Early G1 Cyclin-Dependent Kinases as Prognostic Markers and Potential Therapeutic Targets in Esophageal Adenocarcinoma

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Abstract

**Purpose:** Chromosomal gain at 7q21 is a frequent event in esophageal adenocarcinoma (EAC). However, this event has not been mapped with fine resolution in a large EAC cohort, and its association with clinical endpoints and functional relevance are unclear.

**Experimental Design:** We used a cohort of 116 patients to fine map the 7q21 amplification using SNP microarrays. Prognostic significance and functional role of 7q21 amplification and its gene expression were explored.

**Results:** Amplification of the 7q21 region was observed in 35% of tumors with a focal, minimal amplicon containing six genes. 7q21 amplification was associated with poor survival and analysis of gene expression identified cyclin-dependent kinase 6 (CDK6) as the only gene in the minimal amplicon whose expression was also associated with poor survival. A low-level amplification (10%) was observed at the 12q13 region containing the CDK6 homologue cyclin-dependent kinase 4 (CDK4). Both amplification and expression of CDK4 correlated with poor survival. A combined model of both CDK6 and CDK4 expressions is a superior predictor of survival than either alone. Specific knockdown of CDK4 and/or CDK6 by siRNAs shows that they are required for proliferation of EAC cells and that their function is additive. PD-0332991 targets the kinase activity of both molecules and suppresses proliferation and anchorage independence of EAC cells through activation of the pRb pathway.

**Conclusions:** We suggest that CDK6 is the driver of 7q21 amplification and that both CDK4 and CDK6 are prognostic markers and bona fide oncogenes in EAC. Targeting these molecules may constitute a viable new therapy for this disease. *Clin Cancer Res; 17(13); 4513–22. ©2011 AACR.*
CDK6. Finally, functional analysis of both CDK4 and CDK6 in EAC cell lines shows that siRNA knockdown or inhibition of activity of both CDK4 and CDK6 with the small molecule inhibitor PD-0332991 suppresses proliferation and anchorage-independent growth. Thus, our data provide direct evidence that CDK6 is the functional target of 7q21 amplification in EAC and expression of both CDK6 and CDK4 is a strong predictor of patient survival. Specific inhibition of both molecules is thus a viable therapeutic target in this disease.

Materials and Methods

EAC patients and tissues

Frozen tumors were obtained from 116 patients undergoing esophagectomy at the University of Pittsburgh Medical Center, Pittsburgh, PA, between 2002 and 2008. Three patients suffered perioperative death, and 1 patient received neoadjuvant chemotherapy. These patients were excluded from survival analyses. Normal DNA was obtained from the blood of 15 individuals from the same patient population. All patients signed Institutional Review Board–approved consent and all research was conducted under protocols approved at the participating institutions. Patient age ranged from 43 to 88, and the cohort consisted of 95 men and 21 women. Final pathologic stages were stage I (n = 28), stage II (n = 31), stage III (n = 49), stage IV (n = 7), and unknown stage (n = 1). All tumor specimens were evaluated by a pathologist and were determined to be more than 70% tumor cell representation. A description of the patient cohort is provided in Supplementary Table S1.

DNA copy number analysis

Genomic DNA was isolated using the QiaAmp DNA Mini Kit (Qiagen) and its 600 ng was used for labeling and hybridization to Affymetrix SNP 6.0 arrays at the SUNY Upstate Medical University Microarray Core Facility (Syracuse, NY), using kits and protocols obtained from Affymetrix. Array data quality was assessed using Affymetrix Genotyping Console (GTC) 3.0, and all further data processing and analysis were conducted using Nexus 5.0 Copy Number Analysis software (Biodiscovery, Inc.). Ratios for log, DNA copy number were generated for each probeset on the arrays by using a reference file generated from 15 normal DNA samples. These data were then segmented using the SNP-rank algorithm with a minimum requirement of 8 probes and a significance threshold P value of 10−5. Amplification was defined as any segment with a log2 copy number ratio greater than 0.15 (2.2 copies), with high copy gain defined as greater than 0.5 (2.8 copies).

Gene expression analysis

Total RNA was extracted from 107 (of the original 116) tumors, using the Stratagene RNA Isolation Kit (Stratagene). Labeling, hybridization, washing, and scanning for Affymetrix U133 Plus 2 GeneChip expression arrays were carried out in accordance with the manufacturer’s protocols. Raw data were processed in Partek Genomic

Translational Relevance

Current chemotherapy treatment protocols for the management of esophageal adenocarcinoma (EAC) are not very effective; therefore, the search for new therapeutic targets is of high priority. In this study, we present evidence that the interphase kinases cyclin-dependent kinase 4 (CDK4) and cyclin-dependent kinase 6 (CDK6) are bona fide oncogenes in this disease. Both genes are amplified in a subset of EAC patients at 10% and 35% frequency, respectively. The expression of both kinases is strongly associated with patient survival. CDK4 and CDK6 show redundant and additive mitogenic effect on EAC cells in vitro. We introduce the CDK inhibitor PD-0332991 as a potential therapeutic agent in EAC.
Defining the 7q21 amplicon in EAC

Amplification of 7q21 was observed in 35% (40 of 116) of tumors (Fig. 1A). Although some events are very large (spanning most or all of 7q), many focal aberrations were also identified that help to define the boundaries of the amplicon. Many (17 of 116) of these events were also high copy gains as indicated by the darker blue region in Figure 1A. Definition of specific boundaries is somewhat arbitrary but, conservatively, the amplicon contains 17 genes (MTERF, AKAP9, CYPS1A1, LOC401387, KRIT1, ANKK1, GATAD1, PEX1, C7ORF64, MGC16142, FAM133B, CDK6, SAMD9, SAMD9L, HEPACAM2, CCDC132, and CALC1) with the minimal region of amplification (dotted line in Fig. 1A) defined by only 6 genes (GATAD1, PEX1, C7ORF64, MGC16142, FAM133B, and CDK6).

Association of 7q21 amplification with survival

To assess the prognostic value of amplification at 7q21, we determined whether this amplification correlated with overall and recurrence-free survival of patients. Kaplan–Meier survival estimates indicate a modest association of 7q21 amplification at 7q21 with increased risk of death (P = 0.049) but no statistically significant association with disease recurrence (P = 0.23, Fig. 1B).

Association between 7q21 amplification, gene expression, and patient survival

To identify those genes that are expressed at higher levels in concordance with their amplification status, we analyzed gene expression data from 107 tumors, using Affymetrix U133 Plus 2.0 gene expression arrays. We compared expression level of all U133 probesets corresponding to the seventeen 7q21 candidate genes in amplified versus non-amplified tumors. We found significantly elevated expression (unadjusted P ≤ 0.05 and false discovery rate ≤ 0.10) of at least 1 probeset for 13 of 17 genes in the amplicon as indicated by the short black lines in Figure 2A (note that lines above the P = 0.05 threshold line indicate P < 0.05). Expression data for each of the 7 probesets mapping to CDK6 are also shown for CDK6-positive and CDK6-negative
Because the amplification event is associated with patient survival, we also explored whether expression of genes in the amplicon is associated with survival. Cox regression analysis showed association of gene expression with either overall survival (blue lines above the threshold in Fig. 2A) or recurrence-free survival (red lines above the threshold in Fig. 2A) for 13 probesets. However, associations with overall and recurrence-free survival were present for only 7 probesets representing 2 genes: CDK6 and ANKIB1 (ankyrin repeat and IBR domain containing 1) all 7 significant probesets for overall and recurrence-free survival (2 for ANKIB1 and 5 for CDK6) have a false discovery rate less than 10%. However, ANKIB1 is excluded from the minimal amplicon by 1 tumor where the proximal boundary is within the ANKIB1 gene (Fig. 1A). Therefore, although we cannot rule out a potential role for ANKIB1 in 7q21 amplification and EAC, our data indicate that CDK6 is the major driver of this event and the subsequent prognostic implications. Five of the 7 probesets mapping to the CDK6 gene were associated with overall and recurrence-free survival at $P \leq 0.05$. We therefore conducted a permutation test of the randomness of the distribution of 7 total significant probesets to test whether the observed CDK6 clustering could be explained by random variation. Using 100,000 positional permutations of the 45 probesets, 7 of which were associated with both overall and recurrence-free survival, we calculated the probability ($P$ value) of finding 5 or more significant probeset associations among the 7 contiguous CDK6 probesets. The permutation $P$ value was 0.00035. Thus, the clustering of significantly associated probesets representing the CDK6 gene is unlikely to be random. Kaplan–Meier plots of overall and recurrence-free survival

Figure 1. A, copy number gain (blue) and loss (red) at 7q21 observed in 116 EAC tumors. Dark blue represents high-level amplification. The amplified samples represent 35% of tumors. Solid white lines show the arbitrary defined border of the amplicon (containing 17 genes shown below the figure), and the dotted white lines define the minimal amplicon (containing 6 genes, underlined below the figure). B, Kaplan–Meier survival curves showing correlation of 7q21 amplification with overall (left) and recurrence-free (right) patient survival.

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CDK6 and CDK4 Are Potential Therapeutic Targets in EAC

Effect of CDK6 and CDK4 knockdown on growth of EAC cells

To study CDK6 function in EAC, we used siRNA transfection to knockdown its expression in OE19, OE33, and FlO1A cells. In addition, we evaluated knockdown of the CDK6 homologue CDK4, as these molecules are known to have functional redundancy. To avoid potential off-target artifacts, 2 different siRNA sequences for each molecule were used. Western blot analysis revealed endogenous expression of CDK6 and CDK4 in all 3 cell lines tested and confirmed the almost complete silencing of CDK6 and CDK4 by both siRNAs (Fig. 3A). Monolayer growth analysis showed that CDK6 knockdown reduced cell proliferation in OE19 and FlO1A but not in OE33 (Fig. 3B), whereas CDK4 silencing reduced cell proliferation in all 3 cell lines. Targeting both molecules has an additive effect in suppressing cell proliferation. To confirm the functional mechanism of siRNA-mediated CDK knockdown, we measured the phosphorylation status of the retinoblastoma protein pRB, which is a target of CDKs. As shown in Figure 3C, knocking down CDK6 in EAC cells reduced phosphorylation of pRB at Ser780 and Ser795 (both are known targets of CDK6–cyclin D1 complex, ref. 15), although with much lesser effect in OE33.

Association of CDK4 and CDK6 expression with survival

Given the observation that EAC cell lines seemed to respond most strongly to combined CDK4 and CDK6

![Graph](https://example.com/graph.png)

Figure 2. A, comparison of the expression level (amplified vs. nonamplified tumors) and association with overall (OS) and recurrence-free (RFS) survival for each probeset on the Affymetrix U133 microarray corresponding to the 7q21 genes. The y-axis represents P values for 3 statistical tests: t-test raw P values of gene expression in 7q21 amplified versus nonamplified tumors (black lines), and Cox regression P values for the association of gene expression with overall (blue lines) and with recurrence-free (red lines) survival. The horizontal line cutting the y-axis indicates P = 0.05. Genes mapping to the core amplicon are underlined. B, box plot representation showing increased expression of CDK6 probesets in U133 microarray. *, P = 0.022; **, P = 0.001; ***, P < 0.0001. Note that probeset 231198_at seems to be nonresponsive. C, Kaplan–Meier plots of overall and recurrence-free survival by a median split of the expression levels of CDK6 based on probeset 224848_at. P values for the log-rank test are shown for each plot.

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knockdown, we explored CDK4 amplification and expression in our primary tumors. Amplification of the 12q13 region containing CDK4 was observed in 12 of 116 tumors (10%) and was associated with worse overall survival \( (P = 0.019; \text{Supplementary Fig. S1}) \). Only 4 tumors showed amplification of both CDK4 and CDK6. High expression of CDK4 was also found to correlate with poor prognosis (Fig. 3D). Next, we explored the association of patient survival with expression of CDK4 and CDK6 alone versus in combination. A likelihood ratio test for the addition of CDK6 to a model consisting of CDK4 yielded a likelihood ratio statistic of 4.40 with 1 degree of freedom \( (P = 0.0377) \), thereby indicating improved prognostic value when considering both genes. As an illustration, Kaplan–Meier plots based on median expression levels of predicted log relative hazard in Figure 3D support improved discrimination in overall survival by using both genes. The addition of CDK4 to a model of recurrence-free survival with CDK6 did not show similar improvement (likelihood ratio \( P = 0.1839 \)).

The CDK inhibitor PD-0332991 reduces cell proliferation in EAC cells

Because the combination of CDK4 and CDK6 expression seems to be functionally important both in vitro and in vivo, we tested the effect of the CDK4/6 inhibitor PD-0332991 in EAC cells. PD-0332991 inhibited cell proliferation in a dose-dependent manner in all 3 cell lines tested (Fig. 4A) and, as with the siRNA knockdown of CDK6, this was associated with hypophosphorylation of pRB at Ser780 and Ser795 (Fig. 4D). To confirm that the effects of PD-0332991 are by activation of the pRB tumor suppressor pathway, we determined the effect of the drug on the known pRB target, cyclin A (in the RB axis where CDK4/6 inhibition activates RB and stabilizes the RB–E2F1 complex leading to inhibition of the transcriptional activation of cyclin A by E2F1). As shown in Figure 4D (bottom), PD-0332991 reduces expression of cyclin A in all 3 cell lines. PD-0332991 has no effect on the expression level of either total pRB or RB2 (p130) in EAC cells (Fig. 4D, bottom). PD-0332991 delays cell-cycle progression at the G1–S boundary and is not cytotoxic to a variety of cancer cell lines.
To confirm the same effect in EAC cells, we conducted FACS analysis to measure progression through the cell cycle and to assess hypodiploidy as a measure of apoptotic response. As shown in Figure 4B and C, in all 3 cell lines tested, there is a decrease in S–G2 population compared with G0–G1 5 days after incubation with PD-0332991 and this is without any remarkable hypodiploidy.

The CDK4/6 inhibitor PD-0332991 reduces anchorage-independent growth in EAC cells

Given the effect of PD-0332991 on cell proliferation, we tested whether the drug will reduce the transformation potential of EAC cells. To this end, we assessed anchorage-independent cell growth. Cells were incubated for 3 weeks in semisolid (0.4%) agar in the presence or absence of 125 nmol/L PD-0332991. As shown in Figure 4E, PD-0332991 caused a significant reduction in colony formation in soft agar in all 3 cell lines tested, indicative of a reduction in anchorage independence.

Discussion

Chromosomal abnormalities resulting in gene amplification or deletion are a common mechanism to confer mitogenic advantage in tumors (18). Many targeted therapeutics for cancer are based on inhibitors that block the effect of an upregulated or activated oncogene (19). The classic example of this is the identification of locus-specific...
amplification and overexpression of ERBB2 in breast cancer that led to the successful development of targeted therapy, trastuzumab (20). Development of inhibitors and clinical trials are currently ongoing targeting mitogens and proto-oncogenes such as EGF (21), PI3KCA (22), VEGF (23) c-MYC (19), and MET (24). In tumors such as EAC in which standard cytotoxic chemotherapies have limited effect, identification of targets for novel drug development is of high importance but has been limited by access to tissues from large cohorts of patients.

Amplification at 7q21 has been observed in T-cell lymphoma (25) and gliomas (26), as well as in medulloblastoma, in which CDK6 expression is associated with poor prognosis (27). Chromosome 7 and 7q21 amplifications have also been reported in EAC (10, 11), but only one study has addressed the possible involvement of CDK6 as the driver of the amplicon (12). However, the limited number of tumors (n = 14) along with the low resolution of the comparative genomic hybridization array used in this study made it impossible to fine map the genes in the amplified region or to determine any prognostic significance. Here we used high-density Affymetrix SNP 6.0 microarrays and tissues from a cohort of 116 patients to fine map the genomic alterations at 7q21 in EAC and explored 7q21 gene expression in a subset of 107 tumors. We refined the minimal 7q21amplicon in EAC to only 6 genes, identified prognostic implications of both 7q21 amplification and CDK6 gene expression, and showed that inhibition of CDK6 activity reduced both proliferation- and anchorage-independent growth in EAC cell lines.

We also identified a large amplification event at 12q13 spanning 102 genes (see Supplementary Data) including CDK4, which is a homologue of CDK6. Despite its low frequency (10%), 12q13 amplification correlated with poor survival (Supplementary Fig. S1). Moreover, expression of CDK4 was also associated with survival and the combination of expression of both CDK6 and CDK4 was a superior predictor of survival than either alone. Together, these data strongly implicate CDK6 as the driver of 7q21 amplification in EAC and suggest that the prognosis of the disease is dependent on activity of the other member of the retinoblastoma family RB2 (p130) but not RB1 (p107) tumor suppressor protein (37). However, this study showed that CDK6 expression was reduced in breast tumors compared with normal tissues. In our study, expression of CDK6 was higher in tumors with 7q21 amplification but CDK6 expression correlated more strongly with poor prognosis than amplification alone (Figs. 1B and 2C). This indicates that, in EAC, CDK6 overexpression may also result from mechanisms independent of its amplification. Examination of our array data identified a DNA copy number loss at the Hsa-mir-124a locus in 13% of samples, but no correlation with CDK6 expression was observed.

Targeting CDK6 with siRNA confers proliferative disadvantage to OE19 and Flo1A cells but not OE33 (Fig. 3B). Both CDK4 and CDK6 bind to and are activated by cyclin D1 in early G1 phase and, in this respect, their functions may be redundant. The negative response of OE33 cells to the knockdown of CDK6 by siRNA may therefore be explained by a compensatory effect of CDK4 in these cells. This hypothesis is based on the following observations: First, and as shown in Figure 3B, the combined knockdown of both CDK6 and CDK4 strongly suppresses proliferation in OE33; second, the reduction in phosphorylation of pRB at Ser780 and Ser795 (16, 17). Alteration in expression of total pRB or pRB protein (33) and pRB (34). However, regulation of gene expression is controlled in many ways independent of gene dosage. For example, CDK6 expression is also downregulated by microRNAs Hsa-mir-129 and Hsa-mir-124a. Downregulation of CDK6 in vitro by mir-129 induces cell-cycle arrest in lung adenocarcinoma cells (35), whereas epigenetic silencing of Hsa-mir-124a in acute lymphoblastic leukemia results in CDK6 upregulation, hyperproliferation of cells, and poor prognosis (36). In contrast, ectopic expression of CDK6 in vitro suppresses proliferation of breast cancer cells presumably through activation of RB2 (p103) tumor suppressor protein (37).

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following PD-0332991 treatment has been reported (40). In EAC cells, however, PD-0332991 treatment reduces phosphorylation of pRb but does not alter expression of total pRb or Rb2 (Fig. 4D). The suppression of proliferation (Fig. 4A) and anchorage independence caused by PD-0332991 (Fig. 4E) and the parallel cell-cycle blockage (Fig. 4B and C) and hypophosphorylation of pRb (Fig. 4D) suggest that PD-0332991 may be an attractive therapeutic candidate for EAC. The drug is currently in phase II clinical trials in several solid tumors in which it has proven to be well tolerated (41, 42). Furthermore, the use of CDK inhibitors in combination with cisplatin, the primary component of chemotherapy for EAC, has been suggested (43, 44). Finally, in the CDK6-amplified tumors, we found concurrent amplification of ERBB2 (13 tumors), EGFR (22 tumors), PIK3CA (7 tumors), MET (15 tumors), and VEGFA (14 tumors). This opens the possibility of using combination therapy with agents targeting these genes along with PD-0332991 in clinical trials.

Taken together, our data strongly suggest that CDK6 drives the 7q21 amplification in EAC and its expression carries prognostic significance. Expression of both CDK4 and CDK6 is a superior predictor of prognosis in EAC than either alone. Both CDK4 and CDK6 are functionally mitogenic to EAC cell lines through inhibition of the pRb tumor suppressor pathway. We propose that inhibition of CDK4/6 activity should be considered as potential therapy, particularly in tumors with high CDK4/6 expression.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

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