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Increased Cdc7 expression is a marker of oral squamous cell carcinoma and overexpression of Cdc7 contributes to the resistance to DNA-damaging agents

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ABSTRACT

Cdc7-Dbf4 kinase (Dbf4-dependent kinase, DDK) is an essential factor of DNA replication and DNA damage response (DDR), which is associated with tumorigenesis. However, Cdc7 expression has never been associated to the outcome of oral squamous cell carcinoma (OSCC) patients, and the mechanism underlying cancer cell survival mediated by Cdc7 remains unclear. The Cdc7 protein expression of 105 OSCC tumor and 30 benign tissues was examined by immunohistochemistry assay. Overall survival rates of 80 OSCC patients were measured using Kaplan–Meier estimates and the log-rank tests. Cdc7 overexpression by adenovirus system was used to scrutinize the underlying mechanism contributed to cancer cell survival upon DDR. *In silico* analysis showed that increased Cdc7 is a common feature of cancer. Cdc7 overexpression was found in 96 of 105 (91.4%) studied cases of OSCC patients. Patients with higher Cdc7 expression, either categorized into two groups: Cdc7 high expression (2+ to 3+) versus Cdc7 low expression (0 to 1+) [hazard ratios (HR) = 2.6; 95% confidence interval (CI) = 1.28–5.43; $P = 0.0087$] or four groups (0 to 3+) [HR = 1.71; 95% CI = 1.20–2.44; $P = 0.0032$], exhibited a poorer outcome. Multivariate analysis showed that Cdc7 is an independent marker for survival prediction. Overexpressed Cdc7 inhibits genotoxin-induced apoptosis to increase the survival of cancer cells. In summary, Cdc7 expression, which is universally upregulated in cancer, is an independent prognostic marker of OSCC. Cdc7 inhibits genotoxin-induced apoptosis and increases survival in cancer cells upon DDR, suggesting that high expression of Cdc7 enhances the resistance to chemotherapy.

1. Introduction

Cdc7-Dbf4 kinase (Dbf4-dependent kinase, DDK) is required for the initiation of DNA replication by targeting MCM2-7complex and recruiting replication initiation proteins, such as Cdc45 and GINS, to origins [1,2]. Cdc7 is a serine/threonine kinase, conserved from yeast to humans, and its kinase activity is stimulated by binding to its regulatory protein Dbf4 [3]. DDK is critical for the initiation of DNA replication from each individual origin, including both early and late firing origins [1,4]. In addition to the role in DNA replication, DDK is involved in other cell cycle events, such as DNA damage response (DDR) including S phase checkpoint and DNA repair [5–8]. Aberrations in DNA replication and DDR are major causes to tumorigenesis and genome instability, a hallmark of cancer cells [9]. Indeed, Cdc7 and Dbf4 are overexpressed in many cancer cell lines and in certain primary tumors [10,11]. Cdc7 overexpression is associated with tumor advanced clinical stage, cell cycle deregulation, and genomic instability in ovarian [12] and breast cancer [13,14]. Like other replication factors, increased

Abbreviations: DDK, Dbf4-dependent kinase; DDR, DNA damage response; OSCC, oral squamous cell carcinoma; IHC, immunohistochemistry; HU, hydroxyurea; CPT, camptothecin; CI, confidence interval; HR, hazard ratios; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

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Cdc7 levels are thought to link to proliferation of tumor cells [15]. However, increased Cdc7 levels do not always associate with cell proliferative status. A previous report showed that Cdc7 and Dbf4 overexpression cause cell-cycle arrest in S or G2/M phase depends on their expression level [16]. On the other hand, knockdown of Cdc7 was shown to cause cell death in cancer cells, but not in normal cells in which p53-dependent pathways arrest the cell cycle in G1 phase. In addition, the apoptotic response induced in cancer cells by Cdc7 depletion is not mediated by p53 [17]. Thus, the fact that specific tumor-killing activity of Cdc7 inhibition has allowed for the development of small molecules targeting Cdc7 kinase for cancer therapy [11,18–21].

Head and neck cancer including oral cancer is the sixth most common cancer worldwide and has become a major concern with the rising trend of incidence in young and middle-aged men [22,23]. In Taiwan, oral cancer is a leading cause of cancer-related death, ranked the fourth most prevalent carcinoma, and it has the fastest increasing incidence among all cancers in adult males [24]. The most common subtype of oral cancer is squamous cell carcinoma (OSCC) [23]. So far, the most commonly used prognostic markers of OSCC are tumor stage and lymph node status [23]. However, the reliability of these clinical prognostic markers is not as available as expected at time of diagnosis [25]. To date, there is no report to mention Cdc7-Dbf4 in oral cancer. In these regards, we investigated Cdc7 expression in oral cancer and identified Cdc7 as an independent prognostic marker in OSCC patient outcome. In addition, upregulated Cdc7 increases the resistance to DNA-damaging agents and enhances the survival of tumor cells.

2. Materials and methods

2.1. Patients and clinical sample

A total of 135 cases were chosen for immunohistochemistry (IHC) analysis based on availability of archival human oral tissue blocks from diagnostic resection specimens. Specimens including 105 OSCC tumor tissues and 30 benign tissues of epithelium were retrieved from the archives of the Departments of Pathology at Mackay Memorial Hospital, Taipei, Taiwan from January 1999 to December 2006 with approval from the Institutional Review Board (IRB number: 11MMHIS067). The cases that were lost to follow-up or had insufficient clinicopathological data for analysis were therefore excluded. Thus, a total of 80 cases of OSCC were included in the study. The main clinical characteristics of the 80 patients selected for this study are detailed in Table S1.

2.2. Cell culture

HSC-3, FADU, and KB cells were cultured in medium containing Dulbecco's modified Eagle's essential medium (DMEM), supplemented with 5% fetal bovine serum (FBS) and 5% super calf serum. The all medium contain penicillin 100 Units/mL, streptomycin 100 μg/mL except mentioned. SCC-4, SCC-9, SCC-15, SCC-25, and SAS cells were cultured in medium containing a 1:1 mixture of DMEM/F12 medium, supplemented with 10% FBS. OC3 cells was cultured in medium containing a 1:1 mixture of DMEM/K-SFM medium, supplemented with 10% FBS and OEC-M1 and YD-15 cells were cultured in medium containing RPMI 1640 medium, supplemented with 10% FBS. Oral pre-cancer lesion cell DOK (dysplastic oral keratinocyte) was cultured in medium containing DMEM, supplemented with 10% FBS [26]. Oral keratinocyte normal cell lines, CGHNK2 and CGK6 [27], were grown in a culture medium containing K-SFM medium, supplements for K-SFM, supplemented with G418 (400 μg/mL).

2.3. Short hairpin RNA (shRNA) and retroviral Infection

Silencing of endogenous Cdc7 in SCC-15 cells was performed by retroviral infection using the vector pMKO expressing corresponding shRNAs. [28]. pMKO-based shRNA plasmids were constructed by inserting the annealed and phosphorylated shRNA target sequences into pMKO. The shRNA target sequence used was Cdc7: GCTCAGCAGGAAAGGTGTTCA. The plasmids, Cdc7-ShRNA-pMKO-puro along with packaging plasmid gag-pol and envelope plasmid VSV-G, were transfected into 293T cells by TurboFect. Retroviral supernatant was collected at 24 and 48 h post-transfection and used to infect the target cells SCC-15 for 48 h. Polybrene (hexadimethrine bromide) was added to the medium for improving infection efficacy. After 24 h recovery, puromycin (Sigma–Aldrich) was used to select the successfully infected cells at a final concentration of 2 μg/mL and the survived cells were collected to check the expression of Cdc7 by Western blotting.

2.4. Whole cell lysate

The cell lysate preparation was performed as described previously [8]. Cells were lysed in NETN (150 mM NaCl, 1 mM EDTA, 20 mM Tris–Cl pH 8.0, 0.5% NP-40 (v/v)) containing protease and phosphatase inhibitors (50 mM sodium fluoride, NaF, 0.1 mM sodium orthovanadate, $NaVO_4$).

2.5. Antibodies

Antibodies to human Dbf4 were generated by immunizing rabbits with using OVA-conjugated peptide (SDFSTDNSGSQPKQKSDTV) [8]. Antibodies used in this study were purchased as indicated: Antibody to Cdc7 (DCS-341) from Thermo Scientific; cleaved Caspase-3 (Asp175, 5A1E) antibody from Cell Signaling Technology; Bax and Bcl-2, from Santa Cruz Biotechnology, Inc.; Myc (9E10) from Millipore and Actin from GeneTex.

2.6. Western blot analysis

The cells were harvested by trypsinization and lysed with NETN buffer (20 mM Tris (pH 8.0), 1 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40 (NP-40)) containing protease inhibitor cocktail (Roche). The cell lysates were then centrifuged at 10,000 g at 4 °C to obtain solubilized cellular proteins. Protein was quantified with a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) according to the manufacturer's instructions. Proteins were separated by 8% or 10% or 12% SDS–PAGE and electrotransfered to a polyvinylidene fluoride membrane. Blots were probed with primary antibodies, followed by HRP-conjugated goat anti-rabbit IgG (1:10000, v/v) (GeneTex, Hsinchu, Taiwan) or HRP-conjugated goat anti-mouse IgG (1:10000, v/v) (GeneTex, Hsinchu, Taiwan). After washing with PBS containing 0.5% Tween-20, peroxidase activity was assessed using enhanced chemiluminescence (PerkinElmer Life Science, Boston, MA, USA). For an internal control, the same membrane was re-probed with a monoclonal antibody directed against β-actin (1:10000, v/v). The intensities of the reaction bands were analyzed with the Image Gauge System (Fuji, Tokyo, Japan).

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) and reverse-transcribed at 37 °C with SuperScript II reverse transcriptase (Invitrogen). The resulting cDNA was used as the template for PCR reactions. Real-time PCR (qPCR) reactions were performed on a RotorGene 3000 system (Corbett Research, Mortlake, Australia) using SYBR Green PCR Master Mix (Cambrex Co., East Rutherford, NJ). The sets of forward and reverse primers, the corresponding PCR conditions, and the lengths of PCR products were described as follows: Cdc7 [5′-TGCTATG CAACA GATAAAGTTTGTAG-3′, 5′-TCCTGG TGTA CCTGCCCTA-3′; 94 °C (30 s), 60 °C (60 s) and 72 °C (30 s) for 40 cycles, 72 bp]; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [5′-GAAGGTGAAG GTCGGAGT-3′, 5′-GAAGAT GGTGATGGGATTTC-3′; 94 °C (30 s), 52 °C (30 s) and 72 °C (1 min) for 28 cycles, 220 bp]. All the PCR reactions were started at 94 °C for 5 min and terminated at 72 °C for 5 min. Finally, the data were analyzed using RotorGene software v5.0 (Corbett Research). Differential RNA expressions between various samples were calculated using GAPDH as an internal control.

2.8. Immunohistochemistry (IHC) staining

IHC analysis was performed on an automatic staining machine (BenchMark XT, Ventana Medical Systems, Tucson, AZ, USA) using the iVIEW 3,3-diaminobenzidine (DAB) detection kit (Ventana Medical Systems). Paraffin sections (4 μm) containing human of the 135 OSCC tissues were routinely deparaffinized, hydrated, and heated to 95–100 °C for 4 min to induce antigen retrieval. After inactive the endogenous peroxidase activity, IHC staining was performed with anti-Cdc7 (Thermo Scientific DCS-341, 1:20). All sections were finally incubated with iVIEW copper for 4 min to enhance the signal intensity, then counterstained with hematoxylin, dehydrated, mounted, and observed by a Nikon Eclipse E600 light microscope (Tokyo, Japan). Pictures were acquired using Tissue-Faxs software (TissueGnostics). The staining intensity was estimated in a 4-scored scale (0, Negative staining; 1+, weak; 2+, moderate; 3+, strong intensity). The fraction of stained cells was scored according to the following criteria: Score 0 (no stained or <10% stained cells), Score 1 (11–50% stained cells), Score 2 (51–80% stained cells), Score 3 (>80% stained cells).