Clinical Cancer Research

Less Can Be More for Gene Dose and Drug Sensitivity

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CDK4 is preclinically validated as a therapeutic target in *PAX3–FOXO1* fusion gene-positive rhabdomyosarcomas. Pharmacologic targeting showed sensitivity but, contrary to expectation, *CDK4* genomic amplification and overexpression associated with 25% of cases that exhibited the lowest sensitivities. This emphasizes the importance of tumor-specific preclinical studies to define and understand drug sensitivity. *Clin Cancer Res; 21(21); 4750–2. ©2015 AACR.*

See related article by Olanich et al., p. 4947

In this issue of *Clinical Cancer Research*, Olanich and colleagues (1) explore the dependency of *PAX3–FOXO1* fusion gene-positive alveolar rhabdomyosarcoma (aRMS) on cyclin-dependent kinase 4 (CDK4) and the potential for using small-molecule inhibitors of CDK4 activity to treat patients. *PAX3–FOXO1* fusion gene-positive aRMS represents an aggressive subgroup of soft tissue sarcoma in childhood and young adulthood (2), 25% of which carry an amplification of the chromosomal region 12q13-q14 containing *CDK4* (3). Genomic amplification is the selective increase in copies of regions of DNA and in a tumor sample can be used as a criterion for treatment of patients using therapies that target the overexpressed product of an amplified gene (4). Examples are *HER2* amplification and overexpression in breast cancer, which is targeted by the HER2 antibody trastuzumab, and several other amplified genes whose products are therapeutic targets in clinical development, including FGFR1 in lung and breast cancer (4). Although gene amplification and overexpression may imply high dependence on a gene product, the relationship between this and effective therapeutic targeting may not be straightforward, as illustrated for CDK4 by Olanich and colleagues (1).

Mitogenic stimulus of cells promotes CDK4 or CDK6 to form complexes with D-type cyclins that have kinase activity which inhibits the retinoblastoma protein (RB). This results in transcriptional activation of the E2F family of transcription factors leading to G_1–S progression (Fig. 1). CDK4/6 are negatively regulated by the INK4 family of proteins including p16 that inhibit assembly and activation of cyclin D–CDK4/6 complexes (5). The CDK4/6-RB pathway is aberrantly activated in many cancers with activating alterations described including amplification of *CDK4* or *CDK6*, translocation-driven increased expression of cyclin D1, mutations in *CDK4*, deletion of *CDKN2A* encoding p16 or loss of RB (5).

Olanich and colleagues demonstrate that amplification of 12q13-q14 in RMS is associated with overexpression of CDK4 at the protein level in cell lines, cell line xenografts, and RMS samples from patients (1). Stable CDK4 knockdown in an RMS cell line with amplification led to reduced phosphorylation of RB, reduced RB protein levels, and reduced E2F target expression at the mRNA level. This was linked to reduced proliferation and transformation (foci formation) *in vitro* and tumor growth retardation *in vivo*. Investigation of an RMS cell line without CDK4 amplification also showed similar dependency on CDK4 for proliferation and transformation.

Olanich and colleagues then tested the two most advanced CDK4/6 inhibitors currently in clinical trials. Palbociclib (PD-0332991) recently received accelerated FDA approval for treatment of advanced ER-positive, HER2-negative breast cancer and is being tested in several ongoing studies, including in *CDK4*-amplified liposarcomas (NCT01209598; ref. 6). Ribociclib (LEE011) is another promising candidate in a phase III trial in breast cancer (NCT01958021) and in phase I trial in pediatric solid tumors (NCT01747876). In five different fusion gene-positive aRMS cell lines, Olanich and colleagues demonstrate variable sensitivities to LEE011 and PD-0332991 *in vitro* (1). Surprisingly, the level of sensitivity, assessed by proliferation and G_1 arrest, was least in cell lines with *CDK4* amplification and high levels of protein expression. No morphologic signs of myogenic differentiation were seen, contrary to a previous study (7). Using 2 different aRMS cell lines and stable overexpression of CDK4 or CDK6, they showed that sensitivity was indeed reduced by elevated CDK4/6 levels. These experiments were consistent with *in vivo* analyses of a *CDK4*-amplified aRMS cell line, which was less responsive to LEE011 than a different cell line with no amplification and low expression of CDK4.

This study raises a number of issues related to the role and therapeutic potential of targeting CDK4 in the fusion gene-positive subgroup of aRMS and other tumor types. The RNA interference results clearly indicate that dependency on CDK4 is irrespective of amplification in fusion-positive RMS. Moreover, Olanich and colleagues were able to show that exogenous overexpression of CDK4, at levels comparable with the 12q13-q14 amplified cell line, did not enhance cyclin D-CDK4/6-RB pathway activity measured by E2F-responsive gene expression and did not influence the cell proliferation and transformation capacity. Importantly, co-immunoprecipitation experiments showed consistent levels of cyclin D1 complexed with CDK4, despite increasing CDK4 levels

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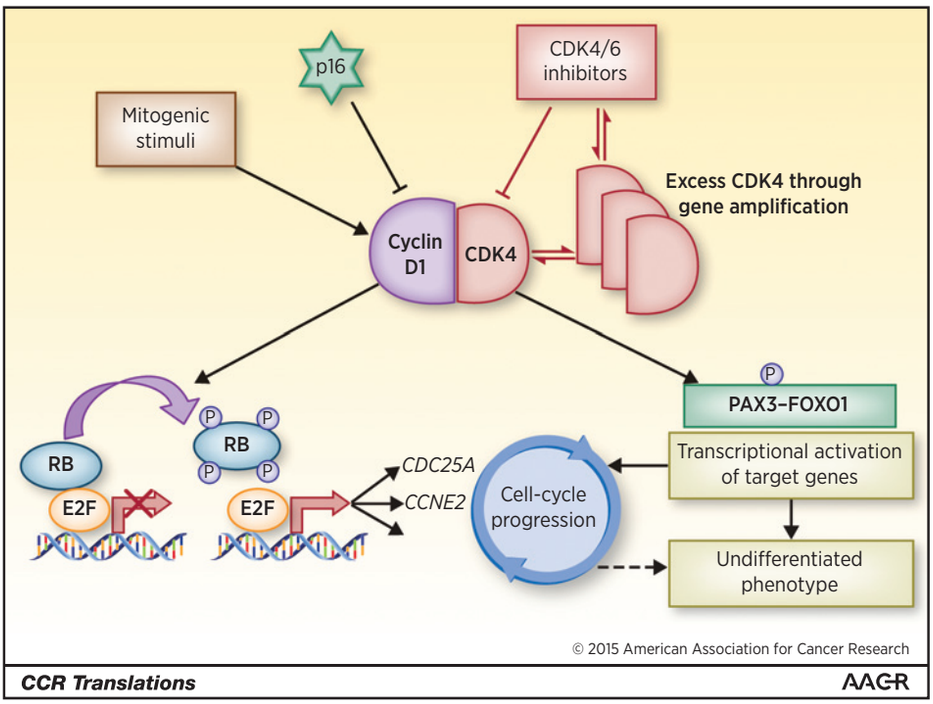

Figure 1.
Simplified schema for the role of CDK4 in *PAX3–FOXO1* fusion gene-positive RMS with and without *CDK4* gene amplification. Mitogenic stimulation promotes cyclin D1/CDK4 complexes which activate the kinase that releases the inhibitory effects of RB resulting in transcriptional activation of the E2F proteins. This leads to G_1–S progression via upregulation of downstream targets such as *CDC25A* and *CCNE2* that were assessed in RMS by Olanich and colleagues (1). Cyclin D1/CDK4 is also reported to phosphorylate (p) the PAX3–FOXO1 fusion protein on Serine 430, contributing to its transcriptional activation of downstream targets (11) that will also drive cell-cycle progression and maintain an undifferentiated phenotype. p16 inhibits CDK4/6 catalytic activity through various conformational changes, and CDK4/6 inhibitors like LEE011 are ATP-competitive inhibitors, binding to the enzymes ATP binding site. Excess uncomplexed CDK4 may stoichiometrically compete with inhibitors and explain the decreased sensitivity of RMS cells with genomic amplification and high levels of CDK4 (1).

(1). This indicates that cyclin D1 availability is the limiting step in the cyclin D-CDK4/6-RB pathway activity in aRMS cell lines (Fig. 1). LEE011 and PD-0332991 are ATP-competitive kinase inhibitors, which bind to the ATP binding pocket of CDK4/6 and inhibit the catalytic activity of the cyclin D–CDK4/6 complex (5). The uncomplexed CDK4 in the highly expressing cell lines may provide stoichiometric competition that reduces sensitivity to the agent, as suggested by the authors (1). This raises concerns for effective therapeutic targeting of highly expressed CDK4 in RMS. Evidence for other tumor types is mixed or lacking. Preclinical studies in glioblastoma indicate resistance to CDK4/6 inhibitor treatment in two cell lines with CDK4 amplification (8). However, investigations of liposarcoma which harbor amplification of CDK4 in > 90% of tumors indicate high sensitivity to pharmacologic inhibition in *CDK4*-amplified cell lines and encouraging results in a phase II trial of *CDK4*-amplified liposarcoma patients with RB pathway activity (6, 9). It will be interesting to see results of ongoing phase II trials with CDK4/6 inhibitors that specifically include *CDK4* amplification as one of the selection criteria [LUNG-MAP trial in squamous cell carcinoma of the lung (NCT02154490), SIGNATURE-trial for "Patients With CDK4/6 Pathway Activated Tumors" (NCT02187783)]. Furthermore, the pediatric phase I study of LEE011 (NCT01747876) included a fusion-positive aRMS patient with *CDK4* amplification that showed progressive disease on trial that may be in-keeping with reduced sensitivity (10).

In a previous study by the authors, 12q13-q14 amplification that includes *CDK4* was found to be associated with significantly poorer survival in fusion gene-positive aRMS patients (3). Levels of CDK4 are also linked to poor prognosis in other cancer types (4). The reason behind these clinical correlations is unclear as functional relevance of increased levels of CDK4 in aRMS cells has not yet been seen. This could be due to the importance of another gene that is amplified at 12q13-q14 or some other cell context–specific association and functional effect. Although there is little evidence to date, CDK4 may also have a role beyond the cyclin D complex that is contributing to the phenotype of RMS.

The dependence on CDK4 in fusion gene-positive aRMS cell lines demonstrated by RNA interference is striking and in contrast to fusion gene-negative RMS (1). However, this is consistent with data from a cell line supporting CDK4-dependent phosphorylation of the PAX3–FOXO1 fusion protein and its increased transcriptional activity (11). Other kinases also phosphorylate the fusion protein, such as PLK1 that has been demonstrated to enhance its stability and activity (12). Therefore, targeting CDK4 may directly affect activity of the fusion protein unique to aRMS as well as inhibiting RB pathway activity (Fig. 1).

Although CDK4 amplification has prognostic marker and is a potential indicator of pathway activation, the compelling results of Olanich and colleagues highlight the need for further studies to better understand the underlying molecular mechanisms and identify predictive biomarkers of response to CDK4/6 inhibitors in RMS and other tumor types.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S.A. Gatz, J.M. Shipley
Writing, review, and/or revision of the manuscript: S.A. Gatz, J.M. Shipley

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References

1. Olanich ME, Sun W, Hewitt SM, Abdullaev Z, Pack SD, Barr FG. *CDK4* amplification reduces sensitivity to CDK4/6 inhibition in fusion-positive rhabdomyosarcoma. Clin Cancer Res 2015;21:4947–59.
2. Missiaglia E, Williamson D, Chisholm J, Wirapati P, Pierron G, Petel F, et al. PAX3/FOXO1 fusion gene status is the key prognostic molecular marker in rhabdomyosarcoma and significantly improves current risk stratification. J Clin Oncol 2012;30:1670–7.
3. Barr FG, Duan F, Smith LM, Gustafson D, Pitts M, Hammond S, et al. Genomic and clinical analyses of 2p24 and 12q13-q14 amplification in alveolar rhabdomyosarcoma: a report from the Children's Oncology Group. Genes Chromosomes Cancer 2009;48:661–72.
4. Santarius T, Shipley J, Brewer D, Stratton MR, Cooper CS. A census of amplified and overexpressed human cancer genes. Nat Rev Cancer 2010;10:59–64.
5. Asghar U, Witkiewicz AK, Turner NC, Knudsen ES. The history and future of targeting cyclin-dependent kinases in cancer therapy. Nat Rev Drug Discov 2015;14:130–46.
6. Dickson MA, Tap WD, Keohan ML, D'Angelo SP, Gounder MM, Antonescu CR, et al. Phase II trial of the CDK4 inhibitor PD0332991 in patients with advanced CDK4-amplified well-differentiated or dedifferentiated liposarcoma. J Clin Oncol 2013;31:2024–8.
7. Saab R, Bills JL, Miceli AP, Anderson CM, Khoury JD, Fry DW, et al. Pharmacologic inhibition of cyclin-dependent kinase 4/6 activity arrests proliferation in myoblasts and rhabdomyosarcoma-derived cells. Mol Cancer Ther 2006;5:1299–308.
8. Cen L, Carlson BL, Schroeder MA, Ostrem JL, Kitange GJ, Mladek AC, et al. p16-Cdk4-Rb axis controls sensitivity to a cyclin-dependent kinase inhibitor PD0332991 in glioblastoma xenograft cells. Neuro Oncol 2012;14:870–81.
9. Zhang YX, Sicinska E, Czaplinski JT, Remillard SP, Moss S, Wang Y, et al. Antiproliferative effects of CDK4/6 inhibition in CDK4-amplified human liposarcoma in vitro and in vivo. Mol Cancer Ther 2014;13:2184–93.
10. Geoerger B, Bourdeaut F, DuBois SG, Fischer M, DeWire M, Marabelle A, et al. Phase I study of LEE011 (CDK4/6 inhibitor) in patients with malignant rhabdoid tumors, neuroblastoma, and cyclin D–CDK4/6 pathway-activated tumors [abstract]. In: Proceedings of the ESMO 2014 Congress; 2014 Sep 26–30; Madrid, Spain; Lugano (Switzerland): European Society for Medical Oncology; 2014. Abstract nr 445.
11. Liu L, Wu J, Ong SS, Chen T. Cyclin-dependent kinase 4 phosphorylates and positively regulates PAX3-FOXO1 in human alveolar rhabdomyosarcoma cells. PLoS One 2013;8:e58193.
12. Thalhammer V, Lopez-Garcia LA, Herrero-Martin D, Hecker R, Laubscher D, Gierisch ME, et al. PLK1 phosphorylates PAX3-FOXO1, the inhibition of which triggers regression of alveolar Rhabdomyosarcoma. Cancer Res 2015;75:98–110.