Keratin-17 Promotes p27KIP1 Nuclear Export and Degradation and Offers Potential Prognostic Utility

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Abstract

Keratins that are overexpressed selectively in human carcinomas may offer diagnostic and prognostic utility. In this study, we show that high expression of keratin-17 (K17) predicts poor outcome in patients with cervical cancer, at early or late stages of disease, surpassing in accuracy either tumor staging or loss of p27KIP1 as a negative prognostic marker in this setting. We investigated the mechanistic basis for the biologic impact of K17 through loss- and gain-of-function experiments in human cervix, breast, and pancreatic cancer cells. Specifically, we determined that K17 functions as an oncoprotein by regulating the subcellular localization and degradation of p27KIP1. We found that K17 was released from intermediate filaments and translocated into the nucleus via a nuclear localization signal (NLS), specific among keratins, where it bound p27KIP1 during G1 phase of the cell cycle. p27KIP1 lacks a nuclear export signal (NES) and requires an adaptor for CRM1 binding for nuclear export. In K17, we defined and validated a leucine-rich NES thatmediated CRM1 binding for export. Cervical cancer cells expressing K17 mutations in its NLS or NES signals exhibited an increase in levels of nuclear p27KIP1, whereas cells expressing wild-type K17 exhibited a depletion in total endogenous p27KIP1. In clinical specimens of cervical cancer, we confirmed that the expressions of K17 and p27KIP1 were inversely correlated, both across tumors and within individual tumors. Overall, our findings establish that K17 functions specially among keratins as an oncoprotein by controlling the ability of p27KIP1 to influence cervical cancer pathogenesis. Cancer Res; 75(17); 1–13. © 2015 AACR.

Introduction

Keratins are intermediate filaments that display a broad range of molecular diversity and undergo tight regulation in a tissue-specific, differentiation-related, and context-dependent manner. Keratin 17 (K17), although not present in normal mature epithelium, is expressed in stem cells of embryonic ectoderm, skin appendages, and the endocervical mucosa, and it is re-expressed in carcinomas (1–8).

In nontumor epithelial cells, p27KIP1 localization is tightly regulated during cell-cycle progression, and this plays a pivotal role in governing its function as a cell-cycle inhibitor. As a negative regulator of G1 phase progression, when localized in the nucleus, p27KIP1 inhibits the activity of cyclin-dependent kinases in complex with cyclins, preventing G1–G0 to S-phase transitions. After mitogen signaling during early G1, p27KIP1 is actively exported from the nucleus in a CRM1-dependent manner and is degraded in the cytoplasm of tumors (11). Deregulation of tumor suppressor p27KIP1 levels is a hallmark of neoplasia because p27KIP1 is progressively lost or sequestered in the cytoplasm during malignant transformation (13, 14). It has been reported that deregulated SRC, MAPK, or PI3K/AKT contribute to oncogenic signaling pathways by either increasing degradation or cytoplasmic sequestration of p27KIP1 in cancer cells (11).

Herein, we show that K17 is a powerful prognostic marker for cervical cancer mortality and investigate the roles of K17 in sustained proliferative signaling and tumor growth using in vitro and in vivo techniques in cervical cancer and other cancer-derived cell lines. Our data suggest that K17 mediates cancer cell-cycle progression and tumor growth by promoting p27KIP1 nuclear export and degradation.

Materials and Methods

Patient tissue sampling and survival analyses

This study included 74 formalin-fixed, paraffin-embedded surgical tissue blocks retrospectively selected from the archival
collections of the UMass Memorial Medical Center (validation dataset). In addition, for the multivariate analyses, we included the 65 formalin-fixed, paraffin-embedded surgical tissue blocks originally used for the discovery of K17 as a prognostic marker (discovery set; Supplementary Table S1; ref. 1). Stony Brook Medicine and UMass Memorial Medical Center institutional review boards approved all protocols. The criteria for selection were (i) diagnosis of primary squamous cell carcinoma of the cervix (SCC) and (ii) ≥18 years at time of diagnosis. Patients with a diagnosis of cancer at other anatomic sites were excluded. SCCs were classified by tumor stage and histologic grade, according to the original pathology report. Survival data were obtained from UMass Memorial Cancer Registry and Stony Brook Medicine Cancer Registry.

Discovery and validation datasets were found to be comparable in tumor stage and patient status (Supplementary Table S1). We validated K17 status, by determining a 50% of positive cells, as the best K17 score cutoff point for high- or low-K17 status in the validation set, as previously determined in the discovery set, according to the minimum Akaike’s Information Criterion (Supplementary Table S1; ref. 1). Overall survival analyses were performed to validate the relationship between K17, p27kip1, and clinical outcomes. The survival curves were generated using the Kaplan–Meier method and compared by log-rank test.

Multivariate analyses were performed by Cox proportional hazards model to examine overall survival rates while adjusting for potential confounders such as stage of the cancer. We grouped cases into two general categories, including tumors limited to the cervix (stages I and II) versus tumors that invaded beyond the cervix or metastasized (stages III and IV). REMARK recommendations for tumor marker prognostic studies were followed (15).

Cell culture

Human cervical cancer cell lines were obtained from ATCC. Pancreatic cancer cell line L3.6 was kindly provided by Dr. Richard Lin, and triple-negative breast cancer cell lines, MBA-MD-231 and 468, were kindly provided by Dr Natasha Marchenko at Stony Brook University (New York, NY). All cell lines were cultured as recommended by ATCC.

Immunohistochemical and immunofluorescent stains and scoring methods

Immunohistochemistry was performed by an indirect immunoperoxidase method, as previously described (Protocol 1; ref. 1). Briefly, after incubation at 60°C, slices were deparaffinized in xylene and rehydrated in alcohols. Antigen retrieval was performed in citrate buffer at 120°C for 10 minutes in a decloaking chamber. Endogenous peroxidase was blocked by 3% hydrogen peroxide, and sections were incubated overnight at 4°C with: mouse monoclonal-[E3] anti-human K17 antibody (Abcam) for SCCs and rabbit- anti-p27kip1 (GenTex), anti-Ki67 (Dako), and anti-PCNA (Abcam) for xenografts. After primary antibody, biotinylated horse secondary antibodies (R.T.U. Vectastain ABC kit; Vector Laboratories) were added. Development was done with 3,3'-diaminobenzidine (DAB; Dako), and counterstain was done with hematoxylin. Negative controls were performed on all runs using an equivalent concentration of a subclass-matched immunoglobulin. Immunohistochemical stains for K17 in SCC were scored by PathSQ, a manual semiquantitative scoring system, which quantifies the percentage of strongly stained tumor cells, blinded to corresponding clinical data (1). Immunohistochemical stains on xenografts were scored using Image64 (NIH), by means of the color deconvolution DAB-Hem plugin (16).

Immunofluorescent stains for SCCs were performed similar to immunohistochemical stains but with antigen retrieval at high pH buffer (Protocol 2). For immunofluorescent stains on cancer cells, cells were seeded on coverslips 24 hours before staining. Cells were fixed in 100% methanol and washed in PBS. After blocking and permeabilizing with 1% donkey serum in PBST (PBS + 0.1% Triton X), primary antibodies were added. Rabbit monoclonal anti-human K17 (Abcam) and mouse monoclonal anti-p27kip1 (BD Transduction Laboratories) antibodies were used in all immunofluorescent stains and incubated overnight at 4°C. Subsequently, cells were washed and incubated with Alexa Fluor fluorescent-labeled secondary antibodies (Life Technologies) for 1 hour. After washing, slides were mounted in Vectashield with DAPI (Vector Labs). Images were detected with a Nikon 2D-Structured Illumination Microscope (SIM-I) or Zeiss confocal microscopes. Immunofluorescence and colocalization were quantified using Image64 (NIH).

siRNA, shRNA, and KRT17-encoding plasmids

For transient knockdown, ON-TARGETplus Human KRT17 gene (3872) siRNA SMART pool (Thermo Scientific) of 4 siRNAs was used (siKRT17). Sequences as follows (5'–3'): AGAAA-GAACCCGUGUACCA, CGUCAGGUGCGCCCAUHIUG, GGUCC-ACAGAUGGAAAGGCU, GGAGAAUAGCCACCAUGCA. ON-TARGETplus Nontargeting Control siRNAs (Thermo Scientific) was used as control (siRNA). siRNAs were transfected into cancer cells using Oligofectamine 2000 (Life Technologies) according to the standard protocol (Protocol 3).

For stable knockdown of K17, 3 GIPZ lentiviral shRNA (GE Dharmacon; shKRT17) were used for screen to best knock efficiency. Sequences as follows (5'–3'): sh1-TCTTGATCTGACGTCAGGTG, sh2-TCTTTCCTGACTGACGTC, and sh3-CTGGCTC- AAACCTGTGTCG. Nontargeting GIPZ lentiviral shRNA was used as control shRNA. Lentivirus production was carried out following manufacturers’ instructions. Cells were selected with 10 μg/mL of puromycin. sh1-KRT17 was used for SiHa cells and sh2-KRT17 was used for CaSkii cells, based on knockdown efficiency (Supplementary Fig. S1; Protocol 4).

For stable expression of human of K17, the human ORF for K17 gene (KRT17) was cloned into pCDNA4/TO Mammalian Expression Vector (Life Technologies). Empty vector was used as negative control. Plasmids were transfected into cancer cells using Lipofectamine 2000 (Life Technologies) according to the standard protocol. Cells were selected with 100 μg/mL of zeocin (Protocol 5). Point mutations on KRT17 sequence were introduced using site-directed mutagenesis, and residues were replaced by alanines (Protocol 6).

Cell proliferation, cell-cycle analysis, senescence assay, and drug sensitivity

Twenty-four hours after transient transfection with siRNAs, SiHa and CaSkii cells were seeded in 96-well plates at 4,000 cells per well. The cell proliferation assay was performed on days 1, 3, and 5 by incubating with WST-1 (Roche Applied Science; Protocol 7). A cell number absorbance curve was performed to calculate cell per well. Cell-cycle analysis was performed by flow cytometry using propidium iodine (Protocol 8) and acridine orange stains (Protocol 9). Cells were harvested and resuspended at 0.5 × 10^6 to 1 × 10^6 cells/ml in modified Krishan buffer with 0.02 mg/mL.
RNase H (Life Technologies) and 0.05 mg/mL propidium iodide (Sigma-Aldrich; ref. 17). Results were calculated with Modfit LT (Verity Software House). For acridine orange, cell-cycle stain and analyses were performed as previously described (18, 19), with and without RNase H (Life Technologies). Samples were analyzed in FACSCalibur (Becton Dickinson) at the Research Flow Cytometry core at Stony Brook University. The Senescence β-Galactosidase Staining Kit (Cell Signaling) was used to determine the percentage of senescent cells following the manufactures’ instructions (Protocol 10). To test cisplatin sensitivity in combination with K17 knockdown, 24 hours after transient transfection, cells were plated in 36-well plates at 4 × 10^5 cells per well in triplicates in 100 μL of medium. After 24 hours, fresh medium containing cisplatin, ranged from 0 to 200 μmol/L, was added, and cells were cultured for an additional 24 hours. Cell viability was measured using the WST-1 assay, and concentration-dependent curves were generated on the basis of the cell viability of the negative control (0 μmol/L; Protocol 7).

To quantify percentage of apoptotic and viable cells, 24 hours after cisplatin treatment (30 μmol/L) cells treated with control siRNA and siKRT17 were harvested, stained with propidium iodide and anti-Annexin V antibody (BD Biosciences) following the manufacturer’s protocol, and detected by flow cytometry (Protocol 11; ref. 17).

Serum starvation release, cycloheximide, and leptomycin B treatments and lysates preparation
Serum starvation release was used to arrest cancer cells at G1 phase and stabilize p27Kip1. Briefly, cells were plated at 50% confluence and serum-starved for 48 hours. After addition of DMEM containing 20% FBS, to induce cell-cycle progression, cell lysates were collected at different time points. For protein stability analysis, cycloheximide was added at 40 μg/mL (Calbiochem) in mixture with 20% FBS DMEM for serum starve release (Protocol 12). For nuclear export inhibition assays, leptomycin B (LM) was added at 20 nmol/L. (Cell Signaling) in mixture with 20% FBS DMEM for serum starve release (Protocol 13). Whole-cell protein samples were collected with RIPA buffer (Sigma-Aldrich) followed by sonication. Nuclear- and cytosolic-enriched fractions were extracted by the NE-PER Protein Extraction Kit (Pierce-Thermo Scientific), according to the manufacturer’s instructions (Protocol 14). To isolate whole-tumor protein samples from formalin-fixed, paraffin-embedded xenografts, we used the Qproteome FFPE Tissue Kit (Qiagen) following manufacturers’ instructions (Protocol 15). All protein lysates were collected using inhibitors of proteases and phosphatases cocktails (Sigma-Aldrich).

RNA isolation, RT-PCR, and qRT-PCR
Total RNA was extracted with TRIzol reagent (Life Technologies) following the manufacturer’s protocol. RT-PCR was performed with Reverse Transcription System (Promega). In all, 1 μg of RNA was used as a template for cDNA synthesis. cDNA templates were mixed with gene-specific primers for KRT17 (K17), CDKN2A (p16INK4a), CDKN2B (p15INK4b), CDKN2C (p18INK4c), CDKN2D (p19INK4d), CDKN1A (p21CIP1/WAF1), CDKN1B (p27Kip1), GAPDH, b-actin, and 18S. All primers are shown in the Supplementary Table S3. TaqMan 2X universal PCR master mix or SYBR Green PCR Master Mix (Life Technologies) was used depending on the detection system. qRT-PCR was programmed as 95°C for 10 minutes; 95°C for 15 seconds; 60°C for 1 minute and repeated for 40 cycles. Data were normalized by the level of expression in each individual sample as described (Protocol 16; ref. 20).

Western blotting and coimmunoprecipitations
Protein concentrations were determined by the BCA Protein Assay (Biorad). Equal amounts of samples were loaded to SDS-PAGE and transferred to PVDF membrane. The membranes were blocked with 5% non-fat milk in TBS/0.5% Tween 20 for 30 minutes and then probed overnight at 4°C. List of primary antibodies is provided in Supplementary Table S4. Goat anti-rabbit and anti-mouse and rabbit anti-goat horseradish peroxidase–conjugated secondary antibodies (Jackson Immunoresearch) were used at 1:2,000. Horseradish peroxidase activity was detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Expression levels were quantified using ImageJ64 software (NIH) and normalized to loading controls (Protocol 17). Co-immunoprecipitations were performed using the Dynabeads Protein G Immunoprecipitation Kit (Life Technologies) following the manufactures’ instructions. Negative controls were performed on all runs using an equivalent concentration of a subclass-matched immunoglobulin (Protocol 18).

Xenograft models
In all, 10- to 12-week-old NOD/SCID female mice (Harlan Laboratories) were used for tumor implantation. The Stony Brook University Institutional Animal Care and Use Committee approved all animal procedures. The mice were anesthetized by isoflurane inhalation. Cancer cells were subcutaneously injected into the lower back areas of the mice using 2 × 10^6 cells in 100 μL. DMEM with 50% Matrigel (BD Biosciences). The tumor size was measured over a month using a caliper, and tumor volume was calculated using the formula V = length × width^2/2 (Protocol 19; ref. 17). Stony Brook University IACUC approved all animal protocols.

Statistical analysis
Each experiment was independently repeated 3 to 4 times with 1 to 3 replicates per experiment. Categorical data are described using frequencies and percentages. Continuous data are described using means ± SD or SEM. Statistical significance between the means of two groups was determined using the Student t tests or Mann–Whitney U tests. Statistical comparisons of the means of multiple groups were determined using 1-way ANOVA or Kruskal–Wallis ANOVA by ranks. All analyses were performed using SAS 9.3 (SAS Institute, Inc.) and SigmaPlot 11 (Systat Software). Statistical significance was set at P < 0.05 (α).

Results
K17 is a cervical cancer prognostic marker, independent of tumor stage
We recently proposed that K17 could be developed as a diagnostic and prognostic marker for cervical cancer (1). Here, using an independent set of cases sufficiently large for power analysis, we report that patients with elevated expression of K17 (“high-K17,” Fig. A1–C) have a rate of survival that is 40% decreased in comparison to patients with low levels of K17 expression (“low-K17”; log-rank P = 0.0001, HR = 5.98, P = 0.0009; Supplementary Tables S1 and S2). Importantly, high-K17 status predicts reduced survival for early as well as advanced-stage cervical cancer.
cancers (Fig. 1D and E, Table 1) and is a more accurate predictor of death than tumor stage (K17: HR = 5.7, \( P = 0.0002 \); stage: HR = 3.2, \( P = 0.0035 \)). Thus, high-K17 status is a biologic marker of cervical tumor aggression that provides survival information more accurate than current standard-of-care clinicopathologic classification.

K17 knockdown induces cell-cycle arrest and cisplatin sensitivity

To determine whether K17 contributes to sustained proliferation signaling, RNAi was used to target the K17-expressing cervical cancer cell lines SiHa and CaSki (Supplementary Fig. S1A–S1G), resulting in a 50% decrease in proliferation (Fig. 2A). Cell-cycle analyses indicated that K17 silencing induces G1 arrest (Fig. 2B, Supplementary Fig. S2A). In contrast, forced expression of K17 in the non-K17-expressing cervical cancer cell line (C33-A) significantly decreased the G1- to S-phase ratio (Fig. 2C). Furthermore, K17 knockdown in pancreatic and breast cancer cell lines increased G1 phase accumulation (Fig. 2D). The effect of K17 appears to be early in G1 in cervical cancer cells as reflected by an increase in the G1A–G1B ratio (Fig. 2E; ref. 19), a decrease in total RNA (19), and a decrease in cell size (Supplementary Fig. S2B and S2C). No differences were found in sub-G1–G0 (apoptotic) percentage, mitotic entry, or percentage

Table 1. Failure hazard for SCC patients stratified by K17 status and tumor stage using Cox proportional hazards model

<table>
<thead>
<tr>
<th>Effect of changing from</th>
<th>Keeping constant</th>
<th>Wald ( \chi^2 )</th>
<th>DF</th>
<th>HR (95% CI)</th>
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<tr>
<td>K17 low vs. high</td>
<td>Stages I + II</td>
<td>6.96</td>
<td>1</td>
<td>4.77 (1.49–15.23)</td>
<td>0.008*</td>
</tr>
<tr>
<td>K17 low vs. high</td>
<td>Stages III + IV</td>
<td>7.93</td>
<td>1</td>
<td>8.65 (1.93–38.83)</td>
<td>0.005*</td>
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<tr>
<td>Stages I + II vs. III + IV</td>
<td>Low-K17</td>
<td>0.13</td>
<td>1</td>
<td>1.37 (0.25–7.50)</td>
<td>0.717</td>
</tr>
<tr>
<td>Stages I + II vs. III + IV</td>
<td>High-K17</td>
<td>4.48</td>
<td>1</td>
<td>2.48 (1.07–5.76)</td>
<td>0.034*</td>
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NOTE: The proportional hazards assumption was valid.
Abbreviations: CI, confidence interval; DF, degrees of freedom.

\* \( P \leq 0.01 \).
\* \( P \leq 0.05 \).
of senescent cells (Supplementary Fig. S2D–S2F). C33-A cells challenged with K17 RNAi did not arrest in G1 (Supplementary Fig. S2G), providing evidence of on-target action for the effects observed for K17 knockdown in the SiHa and CaSki lines.

Cisplatin is used as first-line therapy in advanced or recurrent cervical cancers and the toxic effects are greatest in cells arrested in G1 (21). As K17 knockdown led to G1 arrest, we predicted that silencing K17 would increase cisplatin sensitivity. The cisplatin concentration required to inhibit cell viability by 50% (IC50) for SiHa with K17 knockdown was half that for control cells. Similarly, the IC50 value for CaSki cells with K17 knockdown was lower than in control cells, although the difference was not significant (Fig. 2F). The percentage of apoptotic cells and expression of cleaved caspase-3 were greater in RNAi-treated cells, whereas expression of phospho-AKTSer472 was reduced (Fig. 2G and H). Overall, K17 knockdown exerted a synergistic effect, providing evidence that K17 drives cell-cycle progression and chemoresistance, potentially explaining its association with poorer prognosis in cervical cancer.

K17 knockdown promotes p27KIP1 nuclear accumulation and stabilization

RNAi targeting of K17 was accompanied by 3- to 5-fold increased p27KIP1 protein but no increase in its mRNA level (Fig. 3A, Supplementary Fig. S3A). Elevation of p27KIP1 is known to inhibit G1 cyclin-dependent kinases. Consistent with this, K17 knockdown decreased Rb phosphorylation by 50% and decreased expression of cyclin A (an S/G2-associated cyclin; Fig. 3B, Supplementary Fig. S3B). We did not detect changes in the G0 marker.

Figure 2. K17 knockdown induces cell-cycle arrest and drug sensitivity. A, effects of K17 knockdown in cell proliferation (Protocols 3 and 7). B–D, G1–S ratio in cervical (SiHa, CaSki, and C33-A), pancreatic (L3.6), and breast (MDA-MB-231 and MDA-MB-468) cancer cells (Protocols 3, 5, and 7). E, effects of K17 knockdown in postmitotic G0–G1B ratio (Protocols 3 and 9). F, K17 knockdown effect on dose–response curves with cisplatin. Table indicates the half-maximal inhibitory concentration (IC50) values for each condition (Protocols 3 and 7). G, Annexin V-positive cells after cisplatin treatment (Protocols 3 and 10). H, cleaved caspase-3 and phosphorylated AKT at Ser473 after cisplatin treatment (Protocols 3 and 17). Quantitative data are presented as averages ± SD of triplicate samples. Relative protein levels quantification by densitometry and normalized to GAPDH loading control. Statistical analyses were carried out by t test or Mann–Whitney U test. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
**Figure 3.**
K17 knockdown correlates with nuclear p27<sup>KIP1</sup> accumulation and stability. A–D, expression of p27<sup>KIP1</sup> (A, C, D) and phospho-pRb (B; Protocols 4, 5, and 17). E, p27<sup>KIP1</sup> expression in nuclear and cytosolic fractions (Protocols 4, 14, and 17). F, nuclear p27<sup>KIP1</sup>-positive cells (Protocols 3 and 2). G, phospho-p27<sup>KIP1</sup> in Ser10 (p-p27<sup>KIP1</sup> Ser10) expression (Protocols 4 and 17). H, relative p27<sup>KIP1</sup> protein levels after 3 hours of serum starvation release. p27<sup>KIP1</sup> levels at time point (0 hour) were set at 100%. (Protocols 4 and 12). Quantitative data are presented as averages ± SD of triplicate samples. Relative protein levels quantification by densitometry and normalized to GAPDH or lamin loading control. Statistical analyses were carried out by t test or Mann–Whitney U test. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 4.
K17 interacts with p27\(^{kip1}\) in the nucleus and promotes its translocation into the cytoplasm. A, K17 unique bipartite NLS among type I keratins in humans (h) and conserved only in primates. SV40 and nucleoplasmin are prototypes of bipartite NLS. B and C, K17 and p27\(^{kip1}\) levels and K17 binding to p27\(^{kip1}\) at different time points of G1 (Protocols 12, 17, and 18). CHX, cycloheximide. D and E, two-dimensional structured illumination microscopic images from immunofluorescent nuclear colocalization of K17 with p27\(^{kip1}\) in cells. Scale bar, 5 \(\mu\)m. C, cytoplasm; N, nucleus. (Continued on the following page.)
p130, demonstrating that K17 knockdown causes cell-cycle arrest rather than cell-cycle exit (Supplementary Fig. S3C). In contrast, expression of K17 in C33-A cells eliminated endogenous p27KIP1 protein expression (Fig. 3C). Similarly, p27KIP1 expression increased in pancreatic and breast cancer cells following K17 knockdown (Fig. 3D). Expression of K17 in HeLa293 cells, derived from benign tissue, also resulted in significantly decreased p27KIP1 expression (Supplementary Fig. S3D). To act as a cell-cycle inhibitor, p27KIP1 must be located in the nucleus, whereas its cytoplasmic sequestration enables cell-cycle progression. In the absence of K17, nuclear but not cytoplasmic levels of p27KIP1 increased significantly (2-3 fold, Fig. 3E and F).

The major phosphorylation site of p27KIP1 is at serine 10 (Ser10) and this is required for nuclear export (22, 23). We found that in the absence of K17, export-tagged p27KIP1 accumulated in the nucleus, correlating to early G1 arrest (Fig. 3G). Although p27KIP1 is one of the key cyclin-dependent kinase inhibitors (CKI) that ensure correct G1 phase timing, we further screened for gene expression of other CKIs in K17-knockdown cells (Supplementary Fig. S3E). shKRT17-treated cell levels of p21(CIP1/WAF1) were significantly decreased only in CaSki, indicating that G1 arrest was solely attributed to p27KIP1. Because HPV promotes aberrant cell-cycle progression in cervical cancer by E6-mediated degradation of p53 and E7-mediated degradation of pRb, we evaluated the levels of E6 and E7; no differences were found in shKRT17-expressing cells compared with controls (Supplementary Fig. S3F). Thus, K17 knockdown results in G1 arrest through p27KIP1 nuclear accumulation, independent of other CKIs and E6 and E7 oncoprotein expression.

p27KIP1 translation and protein stability are maximal during G1 and increased p27KIP1 nuclear export and degradation enables G1–S transition (11). Serum-starved control and K17-knockdown cells in G1 were stimulated with FBS to trigger the G1 to S transition in the presence of cycloheximide to block protein translation. p27KIP1 levels decreased more than 40% in the K17-expressing control cells but were virtually unchanged in K17-knockdown cells (Fig. 3H). These findings indicate that p27KIP1 degradation at G1 is reduced in the absence of K17.

p27KIP1 is targeted by KPC1 and SKP2 ubiquitin-E3 ligases, which function at G1 and S-phases, respectively (24, 25). We thus screened for the expression and ubiquitination activity of KPC1 and SKP2. Only in SiHa cells with K17 knockdown, there was an increase in SKP2 expression, potentially due to G1 phase arrest (Supplementary Fig. 4A–E). Of note, KPC1, but not SKP2, co-immunoprecipitated with K17 and p27KIP1 and colocalized with K17 (Supplementary Fig. S4F–S4H). Overall, these results suggest that p27KIP1 stabilization is mediated by retention or delayed nuclear export, preventing degradation, rather than as a result of deficiency in the ubiquitin-mediated degradation by KPC1 or SKP2.

K17 interacts with p27KIP1 in the nucleus

Intermediate filaments are functionally regulated and reorganized by posttranslational modifications, which cause filament disassembly and formation of “speckles” with different functional properties (26). Consistent with previous reports on the nuclear lamina speckles (26), perinuclear, and nuclear keratin speckles (27, 28), we found that there was an increase of nuclear K17 speckles by immunofluorescent imaging and the presence of K17 in the nuclear-soluble fraction of cells (Supplementary Fig. S5A and S5B). The recognition of nuclear K17 speckles during G1 phase prompted an in silico analysis to further characterize K17 sequence and this subcellular localization. This revealed a classical bipartite nuclear localization signal (NLS) in residues 385 to 400 of K17, specific among type I keratins and present only in primates but not in other species (Fig. 4A). The CNLS-Mapper (29) cutoff score of 3.3 predicts that K17 should be found in both the nuclear and cytoplasmic compartments. In addition, we did not find an NLS in type II keratins (data not shown; ref 29). Hypothesizing that nuclear K17 might bind to p27KIP1 within the nucleus before p27KIP1 degradation, we demonstrated that nuclear K17 binds to p27KIP1 during early G1 phase by immunoprecipitation and colocalizes with it using super-resolution structured illumination analysis (Fig. 4B–D). More than half the cells had at least one colocalized signal for K17 and p27KIP1 (Fig. 4D and E, Supplementary Fig. S5D). Of note, total levels of K17 remained constant (Fig. 4B). K17 did not bind to lamin (Fig. 4C), and p27KIP1 was not co-immunoprecipitated in C33-A cells (Supplementary Fig. S5C).

K17 phosphorylation at serine 44 (Ser44) has been reported as a major posttranslational modification during cellular proliferation (27). We found nuclear K17-phospho Ser 44 immunoprecipitated with p27KIP1 in the nuclear fraction of SiHa and CaSki cells after serum starve release (Fig. 4F, Supplementary Fig. S5E). Nuclear K17 binding to p27KIP1, however, was not dependent on Ser 44 phosphorylation or other posttranslational modifications, as pull-down of wild-type recombinant K17 effectively co-immunoprecipitated with p27KIP1 in the nuclear fraction of K17-knockdown cells as well (Fig. 4C). These results suggest that K17 posttranslational modifications may be required for the release of K17 from intermediate filament and/or nuclear translocation as a speckle but are not required for p27KIP1 binding.

K17 promotes p27KIP1 nuclear export

Nuclear export of p27KIP1 involves an adaptor for CRM1 exportin binding. In silico analysis, using ValidNESs (30), we identified a putative leucine-rich, nuclear export signal (NES) dependent for CRM1 binding in K17, between residues 194 and 199 (Fig. 4H). Treatment of SiHa and CaSki cells with LMB, a chemical inhibitor of the CRM1-dependent nuclear export, caused a more than 2-fold retention of nuclear K17 and

(Continued)
p27^KIP1 (Fig. 4I). To a lesser extent, JAB1, a reported adaptor between p27^KIP1 and CRM1 exportin (31), accumulated in the nucleus after LMB treatment (Fig. 4I). In addition, immunoprecipitation analyses revealed that K17 and export-tagged p27^KIP1 bind to CRM1 (Fig. 4J). These findings are consistent with prior observations that K17 (28) and p27^KIP1 (9) are exported from the nucleus through CRM1.

The three leucines within the K17-NES (L194A, L197A, and L199A) and the two lysines within K17-NLS (K399A and K400A) were replaced with alanines to determine whether manipulations of the NLS and/or NES affected p27^KIP1 export. C33-A cells were transfected with vectors encoding human wild-type K17 with the putative NES and NLS (Wt), or mutated NES (mNES), or mutated NLS (mNLS), and the p27^KIP1 levels in nuclear and cytoplasmic fractions were quantified. Nuclear p27^KIP1 was lost in cells expressing Wt K17. In contrast, nuclear p27^KIP1 levels were 3-fold higher in cells expressing either mNLS- or mNES-K17 mutations (Fig. 4K–M). Furthermore, nuclear localization of K17 was abolished in mNLS cells (Fig. 4K and M). In addition, we determined that nuclear mNES-K17 had a slight increase in apparent molecular weight, compared with nuclear Wt K17 potentially attributed to posttranslational modifications that accumulate on K17 that is retained in the nucleus. The overexpression K17-mNES, which has a putative NLS, did not affect the nuclear localization of p27^KIP1, compared with the overexpression of K17-mNLS (Fig. 4K and L). These findings suggest that K17-NLS does not influence the nuclear localization of p27^KIP1 and favor a model in which K17 works in a unidirectional way, promoting p27^KIP1 nuclear export during G1 phase to promote cell-cycle progression in cancer cells.

K17 knockdown decreases tumor growth

Tumors derived from SiHa or CaSki cells expressing control shRNA were more than twice the size of those derived from shKRT17 cells (Fig. 5A and B). In combination, tumors expressing K17 were more than 3.5 times larger than tumors with silenced K17 (Fig. 5C). Tumors with K17 knockdown showed increased p27^KIP1 expression and decreased expression of proliferating cell nuclear antigen (PCNA) and Ki67, S-phase, and interphase makers, as measured by Western blotting and immunohistochemistry, respectively (Fig. 5D and E). These results suggest that G1 arrest by K17 knockdown significantly decreases tumor growth by retention of nuclear p27^KIP1.

Inverse correlation between K17 and p27^KIP1 expression in human cervical cancer

To investigate the relationship between K17 and p27^KIP1 in human cervical cancer specimens, we examined the co-expression of these proteins in tumors with high- versus low-K17 status. Nuclear p27^KIP1 was significantly higher in low-K17 cases than in high-K17 cases (Fig. 6A and B). Intratumorally, tumor nests with K17-positive cells had decreased nuclear p27^KIP1 compared with
adjacent K17-negative cells (Fig. 6C and D). Of note, cytoplasmic sequestration of p27KIP1 was not detected for any tumor type (data not shown).

Earlier prognostic studies on p27KIP1 in carcinomas reported contradictory results regarding its association with survival and disease recurrence (11, 32). Our study, however, reveals that K17 status is a stronger predictor of survival than p27KIP1 status by Kaplan–Meier analysis (Fig. 6E and F). To determine whether K17 alone or in combination with p27KIP1 performed as a more accurate negative prognostic marker, three groups were distinguished using K17 and nuclear p27KIP1 expression: (i) high-K17/low-p27KIP1, (ii) low-K17/low-p27KIP1, and (iii) predominantly low-K17/high-p27KIP1 (Fig. 6G). Overall survival was significantly decreased only in the high-K17/low-p27KIP1 group compared with low-K17/low-p27KIP1 and predominantly low-K17/high-p27KIP1 (Fig. 6H and I). According to the HRs (Supplementary Table S5), we concluded that high-K17 status, independent of p27KIP1 status, is a better prognostic marker in cervical cancer.

**Discussion**

Here, we show that K17 expression predicts poor patient survival, both within low- and high-stage cervical cancer patient populations. Thus, K17 status provides information that goes beyond classical clinicopathologic parameters that are currently used to guide patient management decisions. Furthermore, we determined that the mechanistic basis for K17 as a negative prognostic biomarker can be explained, at least in part, by the discovery that K17 promotes nuclear export and subsequent degradation of tumor suppressor p27KIP1. We previously identified K17 as a candidate cervical cancer biomarker by proteomic analysis of normal cervical mucosa, cervical intraepithelial neoplasia, and cervical squamous cell carcinoma, and other reports have suggested that K17 expression may be overexpressed in pancreatic, uterine, colorectal, and head and neck carcinomas (5–35). Thus, these prior observations, together with our current in vitro and in vivo data, suggest that K17 expression could in fact be involved in oncogenic signaling of a wide range of cancer types.

Nuclear localization of p27KIP1 controls G1 timing, as accelerated p27KIP1 nuclear export and degradation triggers constitutive proliferation, deficient DNA replication, and increased mutations (39). In the absence of K17, cancer cells arrested at early G1 accompanied with nuclear accumulation and stabilization of tumor suppressor p27KIP1, and consequently pRb phosphorylation was decreased following K17 knockdown, consistent with inhibition of G1 cyclin-dependent kinases by elevated p27KIP1. In addition, in cervical cancer specimens, K17 expression was inversely correlated with p27KIP1 nuclear levels. Previously, a study reported that, in the absence of K17, cell proliferation is reduced in basal cell carcinoma; however, this reduction was not further linked to cell-cycle events (40). Our results suggest that K17 deregulates key tumor suppressor programs in G1, promoting p27KIP1 nuclear export, sustained proliferation, and tumor growth.

p27KIP1 lacks the classical leucine-rich NES and mutation of the suggested NES impairs but does not abolish nuclear export (9), suggesting alternative mechanisms for p27KIP1 and CRM1 binding. We report that K17 serves as a bridge between p27KIP1 and CRM1, as point mutations in K17-NES lead to nuclear accumulation of both K17 and p27KIP1 as does LMB treatment. The slight reduction of IAB1 nuclear export by LMB treatment suggests that K17-NES, rather than IAB1-NES (31), is the primary mediator of p27KIP1 nuclear export in K17-expressing cancer cells. Although p27KIP1 nuclear export seems to be mediated by several mechanisms, our results suggest that K17 promotes p27KIP1 nuclear export in cervical, pancreatic, and breast cancer cells. In contrast to the previous NES sequences reported for K8 (residues 377–386) and K18 (residues 299–308; ref. 28), K17-NES is positioned closer to the C-terminus (residues 194–199) than to the N-terminus part of the protein.

To our knowledge, this is the first report of the existence and mechanistic role of the K17 NLS, a sequence that is found only among primates. Mutations of the NLS prevent K17 nuclear localization and result in p27KIP1 nuclear accumulation. We propose that mitogen stimulation of K17-expressing tumor cells results in posttranslational modifications of K17 that increase its nuclear targeting and binding of p27KIP1 and CRM1, leading to p27KIP1 export. Previous studies on other keratins reported their role in nuclear localization of Egr1 in breast cancer cells (31) and cytoplasmic retention of 14-3-3ε in mouse keratinocytes (32). Therefore, the confirmation that K17 undergoes nuclear translocation casts light on the dynamic role of K17 that extends beyond its role in mechanical support, functioning as a nuclear shuttle for p27KIP1 and potentially other tumor suppressors and nuclear proteins.

Persistent infection with high-risk HPV is a necessary but not sufficient factor for cervical SCC development. K17 is not expressed in normal cervical mucosa and is nearly absent in transient HPV infection (LSIL); however, it is significantly upregulated in high-grade premalignant lesions (HSIL) and cervical cancer (1), suggesting that p27KIP1 nuclear export may start as early as in HSIL. K17 expression is increased in premalignant lesions and carcinomas of several anatomic sites, independent of HPV (37, 43–46). Our data on the transgenic K14-HPV16 animal model showed that K17 upregulation is independent of HPV oncoprotein expression (unpublished data); however, the absence of K17 significantly delays the onset of HPV-induced neoplasia (47) and the transformation of progenitor cells of Ewing sarcoma (48). We found that HPV oncoprotein expression is independent of K17 expression levels; however, K17 silencing bypasses the effects of E7, restoring G1-S checkpoint in cancer cells. Overall, these results contribute to the hypothesis that K17 is an oncoprotein that mediates onset of cellular transformation independent of HPV and cell type, potentially by mediating tumor suppressor p27KIP1 nuclear export and other functions.

The association between K17 and poor prognosis and the 2-fold increase in cisplatin sensitivity that results from K17 silencing suggests that K17 inhibition might increase the effectiveness of cisplatin chemotherapy. It remains unknown, however, whether K17 knockdown contributes to drug sensitivity with other
Figure 6.
Inverse K17 and p27\textsuperscript{kip1} expression in human cervical cancer. A and B, K17 and p27\textsuperscript{kip1} immunofluorescent expression in low- versus high-K17 cervical cancer specimens. Scale bars, 100 μm in merged and 50 μm in magnified (Protocol 2). C and D, K17 and p27\textsuperscript{kip1} immunofluorescent expression on tumor nests from cervical cancer specimens, with K17-positive and -negative cells. Scale bars, 50 μm in merged and 10 μm in magnified. Arrowhead, p27\textsuperscript{kip1}/K17\textsuperscript{−/−} cell (Protocol 2). The horizontal dashed lines represent the mean and the solid lines represent the median. Boxes represent the interquartile range, and the whiskers represent the 10th and the 90th percentiles, and black circles represent the outliers. Mann–Whitney U tests: **, P < 0.05. E and F, Kaplan–Meir curves depicting the probability of overall survival based on p27\textsuperscript{kip1} (E) and K17 (F) expression on the same SCC patient set. Low- and high-p27\textsuperscript{kip1} or K17 statuses were defined as ≤10 arbitrary fluorescent units on ImageJ64. G–I, scatter plot of K17 and p27\textsuperscript{kip1} expression in low- (gray circles) versus high-K17 (green circles) cervical cancer cases as described in A. Three groups defined: (i) high K17/low p27\textsuperscript{kip1}, (ii) low K17/low p27\textsuperscript{kip1}, and (iii) predominately low K17/high p27\textsuperscript{kip1} (G). Kaplan–Meir curves depicting overall survival of each one of the three groups mentioned above (H) and combined groups (I). P values calculated using the log-rank test. J, schematic representation of K17-mediated p27\textsuperscript{kip1} nuclear export.

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K17 Mediates p27\textsuperscript{kip1} Nuclear Export

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chemotherapeutic agents. These results indicate that rapidly growing cancer cells that express K17 are relatively chemoresistant, attenuating cell death, potentially either by sequestering the death adaptor TRADD (49) or by minimizing apoptotic effects (50).

Forced expression of p27KIP1 suppresses tumor growth, favoring cell differentiation and inhibition of tumor metastasis; however, current therapies with proteasome inhibitors that increase p27KIP1, including bortezomib, result in intolerable side effects (14). Because K17 is a prognostic marker in cervical cancer and promotes p27KIP1 nuclear export and degradation, targeted therapy directed against K17 might trigger not only p27KIP1 accumulation but also increased cisplatin sensitivity and therefore reduce drug side effects. Future mechanistic studies may shed light on additional novel functions of cytoplasmic and nuclear K17 in oncogenic transformation, making it an attractive target of anticancer therapies.

Disclosure of Potential Conflicts of Interest

K.R. Shroyer is a consultant/advisory board member for OncoGenesis. No potential conflicts of interest were disclosed by the other authors.

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Keratin-17 Promotes p27^KIP1^ Nuclear Export and Degradation and Offers Potential Prognostic Utility

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