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Onconeuronal antigen Cdr2 correlates with HIF prolyl-4-hydroxylase PHD1 and worse prognosis in renal cell carcinoma

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ABSTRACT

Neoplastic expression of the onconeuronal cerebellar degeneration-related antigen Cdr2 in ovary and breast tumors is associated with paraneoplastic cerebellar degeneration (PCD). Cdr2 protein expression is normally restricted to neurons, but aberrant Cdr2 expression has mainly been described for breast and ovarian tumors. Previously, we found strong Cdr2 protein expression in the papillary subtype of renal cell carcinoma (pRCC) and showed that Cdr2 interacts with the hypoxia-inducible factor (HIF) prolyl-4-hydroxylase PHD1. High Cdr2 protein levels are associated with decreased HIF-dependent gene expression in cells as well as in clinical pRCC samples, providing a possible explanation why pRCCs are the most hypovascular renal tumors. Here, we demonstrate that strong Cdr2 protein expression in clinical samples from pRCC patients correlates with elevated PHD1 protein levels, suggesting that increased PHD1 activity attenuates HIF-dependent gene expression. Interestingly, survival analysis revealed a significant correlation between high levels of Cdr2 expression and worse patient outcome in clear cell (cc) RCC patients. These findings provide evidence that Cdr2 might represent an important tumor antigen in kidney cancer and possibly in other cancer types as well. In contrast to ovary and breast tumor patients who develop PCD, no Cdr2 auto-antibodies were detected in the serum of pRCC patients, which is in line with the fact that pRCC patients have not been reported to display paraneoplastic neurodegenerative syndromes. This suggests that, despite a shared target antigen, tumor immunity and autoimmunity only partially overlap, and also highlights to which extent immunosurveillance against cancer can be clinically silent.

Introduction

Paraneoplastic cerebellar degeneration (PCD) is often associated with lung and gynecologic tumors and is thought to develop as a consequence of an autoimmune reaction directed against Purkinje cells (Darnell and Posner, 2006). It has been suggested that the neuron-specific cerebellar degeneration-related protein Cdr2 is expressed by breast and ovarian cancers and thereby elicits an anti-tumor response mediated by Cdr2-specific antibodies and T lymphocytes resulting in neurological symptoms (Albert and Darnell, 2004; Darnell and Posner, 2003). The anti-tumor effectiveness has been questioned as malignancies are finally detected in most anti-Cdr2 sera positive patients (Mathew et al., 2006). Although the *CDR2* gene is ubiquitously expressed, post-transcriptional regulation has been suggested to restrict Cdr2 protein expression physiologically to immune-privileged organs, such as brain and testis, and pathologically to ovarian cancers (Corradi et al., 1997; Darnell et al., 2000). However, Cdr2 protein levels were recently found to be comparably expressed in ovarian cancers and normal ovary tissue, as well as in tumors of other tissue origin. This suggests that Cdr2 antibody response in PCD might also be dependent on dysregulation of the immune system (Totland et al., 2011).

The physiological function of Cdr2 is incompletely understood. Cdr2 was found to interact with c-Myc in yeast and in vitro (Okano et al., 1999). Immunohistochemical analysis of rat brain sections showed a significant co-localization of Cdr2 and c-Myc in the cytoplasm of Purkinje neurons and overexpression of Cdr2 was shown to lead to a redistribution of c-Myc into the cytoplasm where it co-localized with Cdr2 (Okano et al., 1999). More recently, Cdr2 was found to be regulated in a cell cycle dependent manner in cancer cells with highest protein levels during mitosis (O'Donovan et al., 2010).

Regions of low oxygen partial pressures (hypoxia) are found in many tumor types and contribute to malignant progression, therapy resistance and poor patient's prognosis (Brown, 1998; Pouysségur et al., 2006). The master regulators of oxygen homeostasis are the heterodimeric hypoxia-inducible transcription factors (HIFs) and many of its target genes are involved in the adaptation of cancer cells

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to their hypoxic microenvironment (Wenger, 2002; Wenger et al., 2005). Under normoxic conditions, a family of prolyl-4-hydroxylase domain (PHD) proteins hydroxylates the HIFα subunits, targeting them for proteasomal degradation in a von Hippel–Lindau (VHL) tumor suppressor dependent manner (Bruick and McKnight, 2001; Maxwell et al., 1999). In hypoxia, HIFα is stabilized, translocates to the nucleus and forms a powerful transcription factor complex together with its counterpart HIF-1β (also known as aryl hydrocarbon receptor nuclear translocator, ARNT) (Kaelin and Ratcliffe, 2008; Schofield and Ratcliffe, 2004).

We previously identified Cdr2 as a novel PHD1 interacting protein and found increased PHD1 protein levels when Cdr2 was concomitantly overexpressed in cells by transient transfection (Balamurugan et al., 2009). However, endogenous PHD1 protein expression in RCC patients awaited further analysis due to the lack of specific anti-PHD1 antibodies. In this study, we found high PHD1 protein levels in pRCC and a strong correlation between PHD1 expression and Cdr2 expression in tumor tissue microarray (TMA) analysis. Despite the high Cdr2 levels, no Cdr2 auto-antibodies could be detected in the sera of pRCC patients in contrast to patients diagnosed with PCD. This supports the notion that Cdr2 protein expression in cancer does not necessarily correlate with the development of paraneoplastic neurodegenerative disease and emphasizes the still poor mechanistic comprehension of these diseases.

Materials and methods

Immunoblotting

Immunoblot analysis was performed as previously described (Kaufmann et al., 2013). Mouse anti-human Cdr2 antibodies were produced as described before (Balamurugan et al., 2009). Other antibodies used were rabbit anti-PHD1 (Novus Biologicals, Cambridge, United Kingdom, NBP1-40773) and mouse anti-β-actin (Sigma, Buchs, Switzerland). Secondary horseradish conjugated polyclonal goat anti-mouse and goat anti-rabbit antibodies were purchased from Pierce (Pierce, Lausanne, Switzerland). Chemiluminescence signals were detected using Supersignal West Dura substrate (Pierce) and signals were recorded with the LAS 4000 imaging system (Fuji, Bucher Biotec, Basel, Switzerland).

ELISA

Sera from 5 PCD, 12 pRCC, 6 ccRCC and 5 control patients were analyzed using 96-well enzyme-linked immunosorbent assay (ELISA) plates (CANDOR Biosciences, Wangen, Germany). Plates were coated overnight at 4 °C with 50 μl Tris–HCl (pH 9.4) containing 200 ng/μl recombinant Cdr2 capture antigen, produced as described previously (Balamurugan et al., 2009). Wells were washed three times with 200 μl 0.05% Tween in PBS, and blocked with 1% BSA in PBS for 1.5 h at room temperature. Horseradish peroxidase conjugated goat anti-human IgG $F(ab)_2$-fragment from Dianova diluted 1:100,000 in 30% LowCrossbuffer (Candor Bioscience) was used as the secondary antibody. All sera were diluted 1:400 in 30% LowCrossbuffer in PBS and analyzed in duplicates. The PCD serum samples were obtained from J. Honnorat (Lyon, France). Ethical approval for the serum samples of RCC patients was obtained from the local ethics committee and Swissmedic (EK-1017 and EK-1634).

Immunohistochemistry

Immunohistochemistry was performed as described before (Balamurugan et al., 2009). Briefly, TMA sections (2.5 μm), containing a total of 384 kidney samples (255 ccRCC, 48 pRCC, 21 oncocytoma, 14 chromophobe RCC and 46 normal kidney specimens) were stained according to the Ventana automat protocols (Roche, Basel, Switzerland). The same antibodies used for immunoblot analysis were applied for the detection of Cdr2 (1:20) and PHD1 (1:10). Analysis was performed with a Leitz Aristoplan microscope (Leica Microsystems, Heerbrugg, Switzerland). Pictures of RCC specimens were taken with a digital camera (JVC, Reinach, Switzerland, KY-070) and intensities of staining were categorized as absent, weak, moderate and strong. Since there were almost no Cdr2 negative tumors, the negative and weak cases were classified as low expressing and the moderate and strong cases were classified as high expressing tumors. Because overall PHD1 staining was rather modest, the RCCs with weak, moderate and strong staining were combined and classified as positive.

Kaplan–Meier survival analysis

The data of 232 ccRCC specimens were compiled with the software package SPSS, version 21.0 (SPSS Software, Munich, Germany). Spearman's Rho was used to assess the statistical significance of the correlation between molecular and clinicopathological parameters. Univariate survival analysis was performed according to the Kaplan–Meier method (Kaplan and Meier, 1958), and differences in survival curves were assessed with the log rank test. A Cox proportional hazard analysis was used to test for independent prognostic information. A p-value < 0.05 was considered statistically significant.

Results

PHD1 expression strongly correlates with Cdr2 expression in pRCC

To analyze PHD1 protein expression in clinical kidney samples that were previously probed for Cdr2 expression, immunohistochemical analysis was performed on 384 RCC and normal kidney samples using TMA technology (Kononen et al., 1998; Struckmann et al., 2008). PHD1 expression was strong in pRCC, weak in ccRCC (Fig. 1a) and absent in chromophobe RCC or benign oncocytoma (data not shown). Furthermore, 74% (17 out of 23) of pRCC PHD1 positive samples showed strong Cdr2 staining and 67% (14 out of 21) of PHD1 negative samples showed weak Cdr2 staining ($p < 0.01$). There were no notable differences between those two Cdr2/PHD1 expression combinations and the pathological parameters tumor stage and nuclear differentiation grade (Fuhrman et al., 1982) (data not shown).

In addition, normal kidney, pRCC and ccRCC tissues were analyzed for PHD1 protein expression by immunoblotting. As shown in Fig. 1b, the newly available anti-PHD1 antibody specifically detected both known PHD1 isoforms (Appelhoff et al., 2004). Interestingly, the expression of the smaller PHD1 isoform (40 kDa) was increased in pRCC and decreased in ccRCC samples compared to normal kidney. Quantification of PHD1 p40 and PHD1 p43 revealed that total PHD1 protein expression in pRCC was slightly higher compared to normal kidney and clearly higher compared to ccRCC (data not shown). As previously published (Balamurugan et al., 2009), Cdr2 protein expression was strongest in pRCC (Fig. 1b).

Sera of PCD but not RCC patients contain Cdr2 auto-antibodies

Our findings indicate that Cdr2 represents an important tumor antigen not only for ovarian and breast cancers but also for pRCC. To investigate whether high Cdr2 expression in pRCC also elicits an autoimmune response and leads to the generation of Cdr2 antibodies, an ELISA was established using recombinant Cdr2 purified from bacteria. Cdr2-coated 96-well plates were incubated with sera from 5 PCD, 12 pRCC, 6 ccRCC and 5 non-cancerous patients to detect Cdr2 antibodies. However, no Cdr2 antibodies could be detected in any of the pRCC, ccRCC or control patients. In contrast, the sera of PCD patients showed high titers of Cdr2 antibodies and served as a positive control (Fig. 2). These findings are in line with the fact that