAGAGACATCTTC-3’) and for the endogenous control gene YW-HAZ mRNA forward: 5’-GATTCTGCCTGGCTTCTTTT-3’ and reverse: 5’-GGATGTTTGTGGTGATTCTCT-3’ primers. The experiment was conducted in duplicates and the levels of gene expression were determined by the 2^ΔΔCT method.

**Cell Culture**

Human embryonic kidney 293T cells (HEK293T) were cultured in Dulbecco’s modified Eagles high glucose medium (DMEM, Thermo Fisher Scientific Waltham, MA, USA), supplemented with 10% (v/v) fetal bovine serum, 100 U ml⁻¹ penicillin, 100 mg ml⁻¹ streptomycin and 1x glutamax (Thermo Fisher Scientific Waltham, MA, USA), in a humidified incubator at 37°C under 5% v/v CO₂.

**Calcium phosphate Transfection**

HEK293T cells were seeded at 3 x 10⁵ cells per well into 6 well-plates and were incubated with 2 ml per well complete medium DMEM, at 37°C under 5% v/v CO₂, 24 hrs prior to the transfection. Fresh medium was added 1 hr before transfection. For the transfection procedure, plasmids expressing CYLD Wild Type (CYLD wt), Cyld C601S or pcDNA3 vector (1 µg), were introduced into cells, using the calcium phosphate protocol, as previously described [30]. In summary, the DNA-Calcium phosphate mix was added to each well, drop by drop, after 10 min incubation. The plate was incubated at 37°C for 4 hrs. Then, fresh medium was added into cells and were collected next day or as described.

**Clonogenic Assay**

The clonogenic assay was performed as previously described [28]. In brief, HEK293T cells were grown in Dulbecco medium in 6 well plates, as described. Next day they were transfected with plasmids encoding the CYLD wt protein, as well as the mutation CYLDC601S. Next day, whole lysate was prepared (1/10 of the cells) in Sodium dodecyl sulfate (SDS) buffer and kept at -20°C, in order to analyze further using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The remaining cells were counted, seeded in new 6-wells plates at a density of 100 cells per well and cultured for 10 days in the presence of 0.5 mg ml⁻¹ G418 (Invivogen San Diego, CA, USA). Afterwards, the colonies were fixed with methanol for 10 min and were stained with crystal violet for 10 min. After that time point, the number of colonies was scored.

**Immunoblotting**

Extracted proteins were resolved in 8.5% SDS-PAGE and transferred to a Hybond–ECL nitrocellulose membrane (Amerham Bioscences, Piscataway, NJ, USA). Immunodetection was performed using mouse monoclonal antibodies against Cyld (E4, Santa Cruz 1:200), and the secondary ab m-IgGk BP – HRP SC 516102 (1:5000, Santa Cruz Biotechnology, Santa Cruz, USA) or β-actin as a control (1:1000, Santa Cruz Biotechnology, Santa Cruz, USA). The blots were incubated at room temperature for 1 hr and the bands were visualized by ECL-chemiluminescence (Pierce™ ECL Plus Western Blotting Substrate, Thermo Fisher Scientific Waltham, MA, USA).

**Statistical Analysis**

Data were analyzed using the statistical software RStudio for MacOS (RStudio for Macintosh, Version 1.1.442, RStudio PBC, USA). For comparisons between normally distributed variables the Student’s t-test was used, For comparisons between non-parametric variables the Mann-Whitney U test was utilized. Statistical significance was set at $p < 0.05$. 
Results

Expression of CYLD in cancerous (ccRCC) and non-cancerous FFPE kidney tissue

In order to determine whether CYLD expression is affected in ccRCC patient samples, immunohistochemistry was performed in FFPE sections from 15 patients with ccRCC. The cancerous tissue and the adjacent normal tissue were used for staining with anti-CYLD antibody, as shown in Figure 1. Our results showed that Cyld expression was lower in the cancerous tissue, versus the adjacent normal tissue, especially as far as the cytoplasmic and membranous IHC staining is concerned (Fig. 1). This difference was proven to be highly statistically significant as shown in Table 1 (p < 0.001).

In addition, the expression of Cyld mRNA in ccRCC patients was analysed, after extraction of RNA from tumor and adjacent physiological tissue of 21 FFPE patient samples, using RT-PCR. Cyld mRNA expression was significantly downregulated in most ccRCC samples (3.68 median fold expression) in comparison to its expression in the adjacent normal tissue (9.41 median fold expression) (Fig. 2). These findings suggest that CYLD can play a tumor suppressing role in ccRCC.

The effect of CYLD overexpression on the growth of a renal cell line

The downregulation of CYLD expression in human ccRCC suggested a possible growth inhibitory role for this protein in renal cancer cells. In order to investigate this hypothesis further, the effect of enzymatically active CYLD on the growth of a transformed human embryonic kidney cell line (HEK293T) was tested, using a clonogenic assay. This experiment showed that exogenous overexpression of CYLD wt led to the formation of significantly fewer colonies compared to the number of colonies formed upon comparable

| Table 1. Average values of final Immunoreactive Score (IRS) for CYLD expression in every cell compartment (cell membrane, cytoplasm and nucleus) of tumor cases and normal renal tissue (p - values were calculated using unpaired t-test). CI: Confidence Interval |
|-----------------|-----------------|-----------------|-----|
| IRS             | TUMOR (Mean [SD]) | NORMAL TISSUE (Mean [SD]) | p [95% CI] |
| Cytoplasmic     | 4.13 [2.7]       | 8.91 [2.74]     | < 0.001 [-7, -2.55] |
| Nuclear         | 3.07 [3.75]      | 3.64 [2.77]     | 0.67 [-3.34, 2.2]   |
| Membranous      | 4.36 [3.75]      | 8.18 [2.6]      | 0.0023 [-5.92, -1.72] |
expression of a catalytically inactive form of CYLD (CYLD-C601S) (Fig. 3). These findings indicate that CYLD has the potential to suppress the growth of transformed kidney cells, in a manner that depends on its catalytic activity.

**Discussion**

The present study aimed to investigate whether the downregulation of the tumor suppressor protein CYLD is implicated in oncogenesis of ccRCC. Our data suggest a tumor suppressing role for CYLD at least in a subset of ccRCC cases, since CYLD was downregulated at protein and mRNA level in ccRCC cells. This is further supported by our clonogenic assay results, which show a growth inhibitory role of CYLD in transformed human embryonic kidney cells. Our results are consistent with the findings of Li et al. who demonstrated that upregulation of miR181b, which suppresses various tumors suppressors including CYLD, promotes growth and metastasis in RCC [36]. Furthermore, CYLD upregulation by a small molecule was correlated with growth inhibition of RCC cell lines [37].

The molecular mechanism of ccRCC growth suppression by CYLD is not clear at present. Previous studies have shown that CYLD is involved in the downregulation of NF-κB, JNK, p38, Notch and Wnt signaling pathways [12, 14, 15, 16]. It is possible that the deregulation of one of these pathways, caused by CYLD downregulation, plays a critical role in the development of ccRCC. Along those lines, it is interesting to note that NF-κB activation has been associated with ccRCC development [32]. Similarly, JNK and p38 activation has been correlated with ccRCC poor prognosis and metastasis [33, 34, 35, 36]. Furthermore, Notch and WNT pathway activation has been shown to play a critical role in ccRCC cancer stem cell proliferation and self-renewal [38]. Therefore, it is conceivable that CYLD downregulation may contribute to ccRCC by augmenting the activity of one or more of these pathways.

Our findings create new perspectives for possible therapeutic protocols. For example, upregulation of CYLD could be beneficial for the treatment of ccRCC cases in which it is downregulated. It can be envisaged that small molecules that induce the expression of CYLD could contribute to the development of new therapeutic
protocols. For example, previous studies have shown that glitazone and histone deacetylase inhibitors can augment CYLD expression in breast cancer and liver cell lines respectively [16, 39]. Such molecules could be tested for their ability to induce CYLD expression in ccRCC and incorporated in the development of novel therapeutic protocols.

It should be noted that our study is the first to compare the protein levels of CYLD in ccRCC and matched normal kidney tissue samples and correlate them with its mRNA expression levels. Therefore, the number of available patient samples was limited and for this reason more studies with additional samples will be needed to explore further the role of CYLD in ccRCC.

Conclusions
Our study provides evidence for a tumor suppressing role for CYLD at least in a subset of ccRCC cases based on the downregulation of CYLD expression in tested ccRCC patient samples. This is further supported by the results of a clonogenic assay, which showed an enzymatic-activity-dependent growth inhibition of renal cell growth. These results can be exploited for the development of novel therapeutic approaches for ccRCC in the future.

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Competing Interests
The authors have no competing interests to declare.

Availability of data
The datasets analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

Authors contributions
DK: Protocol Development, Data Collection, Data Analysis, Manuscript Writing
EC: Protocol Development, Data Collection, Data Analysis, Manuscript Writing
DP: Protocol Development, Data Collection, Data Analysis, Manuscript Writing
DK: Protocol Development, Data Analysis, Manuscript Writing
MB: Data collection, Data Analysis, Manuscript Revision
PH: Data Analysis, Manuscript Revision
TK: Data Collection, Data analysis
EGH: Protocol Development, Data Collection, Data Analysis, Manuscript Writing and Revision

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