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Expression and alternative splicing of the cyclin-dependent kinase inhibitor-3 gene in human cancer

W. Douglas Cress[a], **Peng Yu**[b], and **Jie Wu**[c,d,*]

[a]Department of Molecular Oncology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida, USA

[b]Department of Electrical and Computer Engineering, and TEES-AgriLife Center for Bioinformatics and Genomic Systems Engineering, Texas A&M University, College Station, Texas, USA

[c]Peggy and Charles Stephenson Cancer Center, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, USA

[d]Department of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, USA

Abstract

The cyclin-dependent kinase inhibitor-3 (*CDKN3*) gene encodes a dual-specificity protein tyrosine phosphatase that dephosphorylates CDK1/CDK2 and other proteins. *CDKN3* is often overexpressed in human cancer, and this overexpression correlates with reduced survival in several types of cancer. *CDKN3* transcript variants and mutations have also been reported. The mechanism of *CDKN3* overexpression and the role of *CDKN3* transcript variants in human cancer are not entirely clear. Here, we review the literature and provide additional data to assess the correlation of *CDKN3* expression with patient survival. Besides the full-length CDKN3 encoding transcript and a major transcript that skips exon 2 express in normal and cancer cells, minor aberrant transcript variants have been reported. Aberrant *CDKN3* transcripts were postulated to encode dominant-negative inhibitors of CDKN3 as an explanation for overexpression of the perceived tumor suppressor gene in human cancer. However, while *CDKN3* is often overexpressed in human cancer, aberrant *CDKN3* transcripts occur infrequently and at lower levels. *CDKN3* mutations and copy number alternation are rare in human cancer, implying that neither loss of CDKN3 activity nor constitutive gain of CDKN3 expression offer an advantage to tumorigenesis. Recently, it was found that *CDKN3* transcript and protein levels fluctuate during the cell cycle, peaking in mitosis. Given that rapidly growing tumors have more mitotic cells, the high level of mitotic *CDKN3* expression is the most plausible mechanism of frequent *CDKN3* overexpression

[*]**Corresponding Author**: Jie Wu, Ph.D., Peggy and Charles Stephenson Cancer Center, University of Oklahoma Health Sciences Center, 975 N.E. 10th Street, BRC-413, Oklahoma City, OK 73104, Telephone: (405) 271-8001, ext. 31092, Fax: (405) 271-2141, jie-wu@ouhsc.edu.

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in human cancer. This finding clarifies the mechanism of *CDKN3* overexpression in human cancer and questions the view of CDKN3 as a tumor suppressor.

Keywords

CDKN3; CDK1; splicing; mitosis; phosphatase; cancer

1. CDKN3

Cyclin-dependent kinase (CDK) inhibitor-3 (CDKN3) is a dual-specificity protein tyrosine phosphatase of the CDC14 group (Alonso et al., 2004). CDKN3 was originally isolated from a HeLa cDNA library as a CDK2 binding protein via a yeast two-hybrid screen and was termed CDK-Associated Phosphatase (KAP) (Hannon et al., 1994). Although CDKN3 was isolated as a CDK2-binding protein (since experiment was designed to isolate CDK2 partners), it was observed that CDKN3 bound preferentially to CDK1 (CDC2), which functions in the mitotic phase of the cell cycle (Hannon et al., 1994).

As major regulators of cell cycle progression, CDK1 and CDK2 activities are tightly regulated. In addition to the required interaction with cyclins that oscillate through cell cycle, CDK1/CDK2 Ser/Thr kinase activity is further regulated by phosphorylation at three residues; Thr-14 and Tyr-15 of both proteins and Thr-161 of CDK1 and Thr-160 of CDK2 (Gu et al., 1992). Phosphorylation at the dual Thr-14/Tyr-15 sites inhibits kinase activity and is regulated by the PKMYT1 (MYT1)/WEE1 kinase and the dual-specificity protein tyrosine phosphatase CDC25. Thr-161/Thr-160 are activating sites of phosphorylation in the kinase activating loop (Gu et al., 1992) in each protein. CDK7 (CAK) phosphorylates Thr-161/160 thus activating each protein. Phosphorylation of Thr-160 in CDK2 increases its binding affinity for the kinase substrates, but has no effect on cyclin A binding (Brown et al., 1999). CDKN3 is responsible for dephosphorylating Thr-161/Thr-160. Mechanistically, CDKN3 dephosphorylates monomeric CDK2; whereas cyclin A protects CDK2 Thr-160 from being dephosphorylated by CDKN3 (Poon and Hunter, 1995). Thus, the cyclin A-bound CDK2 that drives S phase is resistant to CDKN3. CDKN3 dephosphorylates CDK1 during mitotic exit (Nalepa et al., 2013).

The human *CDKN3* gene is located at chromosome 5q13.2 and has 8 exons. The CDKN3 coding mRNA (Genbank accession: NM_005192) has 906 nucleotide residues and encodes a protein of 212 amino acid residues (Fig. 1). In addition to this 212-amino acid residue encoding *CDKN3* transcript, several splicing variants have been reported (Barron et al., 2015; Fan et al., 2015; Yeh et al., 2000; Yu et al., 2007). An exon 2-skipping alternative splicing transcript (Fig. 1) was consistently detected in both normal and tumor cells and tissues (Barron et al., 2015; Fan et al., 2015; Yu et al., 2007).

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2. *CDKN3* overexpression is associated with poor survival in many types of human cancer

Using differential-display PCR, Stuart Aaronson's laboratory was the first to observe overexpression of *CDKN3* mRNA in various human cancer cell lines (Lee et al., 2000). Suppression of CDKN3 expression by antisense CDKN3 inhibited soft-agar colony growth of LNCaP cells and tumor xenograft growth of HeLa cells, suggesting that CDKN3 plays a tumorigenic role in these cells (Lee et al., 2000). Subsequently, increased *CDKN3* expression has been observed in many types of cancer (Fan et al., 2015; Yang and Sun, 2015). Moreover, high *CDKN3* mRNA levels have been associated with poor survival in glioma (Yu et al., 2007), cervical cancer (Barron et al., 2015; Espinosa et al., 2013), and lung adenocarcinoma (Fan et al., 2015; Zang et al., 2015). Examination of The Cancer Genome Atlas (TCGA) RNA-seq data (via cBioPortal) also indicates that high *CDKN3* mRNA levels correlate with poor survival in several types of cancer, including renal clear cell carcinoma, renal papillary cell carcinoma, low grade glioma, and prostate adenocarcinoma (see below).

We found that *CDKN3* expression was elevated in non-small cell lung cancer (NSCLC) (Fan et al., 2015). In three cohorts of lung adenocarcinoma, *CDKN3* overexpression was prognostic for poor overall survival, while no significant differences in *CDKN3* expression were found among different stages of lung adenocarcinoma. Although *CDKN3* was overexpressed in lung squamous cell carcinoma, *CDKN3* overexpression was not prognostic for patient survival. A likely explanation for the lack of difference between high and low *CDKN3* expression to patient survival in lung squamous cell carcinoma is the overall high level of *CDKN3* expression within this histological subtype of lung cancer (Fan et al., 2015).

In the TCGA datasets, *CDKN3* mRNA levels in acute myeloid leukemia, invasive breast carcinoma, and colorectal adenocarcinoma were not prognostic for overall survival, whereas high *CDKN3* mRNA levels were associated with poor overall survival in low grade glioma and renal clear cell carcinoma (Fig. 2A). In prostate adenocarcinoma, while the few deceased cases (2%) limited assessment of a correlation between *CDKN3* levels and the overall survival, high *CDKN3* mRNA expression was associated with poor disease-free survival (Fig. 2A). Acute myeloid leukemia, invasive breast carcinoma, and colorectal adenocarcinoma had high overall *CDKN3* mRNA levels, whereas low grade glioma, renal clear cell carcinoma, and prostate adenocarcinoma had lower overall *CDKN3* mRNA levels (Fig. 2B). This difference is similar to what we observed previously between lung adenocarcinoma and lung squamous cell carcinoma.

3. Normal and aberrant *CDKN3* transcripts

As mentioned above, the full-length 212-amino acid residue CDKN3 is encoded by 906-nucleotide mRNA derived from 8 exons. In addition, an exon 2-skip alternative splicing isoform is present in all human cell lines and tissues that have been analyzed (Barron et al., 2015; Fan et al., 2015; Yu et al., 2007). The exon 2-skip transcript results in a frameshift and encodes a short 23-amino acid residue peptide non-homologous to CDKN3. Since this 23-amino acid residue peptide lacks homology to CDKN3 (Fan et al., 2015), it is unlikely that it

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has any function related to CDKN3. Because these two transcripts are present in all cell lines and tissues that we have examined, we consider these two alternative splicing isoforms to be the normal human *CDKN3* transcripts.

Yeh *et al.* (Yeh et al., 2000) were the first to report 15 aberrant transcripts in 27 hepatocellular carcinoma tissue samples and six aberrant transcripts in 12 noncancerous liver tissues. That is, ~50% of cancerous and noncancerous liver tissues in this study had abnormal *CDKN3* transcripts (although these alterations were not validated in the corresponding genomic DNA). These aberrant *CDKN3* transcripts include nonsense mutations and deletions that resulted in truncated CDKN3 and CDKN3 with missense or internal deletion mutations. Potentially, some of these aberrant transcripts may result in dominant-negative CDKN3 mutants that could interfere with normal CDKN3 function. We found no *CDKN3* mutations among 30 cases of hepatocellular carcinoma from Total Cancer Care™ (TCC) data (Fan et al., 2015). Among 442 cases of hepatocellular carcinoma in TCGA and 231 cases of hepatocellular carcinoma reported by Ahn *et al.* (Ahn et al., 2014), there were two cases with missense mutations (I72T, E17K) (cbioportal.org). Therefore, the high incidence of *CDKN3* missense and nonsense mutations reported by Yeh *et al.* (Yeh et al., 2000) appeared inconsistent with genomic data in hepatocellular carcinoma from other laboratories, and some of the deletion mutations were probably generated post-transcriptionally via alternative splicing. Subsequently, Yu *et al.* (Yu et al., 2007) reported two aberrant splicing variants of *CDKN3* "b" and "d" involving exons 2 and 3, respectively, in glioblastoma. The b variant resulted in an 8-amino acid peptide. The d variant was derived from using an alternative splice site in exon 2, resulting in a 179-amino acid protein skipping part of exon 2 and the entire exon 3. This d variant is the same as the BX-09 transcript previously detected by Yeh et al. (Yeh et al., 2000) in hepatocellular carcinoma and it could interfere with CDKN3 activity, since it lacks a functional catalytic domain, but may compete for CDK binding (Yu et al., 2007).

Barron *et al.* (Barron et al., 2015) reported five minor aberrant transcripts in cervical cancer and control tissues. These transcripts were generated from alternative splicing lacking internal exons or distal exons, but retaining exons 4, 6, 7, and/or 8 that encode CDK2 interacting regions of CDKN3 (Song et al., 2001). Thus, proteins encoded by these aberrant transcripts are predicted to be able to interfere with the wildtype CDKN3 function. These transcripts were detected in normal and cervical cancer samples with similar frequencies (Barron et al., 2015). However, the qPCR measurement of *CDKN3* levels in control and tumor samples mainly represented the wildtype CDKN3 transcript (Barron et al., 2015). In all cases, the *CDKN3* transcript encoding the wildtype CDKN3 is the predominant transcript.

We examined *CDKN3* transcripts in six non-small cell lung cancer tissues, four non-cancerous cell culture or cell lines, and 14 cancer cell lines that include lung, breast, cervical, colorectal, liver, pancreatic, and prostate cancer and osteosarcoma (Fan et al., 2015). Although we used RT-PCR primer pairs that were designed to detect potential alternative splicing in every exon of *CDKN3*, no aberrant alternative splicing or mutation was found in these 24 samples.

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The reasons that others detected minor aberrant CDKN3 transcripts in their samples and that we did not remain to be determined. One possibility is the difference in tissue types and samples. Another possibility is that others used more sensitive methods. For instance, Barron *et al.* (Barron et al., 2015) used nested primers to re-amplify RT-PCR products. Yeh et al. (Yeh et al., 2000) cloned PCR products and sequenced cloned cDNA. We used the same primers to amplify the PT-PCR products and sequenced these PCR products directly. Thus, we did not exclude the possibility that minor aberrant transcripts might exist in our samples, but focused on major transcripts.

4. CDKN3 expression is elevated in the mitotic phase and controls cell division

While *CDKN3* is often overexpressed in human cancer, *CDKN3* gene amplification occurs infrequently. For instance, among the 516 cases of TCGA lung adenocarcinoma for which copy number alteration data were available, 10 cases (2%) had *CDKN3* gene amplification. Thus, the frequent overexpression of *CDKN3* in human cancer is unlikely to be caused by gene amplification.

In a panel of synchronized cell lines, we found that the CDKN3 protein level fluctuated during the cell cycle (Fan et al., 2015). CDKN3 protein was expressed at low levels in $G_{0/1}$ and S phase and was increased in M phase in parallel with phosphorylation of histone H3 Ser-10, which is a marker of M phase. RT-qPCR analysis indicated that M phase cells consistently had high levels of *CDKN3* mRNA and $G_{0/1}$-arrested cells had low levels of *CDKN3* mRNA (Fan et al., 2015). In some cell lines (HCC827, H2228, H1299), high levels of *CDKN3* mRNA were also detected in S phase although we did not observe a corresponding increase at the protein level (Fan et al., 2015). These data suggest that the frequent CDKN3 overexpression in human cancer is primarily due to the increased number of mitotic cells.

In a spindle assembly checkpoint (SAC) assay, in which SAC failure produced a multinucleated phenotype when HeLa cells were treated with taxol, *CDKN3* siRNAs yielded the stronger SAC failure phenotype among 801 siRNAs targeting human phosphatases (Nalepa et al., 2013). Further analysis indicated that the centrosome-located CDK1 was phosphorylated at Thr-161 during mitotic entry. CDK1 Thr-161 was dephosphorylated in anaphase until completion of mitosis, and CDKN3 was responsible for dephosphorylation of CDK1 Thr-161 in late mitosis (Nalepa et al., 2013). Mitotic exit is mediated by inactivation of CDK1/cyclin B. Inhibition of residual CDK1 activity by dephosphorylating CDK1 Thr-161 may be a mechanism to ensure normal mitotic exit (Nalepa et al., 2013).

During S phase, CDK2/cyclin A phosphorylates and activates monopolar spindle 1 (MPS1), which is a dual-specificity kinase that promotes centrosome duplication. Elevated MPS1 results in overduplication of centrosomes that causes abnormal spindle assembly and interferes with cell division. It was found that CDK2/cyclin A phosphorylated MPS1 at Thr-468 and possibly at Thr453, which promotes its centrosomal stability, whereas CDKN3 dephosphorylated MPS1 to induce proteasome-mediated degradation of MPS1 at centrosomes (Srinivas et al., 2015). Recall that CDK2 Thr-160 phosphorylation state does

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not affect cyclin A binding (Brown et al., 1999), and that only after cyclin A is degraded post S phase, can CDK2 Thr-160 be dephosphorylated by CDKN3 (Poon and Hunter, 1995). Thus, it has been postulated that, after cyclin A degradation, CDKN3 dephosphorylates CDK2 and MPS1 to prevent centrosome overduplication (Srinivas et al., 2015). Taken together, these results indicate that CDKN3 has positive roles in regulating cell division by controlling mitotic exit and centrosome duplication.

5. Conclusion and perspective

CDKN3 was perceived as a tumor suppressor gene analogous to *CDKN2A/CDKN2B*. Counter- intuitively, *CDKN3* is often overexpressed in human cancer, whereas *CDKN2A/CDKN2B* are often deleted in certain types of human cancer, such as non-small cell lung cancer. Early studies attributed *CDKN3* overexpression to dominant-negative protein, caused by either mutations or aberrant splicing transcripts. However, recent cancer genomic data indicate that CDKN3 mutations are rare and likely non-disruptive. Aberrant transcripts, when detected, were always present as minor transcripts co-existing with the more abundant full-length normal CDKN3 transcript. Truncated proteins are often rapidly degraded in the cells (Lykke-Andersen and Jensen, 2015). Whether any of the reported aberrant transcripts of *CDKN3* yield a detectable endogenous protein remains to be seen.

While *CDKN3* is often overexpressed in human cancer, few cases have *CDKN3* gene amplification. Gene amplification is predicted to result in constitutively elevated expression. Thus, the fact that the *CDKN3* gene is rarely amplified in human cancer suggests that a constitutive increase in CDKN3 level does not give cancer cells an advantage. We have found that *CDKN3* transcript and protein levels fluctuate during the cell cycle and peak at mitosis. Since rapidly growing tumors have more mitotic cells, the high level of CDKN3 in mitotic phase provides the best plausible explanation for the frequent CDKN3 overexpression in human cancer. While inactivating CDK1 to ensure mitotic exit may explain the need of a high level CDKN3 in the mitotic phase, the mechanisms of dynamic control of *CDKN3* mRNA and protein expression during cell cycle remain to be investigated.

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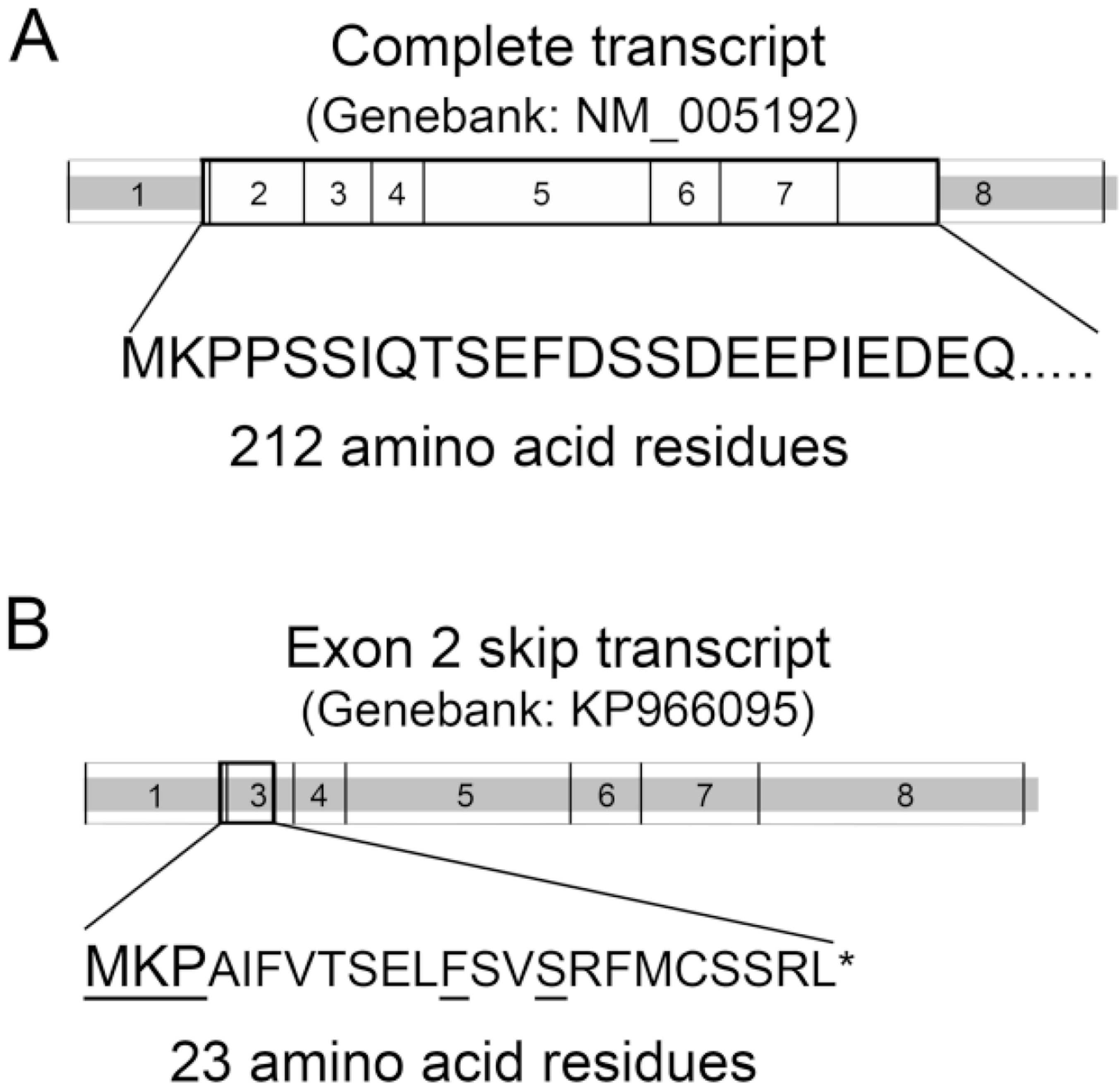

Fig. 1.

Full length and exon 2-skip *CDKN3* transcripts. Full length *CDKN3* mRNA (Genbank: NM-005192) (A) and exon 2-skip splicing variant (Genbank: KP966095) (B) are shown. Heavily outlined boxes indicate protein coding region. Thin bordered boxes indicate exons. Large letters indicate the N-terminal amino acid sequence of CDKN3. Small letters indicate the frame-shift amino acid sequence in the exon 2-skip transcript. Underlined residues are identical to CDKN3 amino acid residues.

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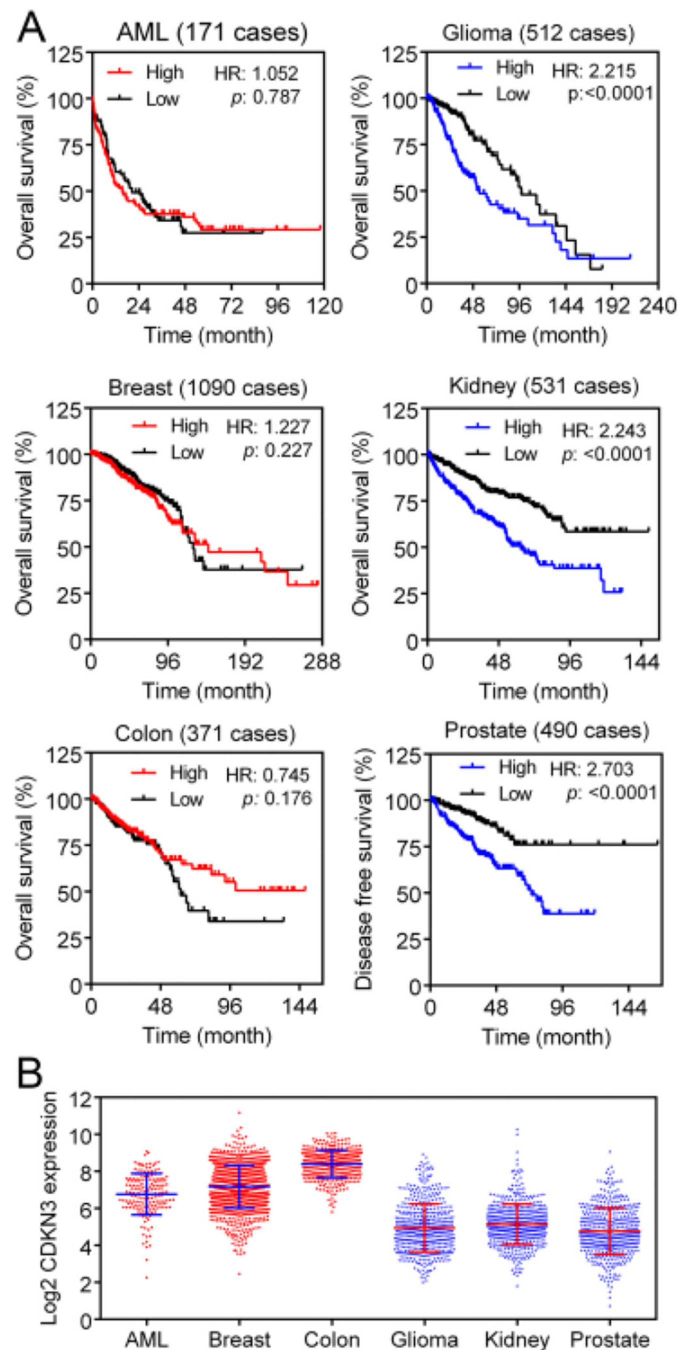

Fig. 2.

Correlation between *CDKN3* levels and cancer patient survival in TCGA datasets. (A) Correlation between *CDKN3* mRNA levels and overall or disease-free survival in the indicated types of cancer. High/low *CDKN3* mRNA levels were dichotomized on the mean expression value. (B) *CDKN3* mRNA levels in six cohorts of cancer from TCGA. TCGA data were obtained via cbioportal.org (Cerami et al., 2012; Gao et al., 2013). AML, acute myeloid leukemia; breast, invasive breast carcinoma; colon, colorectal adenocarcinoma;

glioma, low grade glioma; kidney, renal clear cell carcinoma; prostate, prostate adenocarcinoma.

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