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Reduced expression of CYLD in human colon and hepatocellular carcinomas

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CYLD was originally identified as a tumor suppressor that is mutated in familial cylindromatosis. Recent studies suggested a role for CYLD in nuclear factor-kappaB (NF-κB) regulation. NF-κB activation has been connected with multiple aspects of oncogenesis but the underlying molecular mechanisms of persistent NF-κB activation in tumors remain largely unknown. Thus, we evaluated CYLD transcription in different colon and hepatocellular carcinoma cell lines and tissue samples, respectively. CYLD was downregulated or lost in all tumor cell lines investigated as compared with primary human colonic epithelial cells and hepatocytes, respectively. Further, quantitative PCR analysis revealed reduced CYLD mRNA expression in most tumor samples compared with non-tumorous tissue. Analysis on protein level confirmed these findings. Functional assays with CYLD transfected cell lines revealed that CYLD expression decreased NF-κB activity. Thus, functional relevant loss of CYLD expression may contribute to tumor development and progression, and may provide a new target for therapeutic strategies.

Introduction

The transcription factor nuclear factor-kappaB (NF-κB) has been studied intensively for its role in controlling expression of genes involved in immune and inflammatory function. However, more recently, NF-κB has been implicated in controlling cell growth and oncogenesis (1). The NF-κB signaling pathway has been recognized as 'cell survival and anti-apoptosis signaling' through upregulation of several genes involved in cell proliferation and cell transformation (2,3).

Persistent NF-κB activation was suggested to contribute to cancer development and progression (4). NF-κB activation or overexpression of NF-κB subunits were found in lymphoid malignancies (5,6). Strong and constitutive activity of NF-κB was also detected in many solid human tumors, including hepatocellular carcinoma (HCC) (7–9) and colon cancer (10,11).

NF-κB consists of a number of closely related protein dimers of the Rel family that bind a common sequence motif located in the chromatin. In resting, non-stimulated cells NF-κB dimers are cytoplasmatic. Upon activation NF-κB translocates to the nucleus, binds to κB-dependent gene promoters and induces transcription (12). The main integrator of signal-induced NF-κB activation is the IκB kinase (IKK) complex. The subunit IKKβ is responsible for IκBα phosphorylation, a process that marks the protein for ubiquitination and subsequent proteolytic degradation through the proteasome pathway. Destruction of the NF-κB inhibitor releases the transcription factor, which translocates to the nucleus (13). In addition to this canonical pathway, one second major pathway, the alternative pathway, accounts for nuclear translocation (i.e. activation) of NF-κB. It depends on processing of the NF-κB2 precursor protein, which preferentially binds to RelB in the cytoplasm, resulting in release of RelB/p52 dimers (13). This pathway depends on the IKKα subunit and is IKKβ-independent (14,15).

CYLD was originally identified as a tumor suppressor that is mutated in familial cylindromatosis (Brooke–Spiegler syndrome) (16), an autosomal dominant predisposition to multiple tumors of the skin appendages. The CYLD gene encodes a protein with three cytoskeletal-associated protein-glycine-conserved (CAP-GLY) domains, which are found in proteins that coordinate the attachment of organelles to microtubules (16). CYLD also has sequence homology to the catalytic domain of ubiquitin C-terminal hydrolases. The CYLD gene is composed of 20 exons, of which the first 3 are untranslated, and extends over ~56 kb of genomic DNA (16). The full-length cDNA was predicted to encode a protein of 956 amino acids, but several splice variants exist. By real-time polymerase chain reaction (RT–PCR), expression of the CYLD gene was detected in fetal brain, testis and skeletal muscle, and at a lower level in adult brain, leukocytes, liver, heart, kidney, spleen, ovary and lung (16).

Several studies suggest CYLD as a negative regulator of activation of NF-κB. Brummelkamp *et al.* (17) demonstrated that inhibition of CYLD by RNA interference vectors enhanced NF-κB activation. CYLD binds to the NEMO component of the IKK complex (17) and inhibits IKK activation, but this function of CYLD is receptor-dependent (18). The inhibition of NF-κB activation by CYLD is mediated, at least in part, by the deubiquitination and inactivation of TRAF2 and, to a lesser extent, TRAF6 (17,19).

So far, CYLD expression has not been analyzed in human tumors despite in patients with familial cylindromatosis, a rare autosomal dominant inherited disease characterized by the development of adnexal tumors of the skin.

The present study was performed to evaluate the expression and function of CYLD in two of the most common

Abbreviations: CEC, colonic epithelial cells; CYLD, gene for familial cylindromatosis; HIC1, hypermethylated in cancer 1; HCC, hepatocellular carcinoma; IKK, IκB kinase; PHH, primary human hepatocytes; RT–PCR, reverse transcription polymerase chain reaction; ZNF202, zinc finger protein 202.

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human carcinomas, to investigate whether the function of CYLD is restricted to the skin or whether it plays an active role also in other tumors.

We screened the transcription profile of CYLD in three HCC cell lines and eight colon carcinoma cell lines. Furthermore, we analyzed CYLD expression in tissue samples of human liver and colon cancer compared with non-neoplastic tissue. In addition, functional assays with CYLD re-expressing tumor cell lines were performed to characterize the biological effects of CYLD.

Materials and methods

Cells and cell culture

The colon carcinoma cell lines CaCo2 (ATCC HTB-37), HT29 (ATCC HTB-38), SW48 (ATCC CCL-231), SW480 (ATCC CCL-228), HCT116 (ATCC CCL-247) and LoVo (ATCC CCL-229), the hepatoma cell line HepG2 (ATCC HB-8065), and the HCC cell lines PLC (ATCC CRL-8024) and Hep3B (ATCC HB-8064) were used. Cells were maintained in DMEM supplemented with penicillin (400 U/ml), streptomycine (50 µg/ml), L-glutamine (300 µg/ml) and 10% fetal calf serum (FCS; Sigma, Deisenhofen, Germany) and passaged at a 1 : 5 ratio every 3 days.

Primary normal human colonic epithelial cells (CEC) and hepatocytes (PHH) were isolated and cultured as described previously (20–22).

5-Azacytosine treatment for analysis of promoter methylation was performed as described previously (22).

Tumorous and non-neoplastic human tissues

HCC tissue and non-neoplastic liver tissue of the same patient were obtained from nine HCC patients undergoing partial hepatectomy. Furthermore, tissue samples from 10 colon carcinomas and corresponding normal colon tissue were analyzed. Tissue samples were immediately snap frozen and stored at −80°C. Informed consent was obtained from all patients and the study was approved by the local Ethics Committee.

RNA isolation and reverse transcription

Total cellular RNA was isolated from cultured cells or tissues using the RNeasy kit (QIAGEN, Hilden, Germany) and cDNAs were generated by reverse transcriptase reaction performed in 20 µl reaction volume containing 2 µg of total cellular RNA, 4 µl of 5× first strand buffer (Invitrogen, Groningen, The Netherlands), 2 µl of 0.1 M DTT, 1 µl of dN_6-primer (10 mM), 1 µl of dNTPs (10 mM) and DEPC water. The reaction mixture was incubated for 10 min at 70°C, 200 U of Superscript II reverse transcriptase (Invitrogen) were added and RNAs were transcribed for 1 h at 37°C. Reverse transcriptase was inactivated at 70°C for 10 min and the RNA was degraded by digestion with 1 µl RNase A (10 mg/ml) at 37°C for 30 min.

Expression analysis

RT–PCR analysis of CYLD was performed using specific primers. To ensure expression of the full-length mRNA three primer combinations were used: CYLD forward 67 (exon 2): 5′-GGTGAGGATGGTTCTACACAG-3′ and CYLD reverse 637 (exon 4): 5′-GAGAACTGCATGAGGTTGC-3′; CYLD forward 223 (exon 3): 5′-TGCCTTCCAACTCTCGTCTTG-3′ and CYLD reverse 1898 (exon 9): 5′-CAGCGAGCACTTCATTCAGTC-3′; and CYLD forward 1577 (exon 9): 5′-GACCGTTCTTCACCACCACT-3′ and CYLD reverse 2647 (exon 16): 5′-CAGACATGATGGTGCCTCT-3′. Furthermore, β-actin was amplified by PCR to demonstrate the integrity and the use of equal amounts of RNA. Here, the following pair of primers was used: β-actin forward: 5′-CTACGTGGCCCTGGACTTCGAGC-3′ and β-actin forward: 5′-GATGGAGCCGCCGATCCACACGG-3′.

The PCR was performed in a 100 µl reaction volume containing 5 µl 10× Taq-buffer, 1 µl of cDNA, 0.5 µl of each primer (20 mM), 0.5 µl of dNTPs (10 mM), 0.5 U of *Taq* polymerase and 41 µl of water. The amplification reactions were performed by 33 cycles (and 30 cycles for β-actin, respectively) for 1 min at 94°C, 1 min at 62°C and a final extension step at 72°C for 1.5 min. The PCR products were resolved on 1.5% agarose gels.

Analysis of expression by quantitative PCR

Quantitative real-time PCR was performed on a LightCycler (Roche, Mannheim, Germany) in a total volume of 20 µl combining cDNA template (2 µl), 2 µl 25 mM $MgCl_2$, 0.5 µl (20 mM), 2µl of SybrGreen LightCycler Mix and the following pairs of primers: CYLC forward: 5′-TGCCTTCCAA-CTCTCGTCTTG-3′ and CYLD reverse: 5′-AATCCGCTCTTCCCAGT-AGG-3′; β-actin forward: 5′-CTACGTCGCCCTGGACTTCGAGC, and β-actin reverse: 5′-GATGGAGCCGCCGATCCACACGG. The reaction mix was applied to the following PCR program: 95°C for 30 s (initial denaturation); 20°C/s temperature transition rate up to 95°C for 15 s, 68°C for 3 s, 72°C for 5 s, 81°C acquisition mode single, repeated for 40 times (amplification). The PCR was evaluated by melting curve analysis and checking the PCR products on 1.8% agarose gels.

Protein analysis in vitro (western blotting)

For protein isolation 2×10^6 cells were washed in 1× PBS and lysed in 200 µl RIPA-buffer (Roche). The protein concentration was determined using the BCA protein assay reagent (Pierce, USA). Balanced amounts of cell proteins (20 µg) were denatured at 94°C for 10 min after addition of Roti-load-buffer (Roth, Karlsruhe, Germany) and subsequently separated on NuPage-SDS-gels (Invitrogen). After transferring the proteins onto PVDF membranes (Bio-Rad, Richmond, USA), the membranes were blocked in 3% BSA/PBS for 1 h and incubated with a 1 : 1000 dilution of primary polyclonal anti-CYLD antibody overnight at 4°C. CYLD antibody was generated by immunizing rabbits with a bacterially expressed GST-fusion protein with the N-terminal 323 amino acids of mouse CYLD and affinity purified against the full-length CYLD protein (23). A 1 : 20 000 dilution of AP-conjugated anti-rabbit antibody (Sigma) was used as secondary antibody. Staining was performed using BCIP/NBT-tablets (Sigma).

Immunohistochemistry

Paraffin-embedded preparations of tissues from patients with colon carcinomas ($n = 10$) and HCCs ($n = 10$) were screened for CYLD protein expression by immunohistochemistry. Normal colon and liver tissue (each $n = 5$) served as control. The tissues were deparaffinated, rehydrated and subsequently incubated with anti-CYLD antibody (1 : 1500) overnight at 4°C. Antibody binding was visualized using AEC-solution (DAKO, Hamburg, Germany). Finally, the tissues were counterstained by hemalaun.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts (5 µg) were incubated with a radiolabeled double-stranded oligonucleotide containing class I MHC κB binding site (GGCTGG-GATTCCCCATCT), separated by electrophoresis and analyzed by autoradiography as described previously (21). The specificity of the probe was evaluated by incubating the nuclear extracts with an excess (100×) of unlabeled oligonucleotide.

Transient transfection of carcinoma cell lines with CYLD

Cell clones expressing CYLD were established by transient transfection of PLC and HCT116 cells, respectively, with sense expression plasmid. Transfections were performed using lipofectamine plus (Invitrogen). Two days after transfection, cells were analyzed.

Reporter gene assays

A NF-κB responsive luciferase vector (NFκB-luc, Promega, Mannheim, Germany) was used to analyze NF-κB activity. Activity of the construct was measured in each of the cell lines. For transient transfections 2×10^5 cells per well were seeded into 6-well plates and transfected with 0.5 µg of NFκB-luc plasmids using the lipofectamine plus method (Invitrogen) according to the manufacturer's instructions. For co-transfection 0.1 µg/well of CYLD expression plasmids were used. Forty-eight hours after transfection the cells were lysed and the luciferase activity in the lysate was measured. To normalize transfection efficiency, 0.2 µg of a pRL-TK plasmid (Promega) was co-transfected and renilla luciferase activity was measured by a luminometric assay (Promega). All transfections experiments were repeated at least three times.

Results

Downregulation of CYLD transcription in tumor cells

Initially, we evaluated CYLD mRNA expression in human tumor cell lines compared with primary non-tumorous parenchymal control cells using RT–PCR analysis. To ensure expression of the full-length mRNA, three primer combinations were used. Three HCC cell lines (Figure 1A) and six colon carcinoma cell lines (Figure 1B) were analyzed and compared with primary human hepatocytes (PHH) and CECs, respectively. Strong reduction of expression of CYLD was found in all tumor cell lines compared with their normal

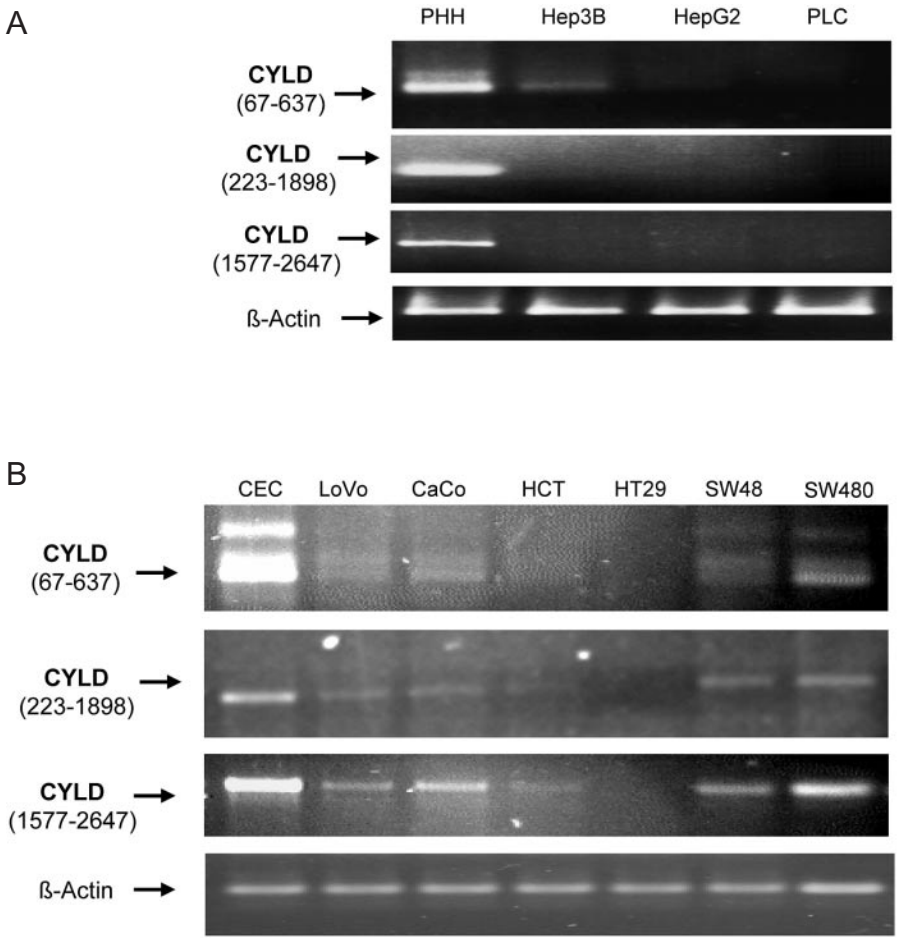

Fig. 1. CYLD expression in tumor cell lines compared with corresponding normal cells. CYLD mRNA expression was analyzed in (**A**) PHH and three HCC cell lines, and (**B**) primary human colon epithelial cells (CEC) and six colon carcinoma cell lines. To analyze the expression of the full-length mRNA, three primer combinations were used amplifying the coding region 67–637, 223–1898 and 1577–2647, respectively (relative to the transcriptional start site). Using primers amplifying the coding region 67–637 bp (relative to the transcriptional start site) resulted in two bands (upper panel in A and B). However, sequencing of the PCR products extracted from both bands revealed that only the lower band is specific (data not shown).

control. Analyzing CYLD mRNA expression by quantitative RT–PCR confirmed these results (data not shown).

Since loss of expression of CYLD gene by promoter methylation can be speculated, we analyzed silenced gene expression in the tumor cell lines. Cells were exposed to 5-azacytidine for demethylation and CYLD expression was quantified. In none of the cell lines upregulation of CYLD expression was observed after demethylation (data not shown). This indicates that expression of CYLD is most likely downregulated by transcriptional control of the promoter.

To identify possible transcription mechanisms involved in downregulation of CYLD in cancers, we searched for putative binding sites for transcriptional repressors on the CYLD promotor using the following databases of known transcription factors: Genomatix (http://www.genomatix.de) and TESS (http://www.cbil.upenn.edu/tess). The analysis revealed potential binding sites of the transcriptional repressors hypermethylated in cancer 1 (HIC1) and zinc finger protein 202 (ZNF202). HIC1 is a tumor suppressor gene that encodes a transcriptional repressor with five Kruppel-like C2H2 zinc finger motifs and an N-terminal BTB/POZ domain and is epigenetically inactivated in several cancers including HCC and colon cancer (24–26). ZNF202 is a transcriptional repressor of genes affecting the vascular endothelium as well as lipid metabolism but its expression and function in cancer has not been analyzed so far (27). Interestingly, the analysis revealed also potential NF-κB binding sites, confirming a recently identified autoregulatory feedback pathway through which activation of NF-κB by TNF induced CYLD in non-neoplastic epithelial cells (28).

Reduced CYLD mRNA expression in tumorous tissue

To address CYLD mRNA expression in tumors *in vivo* we analyzed a panel of nine tissue samples obtained from patients with HCC. From each HCC patient RNA was isolated from cancerous tissue and surrounding non-neoplastic liver tissue, and CYLD mRNA expression was measured by quantitative RT–PCR (Figure 2A). In seven of the nine HCC specimens, CYLD mRNA expression was reduced when compared with matched non-neoplastic liver tissue. Furthermore, we analyzed 10 tissue samples of colon carcinoma in comparison with normal, non-neoplastic colonic tissue (Figure 2B). Here, reduced CYLD mRNA expression was found in eight carcinomas, and in two colon carcinomas (#6 and #7) no CYLD mRNA expression was detectable.

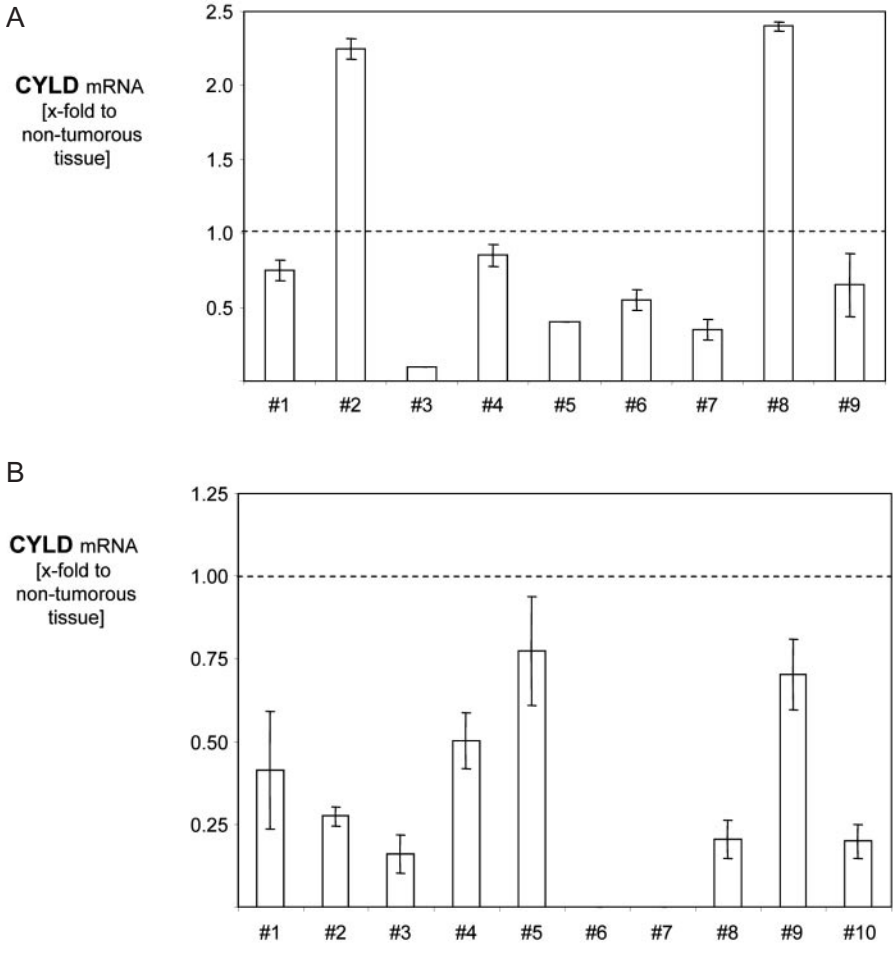

Fig. 2. CYLD mRNA expression in tumorous tissue. RNA was isolated from cancerous and non-neoplastic tissues and analyzed for CYLD mRNA by real-time PCR. CYLD expression was analyzed in cancerous and non-tumorous liver tissue of 9 patients with hepatocellular carcinoma (**A**) and 10 patients with colon carcinoma (**B**), respectively.

Reduced CYLD protein expression in tumor cell lines and tumorous tissue

To address CYLD protein expression in tumors *in vitro* and *in vivo* we analyzed tumor cell lines in comparison with isolated primary cells and a panel of tissue samples obtained from patients with colon carcinoma and HCC, respectively.

Western blot analysis of two HCC cell lines (PLC and HepG2) and two colon carcinoma cell lines (LoVo and CaCo2) compared with PHHs and CECs, respectively, revealed loss of CYLD expression in all tumor cell lines analyzed, while strong bands were visible in non-neoplastic cells (Figure 3A).

Furthermore, CYLD protein expression was analyzed in HCC and colon carcinoma tissue samples and non-neoplastic liver and colon tissue by immunohistochemistry. Representative immunostaining results are shown in Figure 3B, revealing a strong CYLD immunosignal in normal tissues (I: colon; II: liver). In contrast, reduced or lost CYLD expression was found in colon carcinoma (III) and HCC (IV).

Downregulation of NF-κB activity following CYLD expression

Next, we sought to evaluate the functional role of CYLD in tumor cells and to determine whether CYLD acts as a negative regulator for NF-κB activation. CYLD expression was restored in tumor cell lines by transient transfection with a CYLD expression construct. The tumor cell lines PLC (HCC) and HCT116 (colon carcinoma) were analyzed. Successful re-expression of CYLD was verified by quantitative RT–PCR in cells transfected with the CYLD expression construct, whereas no changes of CYLD expression were seen in control transfected cells (data not shown).

A luciferase reporter assay was used to measure NF-κB activity. As expected the three tumor cell lines analyzed displayed strong NF-κB activity (Figure 4). However, after re-expression of CYLD NF-κB activity was markedly reduced in both tumor cell lines (Figure 4).

Furthermore, we analyzed NF-κB activity in six colon carcinoma cell lines by EMSA (Figure 5). Interestingly, cell lines with low CYLD mRNA expression levels (HCT and HT29) (Figure 1) revealed the highest NF-κB activity as compared with SW48 or SW480 cells, which showed relatively higher CYLD mRNA expression. Similarly, HepG2 and PLC cells with almost missing CYLD mRNA expression had higher basal NF-κB activity than Hep3B cells, which revealed relatively higher CYLD mRNA expression (data not shown).

Discussion

The tumor suppressor CYLD is a newly identified member of the deubiquitinating enzyme family that negatively regulates

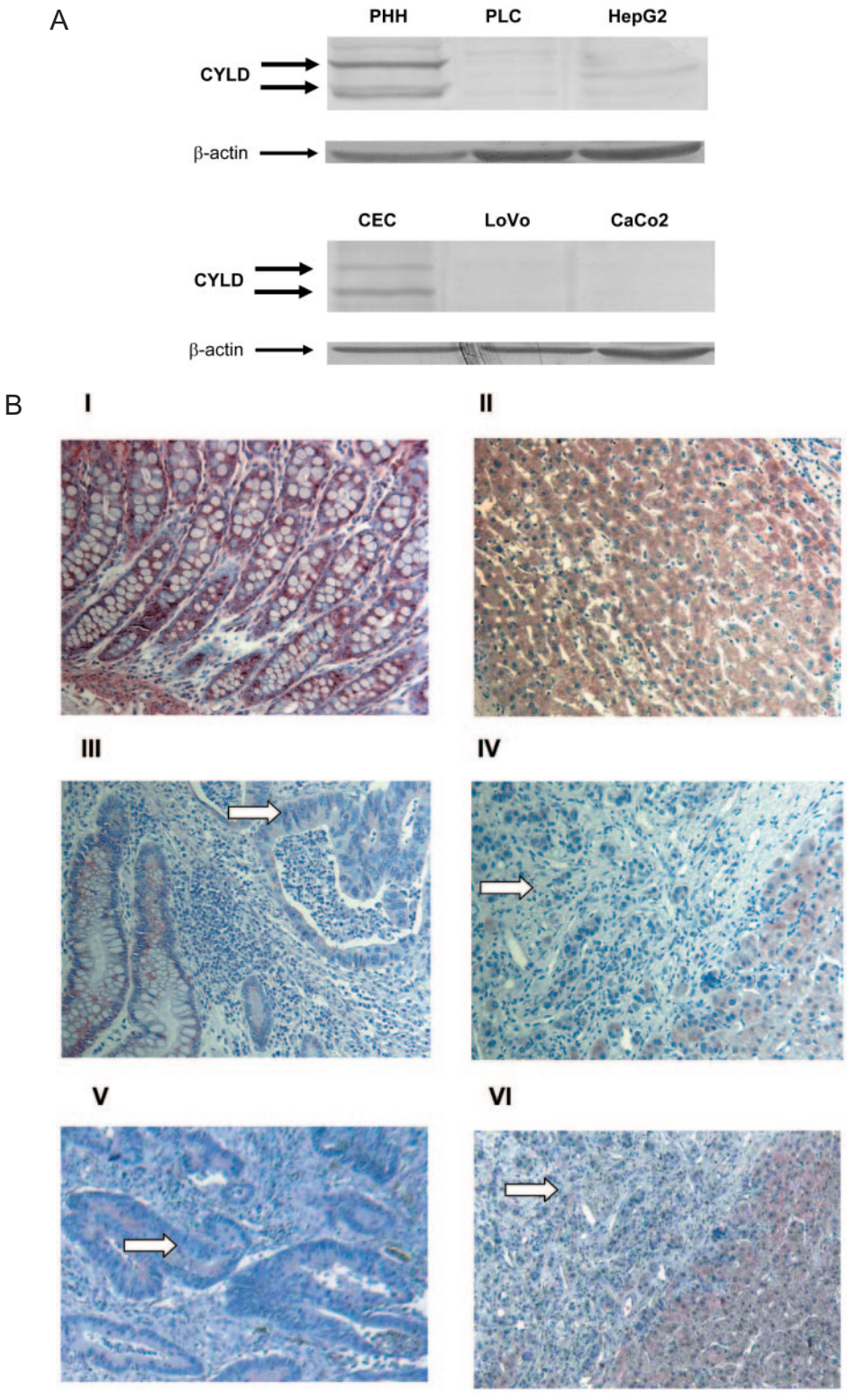

Fig. 3. CYLD protein expression in tumor cell lines and tumorous tissue. (**A**) Western blot analysis of CYLD in PHH and two HCC cell lines (PLC and HepG2) (**upper panel**), and primary human colon epithelial cells (CEC) and two colon carcinoma cell lines (LoVo and CaCo2) (**lower panel**). In normal, non-tumorous cells two bands appear which might correspond to phosphorylated and non-phosphorylated CYLD, respectively. As loading control the blot was counterstained with a β-actin antibody. (**B**) Immunostaining of CYLD revealed strong immunosignals in normal tissues (I: colon; II: liver) and reduced or lost expression in colon carcinoma (III and V; white arrows) and HCC (IV and VI; white arrows).

activation of NF-κB (17,19), a transcription factor suggested to promote carcinogenesis (4).

In this study, we investigated the transcription profile of CYLD in HCC and colon carcinoma. We initiated this study to explore the possibility that CYLD expression may play a role in tumor development or progression not only in the skin but cancer in general. Specifically, we were interested to know whether CYLD transcription is altered in human

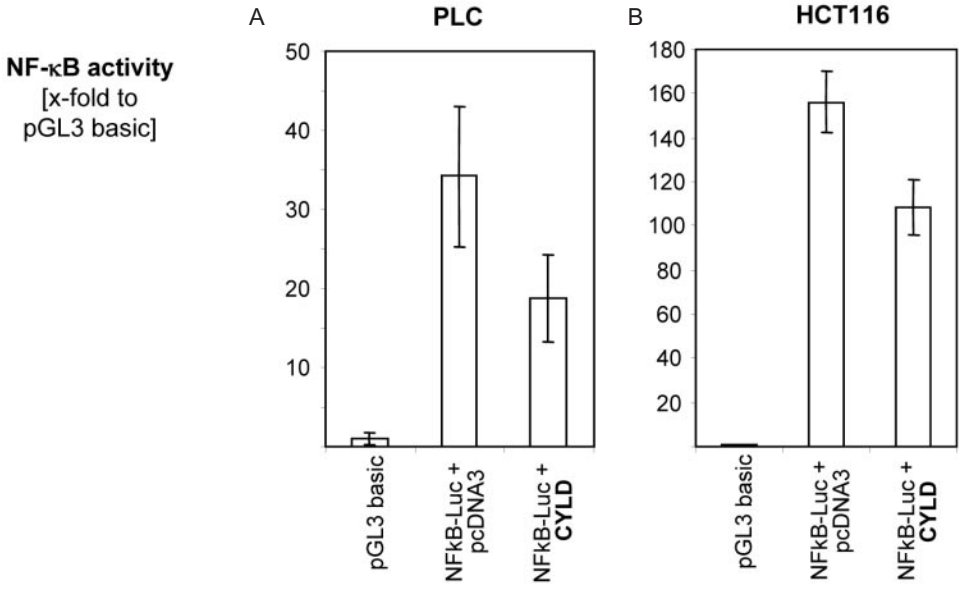

Fig. 4. Effect of CYLD expression on NF-κB activity in tumor cell lines. The cell lines PLC (**A**) and HCT116 (**B**) were transiently transfected with a reporter gene plasmid for NF-κB activity (NFκB-Luc) and an expression plasmid for CYLD (CYLD). Luciferase activity was measured compared with cells transfected with an empty vector construct (pGL-basic).

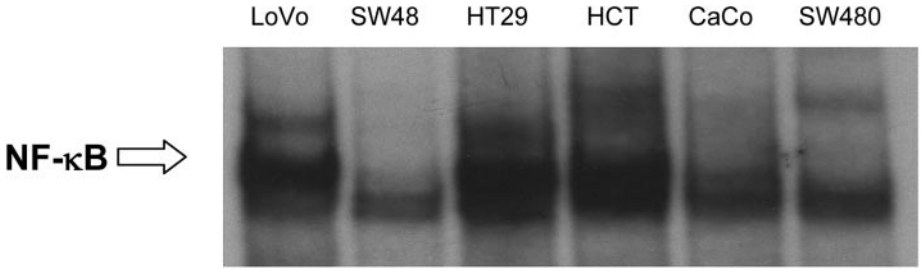

Fig. 5. Comparison of NF-κB activity in tumor cell lines. Nuclear extracts of six colon carcinoma cell lines were prepared and NF-κB binding activity was analyzed by EMSA.

carcinoma and whether CYLD expression levels affect NF-κB activity in tumor cells.

CYLD expression was analyzed in two of the most common human carcinomas worldwide. Colon carcinoma derives from intestinal epithelial cells and HCC derives from hepatocytes. We found reduced CYLD mRNA expression in all three HCC cell lines and eight colon carcinoma cell lines examined compared with normal primary cells. Additionally, reduction or loss of CYLD expression was found *in situ* in most hepatocellular and colon carcinoma compared with non-neoplastic tissue samples.

Colon carcinoma and HCC are common in that they are highly aggressive cancers with a strong and constitutive activity of NF-κB (7–11). Furthermore, recent studies indicate that NF-κB activation correlates with carcinogenesis in both carcinomas (9,11).

Interestingly, in a recent study Costello *et al*. (29) found CYLD mRNA expression downregulated in patients with Crohn disease and ulcerative colitis. These data indicate that decreased CYLD expression may represent an inflammatory control response that is lost or impaired in some way in inflammatory bowel disease (IBD). It is noteworthy that colitis-associated colon cancer represents a serious medical complication for patients suffering from IBD (30). Moreover, the severity of colonic inflammation in patients with ulcerative colitis is a risk factor for colorectal cancer (31). It is intriguing to speculate that reduced CYLD expression may predispose patients with colitis ulcerosa to develop colon carcinoma.

Previous studies have linked inhibition of CYLD with increased resistance to apoptosis, suggesting a mechanism through which loss of CYLD contributes to oncogenesis (17). Trompouki *et al*. (19) identified CYLD as a deubiquitinating enzyme that negatively regulates activation of NF-κB, and loss of the deubiquitinating activity of CYLD correlated with tumorigenesis. The finding that CYLD negatively regulates NF-κB provides insight into the tumor suppressor function of CYLD. The NF-κB pathway is well known for its involvement in cell survival and oncogenic transformation as well as immune responses (32).

In the present study we could demonstrate in functional assays that induction of expression of CYLD by transient transfection resulted in a significant decrease of NF-κB activity in HCC and colon cancer cell lines, respectively.

To identify possible transcription mechanisms involved in downregulation of CYLD in tumors, we performed a database search and identified potential binding sites of the transcriptional repressors HIC1 and ZNF202. However, HIC1 does not seem to be responsible for the reduced CYLD expression in carcinomas since it is epigenetically inactivated

in tumors including HCC and colon cancer (24–26). ZNF202 binds to elements found predominantly in genes that participate in lipid metabolism but its expression and function in cancer is currently unknown (27). Further studies have to be performed to identify the molecular mechanism underlying the CYLD downregulation in cancer.

In summary, taken together with other studies our results indicate that CYLD is affecting NF-κB activity and that the loss of CYLD may be sufficient allowing constitutive NF-κB activation in tumor cells. Since NF-κB activation is suggested to play a critical role in carcinogenesis our study provides the first indication that loss of CYLD expression may contribute to tumor development and progression not only in the skin but in cancer in general, and may provide a new target for therapeutic strategies.

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Conflict of Interest statement: None declared.

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