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Review

CYLD: a novel stratification marker for malignant tumors

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ABSTRACT

In recent years, many molecular-targeted drugs have been developed, and cancer treatment has advanced enormously. Because intratumoral heterogeneity represents genetic and molecular differences affecting patients' response to these therapeutic drugs, establishing personalized therapy based on precise molecular pathogenesis is urgently required to maximize the therapeutic effects. Cylindromatosis (CYLD), a tumor suppressor gene, is closely associated with malignant transformation and poor prognosis in various malignant tumors. Increasing clinical evidence suggests that CYLD dysfunction by loss of its expression may play key roles in the molecular pathogenesis of various tumors. Moreover, we recently discovered that loss of CYLD expression not only be involved in malignant transformation, but also serves as a prognostic and predictive biomarker for molecular diagnosis and cancer treatment. In this review, we introduce the clinical significance of CYLD expression in various malignant tumors, and discuss the possibility of personalized therapy focusing on molecular diagnosis using CYLD expression.

Key words: CYLD, malignant tumor, stratification marker

1. CYLD

Cylindromatosis (CYLD) gene is a tumor suppressor gene that has been identified as the causative gene for familial cylindromatosis (Bignell et al., 2000). CYLD is a systemically expressed gene, located on chromosome 16q12.1, and has been predicted to have deubiquitinating enzyme activity in its C-terminal region (Bignell et al., 2000). It has been shown that CYLD inhibits activity of target molecules by removing lysine-63 (K63)-mediated polyubiquitin chains, which are involved in signal transduction, rather than lysine-48 (K48)-mediated polyubiquitin chains, which are involved in protein degradation (Sun, 2010). Subsequent analysis identified the target molecules for CYLD deubiquitination, such as, tumor necrosis factor receptor-associated factor (TRAF)2, TRAF6, and nuclear factor-κB (NF-κB) essential modulator (NEMO), which are necessary elements for NF-κB activation (Brummelkamp et al., 2003; Kovalenko et al., 2003; Trompouki et al., 2003). Since NF-κB signaling has an anti-apoptotic effect, loss of CYLD function caused by mutation can lead to cell immortalization and tumorigenesis due to NF-κB overactivation. Furthermore, later studies revealed that CYLD targeted a variety of signaling molecules, including transforming growth factor (TGF) β-associated kinase 1 (TAK1), receptor interacting protein 1 (RIP1), B cell lymphoma 3 (Bcl3), and Akt kinase (Lim et al., 2012; Sun, 2010). Since then, further target molecules for CYLD including Mind Bomb homologue 2 (MIB2) (Rajan et al., 2014), Hpo (Chen et al., 2014), and casitas B-lineage lymphoma-b (Cbl-b) (Sanchez-Quiles et al., 2017), have been identified. Through these molecules, CYLD has been shown to regulate not only NF-κB signaling but also various other signaling pathways such as p38 mitogen-activated protein kinase (p38MAPK) (Tesio et al., 2015), Wnt/β-catenin (Tauriello et al., 2010), c-jun N-terminal kinase (JNK) (Reiley et al., 2004), TGF-β (Lim et al., 2012), Notch (Rajan et al., 2014), Hippo (Chen et al., 2014), and epidermal growth factor receptor (EGFR) (Sanchez-Quiles et al., 2017). In addition, it has been reported that CYLD is involved in the regulation of microtubule dynamics (Yang and Zhou, 2016) and DNA damage response (Fernández-Majada et al., 2016). More recently, it has been reported that CYLD regulates PD-L1 expression via IFN-γ, indicating its involvement in immune system signaling (Umemura et al., 2020). These findings suggest that loss of CYLD function may play various and pivotal roles in a variety of diseases caused by abnormal intracellular signaling other than familial cylindromatosis (Sun, 2010).

2. Clinical significance of CYLD expression in malignant tumors

Several clinical studies in patients with familial cylindromatosis have reported many different types of mutations that cause loss of CYLD deubiquitinase activity (Courtois and Gilmore, 2006). On the other hand, recent substantial clinical studies in a variety of tumors reveal that, CYLD dysfunction due to a loss of CYLD protein expression, rather than mutations, may also be crucial factors in the malignant transformation and poor prognosis of various tumors. Hellerbrand et al. reported that the loss of CYLD expression in hepatocellular carcinoma and colon carcinoma tissues was associated with tumor development and progression (Hellerbrand et al., 2007). Massoumi et al. found that, in malignant melanoma, CYLD dysfunction due to a loss of CYLD expression promoted tumor progression through increased proliferation and migration of tumor cells, and patients with loss of CYLD expression exhibited significantly shorter progression-free survival and overall survival (Massoumi et al., 2009). In addition, other groups have also reported loss of CYLD expression in hepatocellular carcinoma (Kinoshita et al., 2013; Urbanik et al., 2010), melanoma (Ishikawa et al., 2012), and basal cell carcinoma (Kuphal et al., 2011). Furthermore, knockdown of CYLD expression in mice indeed promoted carcinogenesis in skin and liver (Massoumi et al., 2006; Nikolaou et al., 2012). Although the factors involved in loss of CYLD expression are still not fully understood, it has been shown that hypoxic condition may trigger CYLD down-regulation (Guo et al., 2014), and that Snail and Hes1, transcription factors up-regulated in hypoxia, directly suppress CYLD expression (Espinosa et al., 2010; Massoumi et al., 2009). Those notable clinical studies had suggested that, even in the absence of mutations, CYLD might be a prognostic factor for cancer patients due to dysfunction caused by loss of expression itself.

Thus, a variety of clinical evidence had brought us the idea conducting a clinical study to elucidate the clinical significance of CYLD expression in tumor progression. As a result, we revealed that dysfunction caused by loss of CYLD expression in tumor tissues significantly deteriorated the life prognosis of patients, in oral squamous cell carcinoma (Shinriki et al., 2018; Suenaga et al., 2019), breast cancer (Hayashi et al., 2014), glioblastoma (Guo et al., 2014), and cholesteatoma (Miyake et al., 2020). In this review, we discuss the clinical significance of CYLD in those tumors and the possibility of personalized therapy based on CYLD expression, as a novel stratification marker.

3. CYLD expression in oral squamous cell carcinoma (OSCC)

Oral cancer is one of the most common malignancies, and more than 90% of oral cancer are oral squamous cell carcinoma (OSCC) (Warnakulasuriya, 2009). Although the diagnosis and treatment of OSCC have been improved in recent years, its 5-year survival rate has remained almost unchanged for the past 30 years (Forastiere et al., 2003). OSCC can easily spread to the lymph nodes, and lymph node metastasis is a factor that significantly worsens the 5-year survival rate (Myers et al., 2001). Although surgical therapy is recommended as the first-line treatment for OSCC, it is highly invasive and carries the risk of compromising the patient's quality of life. Therefore, the development of minimally invasive non-surgical pharmacotherapy based on molecular pathophysiological analysis is highly desirable in the establishment of therapeutic strategies for head and neck cancer including OSCC.

To investigate the clinical significance of CYLD expression in OSCC patients, we first performed immuno-histochemical staining of CYLD in oral tissues. The results showed that CYLD was expressed in normal oral mucosal tissues, while CYLD expression was markedly decreased in the deeply infiltrated areas of OSCC patients (Shinriki et al., 2018). Furthermore, we examined the relationship between CYLD expression and clinical parameters in patients with OSCC, and found that patients with decreased CYLD expression had larger tumor size and impaired prognosis, suggesting that decreased CYLD expression in OSCC tissues has potential to be a poor prognostic factor for OSCC patients and may be a useful prognostic marker at diagnosis.

Subsequently, we determined the relationship between drug resistance and CYLD expression in OSCC. Cisplatin is the key drug for advanced squamous cell carcinoma of the head and neck including OSCC (Browman et al., 2001). However, the resistance to cisplatin and other platinum-based agents has become a serious problem in the pharmacotherapy of patients with OSCC. Although factors associated with cisplatin resistance and poor prognosis in various carcinomas have been explored (Pan et al., 2016; Zheng, 2017), the detailed mechanisms remain unclear. We suppressed CYLD expression in OSCC cells using siRNA, and found that the decrease in CYLD expression induced fibroblast-like morphology and increased migration ability, as well as resistance to various anticancer drugs used in OSCC treatment (Shinriki et al., 2018; Suenaga et al., 2019). In particular, cisplatin treatment markedly reduced the cytotoxic effect in OSCC with decreased CYLD expression, indicating that decreased CYLD expression induces cisplatin resistance in OSCC cells. Moreover, we found that CYLD downregulation-induced cisplatin resistance was induced by both decreased intracellular accumulation of cisplatin and inhibition of apoptosis (Suenaga et al., 2019).

We next investigated the detailed molecular mechanism of cisplatin resistance induced by CYLD downregulation. As mentioned above, CYLD, as a deubiquitinating enzyme, suppressively regulates the NF-κB pathway, which is closely involved in cancer progression. It has also been reported that NF-κB pathway may contribute to develop the resistance to

anti-tumor drugs, such as, cisplatin and 5-FU in oral cancer (Li et al., 2015; Nagata et al., 2011). Therefore, we examined NF-κB activity in OSCC cells, and found that NF-κB activity in OSCC cells was significantly increased with decreased CYLD expression (Suenaga et al., 2019). To test whether this CYLD downregulation-induced NF-κB overactivation contributes to the cisplatin resistance, we treated cells with an NF-κB inhibitor (BAY 11-7085) in addition to cisplatin and evaluated cell viability, and found that NF-κB inhibitor completely abolished cisplatin resistance caused by CYLD downregulation. These results indicate that cisplatin resistance induced by decreased CYLD expression may be due to the overactivation of NF-κB.

In addition, we attempted to overcome cisplatin resistance using drugs currently in clinical use. Bortezomib, a proteasome inhibitor, is approved for the treatment of multiple myeloma and mantle cell lymphoma (Cengiz Seval and Beksac, 2018; Chen et al., 2011). Bortezomib is known to inhibit the activation of NF-κB by inhibiting the proteasomal degradation of IκB, one of the NF-κB signaling molecules. Therefore, we examined the therapeutic effect of bortezomib on cisplatin resistance caused by decreased CYLD expression. As expected, bortezomib treatment significantly ameliorated the decrease in intracellular accumulation of cisplatin and inhibition of apoptosis observed in OSCC cells with downregulation of CYLD. Moreover, the combination of cisplatin and bortezomib showed a marked antitumor effect, decreasing the survival rate of OSCC cells (Suenaga et al., 2019). It is interesting to note that the mechanism by which the combinatory effect of bortezomib and cisplatin improving the reduction of intracellular accumulation of cisplatin may be caused by the change of expression pattern of efflux transporters. It has been reported that efflux transporter (MDR1, MRP1, etc.) expression is up-regulated in cisplatin-resistant cells (Schinkel and Jonker, 2003). It is also documented that MDR1 expression is induced by NF-κB activation (Bentires-Alj et al., 2003), suggesting that bortezomib may suppress the efflux transporter by inhibiting NF-κB, resulting in the decreased excretion of cisplatin. These results suggest that inhibition of NF-κB overactivation may be effective against cisplatin resistance associated with decreased CYLD expression, and that bortezomib, a molecularly targeted drug used in clinical practice, may be a new candidate for pharmacotherapy in patients with cisplatin-resistant OSCC induced by decreased CYLD expression.

4. CYLD expression in breast cancer

Breast cancer is the most common malignant tumor in women, and recognized as a heterogeneous disease composed of molecules with various biological characteristics having diverse genetic abnormalities (Goldhirsch et al., 2011; Sørlie et al., 2001). Since each patient has a different prognosis and response to treatment, it is important to accurately diagnose the type of disease, estimate the risk of recurrence and treatment response, and develop an optimal treatment strategy. To date, several molecules have been used as prognostic and predictive markers of response to treatment in breast cancer (Goldhirsch et al., 2013). However, further subclassification of breast cancer subtypes and the search for factors that predict response to treatment and prognosis are essential for the future development of breast cancer treatment.

We first examined the relationship between CYLD expression and clinicopathological factors and prognosis in breast cancer patients (Hayashi et al., 2014). Immunohistochemical staining of patient tissues revealed that decreased CYLD expression was associated with characteristics of high-grade breast cancer and significantly correlated with poor prognosis. Furthermore, multivariate analysis showed that CYLD expression was an independent prognostic factor for disease-free survival (DFS) (Hayashi et al., 2014). Next, we performed experiments to suppress CYLD expression in breast cancer cell lines, and showed that downregulation of CYLD expression in breast cancer cells may contribute to metastasis and recurrence by promoting increased viability and cell migration through receptor activator of NF-κB ligand (RANKL)-induced NF-κB overactivation (Hayashi et al., 2014). These results indicate that downregulation of CYLD is a new factor causing poor prognosis of breast cancer via overactivation of NF-κB in cancer tissues.

In recent years, the role of RANKL-induced NF-κB overactivation in breast cancer progression has been demonstrated (Jones et al., 2006), and denosumab, a RANKL-targeted molecular drug, has become widely used in clinical practice. Denosumab is approved as a treatment for the suppression of bone events in advanced cancers with bone metastases, but recent studies have shown that it is also expected to have broad tumor suppressive effects in the treatment of breast cancer (Brown and Coleman, 2012). However, there is still no strong predictor of treatment response that identifies NF-κB activation or sensitivity to denosumab. We have obtained preliminarily results that denosumab suppresses NF-κB and reduces cell migration in breast cancer cell lines in which CYLD expression is suppressed (unpublished data). Based on the above-mentioned findings, we expect to use CYLD as an indicator of NF-κB activation to predict the therapeutic effect of denosumab, which may lead to personalized treatment for breast cancer.

5. CYLD expression in glioblastoma multiforme (GBM)

Glioblastoma multiforme (GBM) is an undifferentiated, invasive, and highly proliferative tumor that is the most malignant of gliomas (Westermark, 2012). At present, there is no definitive treatment for GBM. Surgery is the first

choice, but total resection is usually not possible because of its high invasiveness and obscure border with normal tissue. Therefore, postoperative radiation therapy and chemotherapy are used in combination, and temozolomide is commonly used as chemotherapy. However, the prognosis is still poor due to problems such as drug resistance (Furnari et al., 2007). Previous studies have suggested that hypoxia and increased angiogenesis are involved in the development of GBM and resistance to treatment (Binello and Germano, 2011; Yang et al., 2012), but the detailed mechanisms remain unclear. Therefore, there is an urgent need for a more detailed understanding of molecular mechanisms and the development of new therapeutic strategies.

We performed CYLD immunohistochemical staining on GBM patient tissues and found that CYLD expression was markedly decreased in GBM tumor tissues, consistent with hypoxic regions (Guo et al., 2014). Furthermore, consistent with GBM patient tissues, the expression of CYLD in GBM cells was markedly reduced under hypoxic culture. Moreover, the hypoxia-induced inflammatory response was ameliorated by overexpression of CYLD. These results indicate that decreased CYLD expression may be crucial for the expression of pro-inflammatory factors in GBM cells in the hypoxic region.

Chronic anti-angiogenic treatment has been known to induce hypoxia and cause resistance to that treatment (Du et al., 2008; Lucio-Eterovic et al., 2009). And consistent with that, administration of bevacizumab, an antibody against VEGF, to GBM-bearing mice increased hypoxic areas in the tumor and induced the expression of various inflammation-related factors and migration of inflammatory cells into hypoxic areas in the tumor, but these changes were suppressed by overexpression of CYLD (Guo et al., 2014). Furthermore, overexpression of CYLD in GBM-bearing mice significantly prolonged the survival benefit of bevacizumab treatment. Clinical trials on multiple tumors, including GBM, have shown that bevacizumab significantly prolongs recurrence-free survival, but has little impact on overall survival, suggesting adaptation and resistance to the therapy through various mechanisms (Lai et al., 2011; Sennino and McDonald, 2012). Our results indicate that decreased CYLD expression is crucial for the enhancement of pro-inflammatory factors in GBM tissues under hypoxic conditions, and hypoxia enhanced by long-term bevacizumab treatment may promote the establishment of an inflammatory microenvironment and GBM cell infiltration. Therefore, the survival benefit conferred by CYLD overexpression is expected to be a result of the suppression of the inflammatory microenvironment produced by bevacizumab administration. Understanding the molecular mechanisms linking CYLD downregulation and inflammation in hypoxia and the adaptive changes in GBM tissues induced by anti-VEGF therapy are expected to lead to the elucidation of the true nature of GBM and the development of therapeutic strategies.

6. CYLD expression in cholesteatoma

Cholesteatoma is a disease in which keratinized squamous epithelium proliferates into a spherical form in the middle ear, resulting in eventual bone destruction (Olszewska et al., 2004; Semaan and Megerian, 2006). Pathologically, cholesteatoma is not a malignant tumor, but it is a disease involving inflammation and has similarities in the mechanism of development and progression with the various tumors mentioned above, thus we introduce it in this section. The primary treatment of cholesteatoma is surgery (Kuo et al., 2015), but even in cases where cholesteatoma tissue has been completely removed, the number of days to recovery after surgery and whether or not recurrence occurs vary from patient to patient. And the mechanisms that cause these differences are not fully understood. Although many studies have been conducted to understand the pathogenesis of the disease and to improve its treatment, the mechanisms of onset, progression, and recurrence remain unclear. Furthermore, no clinically useful biomarkers to predict prognosis or recurrence have been reported to date.

In order to verify the clinical significance of CYLD expression in cholesteatoma, we evaluated CYLD expression in cholesteatoma tissues by immunohistochemical staining and analyzed the correlation with clinicopathological data (Miyake et al., 2020). First, immunohistochemical staining for CYLD revealed that CYLD expression was mainly found in the spinous and granular layers of epithelial tissues, and the level of expression varied greatly by patient. Subsequent analysis of the correlation between CYLD expression levels in cholesteatoma tissues and clinicopathological data showed that CYLD expression levels were significantly correlated with the grade of otorrhea, one of the clinical symptoms. Furthermore, the number of days to tympanic membrane epithelialization, an indicator of postoperative recovery, was significantly associated with CYLD expression level, suggesting that the lower the CYLD expression level, the shorter the days to recovery. On the other hand, although not statistically significant, CYLD expression levels tended to be lower in patients with recurrence. The results suggested that low CYLD expression level may induce recurrence while postoperative recovery is accelerated (Miyake et al., 2020).

The mechanism by which CYLD expression in cholesteatoma tissues affects postoperative prognosis and recurrence remains to be investigated, but it is possible that the degree of inflammation and CYLD expression levels in cholesteatoma tissues also affect CYLD expression levels in surrounding tissues. In cases of severe inflammation, CYLD expression in the surrounding tissues is induced and works to suppress inflammation, while in cases of weak inflammation, CYLD expression is not induced and cell proliferation is increased, and excessive cell proliferation may cause accelerated recovery and recurrence. It is expected that the expression of NF-κB activity and cell proliferation markers

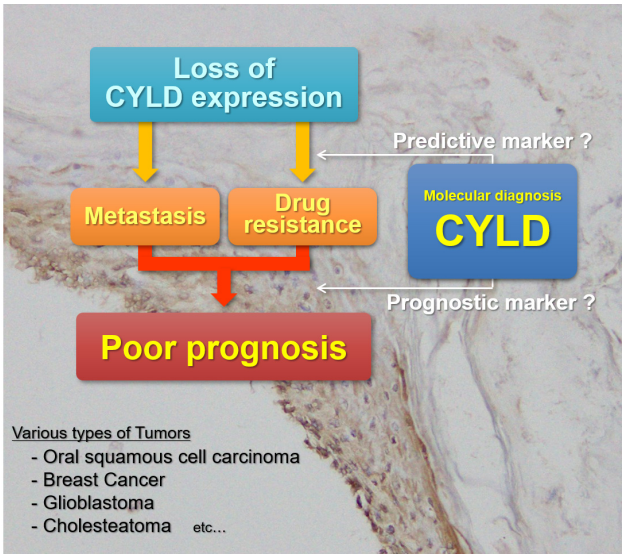

Figure 1. Potential of CYLD as a stratification marker in malignant tumors.

in the cholesteatoma surrounding tissues will be evaluated in the future to elucidate the mechanism in detail.

It should be noted that, the results shown in cholesteatoma first revealed that loss of CYLD expression levels may correlate with accelerated disease recovery. As mentioned above, CYLD expression levels have been examined in various malignant tissues, and in all cases, loss of CYLD expression correlated with tumor malignancy and poor prognosis via activation of NF-κB (Hayashi et al., 2014; Kinoshita et al., 2013; Shinriki et al., 2018). In contrast to these previous reports, the results in cholesteatoma study unveiled and provided a new aspect of CYLD, in which the loss of CYLD expression may be involved in promoting disease recovery in some cases. Moreover, further investigation of the involvement of NF-κB is expected to contribute to the improvement of the treatment of cholesteatoma.

7. Future Prospects

Recent studies keep revealing the novel roles of tumor suppressor gene CYLD in the development and progression of various malignancies, and the potential of CYLD as a prognostic and therapeutic response predictive marker (Figure 1). Even in the absence of mutations, CYLD dysfunction by its loss of expression have been shown to cause malignant transformation and drug resistance in tumor cells, and may be an important factor in determining the prognosis of cancer patients. Further investigation about CYLD function is expected to contribute to elucidate the molecular mechanisms of malignant transformation and drug resistance, which in turn may lead to the discovery of new therapeutic targets for personalized medicine.

Conflict of Interest

No conflicts of interest were declared.

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