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Recurrent cyclin D2 mutations in myeloid neoplasms

V Khanna^{1,2,3}, CA Eide^{1,4,5}, CE Tognon^{1,4,5}, JE Maxson^{1,4,6}, B Wilmot^{1,7}, D Bottomly^{1,7}, S McWeeney^{1,7}, DK Edwards^{1,4,6}, BJ Druker^{1,4,5}, and JW Tyner^{1,4,6}

¹Knight Cancer Institute, Oregon Health and Science University, Portland, OR, USA

²Howard Hughes Medical Research Fellows Program, Howard Hughes Medical Institute, Portland, OR, USA

³Cleveland Clinic Lerner College of Medicine of Case Western Reserve University, Cleveland, OH, USA

⁴Division of Hematology and Medical Oncology, Oregon Health and Science University, Portland, OR, USA

⁵Howard Hughes Medical Institute, Portland, OR, USA

⁶Department of Cell, Developmental and Cancer Biology, Oregon Health and Science University, Portland, OR, USA

⁷Division of Bioinformatics and Computational Biology, Department of Medical Informatics and Clinical Epidemiology, Oregon Health and Science University, Portland, OR, USA

Philadelphia-negative neutrophilic leukemias—atypical chronic myeloid leukemia (aCML), chronic neutrophilic leukemia (CNL), MDS/MPNu (myelodysplastic/myeloproliferative neoplasm, unclassifiable), and MPNu (myeloproliferative neoplasm, unclassifiable)—are rare hematologic neoplasms characterized by leukocytosis, a hypercellular bone marrow comprised predominantly of granulocytic cells, absence of the Philadelphia chromosome (*t(9;22); BCR-ABL1*), and absence of *PDGFRA/B* or *FGFR1* gene rearrangements. Occasional cases of CNL¹ and MDS/MPNu,² as well as a majority of aCML cases, exhibit non-specific cytogenetic abnormalities³ or the *JAK2* V617F mutation,⁴ revealing their clonal nature. The primary genetic basis of these leukemias was unknown until recent work by Maxson *et al.*,⁵ which highlighted mutations in *CSF3R* as key leukemogenic drivers in ~60% of patients with aCML or CNL. Subsequent studies found *CSF3R* mutations in a majority of patients with CNL and only in a minority of patients with aCML,⁶ with parallel studies of aCML revealing the presence of recurrent *SETBP1* and *ETNK1* mutations.^{7,8} Despite these discoveries, knowledge of the full genetic landscape of Philadelphia-negative neutrophilic leukemias remains incomplete. To identify and characterize additional mutations contributing to the pathogenesis of these malignancies, we performed whole exome sequencing (WES) on 116 Philadelphia-negative neutrophilic leukemia samples (Supplementary Materials and Methods).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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We identified 4/116 samples harboring mutations in *CCND2*, the gene encoding the cell cycle regulator cyclin D2. Of these four samples, one was pathologically confirmed as MPNu, while another was confirmed as MDS/MPNu (Supplementary Table 1). Interestingly, all mutated samples harbored variants in codon 281, with three samples possessing a P281S variant and the other carrying a P281L variant (Supplementary Table 1). These changes appeared to occur as heterozygous mutations and were present only in patients lacking *CSF3R* mutations, with one sample (13-00010) harboring a known pathogenic *SETBP1* variant (I871T), and another (14-00247) possessing an SRSF2 P95H variant. Two out of four samples harbored a *TET2* mutation (other observed mutations in Supplementary Table 2). All mutations with additional available DNA were confirmed by Sanger sequencing (Supplementary Figure 1).

To determine the potential incidence of *CCND2* mutations in other hematologic malignancies, we interrogated cohorts of patients with AML ($n = 239$), BCR-ABL1-positive CML (CML; $n = 44$), chronic myelomonocytic leukemia (CMML; $n = 24$), B-cell acute lymphoblastic leukemia (B-ALL; $n = 49$), or T-ALL ($n = 7$) by whole exome sequencing for the presence of variants in *CCND2* (Supplementary Table 2). We identified three AML patients harboring the same *CCND2* variants (two P281S; one P281L), all confirmed as somatic (Supplementary Table 3). These findings are consistent with two recent reports of similar *CCND2* mutations in AML.^{9,10} No mutations in *CCND2* were detected in patients with CML, CMML, B-ALL or T-ALL (Supplementary Table 2). Taken together, these results suggest the presence of *CCND2* mutations may unify a small but recurrent and selective subset of patients with the molecularly heterogeneous myeloid malignancies of Philadelphia-negative neutrophilic leukemias and AML.

Previous work examining a missense variant in *CCND2* (T280A), just one amino acid upstream of P281, demonstrated the variant to confer resistance to degradation.¹¹ To assess whether the nearby variants identified in our patient cohort resulted in similar protein accumulation, NIH-3T3 cell lines stably overexpressing either empty vector, wild-type *CCND2*, or mutant *CCND2* were treated with cycloheximide to block protein translation, and protein extracts from cells were analyzed by Western blotting (Figure 1a). While a rapid reduction in cyclin D2 protein was observed in cells overexpressing wild-type cyclin D2, this was not observed in cells overexpressing the mutant constructs. Therefore, *CCND2* P281 variants result in the accumulation of degradation-resistant cyclin D2.

Interestingly, the *CCND2* T280A variant is also associated with constitutively nuclear localization of the protein.¹¹ Because of the proximity of the *CCND2* P281 variants to the previously reported T280A variant, we hypothesized that *CCND2* P281 variants would be constitutively nuclear in their localization. While overexpressed wild-type *CCND2* exhibited predominantly nuclear localization during G₁ phase of the cell cycle and cytoplasmic staining during S phase, *CCND2* P281 variant protein remained predominantly nuclear in its localization regardless of cell cycle phase (Figure 1b). Quantification of mean pixel density in the nucleus confirmed statistical significance of this difference ($P < 0.0001$; Figure 1c).

Variants in cyclin D1 have been reported at analogous residues to P281S and P281L of cyclin D2, exhibiting predominantly nuclear localization and oncogenic properties.¹² We

therefore assessed the transformative capacity of *CCND2* P281 variants by generating Ba/F3 cell lines stably overexpressing either wild-type *CCND2*, *CCND2* P281S or *CCND2* P281L and removing IL-3 from their growth medium to assess for growth factor independence. As shown in Figure 2a, neither wild-type nor mutant *CCND2* transformed Ba/F3 cells to IL-3-independent growth. However, cell lines that overexpressed *CCND2* relative to cells expressing an empty control vector exhibited clear delays in cell death, suggesting that high levels of *CCND2* expression can support enhanced cell survival (Figure 2b).

Although *CCND2* P281 variants did not confer growth factor independence in the Ba/F3 model, past work had demonstrated a role for *CCND2* in cytokine-mediated proliferation of bone marrow progenitor cells.¹³ We thus examined whether expression of *CCND2* P281 variants promoted the formation of mouse bone marrow progenitors in response to cytokines. Expression of *CCND2* P281 variant constructs significantly enhanced the formation of colonies by mouse bone marrow progenitors relative to progenitors expressing an empty control vector, suggesting that enforced expression of these variants can enhance the proliferative potential of murine hematopoietic progenitors (Figures 2c and d). Notably, however, there were no significant differences in colony formation between wild-type *CCND2*-over-expressing cells and *CCND2* variant-containing cells, presumably due to the wild-type *CCND2* being overexpressed at levels that mimic the phenotype of the P281 variants (Figure 2d).

Cyclin D2 is synthesized in response to mitogens, whereupon it complexes with the cyclin-dependent kinases CDK4 and CDK6, leading to pRb inactivation and E2F-mediated upregulation of genes required for S phase entry.¹⁴ In recent years, inhibition of CDK4/6 has demonstrated effectiveness in various malignancies. Therefore, given the functional connection between cyclin D2 and CDK4/6, we hypothesized that cells expressing *CCND2* P281 variants would be sensitive to CDK4/6 inhibition. While parental NIH-3T3 cells did not exhibit sensitivity to palbociclib, cell lines overexpressing either wild-type cyclin D2 or *CCND2* P281 variants showed relatively increased sensitivity (Figure 2e). The results, therefore, indicate that leukemias that overexpress cyclin D2 may be susceptible to CDK4/6 inhibition.

The study herein provides evidence of *CCND2* mutations in Philadelphia-negative neutrophilic leukemias that result in the overexpression and mislocalization of cyclin D2 protein, as well as increased survival and proliferation of hematopoietic cells. D-type cyclins are arbiters of cell cycle progression, especially between G₁' and S phase, and are frequently dysregulated in cancer. Of the D-type cyclins, cyclin D1 is the best-studied, with past work demonstrating its role in a variety of malignancies. While less is known about cyclin D2, its overexpression occurs in several cancer types.¹⁵⁻¹⁷ Recent work in T-cell acute lymphoblastic leukemias (T-ALL) has demonstrated leukemogenic chromosomal translocations involving cyclin D2 and the T-cell receptor,¹⁸ and over-expression of cyclin D2 has also been reported in chronic myeloid leukemia (CML) and Philadelphia-chromosome positive (Ph+)-ALL.¹⁹ However, to our knowledge, none of the aforementioned leukemias has been found to harbor *CCND2* mutations, consistent with our analysis of CML, T-ALL and B-ALL samples (Supplementary Table 2).

While our work and the recent work of others suggests that the *CCND2* variants contribute to protein degradation resistance, enhanced hematopoietic cell proliferation, and sensitivity to CDK4/6 inhibitors, additional experimental models will be required to further characterize the role of these variants in leukemogenesis. The pathogenic potential of *CCND2* P281 variants may be manifested through promotion of genetic instability, thereby predisposing cells to develop additional deleterious mutations. Work examining the oncogenicity of the constitutively nuclear cyclin D1 variant T286A suggests that its improper nuclear accumulation during S phase triggers DNA damage and activation of the ATM-CHD2-p53 checkpoint pathway, leading to promotion of DNA re-replication.²⁰ If *CCND2* P281 variants do indeed increase genomic instability, one might expect that analysis of serial leukemia samples from patients with *CCND2* mutations would reveal the presence of these mutations at or soon after diagnosis. The presence of these mutations in Philadelphia-negative neutrophilic leukemias, which are known to progress into a blastic ‘AML-like’ phase, may also support this hypothesis. Collectively, future studies that examine the combined effect of *CCND2* mutations with other known deleterious mutations in these leukemias may provide additional insights into the relevance of cyclin D2 to leukemogenesis.

In summary, the present study provides novel insight into the genetics of myeloid malignancies, namely Philadelphia-negative neutrophilic leukemias. Our work suggests that in addition to the commonly recurring classes of genes that are frequently mutated in these malignancies, recurrent mutations in cyclin D2, and perhaps other cell cycle regulators, have biochemical and therapeutic consequences and may play important roles in the pathogenesis of these leukemias.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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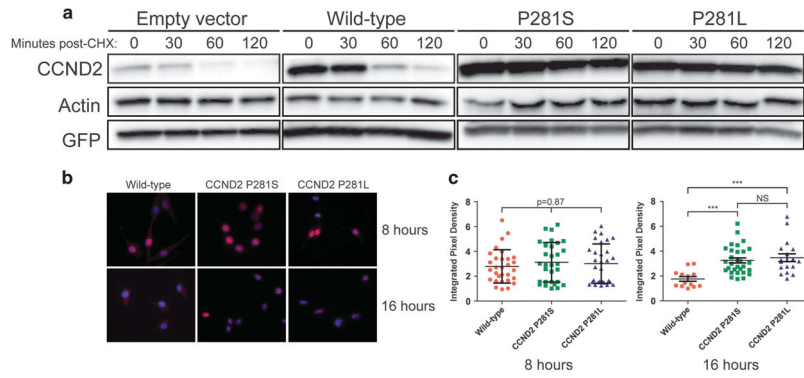


Figure 1.

CCND2 P281 variants are resistant to degradation and nuclear export. **(a)** Protein extracts from NIH-3T3 cells stably expressing each construct (top labels) were treated with cycloheximide (CHX) and were analyzed by western blotting using an antibody against CCND2. The wild-type CCND2 construct shows clear protein reduction by 60 min post-CHX treatment, while mutant constructs show relatively stable levels of protein, even by 120 min post-CHX treatment. **(b)** Representative immunofluorescence images of NIH-3T3 cells stably expressing either wild-type or CCND2 P281 variants. Cells were serum starved for 20 h in RPMI containing 0.1% FBS, reintroduced to serum, and then collected for immunofluorescence analysis at the specified time points post-serum reintroduction. Eight hours and 16 h post-reintroduction were chosen as collect time points because they represent time points at which the majority of wild-type CCND2 is nuclear and cytoplasmic, respectively. Red =CCND2, blue =DAPI. **(c)** Quantification of mean pixel density in the nucleus to establish nuclear localization of CCND2 constructs. At least ten cells were analyzed for each condition. *** $P < 0.0001$ by one-way ANOVA and subsequent Tukey's post hoc tests. Error bars show s.d.

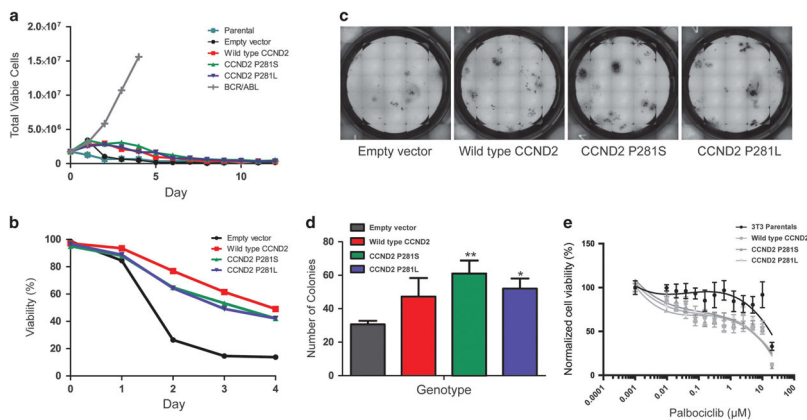


Figure 2. Assessment of the oncogenicity and CDK4/6 inhibitor sensitivity of CCND2 P281 variants. **(a)** IL-3 withdrawal assay demonstrating the dependence of all CCND2-expressing lines on IL-3 for proliferation. **(b)** Normalized cell viability (relative to day 0 of IL-3 withdrawal) over time. Cells expressing CCND2 constructs show delayed decreases in viability relative to cells expressing an empty control vector. **(c)** Representative images of wells containing murine hematopoietic colonies grown in Methocult M3534 (supplemented with murine IL-3, IL-6 and SCF). Images were taken after 14 days of incubation. **(d)** Quantification of colony number, performed in triplicate, for murine hematopoietic cells expressing the labeled constructs. Colony number was compared between groups via one-way ANOVA followed by Tukey’s post hoc tests. ****** $P < 0.01$ for comparison of colony number in empty vector-expressing cells versus the number in P281L-expressing cells. ***** $P < 0.05$ for comparison of colony number in empty vector-expressing cells versus the number in P281L-expressing cells. No significant differences in colony numbers existed between wild-type CCND2-expressing cells and P281 variant-expressing cells. Error bars show s.d. **(e)** Sensitivity of CCND2-expressing NIH-3T3 cells to CDK4/6 inhibition. Cells expressing cyclin D2 constructs show increased sensitivity to palbociclib (a CDK4/6 inhibitor) relative to parental NIH-3T3 cells. Error bars show standard error of the mean for each data point.