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Overexpression of major *CDKN3* transcripts is associated with poor survival in lung adenocarcinoma

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Background: The cyclin-dependent kinase inhibitor 3 (*CDKN3*) has been perceived as a tumour suppressor. Paradoxically, *CDKN3* is often overexpressed in human cancer. It was unclear if *CDKN3* overexpression is linked to alternative splicing variants or mutations that produce dominant-negative *CDKN3*.

Methods: We analysed *CDKN3* expression and its association with patient survival in three cohorts of lung adenocarcinoma. We also examined *CDKN3* mutations in the Cancer Genome Atlas (TCGA) and the Moffitt Cancer Center's Total Cancer Care (TCC) projects. *CDKN3* transcripts were further analysed in a panel of cell lines and lung adenocarcinoma tissues. *CDKN3* mRNA and protein levels in different cell cycle phases were examined.

Results: *CDKN3* is overexpressed in non small cell lung cancer. High *CDKN3* expression is associated with poor overall survival in lung adenocarcinoma. Two *CDKN3* transcripts were detected in all samples. These *CDKN3* transcripts represent the full length *CDKN3* mRNA and a normal transcript lacking exon 2, which encodes an out of frame 23-amino acid peptide with little homology to *CDKN3*. *CDKN3* mutations were found to be very rare. *CDKN3* mRNA and protein were elevated during the mitosis phase of cell cycle.

Conclusions: *CDKN3* overexpression is prognostic of poor overall survival in lung adenocarcinoma. *CDKN3* overexpression in lung adenocarcinoma is not attributed to alternative splicing or mutation but is likely due to increased mitotic activity, arguing against *CDKN3* as a tumour suppressor.

The human *CDKN3* gene encodes the cyclin-dependent kinase inhibitor 3, which is a dual specificity protein tyrosine phosphatase of the CDC14 group. *CDKN3* binds cyclin-dependent kinases CDK1 and CDK2 (Hannon *et al*, 1994) and dephosphorylates their activating loop Thr residues (Poon and Hunter, 1995; Brown *et al*, 1999; Song *et al*, 2001). Dephosphorylation of the activating Thr site reduces substrate and ATP binding activities of the CDKs

(Brown *et al*, 1999). Thus, *CDKN3* is a negative regulator of CDK1 and CDK2.

Since CDK-driven cell cycle is essential for proliferation of cancer cells and *CDKN3* inhibits CDK activities, *CDKN3* has been perceived as a tumour suppressor (Yeh *et al*, 2000; Yu *et al*, 2007; Nalepa *et al*, 2013). Paradoxically, *CDKN3* is often overexpressed in various human cancer tissues and cancer cell lines

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(Lee *et al*, 2000; Yu *et al*, 2007; Espinosa *et al*, 2013). Lee *et al* (2000) found overexpression of *CDKN3* mRNA and protein in breast and prostate cancer. Inhibition of *CDKN3* expression by antisense *CDKN3* suppressed anchorage-independent colony formation *in vitro* and tumour xenograft growth *in vivo* (Lee *et al*, 2000), suggesting that increased *CDKN3* expression in cancer cells plays a positive role in the transformed phenotypes. Yu *et al* (2007) found *CDKN3* mRNA overexpression in astrocytoma and that elevated *CDKN3* mRNA level was significantly associated with shorter patient survival. More recently, Espinosa *et al* (2013) identified *CDKN3* as one of the six most upregulated mitosis pathway genes in cervical cancer. Moreover, *CDKN3* overexpression is linked to the poor survival of these cervical cancer patients (Espinosa *et al*, 2013).

However, based on the perception that *CDKN3* is a potential tumour suppressor, a possible explanation of *CDKN3* mRNA overexpression in cancer is the presence of dominant-negative *CDKN3* mutations, which could not be distinguished from the normal *CDKN3*-encoding mRNA in the gene expression data. Hence, *CDKN3* overexpression may actually result in overexpression of dominant-negative *CDKN3* mutants. It was reported that aberrant *CDKN3* transcripts from alternated splicing, insertion/deletion and nonsense mutations were found in hepatocellular carcinoma (Yeh *et al*, 2000; Yeh *et al*, 2003). In another study (Yu *et al*, 2007), two *CDKN3* transcripts (variant a and c) were identified in all brain samples and two additional transcripts (variants b and d) were found in some glioblastoma samples. Variant a encodes the full length 212-amino acid *CDKN3*. Variants b–c resulted from alternative splicing around exons 2–3. Variant c is an exon 2 skip transcript that results in a short 23-amino acid peptide with little sequence homology to *CDKN3*. Variant b results in a short 8-amino acid peptide. Variant d lacks a part of exon 2 and the entire exon 3, resulting in a 179-amino acid protein. None of variant b–c products has been documented to have any biological activity. Thus, whether *CDKN3* gene overexpression in cancer is linked to alternative splicing or mutations that generate dominant-negative *CDKN3* remains to be further assessed.

Alternatively, the concept that *CDKN3* is a tumour suppressor may be inaccurate. While CDKs drive cell cycle, CDK activities oscillate along the cell cycle. This involves not only CDK activation but also CDK deactivation. Indeed, constitutive CDK2 activation by preventing Thr14/Tyr15 phosphorylation causes problems in DNA replication and genome integrity (Hughes *et al*, 2013). A decrease in CDK1-cyclin B activity is required for mitotic exit (Wurzenberger and Gerlich, 2011; Nalepa *et al*, 2013). Thus, decreasing CDK activity may be just as important as increasing CDK activity in coordinated regulation of cell cycle. Consistent with this notion, knockdown of *CDKN3* leads to mitotic failure (Nalepa *et al*, 2013). Therefore, an alternative explanation of *CDKN3* overexpression in human cancer is that *CDKN3* is important for the safe passage through cell cycle and the elevated *CDKN3* level is attributed to the higher mitotic activity in cancer cells.

In this study, we present evidence that *CDKN3* is overexpressed in human NSCLC and higher *CDKN3* expression level is associated with poor survival of lung adenocarcinoma patients. We detected the full length and the exon 2 skip *CDKN3* transcripts in all cell lines and lung tumour tissues and found no evidence of change in alternative splicing. Furthermore, *CDKN3* mutations are rare in TCGA tumours and in the TCC data set of 3383 tumours. Interestingly, we found that *CDKN3* expression is upregulated during the mitosis (M)-phase of cell cycle in cultured cells.

MATERIALS AND METHODS

Reagents, cells and tissue samples. Monoclonal anti-*CDKN3* antibody 2H10 was purchased from Abcam (Cambridge, MA,

USA). Monoclonal antibody No. 61033410 was from BD Biosciences (San Jose, CA, USA). Rabbit monoclonal antibody (D2C8) to phospho-histone H3 Ser10 (pHH3) was from Cell Signaling Technology (Danvers, MA, USA). Anti-actin antibody was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Thymidine and nocodazole were from Sigma (St Louis, MO, USA).

Lung cancer cell line and hTBE/v cells have been maintained in a central repository at the Moffitt Cancer Center since 2008. All cell lines in the Moffitt repository had been authenticated by STR analysis (ACTG Inc, Whelming, IL, USA) as of September 2010, and all cells had been routinely tested and were negative for mycoplasma (PlasmoTest, InvivoGen, San Diego, CA, USA). BEAS2B cells were from American Type Culture Collection and cultured according to the supplier's instruction. The primary human endothelium (HUVEC) cells were obtained from Sarah Yuan at the University of South Florida. Other cell lines were from the laboratories of Jin Cheng or Kenji Fukasawa at the Moffitt Cancer Center or have been reported (Ren *et al*, 2010; Ren *et al*, 2013).

Patient data and samples. All work was approved by the University of South Florida Institutional Review Board. Gene expression and survival data from a cohort of 398 lung cancer patients diagnosed with adenocarcinoma recruited from Moffitt Cancer Center's TCC (Fenstermacher *et al*, 2011) between April 2006 and August 2010 were included in this study. This large lung adenocarcinoma cohort has been extensively characterised by Moffitt's Lung Cancer Center for Excellence will be described in detail elsewhere. Briefly, RNA isolated from fresh frozen lung tumours were profiled using a custom Affymetrix GeneChip (Affymetrix, Santa Clara, CA, USA) that measured the expression of ~60 000 distinct transcripts. CEL files were normalised against their median sample using IRON (Welsh *et al*, 2013). An RNA-quality-related batch effect was identified in the resulting normalised data, which was removed by training a partial least squares (PLS) model (Wold *et al*, 1984) to the BioAnalyzer BA_RIN RNA quality metric (Agilent Technologies, Santa Clara, CA, USA), then subtracting the first PLS component. Survival analyses were performed to determine if *CDKN3* expression levels were associated with overall survival using Kaplan–Meier survival curves and the log-rank test. Overall survival was right-censored at 5 years and was calculated from the date of diagnosis until the date of last follow-up or death.

RNA was isolated from six de-identified RNA samples from this cohort RNA from freshly frozen tumour tissue macrodissected to contain >70% tumour tissue and RNA was extracted using the manufacturer's instructions for the Qiagen RNeasy Kit (Valencia, CA, USA) by Moffitt's Tissue Core. The isolated RNA was quantified using Nanodrop (Thermo Scientific, Wilmington, DE, USA) and Qubit instrument (ThermoFisher Scientific, Waltham, MA, USA).

Plasmids. A *CDKN3* lentiviral expression vector was constructed by cloning the *CDKN3* coding sequence (identical to *CDKN3* coding sequence of Genbank entry NM005192) from HCC827 cells into Pme I site of the pWPI lentiviral vector obtained from Addgene (Cambridge, MA, USA). pLKO.1-based *CDKN3* shRNA lentiviral vectors TRCN0000002525 and TRCN0000002527, and a non-target shRNA were obtained from Sigma. Lentiviruses were prepared similar to that described previously (Ren *et al*, 2010).

RT-PCR and RT-qPCR. Total RNA from cells were extracted using Trizol reagent (Life Technologies, Benicia, CA, USA). RT-PCR was performed using SuperScript One-Step RT-PCR Platinum Taq system (Life Technologies) with 50 ng RNA and one of the following primer pairs CK1/CK1R, CK2/CK2R, CK3/CK3R or CK1/CK3R (See Supplementary Information). RT-PCR products were separated on agarose gel, purified and re-amplified by

PCR. PCR products were cleaned with ExoSAP-IT PCR product cleanup reagent (Affymetrix) and sequenced in both strains using BigDye Terminator 3.1 cycle sequencing kit (Life Technologies) as described (Ren *et al*, 2013). Quantitative RT-PCR (RT-qPCR) was performed using CK2/CK2R or CK-T/CK-R primer pairs (Supplementary Information). RT-qPCR was performed using Power SYBR Green reagents (Applied Biosystems, Foster City, CA, USA). Samples were assayed in triplicates as described (Schneeberger *et al*, 2014).

Cell culture, synchronization and immunoblotting. Lung cell lines A549, H2172, H3122, H2228, H1975, HCC827, H3255 and BEAS2B were cultured in RPMI 1640/10% foetal bovine serum (FBS). HeLa, PC-3, PANC-1, U2OS, HCA2, SW480, MCF7 and HepG2 cell lines were cultured in Dulbecco's modified Eagle's medium/10% FBS. HUVEC cells were cultured in endothelial cell growth medium (Lonza, Allendale, NJ, USA). hTBE/v cells were cultured in BEBM with additives (Lonza). Cells were maintained in 5% CO₂ humidified atmosphere at 37 °C.

To enrich cells in G₀/early G₁ phase, cells were serum starved for 48 h. For double thymidine block to enrich cells in later G₁/S phase, cells (30% confluent) were treated with 2 mM thymidine for 18 h. The cells were washed with PBS, incubated with normal cell culture medium for 9 h and then treated with 2 mM thymidine for another 16 h. For nocodazole blockage to enrich M phase cells, cells (80–90% confluent) were treated with 100 ng ml⁻¹ nocodazole for 12 h. After removing medium, round and loosely attached mitotic cells were dislodged by shaking, suspended in medium and collected by low speed centrifugation.

Cell lysates were prepared similar to that described (Ren *et al*, 2013; Schneeberger *et al*, 2014; Schneeberger *et al*, 2015) by lysis in cold lysis buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25 mM NaF, 5 mM sodium pyrophosphate, 1 mM Na₃VO₄, 2 µg ml⁻¹ aprotinin, 2 µg ml⁻¹ leupeptin, 100 µg ml⁻¹ phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 20 mM p-nitrophenyl phosphate, 1% Triton X-100). Cell lysate supernatants were obtained by microcentrifugation (16 000 r.p.m., 10 min at 4 °C). Equal amounts of proteins were separated on 12 or 15% SDS-polyacrylamide gels, transferred to nitrocellulose filters and analysed by immunoblotting with indicated antibodies.

CDKN3 knockdown and cell proliferation assay. Lentiviruses were prepared as described (Ren *et al*, 2010) and used to infect A549 and HeLa cells. Infected cells were selected by puromycin. Puromycin-resistant cells were pooled and analysed. Cell proliferation was assayed as described (Ren *et al*, 2010) in 96-well plate in triplicates. Relative viable cells were measured using Cell TiterGlo reagent (Promega, Madison, WI, USA). Statistical analysis was performed using the nonparametric Mann-Whitney test. $P < 0.05$ was considered significantly different.

RESULTS

CDKN3 is overexpressed in NSCLC. By examination of public databases, we noticed that *CDKN3* is often overexpressed in various tumours (Supplementary Figure 1), including lung cancer. To explore the expression of *CDKN3* mRNA in NSCLC, we first compared *CDKN3* expression in NSCLC tumours and matched normal tissues using data from GSE19188 (Hou *et al*, 2010) as previously described (Engel *et al*, 2013). The Affymetrix U133 plus 2.0 microarrays (Affymetrix) data in GSE19188 contain 87 tumour samples and 58 adjacent normal tissue samples (Figure 1A). The log₂ *CDKN3* expression level was 4.345 ± 0.534 (normal tissues, mean \pm s.d.), 7.048 ± 1.104 (adenocarcinoma), 7.617 ± 1.461 (large cell carcinoma) and 8.000 ± 0.683 (squamous cell carcinoma). Thus, *CDKN3* expression was significantly elevated in all three histological subtypes of NSCLC ($P < 0.0001$). We then expanded

the analysis to a larger cohort of TCGA RNA-seq expression data that contain 490 lung adenocarcinoma, 491 lung squamous cell carcinoma and 108 of their matched normal tissues (Figure 1B). Again, *CDKN3* expression in both adenocarcinoma and squamous cell carcinoma was statistically ($P < 0.0001$) higher than in the matched normal tissues.

It was reported that *CDKN3* mRNA and protein were overexpressed in breast and prostate cancer cell lines (Lee *et al*, 2000). To determine if *CDKN3* expression is also elevated in lung cancer cell lines, we performed RT-qPCR analysis of *CDKN3* mRNA in the immortalised human lung epithelial cell line (hTBE/v)

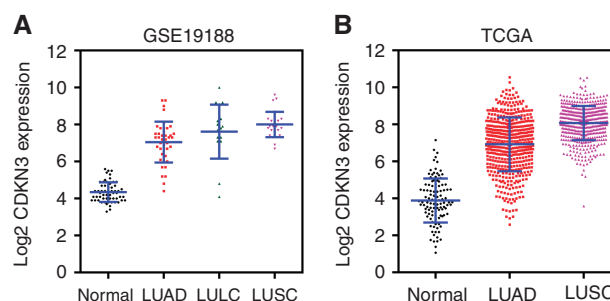


Figure 1. *CDKN3* expression is elevated in NSCLC. (A) *CDKN3* expression in the GSE19188 data set of NSCLC tumour and available adjacent normal samples (Engel *et al*, 2013). LUAD, lung adenocarcinoma. LULC, lung large cell carcinoma. LUSC, lung squamous cell carcinoma. Gene expression was determined via microarray for *CDKN3* using probe 209714_s_at. The horizontal lines are the mean + s.d. of each group of samples. (B) *CDKN3* expression in LUAD, LUSC and matched normal tissues from TCGA. The normal group is the combined data from the matched normal of LUAD and LUSC. The difference between the matched normal of LUAD and LUSC was not statistically significance.

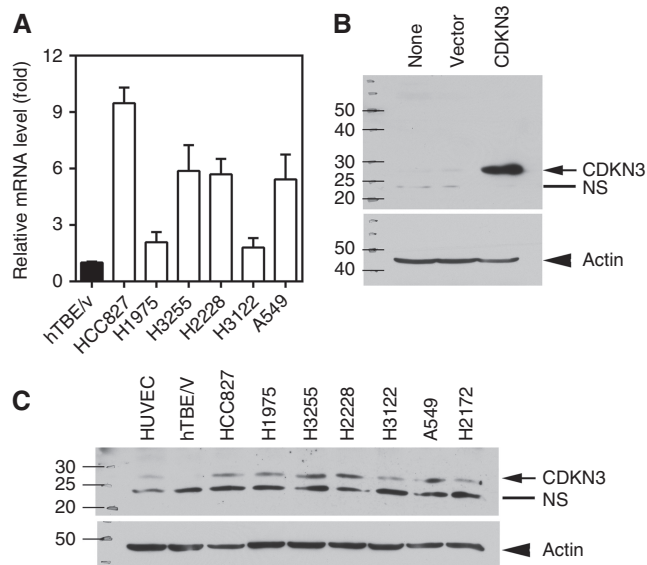


Figure 2. Expression of *CDKN3* mRNA and protein in lung cell lines. (A) RNA was isolated from indicated cells and the relative amounts of *CDKN3* mRNA were determined by RT-qPCR using primers CK2/CK2R. The *CDKN3* mRNA level in hTBE/v cells was arbitrary set as 1. (B) HEK293 cells were transfected with empty or *CDKN3*-encoding lentiviral vector (plasmid). Cell lysates from non-transfected and transfected cells were analysed with antibodies 2H10 or actin. NS, a non-specific reactive band. (C) Cell lysates from indicated cells were analysed by immunoblotting with *CDKN3* antibody 2H10.

and six lung adenocarcinoma cell lines. Compared with hTBE/v cells, higher levels of CDKN3 mRNA were detected in these lung cancer cell lines (Figure 2A).

The identity of CDKN3 protein on immunoblots has been questioned (Yu *et al*, 2007). To correctly identify CDKN3 protein, we cloned the CDKN3 protein coding sequence from HCC827 cells and expressed the non-tagged, 212-amino acid CDKN3 protein coding cDNA (identical to NM_005192.3) in HEK293 cells. Cell lysates from non-transfected cells, empty vector-transfected cells and CDKN3 expression vector-transfected cells were analysed with an anti-CDKN3 mouse monoclonal antibody 2H10 (Figure 2B) or another mouse monoclonal antibody (BD cat. No. 610334, Supplementary Figure 2). Antibody 2H10 correctly detected CDKN3 at 27 kDa and also reacted with a non-specific bands at 23 kDa. Antibody No. 610334 reacted with a 34-kDa band in all three cell lysates (Supplementary Figure 2). Thus, the 27-kDa band reacting with antibody 2H10 is CDKN3. Additional evidence to support the identification of the 27-kDa band as CDKN3 was obtained by knocking down endogenous CDKN3 in cell lines with shRNAs (see below). After establishing the identity of CDKN3 in immunoblots, we probed lung cell lysates for CDKN3 protein. Figure 2C shows that higher levels of CDKN3 protein were present in lung cancer cell lines.

CDKN3 overexpression is associated with poor overall survival in lung adenocarcinoma. We first analysed two public lung adenocarcinoma databases to determine the relationship between CDKN3 expression in tumours and patients outcome in terms of overall survival. The microarray-based Molecular Classification of Lung Adenocarcinoma (MCLA) from the Director's Challenge Consortium contains 478 patients (Shedden *et al*, 2008) (Figure 3A). The RNA-seq-based lung adenocarcinoma cohort from TCGA contains 381 patients (Figure 3B). In both cohorts, CDKN3 expression was prognostic. The overall survival rates for patients with high CDKN3 levels (above the median) were significantly lower than those with low CDKN3 levels (below the median (P -values of 0.0046 and 0.0059, respectively)). We also examined the 390 patient lung squamous cell carcinoma cohort from TCGA. Unlike the lung adenocarcinoma cohorts, CDKN3 expression was not significantly prognostic in the TCGA lung squamous cell carcinoma cohort (Figure 3C). However, comparing with lung adenocarcinoma, lung squamous cell carcinoma has an overall significantly ($P < 0.0001$) higher CDKN3 transcript level (Figure 1). Thus, the 'low CDKN3' expression levels in lung squamous cell carcinoma are very high already.

Next, we examined CDKN3 expression in a cohort of 398 lung adenocarcinomas developed at the Moffitt Cancer Center.

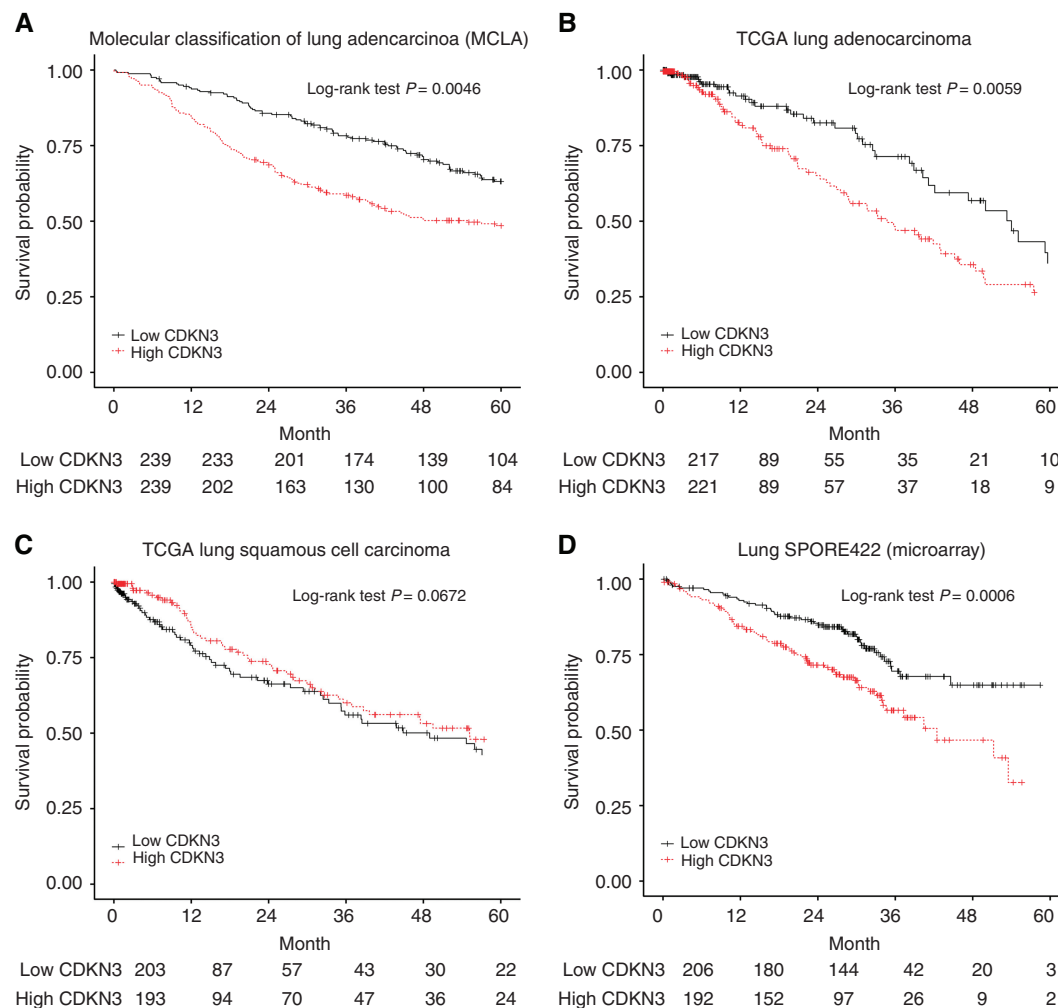


Figure 3. CDKN3 expression is highly prognostic in three lung adenocarcinoma cohorts. CDKN3 mRNA level was correlated with patient outcome by dichotomizing patients into low and high expression groups based on the median expression value. The overall survival of the two CDKN3 groups was compared by Kaplan–Meier estimate and two-sided log-rank test. CDKN3 mRNA and patient survival data were from (A) MCLA, (B, C) TCGA, (D) Moffitt's Lung SPORE 422.

Figure 3D shows that the Lung SPORE422 patients with high *CDKN3* levels had significantly shorter overall survival than those with lower *CDKN3* levels ($P < 0.0006$). No significant differences in *CDKN3* mRNA levels were found among different stages of lung adenocarcinoma (Supplementary Figure 3A and B). Because more of stage I patient survival and *CDKN3* mRNA data were available than other stages of lung adenocarcinoma, we next analysed those stage I patients whose survival data are available ($n = 254$). Again, among stage I patients, those with high *CDKN3* levels had a shorter survival probability (Supplementary Fig. 3C). Taken together, these

data indicate that high *CDKN3* levels are associated with poor overall survival in lung adenocarcinoma patients.

Two *CDKN3* transcripts are present in normal and tumour cells and lung tumour tissues. We designed RT-PCR primer pairs CK1/CK1R located in exons 1 and 5 (Figure 4) that allowed us to detect both the full length *CDKN3* transcript and splicing variants between exons 2–5 reported previously by other investigators (Yeh *et al*, 2000; Yu *et al*, 2007). RNA from primary HUVEC cells, 3 non-transformed, immortalised cell lines and 14 cancer cell lines

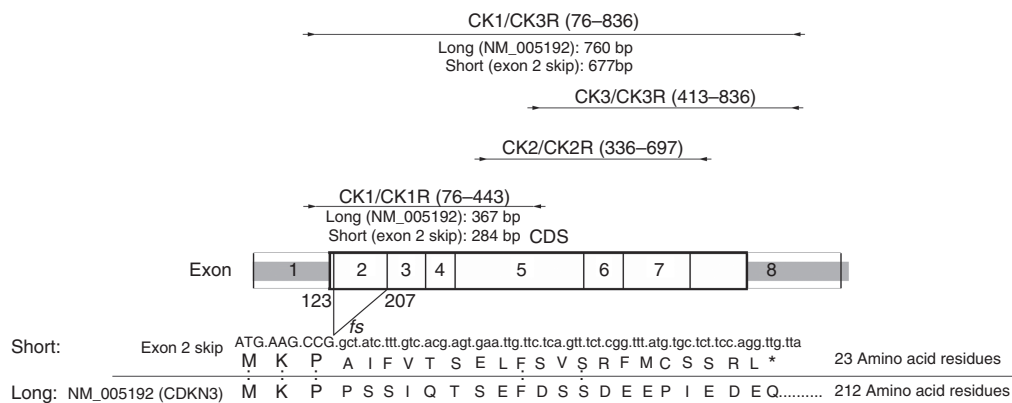


Figure 4. Schematic presentation of *CDKN3* transcripts, exon boundaries and PCR primers. The *CDKN3* mRNA (Genebank NM_005192.3) is shown. Heavy box indicates the protein coding sequence. Exons 1–8 are according to the notation of Genebank NM_005192.3. The amino acid sequence encoded by the short (exon 2 skip) splicing variant is shown and aligned with the long, *CDKN3*-encoding transcript. fs, frame shift. Also shown are locations of three pairs of PCR primers.

Table 1. List of cells and tissues analysed for <i>CDKN3</i> transcripts		PCR primer pairs			
Sample	Description	CK1/CK1R (long)	CK1/CK1R (short)	CK2/CK2R	CK3/CK3R
Non-cancer cells^a					
HUVEC	Primary endothelium	Identical	Exon 2 skip ^b	Identical	Identical
HCA2	Forskin fibroblasts	Identical	Exon 2 skip	Identical	Identical
hTBE	Tracheobronchial epithelium	Identical	Exon 2 skip	Identical	Identical
BEAS2B	Bronchial epithelium	Identical	Exon 2 skip	Identical	Identical
Cancer cells^a					
A549	Lung adenocarcinoma	Identical	Exon 2 skip	Identical	Identical
H2172	Lung adenocarcinoma	Identical	Exon 2 skip	Identical	Identical
HCC827	Lung adenocarcinoma	Identical	Exon 2 skip	Identical	Identical
H1975	Lung adenocarcinoma	Identical	Exon 2 skip	Identical	Identical
H3255	Lung adenocarcinoma	Identical	Exon 2 skip	Identical	Identical
H3122	Lung adenocarcinoma	Identical	Exon 2 skip	Identical	Identical
H2228	Lung adenocarcinoma	Identical	Exon 2 skip	Identical	Identical
MCF7	Breast adenocarcinoma	Identical	Exon 2 skip	Identical	Identical
HeLa	Cervical adenocarcinoma	Identical	Exon 2 skip	Identical	Identical
SW480	Colorectal adenocarcinoma	Identical	Exon 2 skip	Identical	Identical
HepG2	Hepatocellular carcinoma	Identical	Exon 2 skip	Identical	Identical
PANC-1	Pancreatic carcinoma	Identical	Exon 2 skip	Identical	Identical
PC-3	Prostate adenocarcinoma	Identical	Exon 2 skip	Identical	Identical
U-2 OS	Osteosarcoma	Identical	Exon 2 skip	Identical	Identical
Lung tumour tissues					
#10	Primary lung cancer	Identical	Exon 2 skip	Identical	Identical
#42	Primary lung cancer	Identical	Exon 2 skip	Identical	Identical
#3	Primary lung cancer	Identical	Exon 2 skip	Identical	Identical
#16	Primary lung cancer	Identical	Exon 2 skip	Identical	Identical
#20	Primary lung cancer	Identical	Exon 2 skip	Identical	Identical
#41	Primary lung cancer	Identical	Exon 2 skip	Identical	Identical

Abbreviations: *CDKN3* = cyclin-dependent kinase inhibitor 3; HUVEC = human endothelium.
^aExcept HUVEC, other cells are established cell lines.
^bThis transcript has been deposited in Genebank (accession number: KP966095).

were prepared (Table 1). The *CDKN3* transcripts were amplified by RT-PCR. Two RT-PCR products were detected in all samples (Figure 4 and Supplementary Figure 4). These two PCR products were isolated from gels, re-amplified by PCR using the same primers and then sequenced. cDNA sequencing data showed that both the long and the short PCR fragments from all samples were identical (Figure 4, Table 1, and Supplementary Figure 5). The long form is the *CDKN3*-encoding mRNA identical to NM_005192.3. The short RT-PCR product from the CK1/CK1R primer pairs yields a 284-bp fragment resulting from exon 2 skip (equivalent to variant c reported in the study by Yu *et al* (2007), Figure 4). This Exon 2 skip variant results in a frameshift that is predicted to encode a 23-amino acid peptide with little sequence homology to *CDKN3* (Figure 4 and Supplementary Figure 5). No alternative splicing variant in the boundary of exon 2 was detected by cDNA sequencing. No other smaller RT-PCR product, as would be predicted from a longer excision of the mRNA by alternative splicing, was detected. Also, no point mutation was detected. To further evaluate the possibility of the existence of other alternative splicing variants, we used primer pairs CK1/CK3 that cover the entire coding sequence to do RT-PCR. Again, we detected only two PCR products that correspond to the *CDKN3* and the exon 2 skip form. To explore the possibility of point mutation in regions not covered by CK1/CK3 primers, we performed RT-PCR using primer pairs CK2/CK2R and CK3/CK3R (Figure 4), both of them yield a single, predicted PCR product in all samples. No mutation was found in the sequencing data of these PCR products. Next, we obtained mRNAs from six lung adenocarcinoma tissues. The mRNAs from these tissue samples were analysed as above. Data from these lung tumour samples were identical to those obtained from cell lines (Table 1). Taken together, these results show that two *CDKN3* transcripts are present in cells and tissues that we have analysed. No mutation or alternative splicing is found in our cohort of cancer cells and tissues.

CDKN3 gene mutations are rare in human cancer. To further assess the possibility of *CDKN3* mutations in human cancer, we examined the massively parallel sequencing data set of 3383 tumour tissues from 48 tumour types in the TCC project (Fenstermacher *et al*, 2011; Ren *et al*, 2013). Twenty-three *CDKN3* mutation cases were found in 11 tumour types (Table 2). No disruptive changes (frameshift insertion/deletion, nonsense mutation, splice site mutation) were found. Of these 23 mutations, 13 mutations at 3 unique positions of Q8R, I108V and T127I were observed in either 1000 Genomes (Genomes Project C *et al*, 2012) or Exome Sequencing Project (Exome Variant Server, <http://evs.gs.washington.edu/EVS/>, accessed February 2015) of healthy people, and are likely inherited variants. They are all rare, and none were observed in the homozygous state. Therefore, few mutations are observed in this gene: at most 10 in 3383 individual across many tumour types. In addition, there are low levels (1–2%) of *CDKN3* gene amplification in TCGA lung adenocarcinoma.

We next extend the examination to lung adenocarcinoma in the TCGA research network (Cancer Genome Atlas Research N, 2014) and the Cancer Cell Line Encyclopedia (CCLE) (Barretina *et al*, 2012) data sets (www.cbiportal.org (Cerami *et al*, 2012; Gao *et al*, 2013)). No *CDKN3* mutation was detected in lung adenocarcinoma tumour tissues in the TCGA cohort or in lung cancer cell lines in the CCLE cohort. Also, no *CDKN3* mutation in lung cancer is found in the Broad Institute's tumorportal database (www.tumorportal.org) (Lawrence *et al*, 2014). Thus, the frequent overexpression of *CDKN3* in lung cancer is not associated with gene mutation.

Cells in the mitotic phase have high levels of CDKN3. To determine if *CDKN3* expression fluctuates during the cell cycle, we synchronized HeLa and A549 cells in later G1/S phase by double thymidine blockage. Cells at various time points following release

Table 2. CDKN3 mutations in the TCC project

Type	Case	CDKN3 mutation ^a
Adrenal	1	
Ampulla of Vater	1	
Bladder	6	
Bone	2	
Brain	79	
Breast	427	D115N; T127I
Cervix	49	
Endometrium	200	N91K, S177P
Oesophagus	44	T9A
Gallbladder	2	
Gynaecologic	1	
Haem-aml	36	
Haem-cll	94	T127I
Kidney	243	200_201del; Q8R(2); T127I
Large bowel	460	C129Y; R36Q
Larynx	24	T127I
Liver	30	
Lung	603	T127I(2)
Lymph nodes	2	
Mandible	3	
Maxilla	2	
Mesenteric	3	
Nose	2	
Oral cavity	30	
Ovary	235	I108V; K62Q; Q8R; R58I
Pancreas	161	
Penis-scrotum	1	
Peritoneum	9	
Pharynx	3	
Pleura	3	
Prostate	52	
Rectum-anus	72	
Renal pelvis	5	
Retroperitoneum	3	
Salivary gland	7	
Skin	209	
Small intestine	7	
Soft tissue	45	Q8R
Spleen	2	
Stomach	55	
Testes	1	
Thoracic	1	
Thyroid	12	
Tongue	5	
Tonsils	1	
Uterus	148	A109T; I108V; T127I
Vagina	1	
Vulva	1	
Total case	3383	23

Abbreviations: CDKN3 = cyclin-dependent kinase inhibitor 3; TCC = total cancer care. T127I and I108V are seen in 1000 Genomes Project and Exome Sequencing Project (ESP). Q8R is seen in the ESP.

^aMutations are single case unless indicated in the parentheses.

from the second thymidine block were collected. Cell lysates were analysed by immunoblotting with antibodies to *CDKN3* and the mitotic-specific histone H3 Ser10 phosphorylation (pHH3) (Hendzel *et al*, 1997). As shown in Figure 5A and B, low levels of *CDKN3* protein were present in double thymidine-blocked HeLa and A549 cells. The increase in *CDKN3* protein precedes the appearance of pHH3 and peaked at the same time point as pHH3 did. We next extended the analysis to additional lung cancer cell lines as well as HeLa cells by blocking cells in G0/early G1 phase with serum deprivation, in later G1/S phase with double thymidine block and in M phase with nocodazole. Cell lysates were prepared from these cells and analysed by immunoblotting. As shown in Figure 5A–C, cells in the M phase consistently showed the highest *CDKN3* protein level.

To assess if the difference in *CDKN3* protein level was related to the mRNA level, RT-qPCR was performed. Consistent with the results from protein analysis, serum-starved cells have

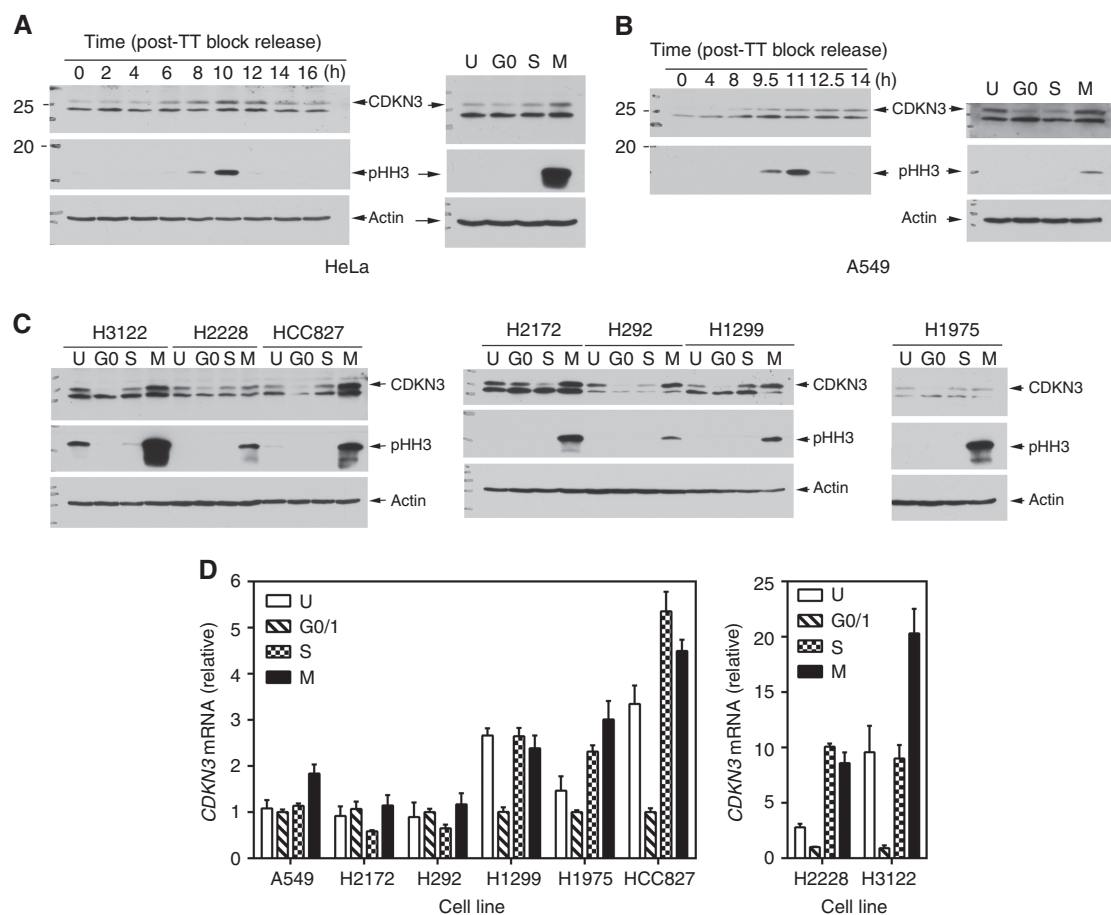


Figure 5. *CDKN3* expression is elevated in M phase. (**A** and **B**, left panels) HeLa and A549 cells were subjected to double thymidine blockage. At the indicated time after release from the second thymidine block, cell lysates were collected and analysed by immunoblotting with indicated antibodies. (**A** and **B**, right panels, and **C**) Cell lysates from unsynchronized (U), serum-deprived (G0/1), double thymidine-blocked (S) and nocodazole block (M) cells were analysed by immunoblotting with indicated antibodies. (**D**) Cells were treated as in **C**. RNA was isolated and analysed by RT-qPCR using CK-T/CK-R primers. The data were averages from two experiments. The relative amount of *CDKN3* mRNA in the serum-deprived cells from each cell line was set as 1.

the lowest level of *CDKN3* mRNA, whereas cells in the M phase have high levels of *CDKN3* although high level of *CDKN3* mRNA was also detected in some cells blocked in the S phase (Figure 5D). Overall, the relative amount of *CDKN3* protein during the three phases of cell cycles correlates with the mRNA level.

If *CDKN3* expression is increased in the mitotic phase, it is predicted that *CDKN3* expression would correlate with the expression of mitotic-related genes. Consistently, gene expression correlation data of lung adenocarcinoma from TCGA shows that genes with the highest expression correlation with *CDKN3* are mitotic-related genes (Supplementary Table 1).

Knockdown of *CDKN3* reduces cell proliferation. If *CDKN3* plays a positive role in regulating mitotic cell cycle, reducing *CDKN3* would be predicted to attenuate cell proliferation. Supplementary Figure 6 shows that we were able to knockdown *CDKN3* in A549 and HeLa cells. *CDKN3* knockdown in A549 and HeLa cells resulted in significantly reduced cell proliferation of these cells (Supplementary Figure 6).

DISCUSSION

Following the initial observation of *CDKN3* overexpression in various types of human cancer, we performed more detailed

characterisation of *CDKN3* expression in NSCLC. We found *CDKN3* expression is elevated in NSCLC. Three cohorts of lung adenocarcinoma consisted of 1328 patients and used either mRNA microarray or RNA-seq method to measure *CDKN3* expression; high *CDKN3* expression is consistently associated with significantly shorter overall survival of these patients. No such association is found in lung squamous cell carcinoma. A possible explanation is that lung squamous cell carcinoma has an overall high level of *CDKN3* expression. The overall high level of *CDKN3* expression within lung squamous cell carcinoma may render indifference between 'low' and 'high' levels in the prognosis of *CDKN3* for the overall survival in this histological type of lung cancer.

When this paper is under peer review, Zang *et al* (2015) reported a mega-analysis of data sets in Lung Cancer Explorer (<http://qbrc.swmed.edu/lce/>) database using different analytic methods. They also found that *CDKN3* is upregulated in lung adenocarcinoma and lung squamous cell carcinoma and that lung squamous cell carcinoma has an overall higher level of *CDKN3*. Furthermore, the high *CDKN3* levels are associated with poor survival in lung adenocarcinoma but not in lung squamous cell carcinoma. These findings are in agreement with the data that we presented here.

Based on the perception of *CDKN3* as a potential tumour suppressor, a possible mechanistic explanation of *CDKN3* overexpression in the tumours is alternative splicing or mutations that

produce dominant-negative products of *CDKN3*. However, in our cohort of 24 samples, we found that all cells and tumours express the same 2 transcripts. We detected no aberrant splice variant or point mutation in the *CDKN3* transcripts. A difference in our analysis and the previous reports in hepatocellular carcinoma and glioma is that previous studies performed sequencing analyses after cloning the cDNA (Yeh *et al*, 2000; Yu *et al*, 2007), whereas we sequenced the RT-PCR products directly. Sequencing analysis of cDNA clones is more sensitive to detect minute changes, although the origins of these changes are unknown. While less sensitive, data of our direct sequencing analyses of RT-PCR products are better representative of what is present as the whole in the cells and thus the overall functionality of *CDKN3* overexpression in the cells. Similar to the results of Lee *et al* (2000), our experiments showed that knockdown of *CDKN3* inhibited cell proliferation, suggesting that *CDKN3* has a positive role in cell proliferation.

CDKN3 mutation data from TCC, TCGA, CCLE and tumour-portal indicate that *CDKN3* mutations are rare in human cancer. Importantly, no disruptive mutation is found. Recurrent missense Q8R, I108V and T127I mutations were found in TCC tumours. These changes were also present in healthy people and thus may represent polymorphic variants. It remains to be determined if these variants have any functional consequence. Nevertheless, the only two cases of T127I change found in lung tumours cannot account for the poor survival of high *CDKN3* expression patients in approximately one-half of 1328 lung adenocarcinoma cases. Taken together, our analyses indicate that the functional *CDKN3*, not dominant-negative *CDKN3* mutants, is overexpressed in lung adenocarcinoma.

Significantly, we found high level of *CDKN3* expression in mitotic cells, whereas cell enriched in the G0/G1 phase by serum-starvation had the lowest level of *CDKN3*. Consistently, TCGA data show that *CDKN3* has the highest expression correlation with genes involving in mitosis. The study by Espinosa *et al* (2013) also identified *CDKN3* as a mitosis pathway gene in cervical cancer. Our finding of elevated *CDKN3* in mitotic cells is in agreement with the notion that *CDKN3* has an important role in mitosis (Nalepa *et al*, 2013) and suggest that *CDKN3* should be added to the list of phosphatases important for cell cycle through mitosis (Wurzenberger and Gerlich, 2011). It also suggests that the high level of *CDKN3* expression in human cancer is likely to reflect the increased fraction of mitotic cells in the tumours.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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