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.
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 alternative 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, 2003). While CDK2 overexpression may actually result in overexpression of CDKN3, overexpression of CDKN3 may be just as important as increasing CDK activity in 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.

**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 (pH3) 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, Whelingu, 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 RNAeasy Kit (Valencia, CA, USA) by Moffitt’s Tissue Core. The isolated RNA was quantified using Nanodrop (Thermo Scientific, Wilmington, DE, USA) and Qubit instrument (Thermo Fisher Scientific, Waltham, MA, USA).

**Plasmids.** A CDKN3 lentiviral expression vector was constructed by cloning the CDKN3 coding sequence (identical to CDKN3 coding sequence of Genebank entry NM005192) from HCC827 cell lines in the Moffitt repository had been authenticated by STR analysis (ACTG Inc, Whelingu, 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).

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**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 **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.

![Image of Figure 1](image-url)

**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.

![Image of Figure 2](image-url)
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 supporting 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

| Table 1. List of cells and tissues analysed for CDKN3 transcripts |
| --- |
| Sample | Description | PCR primer pairs | Sequence data relatedness to NM_005192 |
| Non-cancer cells | | CK1/CK1R (long) | CK1/CK1R (short) | CK2/CK2R | CK3/CK3R |
| HUVEC | Primary endothelium | Identical | Exon 2 skip | Identical | Identical |
| CA2 | ForeSkin fibroblasts | Identical | Exon 2 skip | Identical | Identical |
| Beas2b | Tracheobronchial epithelium | Identical | Exon 2 skip | Identical | Identical |
| Cancer cells | | | | |
| A549 | Lung adenocarcinoma | Identical | Exon 2 skip | Identical | Identical |
| H12172 | 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 |
| H2125 | Lung adenocarcinoma | Identical | Exon 2 skip | Identical | Identical |
| H1212 | 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 |
| #41 | Primary lung cancer | Identical | Exon 2 skip | Identical | Identical |

Abbreviations: CDKN3 = cyclin-dependent kinase inhibitor 3; HUVEC = human endothelium.

*Except HUVEC, other cells are established cell lines.

*This 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 not covered by CK1/CK3 primers, we performed RT–PCR using the same primer pairs CK1/CK1R and CK2/CK2R (Figure 4, Table 1). Taken together, these results show that point mutation of CDKN3 is rare in human cancer.

CDKN3 expression was examined in the massively parallel sequencing data set of 3383 gene mutations from 3383 human cancer samples. No mutation or alternative splicing is found in our CDKN3 cohort of cancer cells and tissues. Analysed. No mutation or alternative splicing is found in our CDKN3 from cell lines (Table 1). Taken together, these results show that point mutation of CDKN3 is rare in human cancer.

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 TCGA 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 both the CDKN3 gene amplification in TCGA lung adenocarcinoma. No alternative splicing variants were 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 obtained 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.

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We next extended the examination to lung adenocarcinoma in the TCGA research network (Cancer Genome Atlas Research Network (Cancer Genome Atlas Research N, 2014) 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.

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 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 (pH3) (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 pH3 and peaked at the same time point as pH3 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

| Table 2. CDKN3 mutations in the TCC project |
|------------------------------------------|
| Type          | Case | CDKN3 mutation* |
|---------------|------|------------------|
| Adrenal       | 1    |                  |
| Ampulla of vater | 1 | T127I            |
| Bladder       | 6    |                  |
| Bone          | 2    |                  |
| Brain         | 79   |                  |
| Breast        | 427  | D115N, T127I     |
| Cervix        | 49   |                  |
| Endometrium   | 200  | N91K, S177P, T9A |
| Oesophagus    | 44   | T127I            |
| Gallbladder   | 2    |                  |
| Gynaecologic  | 1    |                  |
| Haem-aml      | 36   |                  |
| Haem-cl       | 94   | T127I            |
| Kidney        | 243  | 200_201del, Q8R(2); T127I |
| Large bowel   | 460  | C129Y, R36G      |
| Larynx        | 24   | T127I            |
| Liver         | 30   |                  |
| Lung          | 603  | T127I(2)         |
| Lymph nodes   | 2    |                  |
| Mandible      | 3    |                  |
| Maxilla       | 2    |                  |
| Mesentric     | 3    |                  |
| Nose          | 2    |                  |
| Oral cavity   | 30   |                  |
| Ovary         | 235  | I108V, K62Q, Q8R, R58I |
| Pancreas      | 161  |                  |
| Penis-scutum  | 1    |                  |
| Peritoneum    | 9    |                  |
| Pharynx       | 3    |                  |
| Pleura        | 3    |                  |
| Prostate      | 52   |                  |
| Rectum-anus   | 72   |                  |
| Renal pelvis  | 5    |                  |
| Retropitoneum | 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, 1108V, 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

*Mutations are single case unless indicated in the parentheses.
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 splicing 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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