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Mutations in the *CCND1* and *CCND2* genes are frequent events in adult patients with t(8;21)(q22;q22) acute myeloid leukemia

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Abstract

Core-binding factor acute myeloid leukemia (CBF-AML) is defined by the presence of either t(8;21)(q22;q22)/*RUNX1-RUNX1T1* or inv(16)(p13.1q22)/t(16;16)(p13.1;q22)/*CBFB-MYH11*. The resulting fusion genes require a “second hit” to initiate leukemogenesis. Mutation assessment of 177 adults with CBF-AML, including 68 with t(8;21) and 109 with inv(16)/t(16;16), identified not only mutations well-known in CBF-AML, but also mutations in the *CCND1* and *CCND2* genes, which represent novel frequent molecular alterations in AML with t(8;21). Altogether,

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The authors declare no conflicts of interest.

AUTHORSHIP CONTRIBUTIONS

A-KE, JCB, KM and CDB contributed to the study design; A-KE, AdlC, JCB, SS, CJW, KM and CDB contributed to the data interpretation, A-KE, KM, AdlC and CDB wrote the manuscript; A-KE and SO generated the libraries for the targeted sequencing approach; A-KE analyzed the sequencing data; CS, MB and CJW performed laboratory-based research; JSB and KWK performed the data processing; JK and DN performed statistical analysis; AJC, JCB, JEK, MRB, RMS, BLP, KM and CDB were involved directly or indirectly in the care of patients and/or sample procurement. All authors read and agreed on the final version of the manuscript.

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CCND1 (n=2) and *CCND2* (n=8) mutations were detected in 10 (15%) patients with t(8;21) in our cohort. A single *CCND2* mutation was also found in one (0.9%) patient with inv(16). In contrast, *CCND1* and *CCND2* mutations were detected in only 11 (0.77%) of 1,426 non-CBF-AML patients. All *CCND2* mutations cluster around the highly conserved amino acid residue threonine 280 (Thr280). We show that Thr280Ala mutated *CCND2* leads to increased phosphorylation of the retinoblastoma protein, thereby causing significant cell cycle changes and increased proliferation of AML cell lines. The identification of *CCND1* and *CCND2* mutations as frequent mutational events in t(8;21) AML may provide further justification for cell cycle-directed therapy in this disease.

Keywords

acute myeloid leukemia; core-binding factor; *CCND1*; *CCND2*; mutation

INTRODUCTION

Acute myeloid leukemia (AML) with t(8;21)(q22;q22)/*RUNX1-RUNX1T1* [hereafter referred to as t(8;21)] and AML with inv(16)(p13q22)/*CBFB-MYH11* or t(16;16)(p13;q22)/*CBFB-MYH11* [hereafter referred to as inv(16)] are related at the molecular level. The fusion genes created by the aforementioned chromosome rearrangements disrupt, respectively, subunits α and β of core-binding factor, a heterodimeric transcription factor involved in hematopoietic differentiation, and impair its function.¹ Consequently, both these AML types are together referred to as core-binding factor (CBF) AML,^{1,2} and each of them is recognized by the World Health Organization classification of myeloid neoplasms and acute leukemia as a distinct disease entity.³ CBF-AML is relatively frequent, with t(8;21) detected in approximately 6% and inv(16) in approximately 7% of adults with *de novo* AML.² Patients with CBF-AML, compared with other AML subtypes, have a relatively favorable prognosis, especially with the use of high-dose cytarabine-based postremission chemotherapy. However, almost 40% of CBF-AML patients experience relapses of their disease.^{4,5} Patients with t(8;21) were reported to have shorter overall survival (OS) and survival after first relapse than patients with inv(16).^{4,5}

Although the *RUNX1-RUNX1T1* and *CBFB-MYH11* fusion genes are essential, disease-defining contributors to leukemogenesis, they are not capable of causing leukemia by themselves.^{2,6} The presence of additional genetic alterations, including mutations in the *KIT*, *NRAS*, *KRAS* or *FLT3* [especially mutations in the tyrosine kinase domain of *FLT3* (*FLT3*-TKD)] genes, likely represent a “second hit” to initiate leukemogenesis.^{2,6} Thus, elucidation of the full mutational spectrum of both cytogenetic types of CBF-AML is of high importance. While some previously reported molecular features of CBF-AML, such as *KIT* and *RAS* mutations, are shared by both t(8;21)- and inv(16)-positive CBF-AML, mutations in the cohesin complex and chromatin remodeling genes are more frequently found in AML with t(8;21) than in AML with inv(16).^{7,8} The recent discoveries of recurring mutations in *ASXL2*⁹ and *ZBTB7A*^{10,11} that occur either exclusively (*ASXL2*^{9,11}) or almost exclusively (*ZBTB7A*^{10,11}) in t(8;21)-positive CBF-AML further support the existence of specific mutations that distinguish patients with t(8;21) from those with inv(16).

A recent comprehensive study of gene mutations in CBF-AML⁷ detected the presence of at least one mutation in 85% of the patients studied, thus leaving a possibility that the remaining 15% of patients harbor other, as yet not discovered, mutations. Therefore, we hypothesized that novel, hitherto not reported gene mutations may still exist, and if so, that they might contribute to clinical and molecular differences between CBF-AML patients with t(8;21) and those carrying inv(16). We analyzed samples from a relatively large cohort of adults with CBF-AML for mutations in 84 leukemia- and/or cancer-associated protein coding genes using a customized targeted next-generation sequencing approach.

Herein, we report for the first time recurrent mutations in the *cyclin D1* (*CCND1*) and *cyclin D2* (*CCND2*) genes as the third most common mutational event in patients with t(8;21), but not in those with inv(16). Cyclins CCND1 and CCND2 play a crucial role in the regulation of hematopoietic differentiation and cell proliferation,¹² and their differential activation has been implicated in leukemogenesis.^{12,13} While *CCND1* and *CCND2* mutations are relatively frequent mutational events in solid tumors (as evidenced by TCGA studies), they have not yet been described as frequent recurrent events in hematologic malignancies including t(8;21) AML.

PATIENTS AND METHODS

Patients, treatment, and cytogenetic studies

Pretreatment bone marrow (BM) or peripheral blood (PB) samples suitable for next-generation sequencing were obtained from 177 adult AML patients diagnosed with CBF-AML and treated similarly on Cancer and Leukemia Group B (CALGB)/Alliance for Clinical Trials in Oncology (Alliance) trials. In addition, pretreatment BM or PB samples of 81 adults with CBF-AML who were also treated on the aforementioned CALGB/Alliance trials and whose samples were of insufficient DNA quality (260/280 ratio >1.6) or quantity (100ng DNA), were analyzed for their *CCND1* and *CCND2* mutation status using Sanger sequencing. Details of the treatment studies are provided in the Supplementary Information. Cytogenetic analyses of pretreatment BM and/or PB samples were performed by institutional laboratories approved by the CALGB/Alliance. Cytogenetic results were confirmed by central karyotype review.¹⁴ Patients provided written informed consent to participate in protocols CALGB 8461 (cytogenetic studies), CALGB 9665 (leukemia tissue bank) and CALGB 20202 (molecular studies), which involved collection of pretreatment BM aspirates and PB samples. Study protocols were in accordance with the Declaration of Helsinki and approved by the institutional review boards at each center, and all patients provided written informed consent.

Statistical analyses

Baseline characteristics were compared between patients harboring mutations of the *CCND1* or *CCND2* genes and those with wild-type *CCND1* and *CCND2* using Fisher's exact test for categorical variables and the Wilcoxon rank-sum test for continuous variables. A *P*-value of 0.05 was considered statistically significant. Definitions of the clinical endpoints—complete remission (CR), disease-free survival (DFS) and OS—are provided in the Supplementary

Information. The dataset was locked on January 21, 2016. Data collection and statistical analyses were performed by the Alliance Statistics and Data Center using SAS 9.4.

Molecular analyses

Mononuclear cells were enriched through Ficoll-Hypaque gradient centrifugation and cryopreserved until use. Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany). The mutational status of 84 protein-coding genes was determined centrally at The Ohio State University by targeted amplicon sequencing using two different gene panels on the MiSeq platform (Illumina, San Diego, CA; see Supplementary Information for details). The panels comprised the following genes: *AKT1*, *ARAF*, *ASXL1*, *ASXL2*, *ATM*, *AXL*, *BCL2*, *BCOR*, *BCORL1*, *BIRC6*, *BRAF*, *BRD4*, *BRINP3*, *BTK*, *CALR*, *CBL*, *CCND1*, *CCND2*, *CEBPA* (bi-allelic), *CSNK1A1*, *CTNNB1*, *DHX15*, *DNMT3A*, *ETV6*, *EZH2*, *FBXW7*, *FLT3* (for *FLT3*-TKD), *GATA1*, *GATA2*, *GSK3B*, *HIST1H1E*, *HNRNPK*, *IDH1*, *IDH2*, *IKZF1*, *IKZF3*, *ILR7*, *JAK1*, *JAK2*, *JAK3*, *KIT*, *KLHL6*, *KMT2A*, *KRAS*, *MAPK1*, *MAPK3*, *MED12*, *MYD88*, *NOTCH1*, *NPM1*, *NRAS*, *PHF6*, *PIK3CD*, *PIK3CG*, *PLCG2*, *PLEKHG5*, *PRKCB*, *PRKD3*, *PTEN*, *PTPN11*, *RAD21*, *RAF1*, *RUNX1*, *SAMHD1*, *SETBP1*, *SF1*, *SF3A1*, *SF3B1*, *SMARCA2*, *SMC1*, *SMC3*, *SRSF2*, *STAG2*, *SYK*, *TET2*, *TGM7*, *TP53*, *TYK2*, *U2AF1*, *U2AF2*, *WT1*, *XPO1*, *ZMYM3* and *ZRSR2*. The MuCor program¹⁵ was used for initial data analysis, followed by visual inspection and quality control of the BAM files. If less than 15 reads were present, the gene mutation status was considered as not evaluable. The variant allele fraction (VAF) cut-off for reporting mutations was set to 0.10. *CCND1* and *CCND2* mutations above and below the threshold were additionally validated by Topo-TA cloning of the PCR product, followed by Sanger sequencing. Variants (missense, nonsense or frameshift) were considered as mutations if they were not reported in the 1000 Genome database, dbSNP137, or dbSNP142. Testing for the presence or absence of *FLT3* internal tandem duplication (*FLT3*-ITD) was performed as previously described.¹⁶

Tissue culture

KG1a and Kasumi-1 cells (both *CCND1* and *CCND2* wild-type) were obtained from the American Type Culture Collection (ATCC, Manassas, VA) for studying the effects of the *CCND2* Thr280Ala mutation in AML. Both cell lines were authenticated by ATCC using the cytochrome C oxygenase 1 gene (COI) assay for interspecies determination, and short tandem repeat (STR) analysis for intraspecies determination of the unique DNA profiles. The cells were cultured in RPMI medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% Antibiotic-Antimycotic (Gibco, Big Cabin, OK).

Forced abundance of CCND2 Thr280Ala, wild-type CCND2 and empty vector control

The open reading frame of wild-type *CCND2* and *CCND2* harboring the Thr280Ala mutation were cloned into an HIV-based lentiviral dual promoter vector (pCDH-CMV-MCS-EF1-copGFP+Puro cDNA; System Biosciences, Palo Alto, CA) for stable expression. The mutation was introduced using a 1-basepair mismatch reverse primer. Primer sequences were as follows: *CCND2* clone F, gcgtgctagcatggagctgctgtgccacgag; *CCND2* clone R, gcgtgaattcctcacaggtcgatatcccgacgtctgtagggttg; *CCND2* mutR, cagcaaccctacagacgtgcgggatatcgacctgtgaggat (System Biosciences). The lentiviral construct

(45µg) was transfected into HEK-293TN cells using 45µl pPACKH1 and 55µl PureFection (System Biosciences). The supernatant containing the pseudoviral particles was collected after 48 and 72 hours, and the virus was precipitated at 4°C overnight using 5X PEG-IT virus precipitation solution (System Biosciences). Two hundred microliters of Phosphate Buffered Saline (PBS) and 25µM Hepes Buffer were used for resuspension of the pelleted virus. We infected 200,000 cells in triplicate with 20 IU of virus using 5µl Transdux Infection Reagent (System Biosciences). RNA was harvested after 72 hours, and CCND2 overexpression was confirmed at the RNA and protein level.

TiterGlo assays

Cell viability changes in cell lines infected with *CCND2* Thr280Ala, *CCND2* wild-type or empty vector (EV) control were analyzed using chemiluminescent TiterGlo assays (Promega, Madison, WI) 72h after puromycin selection (cell lines) using 20,000 cells in duplicate from three biological replicates according to the manufacturer's instructions.

Immunofluorescence staining and microscopy

Staining was performed on puromycin-selected cells by standard procedures using the following antibodies: Phospho-HistoneH3 (sc-101752, Santa Cruz Biotechnology, Dallas, TX), and Alexa Fluor 488 goat anti-mouse. Micrographs were captured using a BZ-9000 microscope (Keyence, Osaka, Japan).

Propidium iodide flow cytometry cell cycle analysis

Cell lines expressing CCND2 Thr280Ala, wild-type CCND2 or EV were fixed in ethanol, treated with RNase A, then suspended in propidium iodide. Fluorescence activated cell sorting was performed by BD LSR II cytometry with assistance from The Ohio State University Analytic Cytometry Core. Cell cycle analysis was performed with FlowJo software v7.6.3 (FlowJo LLC, Ashland, OR) using the Watson model and no constraints.

RESULTS

Differences in the molecular landscapes of CBF-AML with t(8;21) and CBF-AML with inv(16)

The baseline clinical characteristics of all 177 CBF-AML cases are listed in Supplementary Table S1. Molecular profiling showed mutational events that were shared by patients with t(8;21) and those with inv(16) with a similar incidence, but also revealed mutational features that were more frequent in one of the two CBF-AML subtypes. As a group, mutations in genes encoding kinases were the most frequent both in patients with t(8;21) and those with inv(16)—they were found in 33% of the former and 32% of the latter (Figure 1A and Supplementary Table S2). Interestingly, mutations in specific chromatin remodeling genes were tightly associated with each cytogenetic type of CBF-AML, with *ASXL2*, *EZH2*, *ASXL1* and *SMARCA2* mutations occurring exclusively or almost exclusively in patients with t(8;21), and *BCORL1* and *BCOR* mutations being found predominantly in patients with inv(16) (Supplementary Table S3). Likewise, mutations in the cohesion complex were observed only in patients with t(8;21) [15% vs 0% in inv(16), $P<0.001$]. This is in line with previous reports,⁷ as is a lower incidence of RAS pathway mutations in patients with t(8;21),

which were found in 19% of patients, as compared with 35% of the inv(16) patients with RAS pathway mutations ($P=0.03$).

Identification of recurrent CCND1 and CCND2 mutations in patients with t(8;21)

Strikingly, in addition to the known mutations, we detected recurrent mutations in the *cyclin D1* (*CCND1*) and *cyclin D2* (*CCND2*) genes. In fact, *CCND1* and *CCND2* mutations were the third most frequent mutational events in patients with t(8;21) (together with *NRAS* mutations), being detected in 10 of 68 (15%) such patients (Figure 1B). The variant allele fractions of the detected *CCND1* and *CCND2* mutations were variable, suggesting that these mutations can represent both early and later mutational events (Table 1). Only one *CCND2* mutation was found in 109 (0.9%) patients with inv(16). Similarly, when we used Sanger sequencing to test for *CCND1* and *CCND2* mutations in pretreatment samples from an additional set of 81 CBF-AML patients for whom next-generation sequencing was not possible [inv(16), n=56; t(8;21), n=25], we detected *CCND1* and *CCND2* mutations in three patients with t(8;21), one of whom had a *CCND1* mutation and two carried *CCND2* mutations. As a result, the overall *CCND1* and *CCND2* mutation frequency among a total of 93 patients with t(8;21) tested was 14%. No additional *CCND1* or *CCND2* mutations were found among 56 patients with inv(16) analyzed using Sanger sequencing. Thus, *CCND1* and *CCND2* mutations represent a novel mutational feature, which further distinguishes CBF-AML with t(8;21) from CBF-AML with inv(16) at the molecular level. Interestingly, among patients with t(8;21), two of the eight *CCND2* mutated patients and one of the two *CCND1* mutated patients had multiple mutations in these genes (Figure 1), which likely occurred in separate subclones, suggesting that *CCND1* and *CCND2* mutations play an important role in leukemogenesis.

Differential activation of cyclin genes is a known contributor to leukemogenesis.^{12,13} The identified molecular mechanism responsible for the stabilization of the CCND2 protein is the inhibition of glycogen synthase kinase 3 beta (GSK3B) through Janus kinase 2 (JAK2) and phosphatidylinositol 3' kinase (PI3K) signaling, which is responsible for phosphorylation of CCND2 at amino acid threonine 280 (Thr280), followed by proteasomal degradation of CCND2.¹⁷ Intriguingly, all *CCND2* mutations we detected in t(8;21) patients either directly affected (n=5) or clustered around the codon Thr280, thereby also involving highly conserved amino acid residues Lys234, Glu274, Gln277, and Pro281 (twice) (Figure 2A). The mutations in codon 280 replace threonine with either alanine or asparagine, both of which are the exact amino acid changes that have been previously found to render CCND2 resistant towards proteasomal degradation,^{17,18} resulting in a constitutively active CCND2 protein. Furthermore, *de novo* germline *CCND2* mutations affecting the identical amino acid residues Thr280 or Pro281 have been recently found to cause megalencephaly-polymicrogyria-polydactyly-hydrocephalus syndrome (MPPH) type 3, an extremely rare neurodegenerative disorder.¹⁸ Although no increased cancer or leukemia risk has been reported to date for this patient population, Mirzaa *et al.*¹⁸ observed strongly increased proliferation of embryonic mouse brain cells after introduction of the *CCND2* Thr280Ala mutant.

The *CCND1* mutations detected in our patients with t(8;21) affected amino acid positions Thr286, Arg291 and Val293. Similarly to the data regarding cyclin D2, it has been previously shown cyclin D1 turnover is dependent on Thr286 phosphorylation and is regulated by ubiquitin-dependent proteasomal degradation. Phosphorylation of cyclin D1 was enhanced by binding to CDK4 and mutation of Thr286 to alanine (Thr286Ala) resulted in increased stability of the cyclin.¹⁹ Furthermore, overexpression of cyclin D1 Thr286Ala also facilitated anchorage independent growth of NIH-3T3 cells in soft agar, suggestive of a transformatory potential.²⁰

Clinical and molecular characterization of CBF-AML patients with t(8;21) and *CCND1* or *CCND2* mutations

Detailed clinical and cytogenetic information on the affected patients, the *CCND1* and *CCND2* mutation locations and observed variant allele fractions, and additional mutations found in the patients (if known) are listed in Table 1. The evaluation of mutations in other genes found in these patients did not reveal any statistically significant co-occurring or mutually exclusive mutations (Figure 2B). The most frequent co-occurring mutation was *ASXL2*, which we detected in four of the 10 (40%) cases with t(8;21). As mutations in the receptor tyrosine kinase/RAS/PI3K pathway are frequent in AML patients with t(8;21), the identification of *CCND2* mutations adds a new key player to already known mutations and further highlights the importance of this pathway in leukemogenesis in t(8;21)-positive patients (Figure 2C).²¹

We also compared pretreatment clinical features and outcomes of patients with t(8;21) with *CCND1* and *CCND2* mutations with those of patients with wild-type *CCND1* and *CCND2* genes (Tables 2 and 3). Patients with *CCND1* and *CCND2* mutations presented with lower percentages of PB ($P=0.02$) and BM ($P=0.05$) blasts than patients without these mutations, but did not differ significantly with regard to other pretreatment characteristics (Table 2). No outcome differences were observed between *CCND1* and *CCND2* mutated and *CCND1* and *CCND2* wild-type patients. All but one patient with *CCND1* and *CCND2* mutations (88%) evaluated for outcome achieved a CR after induction chemotherapy, which is similar to the response rates seen in *CCND1* and *CCND2* wild-type patients (Table 3). Three *CCND1* and *CCND2* mutated patients relapsed within the first year.

Functional consequences of the *CCND2* Thr280Ala mutation in AML

Thr280Ala and Thr280Asn, the most frequently detected mutations in our patient cohort, were both previously found to result in a constitutively active *CCND2* protein.^{17,18} To gain insights into the effects of the *CCND2* mutations in AML, we stably introduced the *CCND2* Thr280Ala mutant into AML cell lines KG1a and Kasumi-1, and compared the effects of the mutant on cell proliferation with wild-type *CCND2* and EV control. Notably, the Kasumi-1 cell line harbors the t(8;21) translocation. Introduction of *CCND2* T280A significantly increased cell growth (as assessed by TiterGlo assays and cell counts, Figure 3A) in all experimental set-ups. Western blotting revealed a strong increase of retinoblastoma gene phosphorylation (P-Rb, Figure 3B),²² indicating increased downstream signaling. Moreover, both propidium iodide mediated cell cycle analysis (Figure 3C) and immunofluorescence

staining for phospho-histone H3 (Figure 3D) indicated an increased mitotic rate and a decreased G1 fraction.

Frequencies of *CCND1* and *CCND2* mutations in adult non-CBF-AML patients

The extensive sequencing study performed by the Cancer Genome Project (TCGA) did not report a single *CCND1* or *CCND2* mutation in 200 AML patients analyzed.²³ The Catalogue of Somatic Mutations in Cancer (COSMIC) lists only nine *CCND2* mutations in hematologic malignancies, five of which were found in patients with AML, whose cytogenetic and clinical features have not been reported.

To determine frequencies of *CCND1* and *CCND2* mutations in non-CBF-AML patients, we performed targeted next-generation sequencing of a large cohort of 1,426 adult patients with AML, which comprised patients with a wide spectrum of cytogenetic findings (with the exception of acute promyelocytic leukemia). We detected only five *CCND1* mutations and six *CCND2* mutations (for an overall *CCND1* and *CCND2* mutation frequency of 0.77%). Pretreatment clinical, cytogenetic and molecular characteristics of these patients are provided in Supplementary Table S4. Four of the six *CCND2* mutations were located at, or in the proximity of, the mutation hotspot Thr280. All but one of the patients with mutations were younger than 60 years. Ten of the 11 cases were cytogenetically normal, whereas one patient had a unique balanced translocation t(2;13)(p23;q14) and trisomy 21. Although nine of 11 (82%) *CCND1* and *CCND2* mutated patients achieved a CR, seven of the nine (78%) patients relapsed within the first year after cytarabine/daunorubicin-based chemotherapy.

DISCUSSION

Enhancing our understanding of the molecular alterations in CBF-AML is of major importance, because it may not only help explain the differences in the clinical features between patients with t(8;21) and those with inv(16), but also lead to identification of new mutated genes encoding abnormal molecules which may be potential therapeutic targets.

In this study, we comprehensively characterized the molecular landscapes of both cytogenetic subgroups of CBF-AML. The salient finding of our study is the discovery of mutations in the cyclin genes *CCND1* and *CCND2* as novel and relatively frequent mutational events, occurring almost exclusively in CBF-AML patients with t(8;21). Two aspects make this discovery especially intriguing. First, the fact that *CCND1* and *CCND2* have long been recognized as important players in hematopoietic development and leukemogenesis,^{12,13,17} but frequent mutations in these genes were not reported in AML so far. This is especially noteworthy since the pathophysiologic importance of the phosphorylation changes at the exact mutation hotspot we identified, at *CCND2* amino acid position Thr280 (and the corresponding phosphorylation site of *CCND1*, Thr286²⁰), have been intensively studied. Thr280 has even been proposed previously as drug targets in AML.¹⁷ Second, the mutations in *CCND1* and *CCND2* seem to be a perfect fit into the mutational landscape of CBF-AML with t(8;21), which is dominated by receptor tyrosine kinase (RTK), RAS, and chromatin remodeling gene mutations—all of which can result in targeting of *CCND1* and *CCND2*.²¹

Another aspect of our discovery of *CCND1* and *CCND2* mutations concerns the fact that several somatic mutations found in AML occur at the exact genomic locations as the germline changes known to be causative of rare, often neurodegenerative, syndromes. Like *PTPN11* mutations in Noonan syndrome,²⁴ *EZH2* mutations in Weaver syndrome,²⁵ *SMARCA2* mutations in Nicolaides-Baraitser syndrome,²⁶ and cohesion complex gene mutations in Cornelia de Lange syndrome,²⁷ *CCND2* mutations affecting the identical amino acids were shown to cause MPPH syndrome.¹⁸ MPPH syndrome is characterized by polymicrogyria, megalencephaly, intellectual disability, seizures, polydactyly, low muscle tone, skin elasticity and hydrocephalus. That no increased risk of leukemia has been reported to date in this patient population seems to support the hypothesis that *CCND2* mutations are not sufficient themselves to cause neoplastic disease, but rather serve as a second hit in CBF-AML with t(8;21). The fact that *CCND2* mutations detected in CBF-AML patients with t(8;21) and the germline mutations causative of MPPH syndrome share the exact genomic location is especially noteworthy since *CCND2* mutations in solid tumors are spread throughout the gene and are not preferentially found at position Thr280 (www.cbiportal.com).

In our study, we did not observe significant differences in the outcomes of t(8;21) patients with and without mutations in *CCND1* and *CCND2*. This may be due to the relatively small sample size, or may reflect the common use of cell cycle affecting high-dose cytarabine-based therapy in CBF-AML patients. However, forty-three percent of *CCND1* and *CCND2* mutated t(8;21) patients, and 78% of *CCND1* and *CCND2* mutated non-CBF-AML patients relapsed with their disease. Given the broad experience with drugs targeting aberrantly activated CCND1 and CCND2 in other cancers,²⁸ exploration of those inhibitors in management of AML patients with *CCND1* and *CCND2* mutations,²⁹ both those with CBF-AML and the rare patients with CN-AML, may be of clinical importance.

In summary, the identification of recurrent *CCND1* and *CCND2* mutations in patients with t(8;21) increases our understanding of the mutational landscape of CBF-AML, and further highlights the critical importance of RTK/RAS/CCND pathway activation in the biology of t(8;21) AML.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
(a) Oncoprint of mutations found in CBF-AML patients with t(8;21) and those with inv(16) showing mutation status of the *CCND1* and *CCND2* genes (red color – mutated, white – wild-type), and of genes classified into the previously described functional groups.²² Each column represents an individual patient. A star indicates the presence of two different *CCND1* or *CCND2* mutations in the same patient. Green color indicates that at least one gene belonging to a given functional group was found to be mutated in the patient, white color indicates wild-type status of all genes from this functional group. The exact numbers of specific gene mutations detected in each functional group are provided in Supplementary Table S3. (b) Bar graphs depicting the frequencies of the five most common gene mutations accompanying t(8;21) (green) and inv(16) (white) in CBF-AML patients. *CCND1* and *CCND2* mutations were the third most frequent gene mutations in patients with t(8;21). In contrast, *CCND1* and *CCND2* mutations were rare in patients with inv(16).

(a) Lollipop plots depicting *CCND1* and *CCND2* mutations found in 10 patients with t(8;21) and one patient with inv(16). No. denotes number; aa, amino acid. **(b)** Oncoprint of mutations found in patients who harbored *CCND1* and *CCND2* mutations. Light grey highlights in the last row indicate patients with t(8;21), dark grey, the patient with inv(16). A star indicates the presence of two *CCND1* or *CCND2* mutations in the same patient. Green color indicates a mutation and white color wild-type status of a given gene (other than *CCND1* and *CCND2*) for each patient. **(c)** Schematic depiction of the receptor tyrosine kinase (RTK)-RAS-MAP-Kinase pathway, which directly or indirectly affects the function/activity of *CCND1* and *CCND2*. MUT indicates pathway components encoded by genes whose mutations can be found in t(8;21) patients.

Figure 3.

(a) Cell viability of KG1a cells and Kasumi-1 cells (upper panel) and cell growth of KG1a cells (lower panel), stably expressing *CCND2* wild-type (WT) and *CCND2*Thr280Ala mutant (MUT) as determined by TiterGlo chemiluminescence and trypan blue staining, depicted relative to empty vector (EV) control. $n=3$ biological replicates, displayed as mean $\pm$ standard deviation. “*” indicates a P -value of 0.05 and “**” indicates a P -value 0.005. P -values were determined using 2-tailed Student’s t -tests. (b) Western blots depicting the effects of T280 mutated (MUT) and wild-type (WT) *CCND2* on phosphorylation of the retinoblastoma (Rb) gene. (c) Propidium iodide cell cycle analysis indicating an increased

mitotic rate and a decreased G1 fraction in cells with mutated *CCND2*. **(d)** Amount of phospho-histone H3 detected by immunofluorescence, compared with empty vector (EV) control. n=3 biological replicates, counting of 500 cells/slide.

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Characteristics of patients with core-binding factor acute myeloid leukemia harboring *CCND1* or *CCND2* mutations

Table 1

Patient number	Age (y)/sex	Karyotype	CCND1/2 mutation	VAF	Read depth	Other gene mutations (VAF)	BM blast percentage	CR achievement	Relapse
<i>CCND1</i> mutated t(8;21) patients									
1	24/F	46,XX,t(8;21)(q22;q22)[20]	<i>CCND1</i> Arg291Trp <i>CCND1</i> Arg291Leu	0.42 0.42	112	<i>BCORL1</i> (0.22), <i>GATA2</i> (0.43), <i>JAK1</i> (0.49), <i>RUNX1</i> (0.48)	49	No	NA
2	36/F	46,XX,t(8;21)(q22;q22)[20]	<i>CCND1</i> Val293Glu	0.20	353	<i>KIT</i> (0.14), <i>ASXL2</i> (0.22), <i>DHX15</i> (0.22)	30	Yes	Yes
12 ^a	29/M	46,XX,t(8;21)(q22;q22)[20]	<i>CCND1</i> Arg286Trp	NA	NA	Unknown	81	Yes	No
<i>CCND2</i> mutated t(8;21) patients									
3	69/M	46,XX,t(8;21)(q22;q22), del(11)(q21q23)[17]/46,XY[3]	<i>CCND2</i> Gln274 [*]	0.24	203	none detected	25	Yes	No
4	30/M	45,X,-Y,t(8;21)(q22;q22)[20]	<i>CCND2</i> Gln277 [*] <i>CCND2</i> Pro281Leu	0.10 0.05	334 342	<i>ASXL2</i> (0.52), <i>CALR</i> (0.49), <i>NRAS</i> (0.18), <i>TET2</i> (0.12)	34	Yes	No
5	34/F	45,X,-X,der(8)inv(8)(p21q22)t(8;21)(q22;q22),der(21)t(8;21)(q22;q22)[23]	<i>CCND2</i> Thr280Ala	0.39	280	<i>NRAS</i> (0.30), <i>RAD21</i> (0.42)	85	Yes	Yes
6	25/M	45,X,-Y,t(8;21)(q22;q22)[20]	<i>CCND2</i> Thr280Arg	0.04	377	<i>NRAS</i> (0.12)	40	No	NA
7	59/F	46,XX,t(8;21)(q22;q22)[20]	<i>CCND2</i> Thr280Arg <i>CCND2</i> Lys234Glu	0.37 0.01	194 877	<i>PHF6</i> (0.42)	54	Yes	No
8	41/F	46,XX,t(8;21)(q22;q22), del(9)(q13q*31)[20]	<i>CCND2</i> Thr280Arg	0.36	338	<i>ASXL1</i> (0.49), <i>PTEN</i> (0.41), <i>TYK2</i> (0.48), <i>ZRSR2</i> (0.43)	28	Yes	No
9	37/F	46,XX,t(8;21)(q22;q22)[20]	<i>CCND2</i> Pro281Leu	0.21	157	<i>ASXL2</i> (0.19), <i>ZRSR2</i> (0.39)	44	Yes	Yes
10	38/M	46,XY,t(8;21)(q22;q22)[2]/45,X,-Y,t(8;21)(q22;q22)[18]	<i>CCND2</i> Pro281Arg	0.38	258	<i>FLT3</i> -TKD (0.21), <i>ASXL2</i> (0.43)	32	Yes	No
13 ^a	49/M	45,X,-Y,t(8;21)(q22;q22)[24]/46,XY[1]	<i>CCND2</i> Thr280Arg	NA	NA	Unknown	72	Yes	No
14 ^a	30/F	46,XX,t(8;21)(q22;q22)[9]/46,XX[11]	<i>CCND2</i> Pro281Leu	NA	NA	Unknown	16	Yes	Yes
<i>CCND2</i> mutated inv(16) patient									
11	30/F	47,XX,inv(16)(p13.1q22), +21[16]/46,XX[1]	<i>CCND2</i> Thr280Ala	0.22	333	<i>BCORL1</i> (0.27)	24	Yes	No

Abbreviations: y, years; VAF, variant allele fraction; CR, complete remission; NA, not applicable;

^{*}, frameshift mutation.

^aPatients 12–14 were analyzed for their *CCND1* and *CCND2* mutation status using Sanger sequencing. Thus, for these patients read depth, VAF and information on the presence or absence of additional mutations are not available.

Table 2

Comparison of pretreatment clinical characteristics of t(8;21)-positive CBF-AML patients with and without *CCND1/2* mutations analyzed by targeted next-generation sequencing

Characteristic	CCND1/2 mutated (n=10)	CCND1/2 wild-type (n=58)	P-value ^a
<i>Age, years</i>			0.61
Median	37	40	
Range	24–69	18–72	
<i>Sex, n of females (%)</i>	6 (60)	22 (38)	0.3
<i>Race, n (%)</i>			0.22
White	6 (60)	45 (80)	
Nonwhite	4 (40)	11 (20)	
<i>Hemoglobin, g/dl</i>			0.5
Median	8.9	9.1	
Range	4.5–10.9	5.5–12.6	
<i>Platelet count, x 10⁹/l</i>			0.94
Median	34	38	
Range	Oct-82	7-369	
<i>White blood count, x 10⁹/l</i>			0.32
Median	18.6	12.6	
Range	5.2–46.6	1.4–138.9	
<i>Bone marrow blasts, %</i>			0.05
Median	37	53	
Range	19–85	26–95	
<i>Blood blasts, %</i>			0.02
Median	33	48	
Range	Jan-50	Jun-90	
<i>Extramedullary involvement, n (%)</i>	1 (11)	9 (16)	1

Abbreviation: n, number.

^a P-values for categorical variables are from Fisher's exact test, P-values for continuous variables are from the Wilcoxon rank sum test.

Table 3

Outcomes of t(8;21)-positive CBF-AML patients with and without *CCND1/2* mutations analyzed by targeted next-generation sequencing

Endpoint	CCND1/2 mutated (n=8) ^a	CCND1/2 wild-type (n=43) ^a	P-value ^b
Complete remission, n (%)	7 (88)	40 (93)	0.51
Relapse rate, n (%)	3 (43)	12 (30)	0.66
Disease-free survival			0.95
Median, years	n.r.	n.r.	
% Disease-free at 1 year (95% CI)	71 (26–92)	65 (48–78)	
% Disease-free at 3 years (95% CI)	57 (17–84)	63 (46–75)	
Overall survival			0.41
Median, years	n.r.	n.r.	
% Alive at 1 year (95% CI)	88 (39–98)	86 (72–93)	
% Alive at 3 years (95% CI)	75 (31–93)	65 (49–77)	

Abbreviations: CI, confidence interval; n, number, n.r., not reached.

^a Among patients who achieved a CR, only those who received at least one cycle of postremission chemotherapy according to protocol were included in the outcome analysis.

^b P-values for categorical variables are from Fisher's exact test, P-values for time to event variables are from the log-rank test.