Original Article

CYLD expression in benign, malignant and metastatic lesions of colorectal epithelium and its prognostic role in colorectal carcinoma

Hongying Zhao¹, Feng Xu³, Mulan Jin¹, Guangyu An², Guosheng Feng³

¹Department of Pathology, Beijing Chao-Yang Hospital, Capital Medical University, Beijing, China; ²Department of Oncology, Beijing Chao-Yang Hospital, Capital Medical University, Beijing, China; ³Department of General Surgery, Beijing Chao-Yang Hospital, Capital Medical University, Beijing, China

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Abstract: Dysregulation of the cylindromatosis (CYLD) gene has been involved in various cancer entities, including colorectal carcinoma (CRC). However, the expression profile and clinical significance of CYLD in patients with a series of colorectal lesions are yet to be clear. In this study, we analyzed the difference of CYLD gene expression among 19 normal colorectal mucosae, 60 colorectal adenoma tissues, 170 primary CRC tissues, 20 lymph nodes metastases and 20 liver metastases and investigated the association of CYLD expression in CRC patients with clinicopathological variables and prognostic significance using immunohistochemistry. We observed that CYLD expression was gradually downregulated from normal tissues, through benign colorectal adenoma, as well as invasive carcinoma, to metastatic lesions of CRC. Low CYLD expression in CRC was significantly correlated with poor differentiation, T3/4 invasion depth, positive lymph node metastasis, vessel embolus, perineural invasion and advanced TNM stage (P < 0.01). Kaplan-Meier survival analysis showed that CRC patients with low CYLD expression survived significantly shorter than patients with high CYLD expression (P = 0.001). Cox multivariate analysis revealed that low CYLD expression was an independent poor prognosis factor in CRC patients (HR = 1.723, 95% CI = 1.029-2.884, P = 0.039). In conclusion, CYLD expression is evidently downregulated across both the colorectal adenoma-carcinoma sequence and the metastatic process, suggesting a role in tumorigenesis, tumor progression and metastasis. Moreover, CYLD may serve as a new potential prognostic target in patients with CRC.

Keywords: Colorectal adenoma, colorectal carcinoma, metastatic lesions, CYLD, prognosis

Introduction

Colorectal carcinoma (CRC) is the third most common malignancy and the third leading cause of cancer-related death worldwide, with over 1.23 million new cases and 0.6 million deaths every year [1]. Despite some improvement in current novel monoclonal antibody-targeted therapies, the overall clinical outcome of CRC patients still remains poor [2]. The precise molecular mechanisms of tumorigenesis, progression and metastasis of CRC, which are critical for better treatment strategies, have been unknown completely. In recent years, the colorectal adenoma-carcinoma sequence has been considered as one of the most predominant pathogenic pathway in colorectal carcinogenesis [3]. Furthermore, the evaluation of metastatic lesions of CRC including lymph nodes and liver metastases is of great importance, which represents the strongest prognostic parameters [4]. To a great extent, identification of the diversification of gene expression responsible for the onset, progression and metastasis of CRC may contribute to discover the novel diagnostic and prognostic biomarkers capable of guiding the clinical therapy.

The cylindromatosis (CYLD) gene, which was originally discovered as a tumor suppressor, was germline mutated in familial cylindromatosis, a rare autosomal dominant disorder associated with multiple skin adnexal tumors [5]. Besides familial cylindroma, the same mutations of CYLD have now been identified in patients with other types of skin cancer, such
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as Brooke-Spiegler syndrome (BSS) and multiple familial trichoepithelioma (MFT) [6, 7]. In addition, it was reported that downregulation of CYLD expression promoted tumor progression in the most aggressive cutaneous melanoma [8]. In vivo experiment of CYLD−/− mice also demonstrated that CYLD knockout mice were highly sensitive to chemically induced skin tumors [9]. CYLD possesses an ubiquitin C-terminal hydrolase domain, which encodes a deubiquitinating enzyme that can cleave the lysine 63-linked poly-ubiquitin chains from a series of target proteins regulating some cancer-associated signaling pathways including transcription factor nuclear factor (NF-κB) signaling, transforming growth factor-β (TGF-β) signaling and Wnt/β-catenin signaling [10-13]. Among them, the NF-κB signaling pathway is a major downstream target of CYLD that acts as a negative regulator of NF-κB activation by deubiquitination of NF-κB essential modulator (NEMO), IkB kinase (IKK)-γ, and tumor necrosis factor (TNF) receptor-associated factor (TRAF) -2/-6 [14, 15] in driving the pathogenesis of skin cancers.

In addition to skin tumors caused by CYLD deficiency, reduced CYLD expression has been reported in several types of human solid cancers including hepatocellular carcinoma [16], colon cancer [17], breast cancer [18], cervical cancer [19], renal cell carcinoma [20] and lung cancer [21]. However, the expression profile and clinical significance of CYLD in patients with a series of colorectal lesions remained to be elucidated. In this study, we systematically analyzed the difference of CYLD gene expres-

Figure 1. CYLD expression in normal, benign and malignant colorectal tissues. A, G. Positive expression of CYLD in normal colorectal mucosa. B, H. Positive expression of CYLD in colorectal adenoma. C, I. Positive expression of CYLD in colorectal adenocarcinoma. D, J. Negative expression of CYLD in colorectal adenocarcinoma. E, K. Positive expression of CYLD in lymph node metastasis of CRC. F, L. Positive expression of CYLD in liver metastasis of CRC. (Magnification A-F × 100; G-L × 400). Cells with brown granules in the cytoplasm and nucleus were identified as CYLD positive.
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Methods

Patients and tissue specimens

The formalin-fixed paraffin-embedded tissue specimens were obtained by endoscopic polypectomy and surgical resection from 60 patients with colorectal adenomas, 170 patients with primary colorectal adenocarcinomas and their respective 20 lymph node metastases and 20 liver metastases. These patients were treated at Beijing Chao-Yang Hospital, Capital Medical University, Beijing, China, from January 2007 to December 2009. Five-year overall survival (OS) information was collected by phone and mail. The 19 normal colorectal mucosae were taken at autopsy from individuals without colorectal malignancies. Using hematoxylin and eosin (HE) staining method, colorectal adenomas were classified as mild, moderate and severe dysplasia according to the criteria of Konishi and Morson [22]. Likewise, colorectal carcinomas were histologically classified as well, moderately, poorly differentiated and mucinous adenocarcinomas, using the WHO criteria [23]. The tumor stage was assessed according to the TNM classification of CRC [24]. None of the patients had received preoperative radiotherapy and/or chemotherapy. This study received pre-approval from the Ethics Committee of Capital Medical University.

Immunohistochemistry staining of CYLD

In brief, human colorectal tissues were fixed with formalin, processed and embedded in paraffin wax, and cut into 5 μm-thick sections by microtome. Immunohistochemistry was performed using the PV-6000 One-step plus® Poly-HRP Anti-Mouse/Rabbit IgG Detection System (GIBI, Bothell, WA, USA), according to the manufacturer’s instructions. Each section was dewaxed in xylene and rehydrated by a graded ethanol solution followed by antigen retrieval by heating the tissue in boiling EDTA buffer (diluted 1:50, pH 9.0) for 8 min. The sections were cooled, immersed in 0.3% H₂O₂ for 15 min to block the endogenous peroxidase activity and rinsed in PBS (PH 7.2-7.4) for 5 min. The samples were blocked with 10% goat serum at room temperature for 15 min and incubated with primary antibodies against CYLD (diluted 1:1000, Sigma-Aldrich, St. Louis, MO) at 4°C overnight. After three washes with PBS, the sections were further processed using polyperoxidase-anti-mouse/rabbit IgG (ZSGB-BIO, Beijing, China) according to the instructions provided by the manufacturer. Reactivity was detected using DAB reagent sets (ZSGB-BIO, Beijing, China) for 5 min, and the cells were counterstained with hematoxylin. A negative control was made by replacing the primary antibody with immunoglobulin G.

Evaluation of CYLD staining

Immunostaining of CYLD was independently assessed by two pathologists. Semiquantitative levels of CYLD expression were obtained based on staining intensity and distribution [25]. Staining intensity (I) was graded as 0 (no staining), 1 (weak staining = light yellow), 2 (moderate staining = yellow brown), and 3 (strong staining = brown). The percentage (0-100%) of the extent of reactivity (R) scored as follows: “0” (positive cells rates < 5%), “1” (5-25%), “2” (25-50%), “3” (50-75%) and “4” (>75%). Histochernistry score = I × R. The scores less than 6 were classified as the low expression and the rest as the high expression. The mean optical density was analyzed by Image-Pro Plus 6.0 software.

Statistical analysis

Mann-Whitney U test was used to compare CYLD levels between groups. The correlations between the expression of CYLD and clinicopathological parameters of CRCs were analyzed by the Fisher exact test and χ² test. Overall
the cytoplasm and nucleus. Representative patterns of CYLD expression in normal colorectal mucosa and a series of colorectal lesions were shown in Figure 1. The positive rate of CYLD expression was 94.7%, 80.0%, 62.9%, and 25.0% in normal colorectal mucosa, adenoma, primary CRC tissue, and metastatic lesions of CRC, respectively (Table 1). On the other hand, there was obviously decreasing tendency of CYLD expression from normal colorectal tissues, through benign adenomas, to malignant CRC lesions. Only one normal colorectal tissue showed negative CYLD expression. Six lymph node metastases and four liver metastases showed positive CYLD staining. The positive rates of CYLD expression in primary and metastatic CRC tissues were significantly lower than that in benign colorectal adenoma lesions and normal colorectal tissues (P < 0.01). No significant differences in CYLD expression were observed between normal colorectal mucosa and benign adenomas (P = 0.131).

Relationship between CYLD expression and clinicopathological features in CRC

Furthermore, we analyzed whether there was an association between CYLD expression and the clinicopathological characteristics of CRC patients. Based on the histochemistry score of CYLD expression, CRC patients were divided into high and low CYLD expression subgroups. As shown in Table 2, low expression of CYLD protein was significantly correlated with poor differentiation (P = 0.001), T3/4 invasion depth (P = 0.006), positive lymph node metastasis (P < 0.001), vessel embolus (P = 0.002), perineural invasion (P < 0.001), and advanced TNM stage (P < 0.001). No significant associations were observed between CYLD expression and patient age, sex and tumor location (P > 0.05).

Downregulation of CYLD is an unfavorable prognostic factor in CRC patients

Kaplan-Meier survival analysis with the log-rank test was applied to examine the expression levels of CYLD and survival status in 170 CRC patients. The results showed that patients with low-level CYLD expression had significantly worse OS rates than those with high-level CYLD expression (P = 0.001; Figure 2). The cumulative five-year OS rate was 74.9% for CRC patients in the high-CYLD-expression group, whereas it was only 47.5% for those in the low-expression group.
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Figure 2. Kaplan-Meier curve for overall survival of CRC patients with low expression of CYLD group vs high expression group.

CYLD-expression group. The clinicopathological variables considered to be potential predictors of survival were shown in Table 3. Univariate analyses indicated that factors including tumor differentiation, depth of penetration, lymph node metastasis, TNM stage and CYLD expression were predictors of OS. These factors were further assessed by multivariate analysis using the Cox proportional hazards model. The results revealed that downregulated CYLD expression was an independent adverse prognostic factor for CRC patients (hazard ratio HR 1.723, 95% confidence interval CI 1.029-2.884, \( p = 0.039 \); Table 3).

Discussion

To date, the expression profile of CYLD in a series of colorectal-associated lesions and the clinical significance of CYLD expression in CRC have remained largely unknown, despite previous study showing that both the transcription profile and the protein level of CYLD were downregulated in colon cancer compared with normal colon tissues. In this study, we were the first to explore the difference of CYLD expression in normal colorectal epithelium, benign adenoma, primary CRC and metastatic lesions. Of particular interest, we wondered whether CYLD expression played a part in tumor development, progression or metastasis and whether reduced CYLD expression was a good or poor prognostic factor for CRC patients.

It is known that most colorectal carcinomas are considered to arise from conventional adenoma based on the concept of the colorectal adenoma-carcinoma sequence [26]. We found that there was obviously decreasing tendency of CYLD expression from normal colorectal tissues, through benign adenomas, to malignant CRC lesions. Moreover, the positive rates of CYLD expression in lymph node and liver metastases of CRC were significantly lower compared with corresponding primary CRC tissues. Taken together, our data indicated that decreased levels of CYLD expression in adenomas seemed to be an early event in neoplastic transformation of normal colorectal epithelial cells, which were mostly lost in the fully transformed, even metastatic state.

Regarding the association with CRC, decreased CYLD expression was significantly associated with more aggressive clinicopathological parameters such as poor differentiation, invasion depth, lymph node metastasis, vessel embolus, perineural invasion and advanced TNM stage. These findings strengthened the fact that CYLD functioned as a tumor-suppressor gene not only in the skin tumor but also in CRC. In addition, we firstly demonstrated that reduced CYLD expression was an independent factor for poor prognosis of CRC patients. Based on the evidence above, our results also suggested that downregulation of CYLD might be involved in a series of important biological properties of colorectal cancer cells, such as carcinogenesis, tumor progression and metastasis.

In recent years, loss of the deubiquitinase CYLD has been described in various malignancies other than familial cylindromatosis, such as colorectal carcinoma. As a major downstream target of CYLD, the NF-κB signaling pathway has been recognized as a key player in the initiation and propagation of CRC [27]. The first serious evidence that activated NF-κB correlated with intestinal carcinogenesis came from a CYLD deficient mice model displaying higher
numbers of colitis-associated cancers [28]. It has been reported that constitutively activated NF-κB was observed in 66% of CRC cell lines and 60%-80% of human CRCs [29, 30]. However, clinical trials targeting NF-κB for the cure of CRC patients have not been satisfactory. Moreover, NF-κB was unlikely the sole CYLD regulator in the CRC phenotype. Bcl3 and the JNK/AP1 signaling cascades for cancers associated with reduced CYLD were also recognized as important regulators [9, 31]. Although tumor suppressor gene functions of CYLD are known, the molecular mechanism responsible for CYLD loss-of-function in CRC cells is not entirely understood. Liang et al [32] recently demonstrated that miR-454 directly bound to the 3’-untranslated region (3’-UTR) of CYLD mRNA and repressed expression at both transcriptional and translational levels, which in turn promoted the proliferation of CRCs. Interestingly, Hyeok et al [33] discovered that higher incidences of novel frameshift mutations of CYLD occurred in colorectal cancers with high microsatellite instability (MSI-H) compared with low microsatellite instability (MSI-L) by polymerase chain reaction (PCR)-based single strand conformation polymorphism (SSCP) assay. Thus, more detailed studies needs to be further confirmed.

To our knowledge, it is the first report as to the frequency of CYLD expression in a series of colorectal-associated lesions and its prognostic value in CRC patients. Consistent with previous studies in hepatocellular carcinoma and breast cancer [16, 18], reduced CYLD expression was markedly associated with more aggressive clinicopathological features of CRC and was an independent prognostic factor for poor OS. In particular, it was noteworthy that the expression rate of CYLD in CRC was lower than benign adenoma, but higher than the corresponding metastases. It is suggested that during tumorigenesis, tumor progression and metastasis of colorectal epithelia, CYLD undergo characteristic changes of distribution, resulting in the gradual disappearance of CYLD in the cells of adenoma, adenocarcinoma and metastases. Identification of the molecular mechanism underlying the CYLD downregulation in a particular stage of CRC will provide a solid foundation for diagnosis and lead to the development of novel targets for CRC therapy.

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Disclosure of conflict of interest

None.

Address correspondence to: Feng Xu, Department of General Surgery, Beijing Chao-Yang Hospital, Capital Medical University, 8 Gongti Nan Lu, Chaoyang District, Beijing 100020, China. E-mail: drxufeng@163.com

References


Table 3. Univariate and multivariate analyses of CYLD expression and overall survival of CRC patients

<table>
<thead>
<tr>
<th>Variables</th>
<th>Categories</th>
<th>Univariate Analysis</th>
<th>Multivariate Analysis</th>
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<tr>
<td></td>
<td></td>
<td>HR</td>
<td>95% CI</td>
</tr>
<tr>
<td>Age</td>
<td>&lt; 60/≥ 60</td>
<td>1.175</td>
<td>0.665-2.076</td>
</tr>
<tr>
<td>Gender</td>
<td>Male/Female</td>
<td>0.942</td>
<td>0.569-1.561</td>
</tr>
<tr>
<td>Location</td>
<td>Colon/Rectum</td>
<td>0.885</td>
<td>0.537-1.458</td>
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<td>Differentiation</td>
<td>Poor + Mucinous/Well + Moderate</td>
<td>2.230</td>
<td>1.316-3.777</td>
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<td>Invasive depth</td>
<td>T3 + T4/T1 + T2</td>
<td>3.923</td>
<td>1.423-10.810</td>
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<tr>
<td>Lymph nodes metastasis</td>
<td>N1 + N2/NO</td>
<td>3.009</td>
<td>1.752-5.170</td>
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<td>Vessel embolus</td>
<td>Present/Absent</td>
<td>1.610</td>
<td>0.945-2.744</td>
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<tr>
<td>Perineural invasion</td>
<td>Present/Absent</td>
<td>1.781</td>
<td>0.947-3.348</td>
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<tr>
<td>TNM Stage</td>
<td>III + IV/I + II</td>
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<td>1.758-5.278</td>
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<td>CYLD expression</td>
<td>Low/High</td>
<td>2.357</td>
<td>1.430-3.885</td>
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HR hazard ratio, 95% CI 95% confidence interval.


