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REVIEW

CYLD: a critical regulator of hypoxia-mediated inflammation in tumors

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Cylindromatosis (CYLD) was originally identified as a tumor suppressor, because loss of its function causes a benign human tumor. In the past, multitude of efforts have been made toward elucidating the biological features of CYLD, and uncovered not only its multiple functions as deubiquitinase, but also the clinical significance of CYLD in a wide variety of diseases. At present, dysregulation of CYLD by loss of its expression is believed to play key roles in a multiple of pathological processes, including tumor cell proliferation, survival, and inflammatory responses by regulating their specific cell signaling pathway. Recently, we discovered that loss of CYLD expression in hypoxic regions of human glioblastoma multiforme (GBM), the most aggressive brain tumor, suggesting the clinical significance of CYLD in the pathogenesis of GBM. Here, we reviewed the diverse biological features and clinical significance of CYLD, particularly focusing on the roles of CYLD as a critical regulator of hypoxia-mediated inflammation in GBM.

Keywords: CYLD; glioblastoma; hypoxia; inflammation; bevacizumab

Abbreviations: CYLD, Cylindromatosis; GBM, glioblastoma multiforme; NF-κB, nuclear factor-κB; TNF, tumor necrosis factor; TRAF, TNF receptor-associated factor; NEMO, NF-κB essential modulator; MAP,mitogen-activated protein; JNK, c-jun N-terminal kinase; WT, wild-type; miRNAs,microRNAs; VEGF, vascular endothelial growth factor; PDE4B, phosphodiesterase 4B

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CYLD

In 2000, Bignell and colleagues identified the familial cylindromatosis tumor suppressor gene (CYLD) by determining germline mutations in cylindromatosis families [1]. Tumor suppressor CYLD gene is localized on chromosome 16q12.1 and encodes a protein of 956 amino acids. CYLD was initially reported when it was identified that a mutation in the gene causes the formation of benign tumors in skin appendages [1]. Subsequent studied have revealed that CYLD acts as a negative regulator for nuclear factor-κB (NF-κB) signaling by deubiquitinating tumor necrosis factor (TNF) receptor-associated factor (TRAF) 2, TRAF6, and NF-κB essential modulator (NEMO, also known as IκB kinase γ) [2-4]. The deubiquitinating enzyme CYLD contains ubiquitin carboxy-terminal hydrolases that bind to ubiquitin chain and detach it from a target protein [5]. In patients with familial cylindromatosis, malfunction of deubiquitinating enzyme CYLD increases resistance to apoptosis caused by NF-κB overactivation, suggesting a mechanism through which loss of CYLD leads to tumorigenesis [1-4].

Polymerization of ubiquitin attached to a pre-existing protein typically arises by linking the carboxy-terminal glycine of ubiquitin to internal lysine of another ubiquitin, and regulates diverse biological processes, such as protein degradation, trafficking and signal transduction [6 - 8]. Among the various types of polyubiquitination chains, CYLD specifically binds ubiquitin chains linked by K63 and removes them from the target protein [9]. Since the K63-linked ubiquitin chain associates various non-degradative cell responses, such as cell signal transduction, CYLD is believed to play key roles in not only tumor cell-specific responses, but also in a multiple of biological responses including cell proliferation, survival, and inflammatory responses by regulating their specific cell signaling pathway [5].

Roles of CYLD in inflammatory responses

Although inflammation is primary response to hazardous stimuli and is a crucial part of the innate immune response acting to signal the host to any bodily insult, excessive inflammatory response is obviously harmful to the host, because of severe tissue damage [10-12]. To prevent detrimental excessive inflammatory response, the inflammatory signaling pathways must be tightly controlled. During evolution, the host has developed a variety of biological systems to avoid detrimental inflammatory response. Among such systems, negative feedback regulation is considered to play a crucial role in preventing overactive inflammation by strictly regulating the activation of the key receptor-dependent signaling adaptor molecules [13].

Among various cell signaling pathways, activation of NF-κB, a primary mediator of inflammatory responses, plays key roles in regulating inflammatory and immune responses [14-16]. In addition to well-known roles that induction of IκBα plays in inhibiting the transient nature of NF-κB activation, NF-κB-dependent CYLD up-regulation that in turn leads to the inhibition of NF-κB especially in more delayed or persistent phase in an auto-regulatory feedback manner [17]. In the NF-κB regulatory signaling pathway, CYLD specifically targets and deubiquitinates the upstream kinase of IκBα, such as NEMO, TRAF2, TRAF6, TRAF7, TRIP, and TAK1, leading to its inactivation [2, 3, 4, 18-20]. Because the CYLD expression is itself under the control of NF-κB activation, CYLD participates a negative feedback regulation of NF-κB and is essential for ensuring the proper control of NF-κB activation in the transient and the delayed or persistent phases [17, 18]. Moreover, in review of all known shared signaling upstream transducers, TRAFs targeted by CYLD, have been shown to be critically involved in activation of not only NF-κB, but also various inflammatory signaling molecule, such as p38 mitogen-activated protein (MAP) kinase and c-jun N-terminal kinase (JNK) [18, 21-23]. Thus, controlled CYLD expression plays a critical roles in tight regulation of a wide variety of inflammatory response.

Down-regulation of CYLD

Over the past decade, based on its important roles in cell signaling regulation, a number of progress has been made for determining the biological functions of CYLD, by utilizing molecular biological tools to induce loss of CYLD function. Reiley *et al*. generated CYLD-deficient mice and reported that CYLD served as a positive regulator of proximal T cell receptor signaling in thymocytes by physical interaction with active Lck and promoted recruitment of active Lck to its substrate, Zap70 [24]. CYLD deficiency developed a tendency to chemically induced skin tumors caused by tumor formation and keratinocyte proliferation through overactivation of Bcl-3-Dependent NF-κB Signaling [25]. Lim *et al*. showed that, in CYLD-deficient mice, lung tissue exhibited increased leukocyte infiltration in response to bacterial infections compared to the wild-type (WT) mice [26, 27]. Concurrently, CYLD-deficient mice also had up-regulated proinflammatory cytokines in response to Nontypeable *Haemophilus influenzae* [27]. Moreover, lack of CYLD caused the development of lung fibrosis in mice with *Streptococcus pneumoniae* infection [28]. CYLD suppressed transforming growth factor-β-signaling and prevented lung fibrosis by destabilizing Smad3 in an E3 ligase carboxy terminus of Hsc70-interacting protein-dependent manner, indicating a critical role for CYLD in tightly controlling the resolution of lung injury and preventing lung fibrosis [28]. These lines of evidence unveiled the precise biological roles of CYLD in a variety of diseases, and also implicated the potential possibility that malfunction of CYLD by loss of its expression, rather that its mutant, may trigger the various types of pathogenesis.

In clinical, in addition to the multiple reports showing functional dysregulation of CYLD by various gene deletion or mutation, a growing body of evidence is accumulating to show that loss of CYLD expression can be observed in different types of human cancer. Hellerbrand *et al*. reported that functional relevant loss of CYLD expression contributed to tumor development and progression in human colon and hepatocellular carcinomas [29]. In malignant melanoma, down-regulation of CYLD expression by transcription factor Snail promotes tumor progression [30]. It should be noted that tumor thickness and progression-free and overall survival of patients with malignant melanoma inversely correlated with CYLD expression [30]. Moreover, increasing reports revealed that loss of CYLD expression presumably triggered the pathogenesis of various tumors by dysregulation of

biological responses, including cell proliferation, survival, and inflammatory responses [29 - 35].

Clinical significance of CYLD in glioblastoma

Glioblastoma

Glioblastoma multiforme (GBM), the most common primary tumor of the central nervous system in humans, has features of rapid and invasive growth in the brain [36]. Of the various types of glioma, GBM is the most frequent and aggressive, and characterized by highly malignant features. The median overall survival of patients with GBM who have standard and targeted therapies is still just more than 1 year, mostly because of resistance to therapy [37]. Due to their diffuse infiltrative features, most GBMs are not curable by resection, and they are extremely resistant to radiotherapy and/or chemotherapy. Those characteristics make GBMs extraordinarily lethal [38, 39]. Because few therapeutic targets are available for GBM, better understanding of the molecular mechanisms of GBM progression and therapy resistance is important. Recently, Song *et al*. have shown that, in malignant gliomas, CYLD reduction was found to be associated with glioma aggressiveness and the survival of patients with gliomas [40]. Moreover, Guo *et al*. reported the loss of CYLD expression in hypoxic regions of tissue specimens from GBMs [41].

Hypoxia-induced CYLD down-regulation is associated with the inflammatory microenvironment in GBM

Hypoxic regions often occur in GBM, and increased tumor hypoxia is associated with the resistance to chemotherapy and radiation, and the poor prognosis of GBM patients [42]. Hypoxia, a characteristic and significant biological phenomenon of malignant tumors, frequently outpaces their blood supply [43]. The hypoxic microenvironment promotes invasion and treatment resistance of GBM cells, and glioma-initiating cells possess strong drug resistance and tumorigenicity [42, 44]. Given the well-established clinical relationship between increased hypoxia and GBM progression, targeting hypoxia-induced processes may be essential for developing successful treatment of GBM [42, 44, 45]. Assessment of clinical GBM tissues and *in vitro* analysis revealed that CYLD expression was reduced under hypoxic conditions via transcriptional regulation in human GBM tissues [41]. As of this moment, transcriptional regulation of CYLD in tumors has yet to be determined. Previous reports have shown that transcription of CYLD is directly suppressed by transcription factor Snail and Notch target Hes1, both of which are up-regulated and activated under hypoxic conditions [30, 46-48]. Hypoxia stimulates human papilloma virus-encoded E6 protein to promote

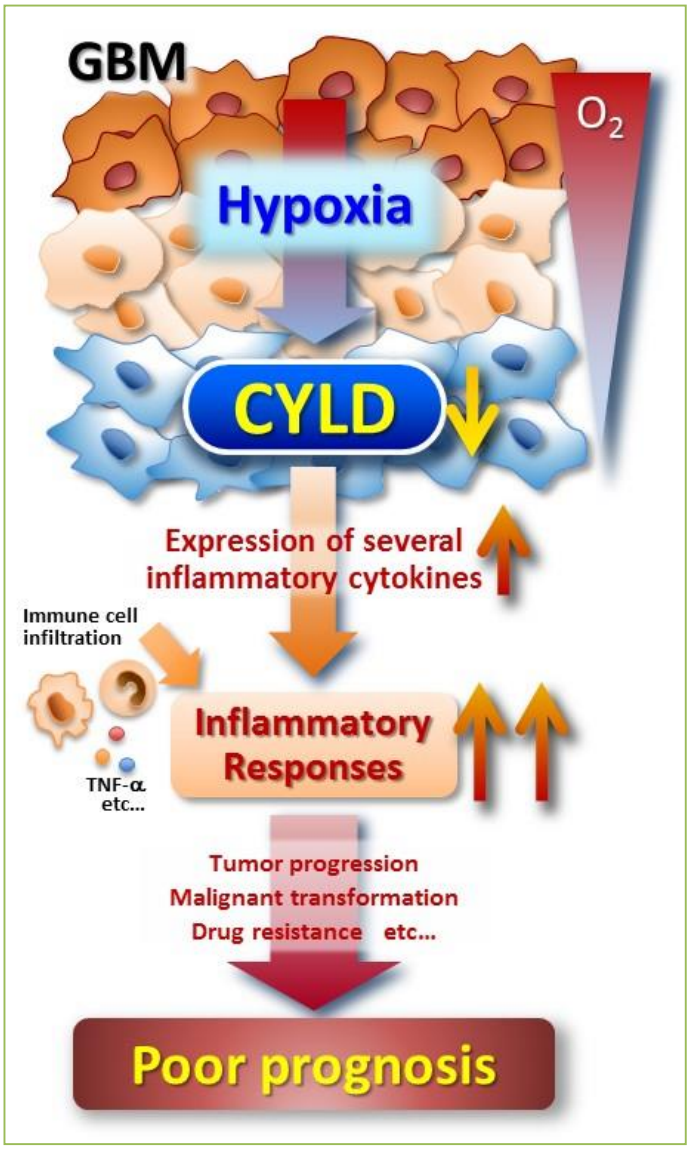

Figure 1. Hypoxia suppresses CYLD expression to promote inflammation in glioblastoma.

ubiquitination and proteasomal degradation of CYLD in human papilloma virus-positive squamous cell carcinoma cell lines [49]. In addition, a number of studies showed that several microRNAs (miRNAs), endogenous small non-coding RNAs 19-24 nucleotide in length known to regulate gene expression, suppressed CYLD expression in multiple tumors [40, 50, 51]. Additional studies are needed to clarify the gene regulatory mechanism underlying the relationship between CYLD down-regulation and hypoxia.

Inflammatory microenvironment, the seventh hallmark of cancer, generally promotes malignant progression [52]. Increasing evidence suggests the clinical significance of hypoxia-elicited inflammation in tumors [53]. As evidenced by the fact that various inflammatory cytokines promote the growth, survival, and invasion of GBM cells [54-56]. Increased

expression of inflammatory cytokines including angiogenic mediators such as vascular endothelial growth factor (VEGF) has been associated with the poor prognosis of GBM [57, 58]. It is particularly worth noting that hypoxia promoted the activation of NF-κB and involved in regulating the inflammatory microenvironment [59-62]. In fact, hypoxia-induced CYLD reduction was critical for inflammatory responses triggered by hypoxia in GBM, and strongly associated with GBM tumor progression including malignant transformation and drug resistance [41]. As stated, it is well-documented that CYLD acts as a negative regulator for NF-κB signaling, a primary mediator of inflammatory responses [2-4]. Because TNF-α rapidly activates the NF-κB pathway [52], an excessive inflammatory response to the cytokine under hypoxic conditions may be due largely to CYLD down-regulation. Inhibition of several cytokine expression by CYLD in a hypoxia-specific manner, which indicated the presence of hypoxia-specific molecular mechanisms regulated by CYLD [41]. Taken together, hypoxia-induced CYLD reduction may promote inflammation in an autocrine and paracrine fashion in GBM tissues, which in turn leads to poor prognosis of GBM patients (Figure 1).

Roles of CYLD in adaptive changes in GBM during anti-angiogenic therapy

GBM is one of the most highly vascularized tumors and expresses high levels of VEGF, which is therefore an attractive target for anti-angiogenic therapies [63]. Bevacizumab, a humanized monoclonal antibody against VEGF, is approved for recurrent and newly diagnosed GBM [64]. Although clinical trials with bevacizumab produced impressive radiographic responses and prolongation of progression-free survival, GBM inevitably progresses within months [65], and the impact of this therapy on overall survival is still not clear [66, 67]. In view of basal tumor-promoting roles of hypoxia in GBM, recent studies suggest that prolonged anti-angiogenic treatment leads to tumors developing progressive hypoxia, which is thought to be critical for resistance to therapy [68, 69]. Guo *et al*. reported that chronic administration of bevacizumab, a monoclonal anti-VEGF antibody, induced expression of proinflammatory cytokines with massive infiltration of immune cells in GBM xenografts. Moreover, CYLD overexpression in GBM cells not only prevented those proinflammatory responses but also significantly improved the prosurvival effect of bevacizumab, which by itself had no impact on survival [41]. Since there was no apparent difference in basal vascularity, tumor growth, and anti-angiogenic efficacy itself by bevacizumab treatment, the prosurvival effect by CYLD overexpression may depend on modulation of phenotypic alterations occurring during bevacizumab treatment.

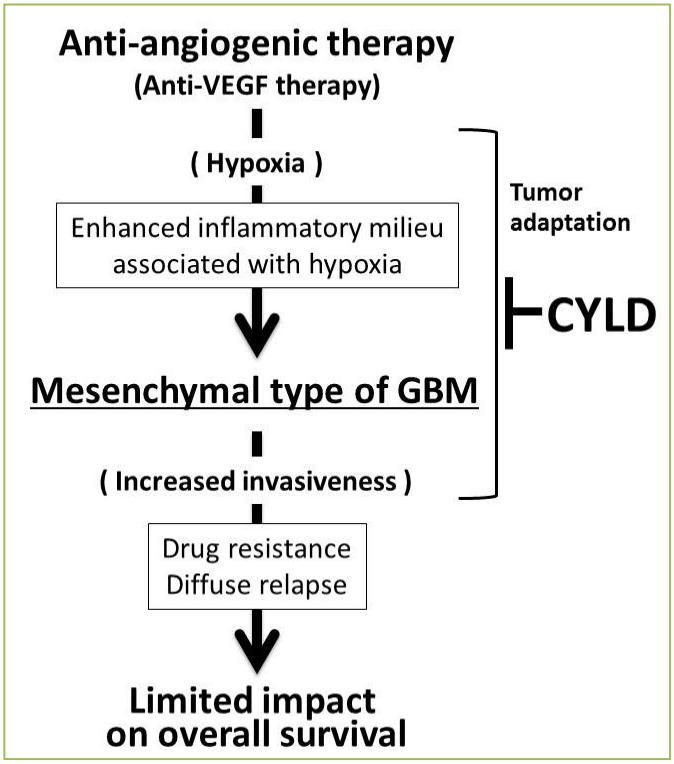

Figure 2. Roles of CYLD in adaptive changes in GBM during anti-angiogenic therapy.

Growing evidence implicate that anti-VEGF treatment has antitumor effects but simultaneously induces tumor adaptation and progression to greater malignancy, with increased invasiveness [65, 70]. Among high-grade gliomas, the mesenchymal type of GBM, characterized as having gene expression associated with tumor invasiveness, shows the worst prognosis with treatment resistance [71, 72]. Indeed, previous studies have shown that mesenchymal transition of GBM cells during chronic anti-VEGF treatment underlies a diffuse relapse, and the predominant biological process occurring during the transition was an inflammatory response [73-76]. Increased infiltration of myeloid cells reflected recurrence after anti-angiogenic therapy, and such an increased myeloid cell influx correlated strongly with the degree of tumor hypoxia [75, 77, 78]. In bevacizumab-treated xenograft GBM model, CYLD clearly prevented massive immune cell infiltration surrounding necrotic regions, and pseudopalisades, a characteristic feature of GBM that is currently thought to indicate tumor cells' actively migrating away from central hypoxic areas [41]. Since the increased invasiveness of GBM cells during anti-VEGF therapy is likely due to an enhanced inflammatory milieu associated with hypoxia induction, inhibition of the GBM cell-derived inflammatory response by CYLD overexpression led to less aggressiveness, including invasion, and ultimately better survival (Figure 2).

Conclusions

In addition to its critical clinical significance during tumor progression, dysregulation of CYLD by loss of its expression, rather that its mutant, may be involved in the various types of pathogenesis. A series of evidence suggests that loss of CYLD expression is deeply associated with a wide variety of diseases, including malignant tumors, inflammatory diseases, infectious diseases, lung fibrosis, neural development, and cardiovascular dysfunction [79-85]. In this review, we described the clinical significance of CYLD, particularly focusing on the roles of CYLD as a critical regulator of hypoxia-mediated inflammation in GBM, which may affect the long-term efficacy of anti-VEGF therapy. Elucidating the mechanisms linking hypoxia-induced CYLD down-regulation and inflammation, and adaptive changes in GBM tissues during anti-VEGF therapy, may provide insights into GBM pathobiology and development of more effective therapeutic approaches to GBM. Recently, Komatsu *et al*. reported that Rolipram, a selective inhibitor for phosphodiesterase 4B (PDE4B), enhanced up-regulation of CYLD expression [86]. This finding suggests that up-regulating the CYLD expression may also have the potential to be promising therapeutic strategies for tumors as the low CYLD expression level has been reported to have an crucial role in the development of tumors in patients [86]. Thus, deeper understanding of more biological features and clinical significance of CYLD may not only bring new insights into the pathogenesis of tumors, but may also open up novel therapeutic strategies for a wide variety of diseases.

Conflict of interest

The authors declare that there is no conflict of interest.

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