**ARTICLE**

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# CDK6 protein expression is associated with disease progression and treatment resistance in multiple myeloma

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## Abstract

Multiple myeloma (MM) is a heterogeneous malignancy of plasma cells. Despite improvement in the prognosis of MM patients after the introduction of many new drugs in the past decades, MM remains incurable since most patients become treatment-resistant. Cyclin-dependent kinase 6 (CDK6) is activated in many types of cancer and has been associated with drug resistance in MM. However, its association with disease stage, genetic alterations, and outcome has not been systematically investigated in large cohorts. Here, we analyzed CDK6 expression using immunohistochemistry in 203 formalin-fixed paraffin-embedded samples of 146 patients and four healthy individuals. We found that 61.5% of all MM specimens express CDK6 at various levels. CDK6 expression increased with the progression of disease with a median of 0% of CDK6-positive plasma cells in monoclonal gammopathy of undetermined significance (MGUS) ($n$ = 10) to 30% in newly diagnosed MM ($n$ = 78) and up to 70% in relapsed cases ($n$ = 55). The highest median CDK6 was observed in extramedullary myeloma ($n$ = 12), a highly aggressive manifestation of MM. Longitudinal analyses revealed that CDK6 is significantly increased in lenalidomide-treated patients but not in those who did not receive lenalidomide. Furthermore, we observed that patients who underwent lenalidomide-comprising induction therapy had significantly shorter progression-free survival when their samples were CDK6 positive. These data support that CDK6 protein expression is a marker for aggressive and drug-resistant disease and describes a potential drug target in MM.

## INTRODUCTION

Multiple myeloma is a genetically and clinically heterogeneous malignancy caused by impaired apoptosis and uncontrolled proliferation of the postgerminal center B-cell-derived plasma cell.[1] The introduction of new drugs, including proteasome inhibitors, immunomodulatory drugs (IMiDs) such as lenalidomide, and monoclonal antibodies in intensive or nonintensive treatment regimens, has greatly improved the prognosis for multiple myeloma over the last two decades.[2] Nevertheless, multiple myeloma is still considered incurable because even patients with deep responses eventually relapse due to pre-existing or acquired drug resistance.[3] Current predictors for a short duration of response and overall survival are serological parameters, such as the International Prognostic Scoring System (ISS) stage III and high lactate dehydrogenase levels, high-risk cytogenetic abnormalities t(4;14), del17p, and amplification of 1q21,

gene mutations in *TP53*, high-risk gene expression profiles, and extramedullary manifestations.[4–7] Extramedullary myeloma is an aggressive and particularly treatment-resistant manifestation of multiple myeloma that is rare at initial diagnosis but is found more frequently in highly pretreated patients.[8]

Progression-free survival and overall survival under intensive treatment regimens in patients with high-risk features is considerably shorter than in patients with standard risk and salvage treatments are less effective.[3] In addition, most patients currently considered to be at standard risk eventually relapse due to acquired drug resistance, highlighting the need for better treatment strategies.

Uncontrolled proliferation by deregulated cell cycle control is a hallmark of cancer, including multiple myeloma.[9] Initiating genetic events lead to upregulation of D-type cyclin signaling, either via hyperdiploidy or via direct (t(11;14) [*CCND1*], t(6;14) [*CCND3*]) or indirect (t(4;14) [*MMSET/FGFR3*], t(14;16) [*c-MAF*], t(14;20) [*MAFB*])

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translocations involving the immunoglobulin heavy-chain enhancer.[10,11] To synergize cell cycle disruption, myeloma cells variably acquire further genetic alterations at the subclonal levels that may be already present at initial stages but increase during disease progression.[12-15] They include chromosome deletions leading to loss of tumor suppressor genes like *TP53*, *RB1*, or *CDKN2C* and secondary translocations resulting in upregulation of c-MYC like the translocation t(8;14).[16-18]

The cell cycle is orchestrated by a balanced interaction of promoting factors, such as cyclins and cyclin-dependent kinases (CDKs), and negative factors like cyclin-dependent kinase inhibitors (CKIs). A critical checkpoint is the restriction point before the G1/S phase transition, since cyclin D and CDK4/6 eventually phosphorylate pRb, leading to E2F transcription factor-dependent cell cycle progression. Disturbed homeostasis of this checkpoint is crucial for the development of hematologic malignancies, including alterations of cyclins, CDKs, CKIs, p53, and pRB.[19,20]

CDK6 has been found activated in a broad spectrum of solid and hematologic malignancies, such as breast cancer, malignant melanoma, glioblastoma, and B and T cell lymphomas.[21-24] CDK4/6 inhibitors are approved for advanced hormone receptor-positive breast carcinoma in combination with endocrine-based therapy.[25]

The mutually exclusive pairing of cyclin D2 with CDK6 and cyclin D1 with CDK4 has shown to be critical for cell cycle dysregulation and multiple myeloma progression.[26] Ely and colleagues[27-29] argued that this is mediated by phosphorylation and thus inactivation of retinoblastoma, a key cell cycle regulator disturbed in most cancers. The CDK4/6 inhibitor palbociclib showed effective cell cycle arrest and growth suppression of myeloma cells and xenografts as monotherapy or in combination with bortezomib. This led to a clinical phase

1/2 trial including relapsed and refractory myeloma patients who received palbociclib and bortezomib.[30] The study showed good response rates with stable disease in 44% of patients. A recent study by Ng et al.[31] demonstrated, using quantitative proteomic analyses, that CDK6, but not CDK4, is upregulated in relapsed and IMiD-resistant multiple myeloma. CDK6 governs a protein resistance signature that includes proteins involved in DNA damage repair, cell cycle, and metabolic pathways. Inhibition of CDK6 resulted in the resensitization of multiple myeloma cells to IMiD treatment. Surprisingly, this effect remained unaffected in the absence of pRb, pointing to cell cycle-independent functions of CDK6. However, little is known about the clinical implications of CDK6 expression in multiple myeloma to date.

Therefore, we sought to evaluate CDK6 expression and its correlation to clinical and genetic parameters in a large cohort of multiple myeloma patients. We applied immunohistochemistry as an established and reliable method for in situ protein detection, as previous studies had found that protein levels diverge from RNA expression levels in several cancers.[31-33]

## METHODS

### Study cohort

We included 203 formalin-fixed paraffin-embedded (FFPE) tissue blocks from 146 patients and four healthy individuals in this study (Figure 1). All patients provided informed consent, which was approved by the ethics commission of Ulm University (136/20). Samples
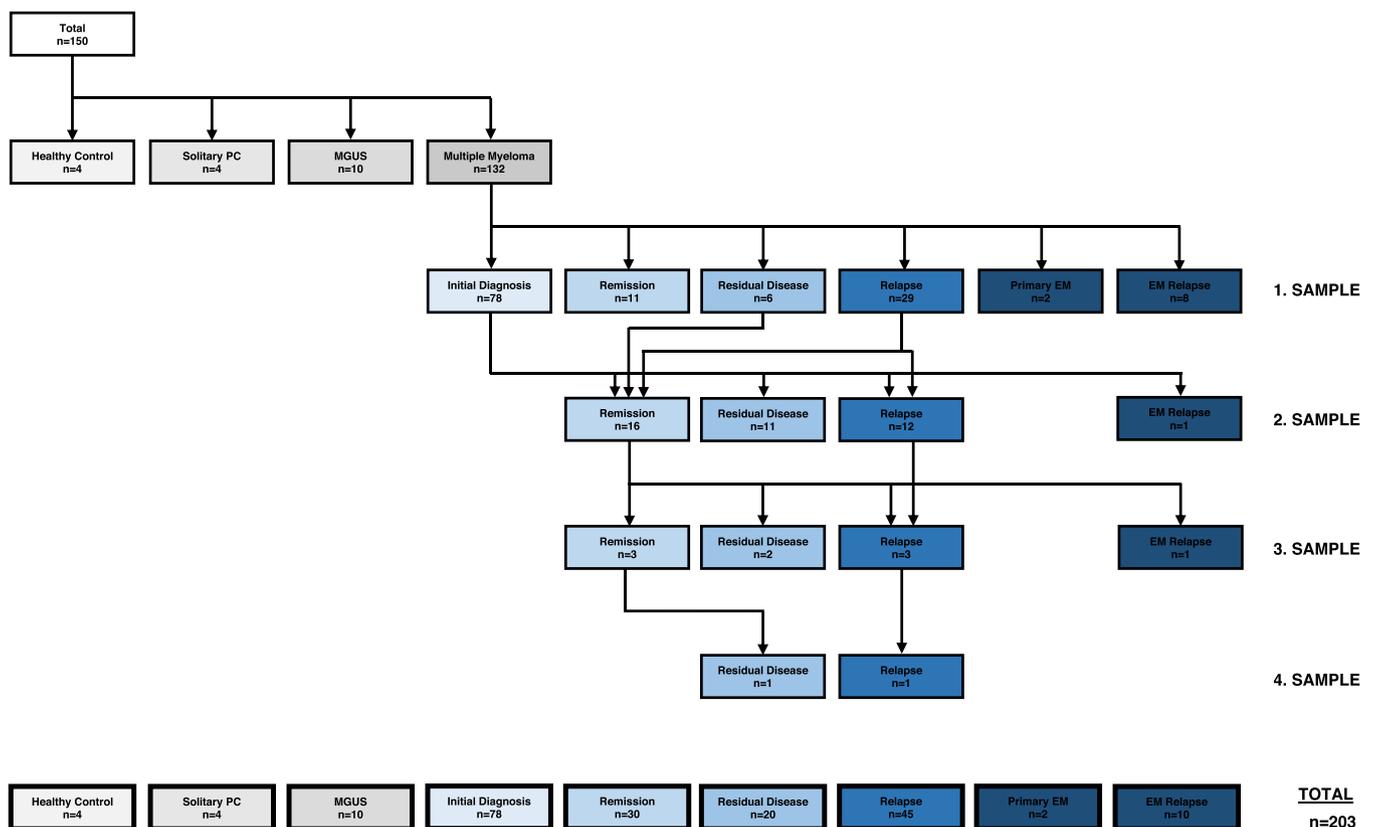


**FIGURE 1** Illustration of the study cohort and all analyzed samples according to disease and disease status.

included bone marrow biopsies (n = 166) and surgical resection specimens (n = 37). Cytoplasmic immunoglobulin-fluorescence in situ hybridization (cIg-FISH) was performed for the detection of structural genetic variants and translocations, according to standardized protocols using commercially available probes in routine diagnostics, and was available for most patients (Abbott Molecular; MetaSystems).[34]

One FFPE sample was available for 102 patients, while 44 patients had two or more samples. The tissue samples were obtained at varying time points. To ensure comparability, we categorized them according to disease status (Figure 1).

We further analyzed 37 patients with other B-cell malignancies classified according to the World Health Organization 5th edition from the tissue bank of Ulm University Hospital as control. We included four B-cell chronic lymphatic leukemias, five diffuse large B-cell lymphomas, four follicular lymphomas, three classical Hodgkin lymphomas, four mantle cell lymphomas, and 17 lymphoplasmacytic lymphomas.

## Immunohistochemistry

FFPE tissue was cut into 4-μm-thick sections by a microtome (RM 2255; Leica Biosystems) and stored in a −20°C freezer. Slides were deparaffinized in a xylene bath and rehydrated by sequential washing steps in ethanol with gradually decreasing concentrations. Antigen retrieval was carried out by heating in citrate buffer (pH = 6.0) in a microwave (ER-5420; Toshiba) at 800 W for 25 min. Specific antigen binding was ensured by incubation of the primary (anti-CDK6; Life Span, Biosciences, mouse monoclonal (8H4), dilution 1:50) and secondary antibodies for half an hour at room temperature. Detection of antibodies was performed by treatment with an alkaline phosphatase/RED detection system (Dako, Agilent Technologies). The pictures were taken in a bright field using a Zeiss Axiophot microscope with an included CCD camera (JVC Digital Camera KY-F75U) connected to the Diskus Viewer software (v.5.0; Carl H. Hilgers). The evaluation was conducted by two different independent pathologists, who were not informed about the clinical parameters. The staining intensity, percentage of positive cells, and subcellular expression pattern were assessed in each sample, with the staining intensity being categorized as absent, medium, or strong.

We used serial sections of Ki-67, CD38, and CD138 stainings (anti-Ki-67; Dako, mouse monoclonal (MIB-1), dilution 1:200; anti-CD38: Abcam, rabbit monoclonal (SP149), ab183326, dilution 1:100; anti-CD138: Dako, mouse monoclonal (MI15), dilution 1:200) for evaluating the proliferation and bone marrow infiltration rates and for visualizing and localizing plasma cells. For samples with a low bone marrow infiltration rate, we established a double-staining method for sequential co-staining of CDK6 and CD38 (n = 87) (see above; dilution 1:50). To prevent further cross-reaction of the second secondary antibody with the first primary antibody, we performed antigen denaturation of the first targeted antigen by treatment on a hot plate (RCT basic, IKA) at 90°C for 10 min.

## RESULTS

## CDK6 expression correlates with advanced disease stage

We performed immunohistochemical stainings of the FFPE tissue of a cohort of multiple myeloma patients at different disease stages. We determined the amount of CDK6-stained plasma cells in relation to all plasma cells, the staining intensity, and the subcellular localization in each sample. Plasma cells were detected by serial sections stained for CD38 and CD138 or by a double-staining method (n = 87) for CD38 and CDK6 (Figure 2A).

CDK6-positive plasma cells were detected in 124 of all 199 (62.3%) samples from patients with a plasma cell disorder. In comparison, CDK6 was generally not detected or expressed at very low levels in normal plasma cells in the bone marrow of individuals without hematologic malignancy (Figure 2A).

Analyzing clinicopathological and demographic variables showed a balanced distribution of most parameters in the CDK6-positive versus CDK6-negative subgroups (Table 1). However, we observed significant differences regarding disease progression and aggressiveness (Table 1).

The median amount of positive plasma cells gradually increased with the disease stage (Figure 2A–C: healthy control, MGUS, initial diagnosis, extramedullary; p = 0.0003; Kruskal–Wallis test). No CDK6-positive plasma cells were detected in the bone marrow of healthy controls. Only two out of 10 MGUS samples had CDK6-positive plasma cells, with amounts of positive plasma cells of 5% and 15% out of all plasma cells. The staining intensity was considered to be medium in both. Of the treatment-naïve multiple myeloma patients, 64.1% (50 of n = 78) had a positive CDK6 signal. The median amount of CDK6-positive plasma cells compared to all plasma cells was 30% (range: 0%–100%). Of those patients, 35 patients had a medium signal and 15 patients had a strong signal. Samples obtained from extramedullary sites showed the highest CDK6 expression, with 10 out of 12 positive cases (83.3%) and a median amount of 72.5% (range: 0%–100%) CDK6-expressing plasma cells. Of these, four samples showed a strong staining intensity, and 10 samples a medium staining intensity.

Specimens of relapsed patients were positive for CDK6 in 72.7% of cases (40 of n = 55), while the median amount of positive plasma cells amounted to 70% (range: 0%–100%). Twenty-five of the 40 CDK6-positive relapsed cases showed a medium signal, whereas 15 had a strong signal. Patients who did not have a biochemical or clinical relapse, but whose samples still had light-chain-restricted plasma cells were categorized as residual disease. In this subgroup, six out of 20 patients had a medium CDK6-positive sample and one patient had a strongly staining CDK6-positive sample. Samples without light-chain restriction were considered as remission. We found CDK6 expression in 11 of 30 samples with a median amount of CDK6-positive plasma cells of 0% (range: 0%–80%).

With regard to the subcellular expression of CDK6, cytoplasmatic CDK6 staining was more common than nuclear CDK6 staining. There were no samples with exclusive nuclear staining. Hence, nuclear staining was only detected as an additional feature in combination with cytoplasmatic staining. A nuclear signal tended to be more frequent in abundant cytoplasmatic-positive samples. Likewise, a nuclear CDK6 signal was more common in relapsed samples than in newly diagnosed samples (Table 1: 38.2% vs. 17.9%; p = 0.0157; Fisher's exact test).

When comparing the median amount of cytoplasmatic CDK6-positive plasma cells, there was an increase from initial diagnosis to relapse. However, the difference was not statistically significant (Figure 3A: median 30% vs. 70%; p = 0.162; Mann–Whitney test). Considering only the nuclear CDK6 signal resulted in a significantly higher median amount of CDK6-positive plasma cells at relapse
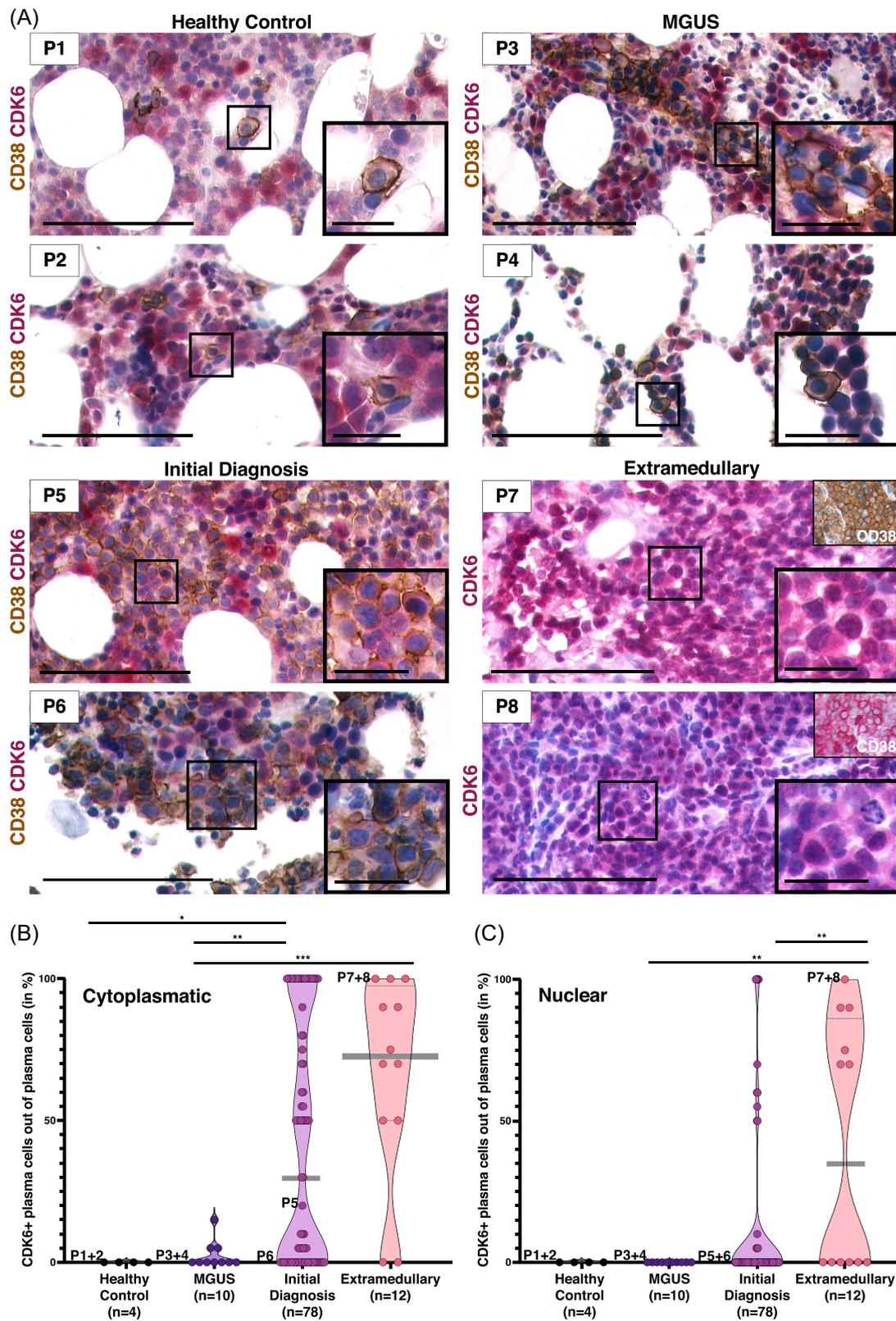
**FIGURE 2** Immunohistochemical stainings reveal differential cyclin-dependent kinase 6 (CDK6) protein expression in myeloma. (A) Representative samples of healthy controls, monoclonal gammopathy of undetermined significance (MGUS) patients, myeloma patients at initial diagnosis, and extramedullary myeloma stained for CD38 and CDK6 by a double-staining method or by single-staining of serial sections. Higher magnification allows evaluation of the cytoplasmatic versus nuclear CDK6 signal at a single-cell level. CDK6 protein expression was approached by measuring the staining intensity and amount of positive plasma cells compared to all plasma cells in each sample. Scale bars = 100 μm; inlet scale bars = 25 μm. (B, C) Violin plot illustrates the cytoplasmatic (B) and nuclear (C) amount of CDK6-positive plasma cells in each sample, compared between the abovementioned subgroups. Circles indicate individual data points; bars show the median amount. Circles of representative patients from (A) are labeled. Differences were analyzed using the Mann–Whitney test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.0001$.

**TABLE 1** Demographic and disease characteristics.

| Variable | CDK6-positive cytoplasmatic | CDK6-negative cytoplasmatic | p Value |
|---|---|---|---|
| Age | | | |
| Median (range) | 61 (31–79) | 56 (33–79) | n.s. |
| Follow-up | | | |
| Median (range) | 3.25 (0.1–9.5) | 4.7 (0.4–14.9) | n.s. |
| Bone marrow infiltration rate | | | |
| Median (range) | 60 (6.0–99.0) | 30 (1.0–70.0) | 0.0002 |
| Gender | | | |
| Men, n (%) | 29 (60.4) | 19 (39.6) | n.s. |
| Women, n (%) | 21 (70.0) | 9 (30.0) | |
| Light chain | | | |
| Kappa, n (%) | 36 (63.2) | 21 (36.8) | n.s. |
| Lambda, n (%) | 14 (66.7) | 7 (33.3) | |
| Location | | | |
| Medullary, n (%) | 110 (60.1) | 73 (39.9) | n.s. |
| Extramedullary, n (%) | 10 (83.3) | 2 (16.7) | |
| Disease status | | | |
| Healthy control, n (%) | 0 (0.0) | 4 (100.0) | |
| MGUS, n (%) | 2 (20.0) | 8 (80.0) | |
| Initial diagnosis, n (%) | 50 (64.1) | 28 (35.9) | |
| Relapse, n (%) | 40 (72.7) | 15 (27.3) | |
| Residue, n (%) | 7 (35.0) | 13 (65.0) | |
| Remission, n (%) | 11 (36.7) | 19 (63.3) | 0.0001 |

| Variable | CDK6-positive nuclear | CDK6-negative nuclear | p Value |
|---|---|---|---|
| Location | | | |
| Medullary, n (%) | 34 (18.6) | 149 (81.4) | 0.0184 |
| Extramedullary, n (%) | 6 (50.0) | 6 (50.0) | |
| Disease status | | | |
| Initial diagnosis, n (%) | 14 (17.9) | 64 (82.1) | 0.0157 |
| Relapse, n (%) | 21 (38.2) | 34 (61.8) | |

Abbreviations: CDK, cyclin-dependent kinase; MGUS, monoclonal gammopathy of undetermined significance; n.s., not significant (at $p < 0.05$).

compared to the initial diagnosis (Figure 3A: $p = 0.002$; Mann–Whitney test). The plasma cells of patients in remission or with residual disease had significantly lower cytoplasmatic CDK6 expression compared to patients at initial diagnosis (Figure 3A: $p = 0.0003$ and $p = 0.013$; Mann–Whitney test).

In summary, these data demonstrate that CDK6 expression correlates with more active disease. CDK6 expression was also found in malignant cells in the bone marrow and lymphatic tissue of other B-cell malignancies (Supporting Information S1: Figure 1), including B-cell chronic lymphatic leukemia (four of four cases, median 65% of malignant cells, range: 5%–90%), diffuse large B-cell lymphoma (five of five cases, median 80% of malignant cells, range: 5%–100%), follicular lymphoma grades 2 and 3 (four of four cases, median 100% of malignant cells), classical Hodgkin lymphoma (three of three cases, median 80% of malignant cells, range: 60%–80%), mantle cell lymphoma (four of four cases, median 100% of malignant cells), and lymphoplasmacytic lymphoma (10 of 17 cases, median 20% of malignant cells, range: 0%–100%). The median amount of CDK6-positive plasma cells was not significantly different between the two plasma cell disorders at initial diagnosis (20% in lymphoplasmacytic lymphoma vs. 30% in multiple myeloma; $p = 0.547$, Mann–Whitney test).

## CDK6 protein expression is linked to deletion 13q but not to genetic high-risk constellations

We next investigated CDK6 expression at initial diagnosis ($n = 78$) in cytogenetic subtypes of multiple myeloma assessed by FISH in routine diagnostics (Figure 4A). Data were available for part of the cohort as follows: standard-risk aberrations, such as amp(9q34) (55%; 22 of $n = 40$), del(13q14) (53.5%; 23 of $n = 43$), t(11;14) (20.7%; six of $n = 29$), and amp (1q21) (38.1%; 16 of $n = 42$); and high-risk aberrations, such as del(17p) (11.9%; five of $n = 42$) and t(4;14) (15.4%; six of $n = 39$).

The median amount of CDK6-positive plasma cells was 40% in amp (9q34) (range: 0%–100%), 50% in del(13q14) (range: 0%–100%), 5% in t (11;14) (range: 0%–100%), 75% in amp(1q21) (range: 0%–100%), 50% in del(17p) (range: 0%–100%), and 37.5% in t(4;14) (range: 0%–100%). In our cohort, there was no significant difference in CDK6 protein expression between patients with standard-risk versus high-risk constellations (Figure 4B: median 50% vs. 27.5%; $p = 0.980$; Mann–Whitney test).

Regarding the single aberrations, there was a statistically significant higher median CDK6 percentage in cases with aberration del(13q) compared to 13q normal (Figure 4C: median 50% vs. 0%; $p = 0.049$; Mann–Whitney test). Furthermore, there were no associations with other cytogenetic events as is exemplarily shown by t(11;14) (Figure 4D: 10% vs. 5%; $p = 0.889$; Mann–Whitney test).

Heterozygous deletion of 13q is one of the most common genetic events in myeloma and leads to monoallelic loss of tumor suppressor RB1.[35,36] At first glance, the opposed incidence of CDK6 and del(13q) seems contradictory. However, high CDK6 could be a sign of increased functional inactivation of the remaining pRb by phosphorylation. Consequently, CDK6 overexpression in del (13q) could be an explanation for the paradox of RB1 being only heterozygously deleted in most cases.[13,37–39]

## Implications of CDK6 on cell cycle and proliferation

Given the role of CDK6 in cell cycle progression, we then analyzed its relationship to proliferation markers (Figure 4E,F). No correlation was seen between the percentage of CDK6-positive cells and the percentage of Ki-67-positive cells (Figure 4E: $r^2 = 0.006$, $p = 0.634$, simple linear regression). Double-immunofluorescence of single cases showed that not all CDK6-expressing cells are cycled, as shown by Ki-67 expression (Figure 4G). However, we saw a high correlation between the percentage of CDK6-positive cells and the bone marrow plasma cell infiltration rate (Figure 4F: $r^2 = 0.142$, $p < 0.0001$, simple linear regression), further demonstrating that CDK6 expression is associated with more advanced disease.

The kinase activity of CDK6 depends on the presence of different cyclins. The majority of myeloma cases show upregulation of D-type cyclins.[9] However, we observed a tendency toward higher CDK6 expression in the group of cyclin D1-negative samples, assessed by immunohistochemistry (Supporting Information S1: Figure 2B: median 20% vs. 5%; $p = 0.086$; Mann–Whitney test). Translocation t(11;14) accounts for approximately 20% of newly diagnosed multiple myeloma and leads to upregulation of cyclin D1.
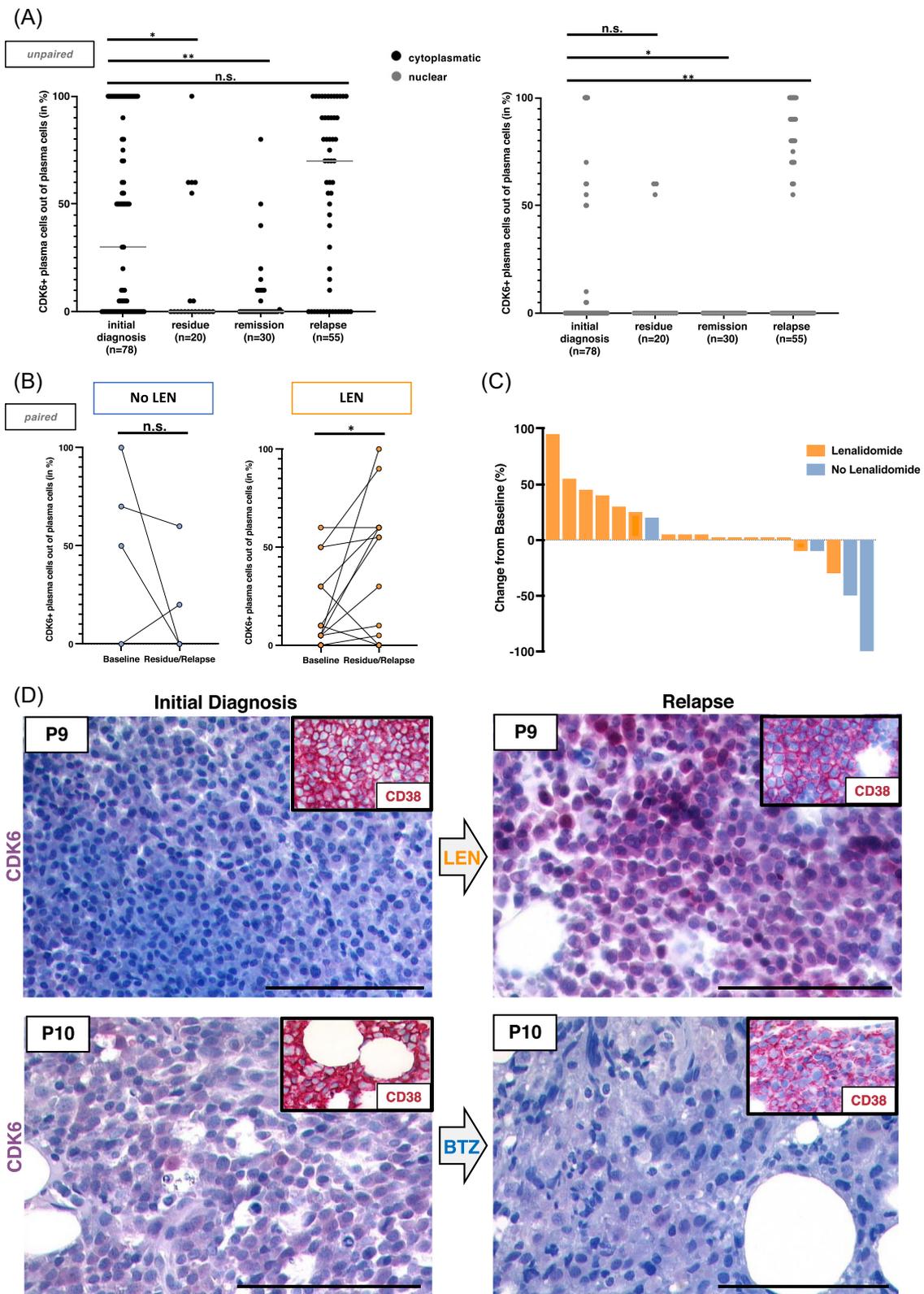
**FIGURE 3** Elevation of cyclin-dependent kinase 6 (CDK6) protein expression under therapy depends on the applied drugs. (A) Comparison of the amount of CDK6-positive plasma cells between unpaired multiple myeloma samples subdivided by disease status. Differences were evaluated by using the Mann–Whitney test. *$p < 0.05$; **$p < 0.01$. n.s. for nonsignificant at $p < 0.05$. (B–D) Analysis of the amount of CDK6-positive plasma cells between paired pretreatment versus posttreatment samples revealed therapy-specific effects. (B) Comparison of the amount of CDK6-positive plasma cells before and after treatment with lenalidomide (orange) and bortezomib (blue) using Wilcoxon's test. *$p < 0.05$. n.s. for nonsignificant at $p < 0.05$. (C) Waterfall plot illustrates the therapy-dependent change of CDK6 expression. (D) Representative immunohistochemical stainings for CDK6 before and after lenalidomide versus bortezomib treatment. Serial sections stained for CD38 show complete bone marrow infiltration by plasma cells, confirming that all depicted cells are plasma cells. BTZ, bortezomib; LEN, lenalidomide. Scale bars = 100 µm.
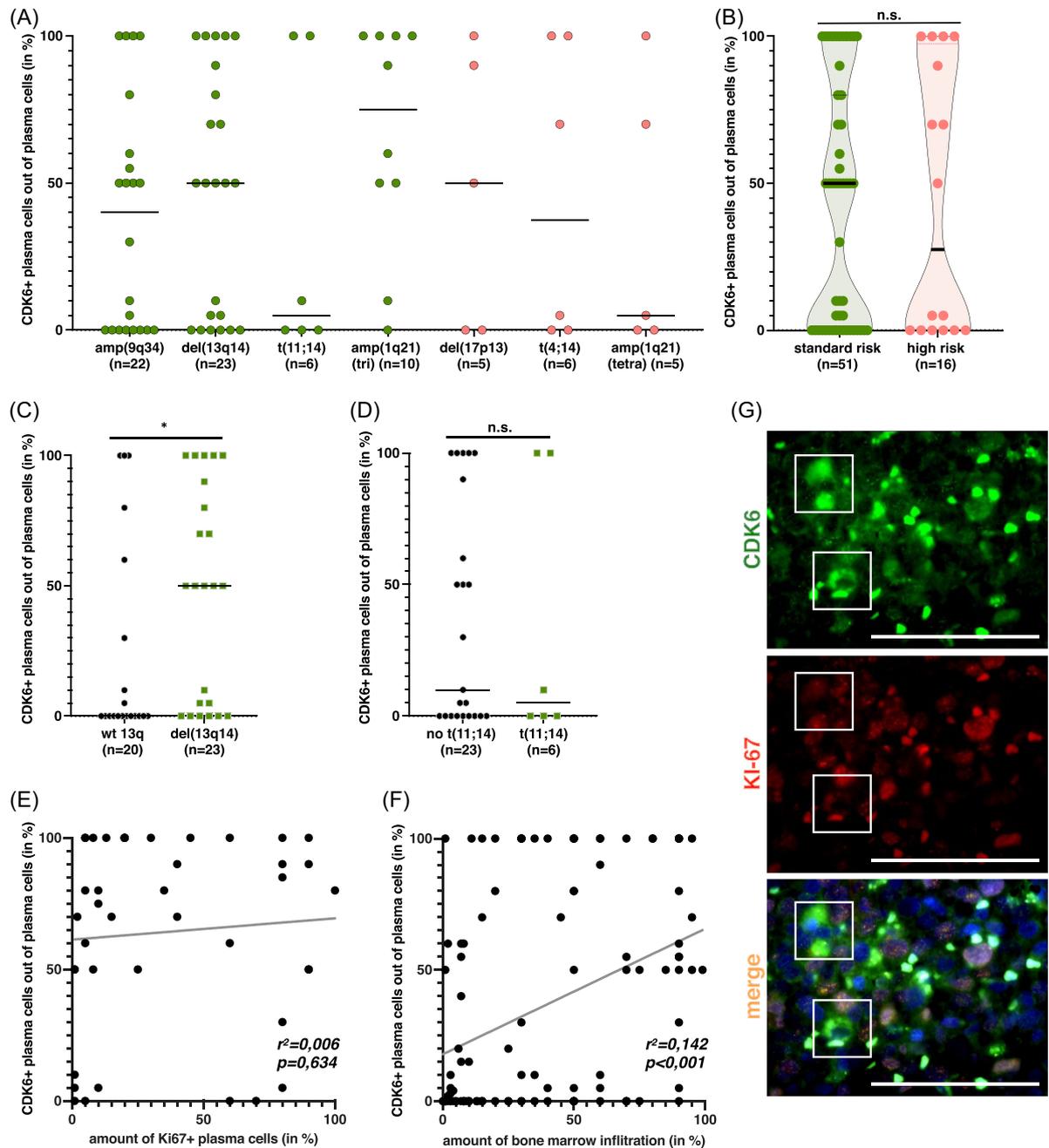
**FIGURE 4** High cyclin-dependent kinase 6 (CDK6) protein expression is independent of high-risk cytogenetics or proliferation. (A) Comparison of the amount of CDK6-positive plasma cells in relation to all plasma cells between different cytogenetic aberrations. (B) Cumulating them to standard- and high-risk cytogenetic aberrations and comparing them using the Mann–Whitney test showed no significant difference at $p < 0.05$. (C, D) Analysis of the CDK6 amount in patients with del(13q14) (B) and t(11;14) (C) compared to its respective wild-type using the Mann–Whitney test. *$p < 0.05$. n.s. for nonsignificant at $p < 0.05$. (E, F) Correlation of the amount of CDK6-positive plasma cells to the amount of Ki-67-positive plasma cells (E) and to bone marrow infiltration rate by plasma cells (F) using simple linear regression. (G) Representative immunofluorescence samples show that abundant CDK6-positive plasma cells do not express the proliferation marker Ki-67. Scale bars = 100 μm.

In line with this, we found cyclin D1 expression in all patients with t(11;14). In contrast, five out of 17 patients without t(11;14) also showed cyclin D1-positive MM cells.

## Longitudinal analysis of CDK6 protein expression

In the overall cohort, there was a trend toward higher cytoplasmatic CDK6 and a significantly higher nuclear CDK6 detection in relapsed cases compared to newly diagnosed cases (Figure 3A: median 30% vs. 70%; $p = 0.162$; median 0% vs. 0%; $p = 0.002$; Mann–Whitney test). To observe intra- and interpatient changes, we next examined the protein expression in a longitudinal cohort of patients with paired pretreatment and posttreatment samples. Twenty patients with a specimen at initial diagnosis and a specimen at the time of relapse or residual disease were included. Altogether, there was a tendency toward an increase in CDK6

protein expression that was not statistically significant (median 7.5% vs. 15%; p = 0.398; Wilcoxon's test). To determine the impact of different therapeutics on CDK6 evolution under and after treatment, we stratified patients by treatment with or without lenalidomide (Figure 3B,C). The group treated with lenalidomide-containing regimens developed significantly higher CDK6 expression during treatment (Figure 3B: median 5% vs. 20%; p = 0.045; Wilcoxon's test). In contrast, the patients treated with other regimens did not have significantly different CDK6 levels at relapse (Figure 3B: p = 0.375; Wilcoxon's test). Figure 3D shows representative samples of each group.

## Extramedullary myeloma exhibits abundant CDK6 expression

Stratification not only by disease status but also by origin of the analyzed tissue had an impact on the CDK6 expression level. In the overall cohort, we observed that, in particular, the samples from extramedullary tissue had substantially higher CDK6 amounts. Biopsies or surgical specimens from solitary plasmacytomas showed the highest CDK6 protein expression among the whole cohort (median amount of positive plasma cells: 87.5%; range: 80–100; n = 4). A larger group of multiple myeloma patients with extramedullary manifestations in addition to bone marrow presented an equally high median cytoplasmatic CDK6 amount (Figure 2A,B: 72.5%; range 0–100; n = 12). Two of the 12 patients already presented with extramedullary manifestations at initial diagnosis, while these manifestations appeared in the other 10 patients at relapse. The median amount differed between those two groups (50% vs. 82.5%). Compared to medullary myeloma, extramedullary myeloma samples had a significantly higher median amount of nuclear CDK6-positive plasma cells compared to medullary myeloma (Figure 2C: 35% vs. 0%; p = 0.007 Mann–Whitney test).

## Impact of CDK6 expression on outcome

To determine whether CDK6 expression could serve as a prognostic marker in multiple myeloma, we analyzed the impact of CDK6 expression on the clinical outcome. Here, we distinguished between patients who had received intensive treatment with high-dose chemotherapy followed by autologous stem cell transplantation (ASCT) and nonintensive treatment regimens. Further, we compared patients who had received lenalidomide as part of their therapy regimen with those who had not.

We divided those cohorts by the presence or absence of CDK6-positive plasma cells. The samples were considered positive if there were at least 5% positive plasma cells in relation to all plasma cells. This resulted in a tendency toward a shorter progression-free survival of patients with CDK6-positive bone marrow samples in the overall cohort (Supporting Information S1: Figure 3A: median survival 4 vs. 3.2 years; p = 0.196, log-rank test). The same trend was observed when we restricted the analysis to patients who were intensively treated with high-dose chemotherapy followed by ASCT (Figure 5A: median survival 8.4 vs. 4.2 years; p = 0.162, log-rank test).

We detected a correlation of CDK6 expression with outcomes for patients treated with lenalidomide-comprising induction therapy. In these patients, we observed a significantly shorter progression-free survival in patients with CDK6-positive samples compared to patients with CDK6-negative samples (Figure 5B: median survival

2.6 vs. 8.4 years; p = 0.029, log-rank test). Regarding overall survival, CDK6 protein expression showed no impact in the overall cohort. However, we observed a tendency toward shorter survival of CDK6-positive cases in the lenalidomide-treated cohort (Supporting Information S1: Figure 3B: median survival 6.2 vs. 11.9 years; p = 0.37, log-rank test).

We further analyzed progression-free survival in relapsed cases. We considered only patient samples obtained at the time of their first relapse after high-dose chemotherapy and ASCT. This cohort was split up into a CDK6 percentage under and above 60%. Kaplan–Meier survival analysis revealed a strong trend for shorter median survival in cases with high CDK6 expression (Figure 5D: median survival 3.4 vs. 0.4 years; p = 0.057; log-rank test).

Moreover, we noticed a significantly shorter overall survival in patients who had more than 20% Ki-67 positive plasma cells (Supporting Information S1: Figure 3D: median survival 1.8 years vs. median not reached; p = 0.0008; log-rank test).

## DISCUSSION

We applied immunohistochemistry to determine CDK6 expression on the protein level in a large cohort of multiple myeloma patients covering a broad spectrum of different tissues and disease stages. The detection of immunohistochemical signals of single plasma cells in bone marrow tissue was challenging, as myeloma typically exhibits random infiltration patterns; we overcame this problem by double-staining CDK6 and CD38 for cases with a low bone marrow infiltration rate. The advantage of immunohistochemistry is the preservation of tissue architecture, allowing the CDK6 signal to be pinpointed at the single-cell level. We found that nuclear staining is less frequent than cytoplasmatic staining and that nuclear staining only appears in addition to cytoplasmatic staining. This is consistent with a study by Kohrt et al.[40] stating that subcellular localization of CDK6 is linked to function. The majority of CDK6 is localized in the cytoplasm, while only the nuclear proportion of CDK6 acts as a kinase for pRb.[41] Explanations for this paradox are possible mechanisms of cell cycle regulation by cytoplasmatic sequestration and additional poorly described cytoplasmatic functions of CDK6.[42–44]

We found that myeloma disease progression is accompanied by elevated CDK6 expression levels. Specimens of relapsed multiple myeloma exhibited stronger signals and higher amounts of CDK6 compared to newly diagnosed multiple myeloma. Even higher CDK6 expression levels were seen in the extramedullary manifestations of multiple myeloma and solitary plasmacytoma, a distinct entity of plasma cell neoplasia. CDK6 protein expression was not linked to high-risk cytogenetic aberrations. However, we observed a higher expression in cases with del13q comprising RB1. CDK6 was not associated with the Ki-67 index, showing that CDK6 is not a simple surrogate marker for proliferation.

Our findings are in line with previous findings of Ely et al.[26] stating that CDK6 expression is linked to advanced multiple myeloma.

The authors describe a mutually exclusive interaction of CDK4/6 with cyclin D1/2 leading to pRb-driven cell cycle progression. However, the clinical implications of CDK6 expression were not investigated in this study.

We compared CDK6 protein expression among different clinically defined subgroups and identified significant differences. We found that CDK6 increased under therapeutic pressure depending on the treatment regimens. We observed that patients treated with lenalidomide-including regimens had a significant increase in CDK6
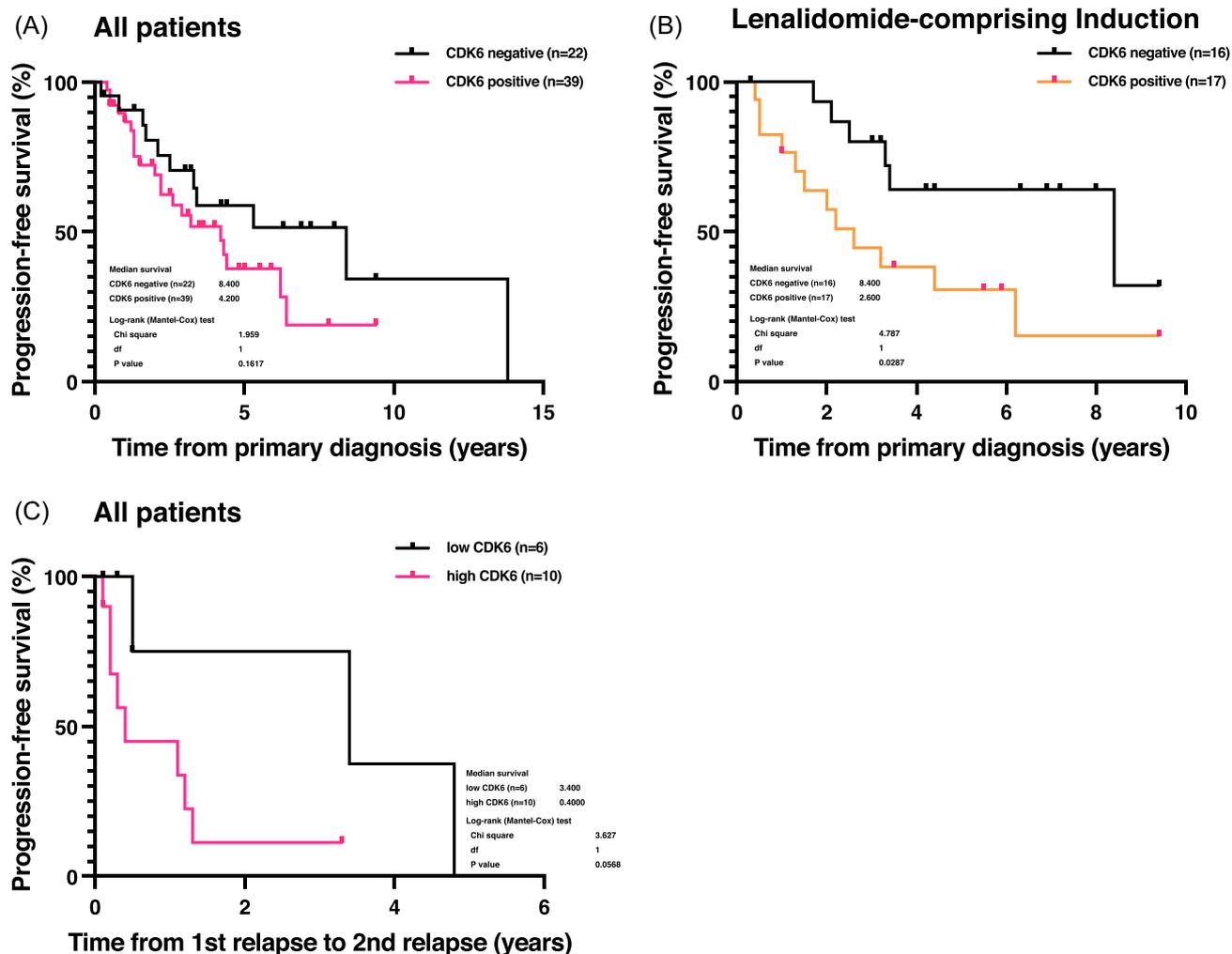
**FIGURE 5** Presence of cyclin-dependent kinase 6 (CDK6)-positive plasma cells has a negative prognostic impact in lenalidomide-treated patients. (A, B) Progression-free survival after high-dose chemotherapy (HDCT) according to CDK6 expression in the overall cohort (A) and in patients with lenalidomide-comprising induction therapy (B). (C) Progression-free survival after second-line therapy in patients who formerly underwent primary HDCT according to high versus low CDK6 with a cut-off of 60%. All survival analyses were carried out by the log-rank test.

expression from the initial diagnosis to relapse, indicating the involvement of CDK6 in IMiD resistance. This is consistent with a recent study applying proteomic analyses to quantify protein in serial samples of a smaller cohort of five patients.[31] Tandem mass tag-based quantitative analyses of paired pre- and postlenalidomide-treated samples identified CDK6 among the top six upregulated proteins in over 6000 analyzed proteins. CDK6 upregulation was verified in a lenalidomide-resistant myeloma cell culture, and inhibition of CDK6 by palbociclib or degradation by proteolysis targeting chimeras (PROTAC) led to a resensitization of myeloma cells to lenalidomide treatment. This effect was remarkably not affected by *RB1* knockout, indicating cell cycle-independent mechanisms of combined lenalidomide and palbociclib treatment.[31]

Comparison with several types of B-cell lymphomas revealed CDK6 to be highly expressed in all entities, underlining its importance in B-cell malignancies. However, multiple myeloma seems to be exceptional, as their expression was at a comparably high level only at relapse, whereas the level at diagnosis was low. Connection to disease progression is further emphasized by our findings of high CDK6 associated with extramedullary disease. The latter is a less frequent but highly aggressive entity of myeloma caused by the bone-marrow-independent growth of plasma cells.[45]

CDK6 measurement in multiple myeloma did not have a statistically significant impact on outcome in the overall cohort but showed a tendency toward shorter progression-free and overall survival in CDK6-positive cases. However, in the subgroup of lenalidomide-treated patients, we saw a significantly shorter progression-free survival for patients with CDK6-positive samples compared to patients with CDK6-negative samples, supporting the hypothesis that CDK6 is involved in IMiD resistance. The short follow-up time is a major limitation that may prevent achieving clearer results regarding the prognostic value of CDK6 expression in multiple myeloma. Future studies with higher case numbers and a longer follow-up, ideally in the setting of clinical trials, are necessary to verify our findings.

High proliferation assessed by Ki-67 expression corresponded to an inferior outcome in our cohort. This has already been shown in several previous studies.[46,47] However, we did not find any correlation between Ki-67 expression and CDK6 expression. This indicates that CDK6 has additional functions for controlling the cell cycle in multiple myeloma. Ng et al.[31] recently found a relapse signature of deregulated proteins linked

to DNA damage repair and cellular metabolism that is governed by CDK6 through phosphorylation or transcriptional regulation. They showed that the action of CDK6 is beyond cell cycle control and that there are further important substrates of CDK6, such as TRIP13 and RRM1.[31] Kollmann et al.[48] described kinase-independent functions of CDK6 like transcription modulation of p16 and VEGF-A. They additionally argue that further alterations like p16 mutation or deletion are required for CDK6 to endow its full proliferation capacity.[48]

In conclusion, we have shown that CDK6 protein expression determined by immunohistochemistry is a marker for aggressive and drug-resistant multiple myeloma. CDK6 thus may represent a potential drug target and palbociclib has already shown efficacy in relapsed and refractory myeloma patients in combination with bortezomib. We demonstrate that CDK6 expression is increased especially in lenalidomide-treated patients and high CDK6 expression has a negative prognostic impact in this subgroup. Our study provides a rationale for evaluating CDK4/6 inhibitors further in lenalidomide-resistant, CDK6-positive multiple myeloma.

## AUTHOR CONTRIBUTIONS
Jan Krönke and Thomas F. E. Barth conceived the idea and designed the study. Thomas F. E. Barth and Peter Möller provided the tissue samples. Johannes Steinhart collected the data. Johannes Steinhart, Jan Krönke, Thomas F. E. Barth, and Peter Möller analyzed and interpreted the data. Johannes Steinhart and Thomas F. E. Barth performed the microscopy and edited the microscopy pictures. Johannes Steinhart, Jan Krönke, Miriam Kull, Thomas F. E. Barth, and Peter Möller designed the figures and wrote and edited the manuscript.

## CONFLICT OF INTEREST STATEMENT
Jan Krönke received compensation for advisory boards from Celgene/BMS, Takeda, Janssen, Amgen, Sanofi, and Abbvie.

## DATA AVAILABILITY STATEMENT
All relevant data generated in this study is included in the article. Detailed information can be obtained from the corresponding author upon reasonable request.

## SUPPORTING INFORMATION
Additional supporting information can be found in the online version of this article.

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