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 6 genes. 7q21 amplification was associated with poor survival and analysis of gene expression identified 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 homolog, CDK4. Both amplification and expression of CDK4 correlated with poor survival. A combined model of both CDK6 and CDK4 expression 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.

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 CDK4 and 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.

Introduction
The epidemiology, early detection and treatment of esophageal cancer are changing rapidly. Considered an uncommon tumor 20 years ago, the incidence of adenocarcinoma of the esophagus and gastroesophageal junction (EAC) has grown faster than any tumor type in the United States, outpacing the next closest cancer by almost 3 times (1). As a result EAC is now the most common esophageal malignancy in the United States. This rising incidence of EAC is associated with the increasing prevalence of obesity and gastroesophageal reflux disease (GERD) in westernized populations. Prolonged reflux of gastric and duodenal contents into the lower
esophagus causes a metaplastic change in the esophageal epithelium from normal stratified squamous to a columnar-type epithelium known as Barrett’s Esophagus (BE). BE is currently the strongest known risk factor for the development of EAC (2). Progression from BE to dysplasia and adenocarcinoma is associated with the accumulation of genetic and epigenetic alterations such as copy number gain and loss, mutations and promoter methylation (3) that presumably lead to oncogenic changes in mRNA and protein expression.

Despite an increase in awareness leading to earlier disease detection, and the evolution of multi-modality therapies, survival for esophageal adenocarcinoma patients remains poor compared with other tumor types. The lack of a serosal layer in the esophagus facilitates early and widespread lymphatic dissemination of tumor cells and current chemotherapy regimens have limited effectiveness against bulky metastatic disease. As a result, overall 5-yr survival for stage III or IV disease is only 5-20%. Even for earlier stage patients, overall 5-yr survival is only 40-50% for stage II and 70-80% for stage I (4). Thus, given the rapid rise in incidence, the continued high prevalence of risk factors and the poor clinical outcomes, it is important that we continue to search for new and more active therapeutic agents for EAC.

One approach for the discovery of new therapeutic targets is through identification of genes whose expression is deregulated by genetic alterations in the tumor (5). Of particular interest are mitogens that are amplified and subsequently highly expressed leading to a proliferative advantage in the tumor. For example HER-2 (Human Epidermal growth factor Receptor 2) is frequently amplified and overexpressed in breast cancer and is effectively targeted by the drug Trastuzumab (Herceptin). Recent studies
have shown that HER-2 is also amplified and overexpressed in a subset of EACs (6) and clinical trials in patients with gastric adenocarcinoma have shown a significant survival advantage for patients treated with a combination of Trastuzumab and standard chemotherapy versus chemotherapy alone (7). Clinical trials using Trastuzumab for EAC have recently been initiated in the United States (8).

Recurrent amplification of the 7q21-22 chromosomal region has been previously reported in EAC (9-11) and Van Dekken et al. suggested that cyclin-dependent kinase 6 (CDK6) may be the key gene targeted by this event (12). However, the 7q21 amplicon contains several genes and this hypothesis is based on the known function of CDK6 as a cell cycle regulator rather than any proven clinical or functional importance of CDK6 amplification or expression in EAC. Here we used high resolution microarrays to map the 7q21 amplicon in 116 cases of EAC. We report refined estimates for amplification frequency and identify a focal, minimal amplicon spanning only six genes. Association of 7q21 amplification and expression of genes in the amplicon were explored for association with clinical outcomes and identified CDK6 as the most likely driver of this amplification event. In addition, we observed that amplification at 12q13-14, spanning 102 genes including the CDK6 homolog, CDK4, was also associated with survival. We thus investigated association of CDK4 expression with survival both alone and in combination with CDK6. Finally, functional analysis of both CDK4 and CDK6 in EAC cell lines shows that siRNA knockdown or inhibition of both CDK4 and CDK6 activity 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 both CDK6 and CDK4 expression is a strong
predictor of patient survival. Specific targeting 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 one 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 IRB approved consent and all research was performed under protocols approved at the participating institutions. Patient age ranged from 43-88 and the cohort consisted of 95 males and 21 females. Final pathologic stages were stage I (28), stage II (31), stage III (49), stage IV (7) and unknown stage (1). All tumor specimens were evaluated by a pathologist and were determined to be >70% tumor cell representation. A description of the patient cohort is provided in supplementary Table 1.

**DNA Copy Number Analysis.** Genomic DNA was isolated using the QiaAmp DNA Mini Kit (Qiagen, Valencia, CA) and 600ng 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 (Santa Clara, CA). Array data quality was assessed using Affymetrix Genotyping Console (GTC) 3.0 and all further data processing and analysis was performed using Nexus 5.0 Copy
Number Analysis software (Biodiscovery, Inc. El Segundo, CA). Log$_2$ DNA copy number ratios were generated for each probeset on the arrays using a reference file generated from 15 normal DNA samples. The data was then segmented using the SNP-Rank algorithm with a minimum requirement of 8 probes and a significance threshold p-value of $10^{-6}$. Amplification was defined as any segment with a log$_2$ copy number ratio >0.15 (2.2 copies) with high copy gain defined as >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, CA). Labeling, hybridization, washing and scanning for Affymetrix U133 Plus 2 GeneChip expression arrays were carried out in accordance with the manufacturers protocols. Raw data was processed in Partek Genomic Suite (Partek Inc., St. Louis, MO) using the GC-RMA approach to provide normalized expression data for each probeset on the arrays.

**Cell lines and targeting CDK6 and CDK4 expression in vitro.** OE 19 was from ECACC, Porton Down, Salisbury, Wiltshire, UK, OE 33 was from Sigma, St Louis, MO and Flo1A was a generous gift from Dr. Steven Hughes, University of Pittsburgh, Pittsburgh, PA. Cells were maintained at 37º C, 5% CO$_2$ in RPMI medium (Invitrogen, Carlsbad, CA) containing 7% FBS. siRNAs targeting CDK6 or CDK4 and control siRNA (an siRNA without homology to any human transcript was from Ambion (Austin, TX). siRNA sequence detail is provided in the supplementary material. 30nM total siRNA was transiently transfected into cells using RNAiMax reagent from Invitrogen as described by the manufacturer. When targeting both CDK4 and CDK6, 15nM of each
siRNA was used. The CDK4/6 inhibitor PD-0332991 was from Selleck Chemicals LLC (Houston, Texas).

**Monolayer and anchorage-independent growth assays.** Monolayer cell growth was assessed over 6 days by incubating 20 x 10³ cells per well in 12 well plates in RPMI media containing 7% FBS. Cells were counted (average of 4 wells per data point) in order to determine total cell number. Anchorage independent growth was assessed by colony formation in soft agar as described (13) (see supplementary material for details).

**Antibodies and western blot.** Antibodies for CDK6, CDK4, Cyclin A, total pRB, RB2 (p130), pRB phospho-serines 780 and 795 and Actin were from Cell Signaling (Danvers, MA). SDS-PAGE running and transfer for Western blots was as described (13). Protein bands were detected using SuperSignal (Pierce Chemical, Rockford, IL, USA).

**Cell Cycle analysis by fluorescence-activated cell sorting (FACS).** Cells (0.3 X 10⁶/well in 6-well plates) were treated with 125 nM of PD-0332991 for 72 hrs then trypsinized and fixed in 70% ethanol overnight at -20°C. Cells were resuspended in PBS containing 50 μg/ml propidium iodide (EMD, Gibbstown, NJ) and 100 Kunitz units/ml RNAse A (Fermentas, Glen Burnie, MD). Cell sorting was performed at the University of Rochester FACS core facility.
**Statistical analysis.** Significance of assays measuring growth rate, colony formation and association of gene expression with amplification status were determined using the Student t-test. Kaplan-Meier survival estimates were plotted for both overall survival (OS) and recurrence-free survival (RFS) based on 7q21 amplification status and gene expression (split at the median) and p-values were calculated using the log-rank test. Associations of gene expression (as a continuous variable) with survival were estimated using Cox proportional hazards regression. P-values were adjusted for false discovery by the method of Storey and Tibshirani (14). To test whether the clustering of significant CDK6 probesets with survival was a non-random observation, we conducted a permutation test for randomness. This test permuted the positions of the significant probesets among the 45 probesets across the amplicon. The combination of two genes, CDK4 and CDK6, was evaluated for association with survival by proportional hazards regression. A recursive portioning model was also constructed but not reported because results were similar to those of the proportional hazards model. A likelihood ratio test was used to evaluate whether the combination of CDK4 and CDK6 significantly improved a model with CDK6 alone. The proportional hazards models were illustrated (Figure 3D) with Kaplan Meier plots of overall survival that were constructed by splitting the log relative hazard at the median.
Results

Defining the 7q21 amplicon in EAC. Amplification of 7q21 was observed in 35% (40/116) of tumors (Figure 1A). While 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 (17/116) of these events were also high copy gains as indicated by the darker green in Figure 1A. Definition of specific boundaries is somewhat arbitrary but conservatively, the amplicon contains 17 genes (MTERF, AKAP9, CYP51A1, LOC401387, KRIT1, ANKIB1, GATAD1, PEX1, C7ORF64, MGC16142, FAM133B, CDK6, SAMD9, SAMD9L, HEPACAM2, CCDC132 and CALCR) with the minimal region of amplification (dotted line in Figure 1A) defined by only 6 genes (GATAD1, PEX1, C7ORF64, MGC16142, FAM133B and CDK6).

Association of 7q21 amplification with survival. In order 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 with increased risk of death (p=0.049) but no statistically significant association with disease recurrence (p=0.23: Figure 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 17, 7q21 candidate genes in amplified versus non-amplified tumors. We found significantly elevated expression (unadjusted \( p \leq 0.05 \) and false discovery rate \( \leq 0.10 \)) of at least one probeset for 13/17 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 \textit{CDK6} is also shown for CDK6-positive and CDK6-negative tumors in Figure 2B. Since 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, OS (blue lines above the threshold in Figure 2A) or recurrence-free survival, RFS (red lines above the threshold in Figure 2A) for thirteen probesets. However, associations with both OS and RFS were present for only seven probesets representing two genes: \textit{CDK6} and \textit{ANKIB1} (ankyrin repeat and IBR domain containing 1). All seven significant probesets for RFS and OS (2 for \textit{ANKIB1} and 5 for \textit{CDK6}) have a false discovery rate <10%. However, \textit{ANKIB1} is excluded from the minimal amplicon by one tumor where the proximal boundary is within the \textit{ANKIB1} gene (Figure 1A). Therefore, while we cannot rule out a potential role for \textit{ANKIB1} in 7q21 amplification and EAC, our data indicates that \textit{CDK6} is the major driver of this event and the subsequent prognostic implications. Five of the 7 probesets mapping to the \textit{CDK6} gene were associated with both OS and RFS 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 \textit{CDK6} clustering could be explained by random variation. Using 100000 positional permutations of the 45 probesets, 7 of which were associated with both OS and RFS, 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 by a median split of the expression levels of probeset X224848_at is shown (Figure 2C).

**Effect of CDK6 and CDK4 knock-down on growth of EAC cells.** To study CDK6 function in EAC we used siRNA transfection to knockdown expression in OE19, OE33 and Flo1A cells. In addition, we evaluated knockdown of the CDK6 homolog, CDK4 since these molecules are known to have functional redundancy. To avoid potential off target artifacts, two different siRNA sequences for each molecule were used. Western blot analysis revealed endogenous expression of CDK6 and CDK4 in all three cell lines tested and confirmed the almost complete silencing of CDK6 and CDK4 by both siRNAs (Figure 3A). Monolayer growth analysis showed that CDK6 knockdown reduced cell proliferation in OE19 and Flo1A but not in OE33 (Figure 3B), whereas CDK4 silencing reduced cell proliferation in all three cell lines. Targeting both molecules has an additive effect in suppressing cell proliferation. In order 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 cyclin-dependent kinases. As shown in Figure 3C, knocking down CDK6 in EAC cells reduced phosphorylation of pRB at serines 780 and 795 (both are known targets of CDK6/Cyclin D1 complex (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 knockdown, we explored CDK4 amplification and expression in our primary tumors. Amplification of the 12q13 region containing CDK4 was observed in 12/116 tumors (10%) and was associated with worse overall survival (p=0.019: supplementary Figure 1). Only 4 tumors demonstrate amplification of both CDK4 and CDK6. High expression of CDK4 was also found to correlate with poor prognosis (Figure 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 CDK4 to a model consisting of CDK6 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 RFS with CDK6 did not show similar improvement (likelihood ratio p=0.1859).

**The CDK inhibitor PD-0332991 reduces cell proliferation in EAC cells.** Since 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 three cell lines tested (Figure 4A) and, as with the siRNA knockdown of CDK6, this was associated with hypophosphorylation of pRB at serines 780 and 795 (Figure 4D). To confirm that the effects of PD-0332991 are through 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 panel), PD-0332991 reduces expression of Cyclin A in all three cell lines. PD-0332991 has no effect on the expression level of either total pRB or RB2 (p130) in EAC cells (Figure 4D, bottom panel).

PD-0332991 delays cell cycle progression at the G1/S boundary and is not cytotoxic to a variety of cancer cell lines (16, 17). to confirm the same effect in EAC cells, we performed 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 three cell lines tested there is a decrease in S/G2 population compared to G0/G1 five 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 three weeks in semisolid (0.4%) agar in the presence or absence of 125nM PD-0332991. As shown in Figure 4E, PD-0332991 caused a significant reduction in colony formation in soft agar in all three cell lines tested indicative of a reduction of 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 which 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 EGFR (21), PIK3CA (22), VEGF (23) c-MYC (19) and MET (24). In tumors such as EAC where 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), gliomas (26) and in medulloblastoma, where CDK6 expression was associated with poor prognosis (27). Chromosome 7q and 7q21 amplification has 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 CGH 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 in order 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 six genes, identified prognostic implications of both 7q21 amplification and CDK6 gene expression and demonstrated 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 homolog of CDK6. Despite its low frequency (10%), 12q13 amplification correlated with poor survival (supplementary Figure 1). 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 early G1 cyclin-dependent kinase activity. We thus provide a rationale for testing of CDK4/6 inhibitors such as PD-0332991 in clinical trials for this cancer.

CDK6 is a serine/therionine kinase whose activity is dependent on binding to D-type cyclins (Cyclin D1, D2 and D3) and, like CDK4, it phosphorylates and inhibits the pRB tumor suppressor allowing cell cycle progression during the mitogen-dependent early G1-phase (reviewed in (28)). Cyclin D1-CDK4/6 activity is inhibited by binding to the cyclin-dependent kinase inhibitors of the INK4 family that includes p16/INK4A. The deregulation of the cell cycle control pathway (D-type cyclins-CDK4/6-pRB) is reported in most, if not all, human cancers (29, 30). This includes overexpression of cyclins (D1 and E1 in particular(31)) and loss of CDK inhibitors (INK4A, INK4B and KIP1) and pRB...
Aberrant expression of these cell cycle regulators has been observed in many tumors including EAC as a result of chromosomal amplification, e.g. cyclin D1 (29), CDK4 (29), CDK6 (25, 26, 29), cyclin E1 (32) or deletion e.g. INK4A and B (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 microRNA’s 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). However this study showed that CDK6 expression was reduced in breast tumors compared to 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 (Figures 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 (Figure 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 knock down 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 of phosphorylation of pRB at Ser780 and 795 when CDK6 is knocked down is less in OE33 compared to OE19 and Flo1A (Figure 3C), and third, PD-0332991 (which specifically inhibits both CDK4 and 6 kinase activity (16, 17)) significantly inhibits proliferation of OE33 cells (Figure 4A). Such compensatory mechanisms among CDKs and also D-type cyclins have previously been suggested to account for several phenotypes resulting from the knockout of these molecules in vitro and in vivo (28, 38, 39). For example, there is evidence that both CDK4 and CDK6 are not required for proper cell cycle progression in vivo. CDK6 knockout mice are viable and exhibit slight hematopoietic impairment (38) while CDK4/CDK6 double knockout embryos die at embryonic day E18.5 due to severe anemia (38). Our data further suggests that CDK4 and CDK6 may be functionally redundant in their impact on patient survival in EAC.

The small molecule PD-0332991 is a specific and reversible inhibitor of CDK4/6 kinase activity (16, 17). When used as a single agent, PD-0332991 is cytostatic causing inhibition of proliferation without remarkable apoptosis in responsive cell lines and its function can be monitored through reduced pRB phosphorylation at serine 780 and 795 (16, 17). Alteration of expression of total pRB or the other member of the retinoblastoma family RB2 (p130) 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
(Figure 4A and B) and anchorage independence caused by PD-0332991 (Figure 4D and E) and the parallel cell cycle blockage (Figure 4B) and hypophosphorylation of pRB (Figure 4C) 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 where it has proven to be well tolerated (41, 42). Furthermore, the use of cyclin-dependent kinase 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.

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References:


**Figure legend:**

**Figure 1:** A. Figure showing 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.

**Figure 2:** A. Comparison of the expression level (amplified versus non-amplified tumors) and association with overall (OS) and recurrence-free survival (RFS) 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 non amplified tumors (black lines), Cox regression p -values for the association of gene expression with overall survival (blue lines) and with recurrence-free survival (red lines). 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 in amplified versus non-amplified tumors for 6 out of 7 different CDK6 probesets in U133 microarray (*, p=0.022, **, p=0.001, ***, p<0.0001). Note that probeset 231198_at seems to be non-responsive. **C.** Kaplan Meier plots of overall and recurrence-free survival by a median split of the expression levels of CDK6 based on probeset X224848_at. Log rank test p-values are shown for each plot.

**Figure 3:** **A.** Western blot analysis confirming efficient knock down of CDK6 and CDK4 after transfection of two different siRNA targeting each molecule in OE19, OE33 and Flo1A cell lines. Cnt corresponds to transfection of a control siRNA targeting no known coding sequence in the human genome. **B.** Cell proliferation 6 days post siRNA transfection. Average of 3-4 independent experiments in triplicates ± SEM. *, p<0.05, **, p<0.01, ***, p<0.001. **C.** Phosphorylation status of pRB at serines 780 and 795 three days after siRNA transfection. Total pRB (RB1) and Actin are loading controls. **D.** Kaplan Meier plots showing overall survival of high and low risk groups formed by a median split of the predicted log relative hazard for three models, CDK4 alone, CDK6 alone and CDK6 + CDK4. The hazard ratio of the two survival curves are shown along with the p value of a log rank test. Expression levels are based on probe set X224848_at for CDK6 and 202246_s_at, the only probe set for CDK4 in the U133 microarray.

**Figure 4:** **A.** PD-0332991 causes inhibition of proliferation of EAC cells in a dose-dependent manner. The three EAC cell lines were incubated for 5 days in the presence or absence (Cnt) of the indicated molar concentration of PD-0332991. N=3, ±SEM, *,
p≤0.05, **, p≤0.05, ***, p<0.001. **B.** FACS analysis of cell cycle progression in OE19, OE33 and Flo1A after 5 days incubation with 125 nM PD-0332991. **C.** Averaged percent of cells in S/G2 phase in the presence and absence of PD-0332991. **D.** Western blot showing the phosphorylation status of pRB at Ser780 and Ser795 after incubation with 125nM PD-0332991 for 24 and 72 hrs. Reduction in Cyclin A expression and the expression of RB2 (p130) tumor suppressor 72 hrs after PD-0332991 treatment are shown (bottom panel). Total pRB (RB1) and Actin are loading controls and T293 cell lysate (293) is a positive control for total pRB and phospho pRB. **E.** Anchorage-independent growth of the three cell lines in the presence or absence (Cnt) of 125nM PD-0332991 for 3 weeks. N=3, ±SEM, **, p=0.0016, ***, p<0.0001.
Figure 1

A

Genes in the amplicon: MTERF, AKAP9, CYP51A1, LOC401387, KRIT1, ANKIB1, GATAD1, PEX1, C7ORF64, MGC16142, FAM133B, CDK6, SAMD9, SAMD9L, HEPACAM2, CCDC132, CALCR

B

p = 0.0489

p = 0.2354

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Figure 2

A. Gene expression levels for various genes after esophagectomy.

B. Core Amplicon diagram showing gene expression levels.

C. Proportion surviving without recurrence and p-values for expression levels.

- **Expression**
- **OS**
- **RFS**

- **Non Amplified**
- **Amplified**

- **p = 0.012**
- **p = 0.0024**
Figure 3

A

OE19  OE33  Flo1A  OE19  OE33  Flo1A
Cnt si  +  -  +  +  +  -  +  +  +  +  +  +  +  +  +  -  +  +  +  +  +  +  +  +
CDK6,si1  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
CDK6,si2  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
CDK6   +  -  +  -  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +
Actin   +  -  +  -  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +

B

%Cell proliferation (Control=100%)

OE19  OE33  Flo1A
Cnt si  +  -  +  +  +  -  +  +  +  +  +  +  +  +  +  -  +  +  +  +  +  +  +  +
CDK6,si1  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
CDK6,si2  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
CDK4   +  -  +  -  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +
Actin   +  -  +  -  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +

C

OE33  OE19  Flo1A
Cnt si  +  -  +  +  +  -  +  +  +  +  +  +  +  +  +  -  +  +  +  +  +  +  +  +
CDK6,si1  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
CDK4,si1  Ph-RB (5795) Ph-RB (5740) RB1
OE19  OE33  Flo1A
Cnt si  +  -  +  +  +  -  +  +  +  +  +  +  +  +  +  -  +  +  +  +  +  +  +  +
CDK6,si1  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

D

CDK6 Alone

Hazard Ratio = 1.8
p = 0.029

CDK4 Alone

Hazard Ratio = 1.9
p = .0067

CDK6 + CDK4

Hazard Ratio = 2.1
p = 0.0008
Figure 4

A

Number of cells X 1000

OE19   OE33   Flo1A

0.125 μM

B

OE19   OE33   Flo1A

0.25 μM

C

Cells in S/G2 (%)

OE19   OE33   Flo1A

0.5 μM

D

PD-0332991

OE33   OE19   Flo1A

Ph-RB (S795)

Ph-RB (S780)

RB1

Actin

Ph-RB (S795)

Ph-RB (S780)

RB1

Actin

CyclinA

RB2

Actin

E

OE19

OE33

Flo1A

24 hrs

72 hrs

Number of colonies

OE19   OE33   Flo1A

PD-0332991 0.125 μM

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Early G1 cyclin-dependent kinases as prognostic markers and potential therapeutic targets in esophageal adenocarcinoma

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