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CK2 Phosphorylates and Inhibits TAp73 Tumor Suppressor Function to Promote Expression of Cancer Stem Cell Genes and Phenotype in Head and Neck Cancer[1,2]

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Abstract

Cancer stem cells (CSC) and genes have been linked to cancer development and therapeutic resistance, but the signaling mechanisms regulating CSC genes and phenotype are incompletely understood. CK2 has emerged as a key signal serine/threonine kinase that modulates diverse signal cascades regulating cell fate and growth. We previously showed that CK2 is often aberrantly expressed and activated in head and neck squamous cell carcinomas (HNSCC), concomitantly with mutant (mt) tumor suppressor TP53, and inactivation of its family member, TAp73. Unexpectedly, we observed that classical stem cell genes *Nanog*, *Sox2*, and *Oct4*, are overexpressed in HNSCC with inactivated TAp73 and mtTP53. However, the potential relationship between CK2, TAp73 inactivation, and CSC phenotype is unknown. We reveal that inhibition of CK2 by pharmacologic inhibitors or siRNA inhibits the expression of CSC genes and side population (SP), while enhancing TAp73 mRNA and protein expression. Conversely, CK2 inhibitor attenuation of CSC protein expression and the SP by was abrogated by TAp73 siRNA. Bioinformatic analysis uncovered a single predicted CK2 threonine phosphorylation site (T27) within the N-terminal transactivation domain of TAp73. Nuclear CK2 and TAp73 interaction, confirmed by co-immunoprecipitation, was attenuated by CK2 inhibitor, or a T27A point-mutation of this predicted CK2 threonine phospho-acceptor site of TAp73. Further, T27A mutation attenuated phosphorylation, while enhancing TAp73 function in repressing CSC gene expression and SP cells. A new CK2 inhibitor, CX-4945, inhibited CSC related SP cells, clonogenic survival, and spheroid formation. Our study unveils a novel regulatory mechanism whereby aberrant CK2 signaling inhibits TAp73 to promote the expression of CSC genes and phenotype.

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Introduction

The development of cancers has recently been linked to a small subset of cells capable of reproducing the cancer cell population and forming tumors, designated as tumor-initiating or cancer stem cells (CSC) [1,2]. In head and neck squamous cell carcinomas (HNSCC), cells with CSC-like phenotype and tumor forming properties have been identified in tumors and cell lines [2–6]. Recently, HNSCC CSC were found to be enriched within the "side population" (SP) of cells

Abbreviations: CK2, Casein Kinase 2; CSC, Cancer Stem Cells; DMAT, 2-Dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole; HNSCC, Head and neck squamous cell carcinoma; HEKA, Human epidermal keratinocytes; HOK, Human oral keratinocytes; mt, Mutant; SP, Side population; TAp73, Transactivating p73; TP53, Transforming Protein p53; UM-SCC, University of Michigan Squamous Cell Carcinoma; wt, Wild-type

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excluding Hoechst dye 33342 by fluorescence activated cell sorter analysis [6], a phenotype also associated with export and resistance to chemotherapy. Such isolated SP cells, when compared to non-SP cells, differentially expressed stem cell gene markers *BMI-1* and *ABCG2* transporter, formed self-replicating spheroids *in vitro*, and initiated tumors, characteristic of CSCs. Genes encoding key stem cell factors that promote the developmental stem cell phenotype, including *Sox2*, *Oct4* and *Nanog*, are also increased within tumors and CSC in HNSCC [7]. Sox2, Oct4, and Nanog activation, target gene regulation, and the CSC phenotype are inducible, supporting their functional importance in HNSCC CSCs. However, the signal and transcription factors orchestrating expression of these genes and the CSC phenotype in HNSCC are incompletely understood.

Among possible candidates, CK2 (formerly casein kinase II) has emerged as a key signal serine/threonine kinase that modulates diverse proteins and target cascades to regulate cell fate and growth [8]. CK2 is dysregulated in most cancers examined, including HNSCC, where it is aberrantly expressed and activated [8–10]. CK2 is detected as a tetrameric complex comprised of catalytic α and/or α′ and regulatory β subunits in the cytoplasm that mediate cell signaling. Additionally, catalytic CK2α subunits have also been found to be localized to the nucleus and complexed with chromatin, suggesting a potential role for CK2α in regulating gene transcription and expression [10]. Supporting this possibility, we demonstrated that CK2α is a key mediator repressing expression and function of the critical transcription factor and tumor suppressor TP53, in a subset of HNSCC with wild type *TP53* genotype [11]. Knockdown of CK2 by siRNA, particularly CK2α, increased TP53 mRNA and protein expression, inducing TP53-mediated growth arrest and apoptosis *in vitro*, and inhibiting tumorigenesis of wtTP53 HNSCC xenografts *in vivo* [11]. Intriguingly, TP53 activated by ultraviolet light-induced DNA damage has also been previously implicated in terminating embryonic stem cell renewal, by suppressing *Nanog* transcription and expression [12]. Unfortunately, TP53 is directly mutated in the majority of epithelial malignancies, and >70% of HNSCC [13], compromising its potential to suppress CSC gene expression and tumorigenesis. However, the TP53 family also includes p63 and p73, which are implicated in regulation of self-renewal and programmed cell death and differentiation of squamous epithelia [14,15]. These observations raise the question whether these TP53 homologues that control physiological epithelial self-renewal and differentiation may also be dysregulated by CK2 to unleash the expression of stem cell genes and phenotype in cancer.

We recently showed that HNSCC with mtTP53 often retain and overexpress related family member, TAp73, which has the potential to replace TP53 function [16]. TAp73 has an N-terminal transactivation (TA) domain which shares homology, transactivating, and tumor suppressor function with TP53. In HNSCC with mtTP53, our studies revealed that TAp73 is capable of repressing expression of key TP53 target growth arrest and apoptotic genes including *p21*, *NOXA* and *PUMA*. However, although overexpressed, TAp73 is inactivated by a reversible mechanism involving inflammatory signaling and displacement from p53 promoter response elements by ΔNp63α, a p63 isoform lacking the full N-terminal TA domain. Whether and how CK2 signaling may contribute to TAp73 inactivation, and CSC gene expression and phenotype, is unknown, but could provide a potential mechanism to target for prevention of malignant progression in cells after mutation of TP53.

In the present study, we noted from gene expression profiling that *Sox2*, *Oct4* and *Nanog* gene expression is increased in HNSCC lines in which TAp73 was increased but inactivated, and in the side population previously demonstrated to contain CSC [6]. Thus, we hypothesized that CK2 signaling may inactivate TAp73 to promote CSC gene expression and phenotype in HNSCC with mtTP53. Here, we examined whether CK2 mediates inactivation of TAp73, to orchestrate expression of key CSC-related transcription factor genes *Nanog*, *Sox2* and *Oct4*, the side population, clonogenic survival, and sphere forming CSC phenotypes in HNSCC expressing TAp73 with mtTP53.

Materials and Methods

Cell Lines

The UM-SCC cell lines were obtained from Dr. Thomas E. Carey, University of Michigan, and re-genotyped and origin confirmed in 2010 [17]. Genotyped stocks were frozen and used within 3 months of thawing. Expression of TP53, p63, and p73 isoforms and TP53 sequence for exons 4 to 9 was confirmed in our laboratory as previously reported [16,18]. Primary human epidermal keratinocytes (HEKA) or Oral Keratinocytes (HOK) were cultured in accordance with the supplier's protocol (Invitrogen) and used within 5 passages.

Reagents, siRNA and Plasmid Transfection

CK2 inhibitor 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT) was from Calbiochem and used as described previously [11]. CX-4945 is a novel selective CK2 inhibitor [19] obtained from Cylene Pharmaceuticals under a Materials Transfer Agreement with NIDCD. The oligonucleotide sequences for TAp73 specific siRNA inhibition were: 5′r(CGGAUUCCAGCAUGGACGU)d(TT)3′and 5′r (ACGUCCAUGCUGGAAUCCG).

d(TT)3′ (Integrated DNA Technologies, IDT). The CK2 specific siRNAs were from Dharmacon/Thermo Scientific, CK2A1, siGENOME SMARTpool (Cat# M-003475-03); CK2A2 ON-TARGET plus SMARTpool (Cat# L-004752-00); CK2B, ON-TARGETplus SMARTpool (Cat# L-007679-00); Control siRNA, ON-TARGET*plus* Non-targeting Pool (Cat# D-001810-10-05). The p53/p73 specific response element pG13-luc, PUMA-luc, and p21/WAF1-luc luciferase reporter genes were kindly provided by Dr. Alex Zaika, Vanderbilt University [20]. The expression vector containing a human Flag-pcDNA3-TAp73 was kindly provided by Dr. Zhi-Min Yuan, Harvard University [21]. The TAp73-T27A mutant, in which Thr-27 was substituted to Ala (T27A), was synthesized by GENEWIZ, Inc, and sequence verified. All transfections were performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen/Life Technology). Each sample was assayed in triplicate and data were presented as mean ± SD.

Western Blot and Coimmunopreciptiations

Western blot analysis and co-immunoprecipitation was performed as previously [16] with antibodies indicated, CK2α (Santa Cruz, sc-6479), CK2α′ (Santa Cruz, sc-6481), Nanog (Cell Signaling, 4903), Oct4 (Cell Signaling, 4286), Sox2 (Cell Signaling, 2748), beta-actin (Cell Signaling, 4967), TAp73 (IMGENEX,IMG-246), p73 (IMGENEX,IMG-259A), Oct-1 (Santa Cruz, sc-53830), Flag antibody(Sigma, M2), PUMA (Cell Signaling, 4976).

Real time RT-PCR

RNA isolation and cDNA synthesis were performed as previously [16]. PCR primers for TAp73(GGCTGCGACGGCTGCAGAGC; GCTCAGCAGATTGAACTGGGCCAT)were synthesized by