

Award Number: W81XWH-21-1-0487

TITLE: Overcoming Resistance to EGFR Inhibitors in Advanced Head and Neck Cancers

PRINCIPAL INVESTIGATOR: Erica Golemis, Ph.D.

CONTRACTING ORGANIZATION: The Research Institute of Fox Chase Cancer Center
Philadelphia, PA

REPORT DATE: July 2022

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The Research Institute of Fox Chase Cancer Center 333 Cottman Avenue Philadelphia, Pennsylvania 19111 E-Mail: osr@fccc.edu				8. PERFORMING ORGANIZATION REPORT NUMBER		
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14. ABSTRACT Head and neck squamous cell carcinomas (HNSCCs) affect more than a half million people annually worldwide. The goal of this proposal is to develop strategies to overcome HNSCC resistance to EGFR inhibitors (EGFRis). Based on extensive published and preliminary data, this proposal tests three hypotheses: 1) that upregulation of AURKA provides a major source of resistance to EGFRis in HPV- HNSCC. 2) that tumors most likely to upregulate AURKA will either have highly damaging LOF mutations in TP53, or upregulation of TPX2 or NEDD9, and that these tumors will have the highest level of resistance to EGFRis. 3) that targeting AURKA either with single agent kinase inhibitors, or in combination therapies that inhibit AURKA and a second protein, WEE1, that collaborates with AURKA to control mitotic progression, will be particularly effective in counteracting resistance to EGFRis. In the first year of funding, we have defined the pattern of TP53 mutations in HNSCCs. We have created HNSCC cell models resistant to EGFR-targeting inhibitors, and have been profiling response to AURKA and WEE1 inhibitors. We have accrued specimens from a clinical trial of EGFR inhibitors in HNSCC, which will support correlating therapeutic resistance with AURKA, TPX2, and NEDD9 expression. Work is ongoing.						
15. SUBJECT TERMS Head and neck cancer, head and neck squamous cell carcinoma, targeted therapy, TP53, AURKA, EGFR, WEE1, resistance, combination therapy.						
16. SECURITY CLASSIFICATION OF:				17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 63	19a. NAME OF RESPONSIBLE PERSON USAMRDC
a. REPORT Unclassified	b. ABSTRACT Unlimited	c. THIS PAGE Unclassified	19b. TELEPHONE NUMBER Include area code)			

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INTRODUCTION: The goal of the project is to address the interactions between TP53 mutation status, and the utility of using inhibitors of AURKA, WEE1, and EGFR to achieve therapeutic benefit. Specifically, we proposed to determine how TP53 mutation class (damaging or benign) affected AURKA expression and sensitivity to inhibition of AURKA and EGFR. We also proposed to explore the relative efficacy of AURKA used alone, or an AURKA-WEE1 inhibitor combination, in EGFR inhibitor-resistant tumors, and to determine whether these drugs could preventing or reverse adaptive resistance to EGFR inhibition. Finally, we proposed to define the relationship between TP53 genotype, AURKA expression, and response to EGFR inhibition using clinical trial samples for HNSCC.

KEYWORDS: Head and Neck Cancer, Head and neck squamous cell carcinoma, targeted therapy, TP53, AURKA, EGFR, WEE1, resistance, combination therapy.

ACCOMPLISHMENTS:

What were the major goals of the project?

The goals of the project as approved in the SOW are as follows:

Specific Aim 1: Determine how TP53 mutation class determines AURKA expression and alters sensitivity to inhibition of AURKA and EGFR.

Specific Aim 2: Explore relative efficacy of AURKA monotherapy and an AURKA-WEE1 inhibitor combination in the setting of and in preventing or reversing adaptive resistance to EGFR inhibition.

Specific Aim 3: Define the relationship between TP53 genotype, AURKA expression, and response to EGFR inhibition using clinical trial samples for HNSCC.

What was accomplished under these goals?

Major task 1 (Aim 1), to be performed over months 1-6 was for the Golemis group to use public resources to establish relationship between TP53 mutation segregated by class, and expression of mRNA for AURKA, NEDD9, and TPX2. This work is substantially completed. As a first step in this analysis, we assembled a dataset of 794 patients with HPV-negative HNSCC for whom data were available in cBioportal from The Cancer Genome Atlas PanCancer and Broad Institute data sets, and from AACR-GENIE public resources. Within this dataset, TP53 mutations have been assigned into different functional classes (e.g., considered damaging based on ACMG criteria, Poeta criteria, or others). This classification analysis has been published, as part of a broader study of TP53 mutation patterns in HPV-negative head and neck squamous cell carcinoma, with DOD funding acknowledged (Deneka et al, Clin Cancer Res 2022; PMID 35491653).

For specimens for which mRNA was available, we then analyzed levels of expression of AURKA, NEDD9, and TPX2, examining average level of expression in specimens with different classes of TP53 mutation, and also correlating expression of AURKA, NEDD9, and TPX2 with each other. Based on this, we found that while AURKA mRNA expression in HPV-negative HNSCC tumors was significantly elevated above that in normal adjacent tissue, as is well established (not shown), mRNA expression did not significantly differ based on whether tumors contained wt TP53, or TP53 mutations of various distinct biological classes, including ACMG criteria for pathogenic, Poeta classification for extremely damaging/pathogenic, or gain-of-function (**Figure 1A**). We also performed analysis to explore all potential correlations or between expression of TP53, AURKA, TPX2, and NEDD9. Of these, a very highly significant correlation was between AURKA and TPX2 mRNA expression (**Figure 1B**); all other genes showed no correlation (not shown). This result strongly suggests an underlying source of resistance to AURKA inhibitors in HNSCC may lie in the common co-expression of TPX2 with AURKA, given a TPX2-AURKA interaction is established to reduce effectiveness of AURKA-targeting inhibitors.

Major tasks 2 (Aim 1) and 3 (Aim 2, to be performed over months 6-14, are to functionally test the relation between *TP53* mutation, AURKA mRNA and protein expression and activity, and resistance to the EGFR inhibitors afatinib and erlotinib. For this, the first step was for the Burtens group to generate cell models in FaDu, Cal27, A431, and Detroit 562 cell lines that were made resistant to these EGFR inhibitors. To date, erlotinib and afatinib lines have been generated in the FaDu and Cal27 models, based on gradual step-up in drug dosage over several months.

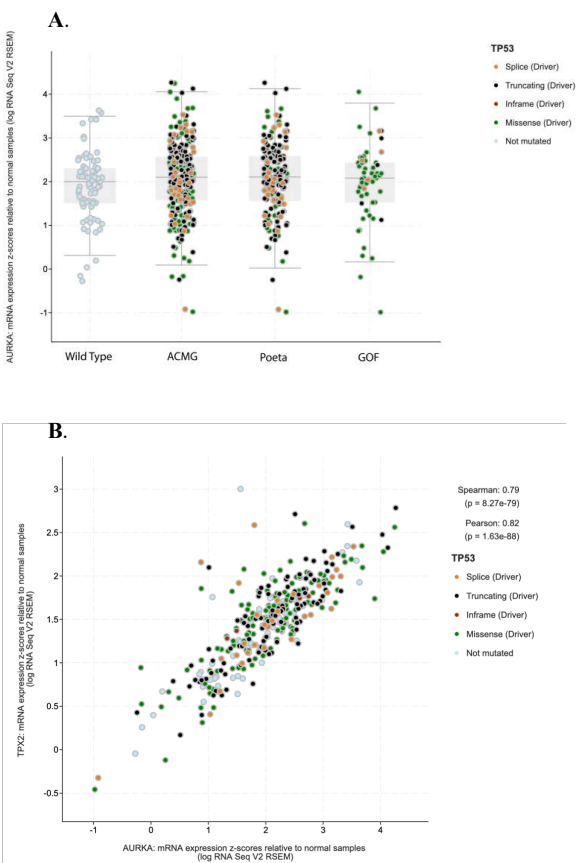


Figure 1. Expression of AURKA based on TP53 mutation class and TPX2 expression. **A.** AURKA expression does not significantly differ in HNSCC tumors bearing distinct classes of TP53 mutations. **B.** AURKA mRNA expression is highly correlated with expression of TPX2.

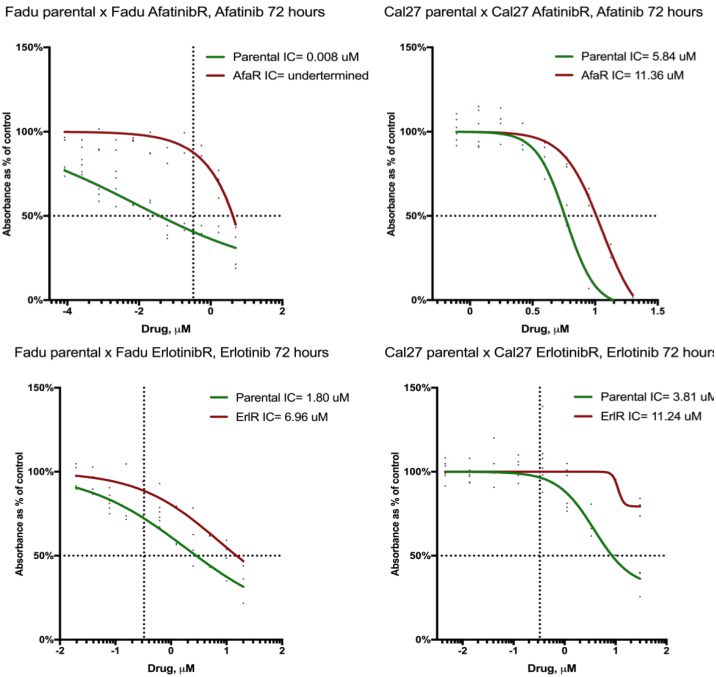


Figure 2. IC₅₀ values of parental FaDu or Cal27 cells treated with erlotinib or afatinib, versus IC₅₀ values of erlotinib- or afatinib-resistant derivatives, as indicated.

Data for the resistant cell lines, reflecting work from the Golemis and Burtneiss groups, are shown in **Figure 2**. For afatinib-resistant FaDu cells, this reflects a difference in IC₅₀ of 8 nM versus >1 μ M; for afatinib-resistant Cal27, 5.8 versus 11.4 μ M; for erlotinib-resistant FaDu, 1.8 versus 7 μ M; and for erlotinib-resistant Cal27, 3.8 versus 11.2 μ M. The establishment of other cell lines resistant to EGFR inhibitors is still in progress, but nearly complete. The selection process has proven to take time because of the need to do very gradual increases in drug dosage to avoid killing the cells, and because of the need to address some unexpected issues that arose (e.g., the tendency of resistant cells to lose attachment to tissue culture plastic). To achieve uniformity of analysis across all cell models, we will perform Western analysis of baseline and EGFR-inhibitor-induced expression of AURKA, TPX2, NEDD9, EGFR, and sentinel effectors once all models are in hand, so they can be analyzed in parallel to achieve greater consistency of findings. This should occur within the next few months.

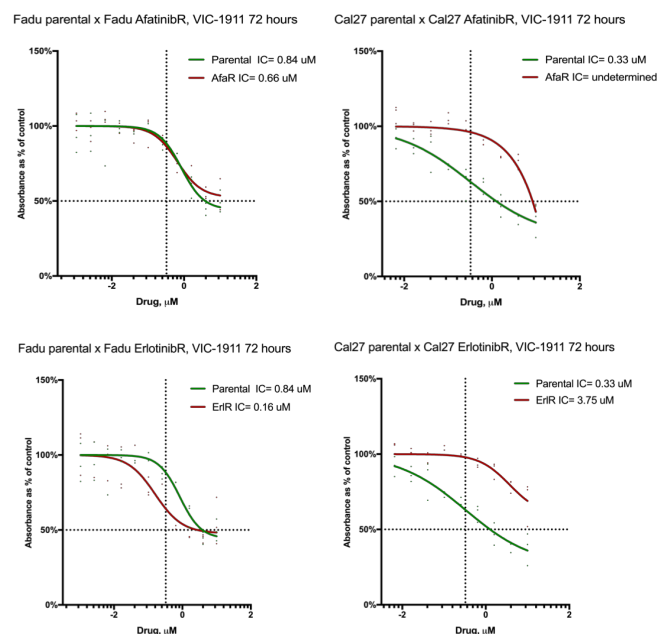


Figure 3. IC₅₀ values of parental FaDu or Cal27 cells treated with VIC-1911, versus IC₅₀ values of erlotinib- or afatinib-resistant derivatives, as indicated.

For Specific Aim 2, the goal is to explore relative efficacy of AURKA monotherapy and an AURKA-WEE1 inhibitor combination in the setting of and in preventing or reversing adaptive resistance to EGFR inhibition. Major task 3, to be addressed by both Burtneiss and Golemis laboratories over months 4-16 of the project, is to determine efficacy of AURKA inhibition with TAS-119 alone or in combination with adavosertib in afatinib- or cetuximab-resistant HNSCC. FaDu, Cal27, Detroit 562 cells and A431 cells. Techniques to be used include cell viability synergy assays, oncosphere, and anchorage independent growth assays, as well as functional assessment with flow cytometry and confocal microscopy. This is well under way with the existing cell models.

Examples of single agent IC₅₀ data for treatment of resistant versus non-resistant lines with TAS-119 (renamed VIC-1911, due to purchase of the compound by another biotechnology company) or adavosertib include **Figures 3 and 4**. All data shown represent the average results from at least three independent experimental replicates.

This yielded somewhat surprising results, with resistance to EGFR targeting agents producing opposing outcomes for resistance to the AURKA inhibitor VIC-1911 in the FaDu versus the Cal27 cell models. In FaDu cells, afatinib or erlotinib resistance had no effect, or increased the sensitivity of cells to VIC-1911; the opposite was observed in Cal27 cells. In contrast, resistance to erlotinib or afatinib increased sensitivity to the WEE1 inhibitor adavosertib, with a modest effect in FaDu cells and a more significant effect in Cal27 cells.

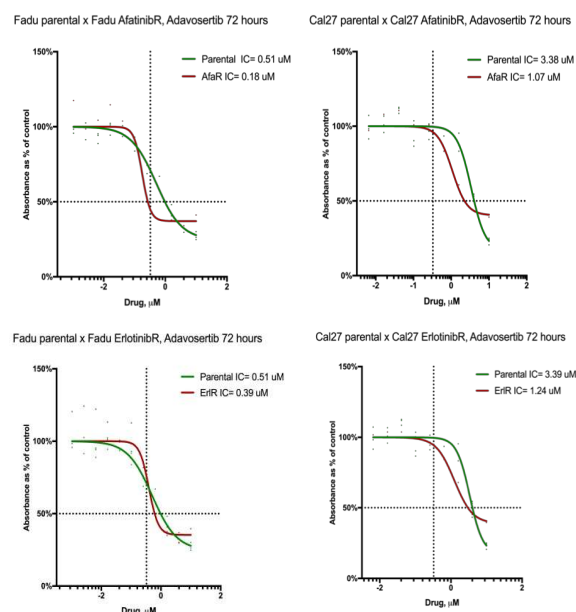


Figure 4. IC₅₀ values of parental FaDu or Cal27 cells treated with adavosertib, versus IC₅₀ values of erlotinib- or afatinib-resistant derivatives, as indicated.

We are in the process of performing synergy tests, using Loewe synergy plot analysis. Examples of analysis of synergy in the parental FaDu and CAL27 cell lines for the combinations of afatinib or erlotinib and VIC-1911, or adavosertib and VIC-1911, are shown in **Figures 5 and 6**. All data shown represent the average results from at least three independent experimental replicates.

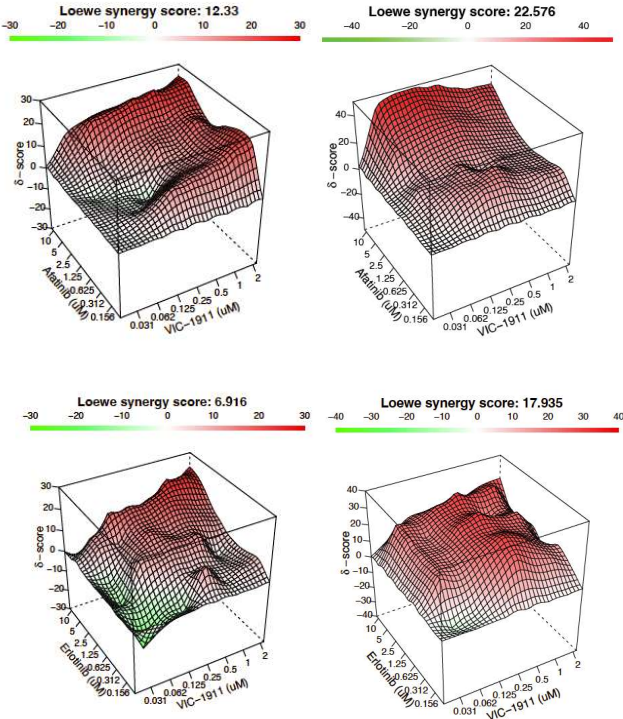


Figure 5. Loewe synergy score for combination of the indicated EGFR inhibitors with VIC-1911 at multiple ratios, as indicated. Left, FaDu; right, Cal27; top, afatinib; bottom, erlotinib.

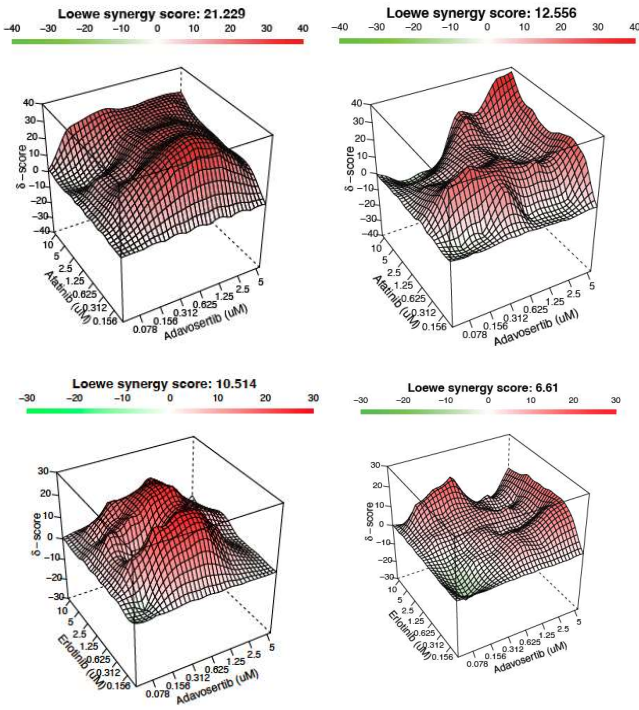


Figure 6. Loewe synergy score for combination of the indicated EGFR inhibitors with adavosertib at multiple ratios, as indicated. Left, FaDu; right, Cal27; top, afatinib; bottom, erlotinib.

Based on this analysis, the most synergistic drug ratios, and degree of synergy for each model are summarized here:

Cell Lines	Drug 1	Drug 2	Loewe synergy score	Most synergistic ratio (Drug 1/ Drug 2)	Optimal Ratio synergy score
Fadu	Afatinib	VIC-1911	12.33	1:3	16.3
	Erlotinib	VIC-1911	6.92	5:1	24.6
Cal27	Afatinib	VIC-1911	22.58	5:1	32.2
	Erlotinib	VIC-1911	17.93	20:1	31.2

Cell Lines	Drug 1	Drug 2	Loewe synergy score (overall)	Most synergistic ratio (Drug 1/ Drug 2)	Optimal Ratio synergy score
FaDu	Afatinib	Adavosertib	21.23	1:2	37.6
	Erlotinib	Adavosertib	10.51	1:1	27
CAL27	Afatinib	Adavosertib	12.56	1:16	28.8
	Erlotinib	Adavosertib	6.61	1:8	19.5

In Loewe analysis, synergy scores above 10 typically reflect synergy, while those below -10 reflect antagonism. Very little antagonism was observed at any concentration or combination ratio, whereas many concentrations and combination ratios demonstrated high levels of synergy. These preliminary data support the idea of using EGFR-targeting drugs in combination with VIC-1911 or adavosertib. Similar experiments are now in progress using the erlotinib and afatinib resistant lines. We are also using the optimal ratio of drugs selected from IC50 data to perform the additional assays (clonogenic, FACS, etc) noted in this aim, with this work in progress.

For Aim 2, major task 3, we are also preparing to perform specified xenograft experiments. For this purpose, the Burtess laboratory has successfully obtained ACURO approval for our animal protocol to perform the described in vivo xenograft experiments.

For Aim 3, Major task 6, the Burtess group will analyze specimens from an ongoing clinical trial of response and progression-free survival in patients treated on a phase II trial of afatinib and cetuximab for HNSCC (NCT02979977). Accrual of specimens to this trial is currently 39 of 50 patients. Accrual of 6 patients over the past year was slightly below the anticipated volume due to COVID-related staffing constraints, now resolved. We anticipate accruing the remaining 11 patients in the next 12 to 18 months. Baseline tissue is available from all patients. Post-treatment biopsies are in hand from 18 patients. A patient-derived xenograft from an on-study biopsy from an immunotherapy-experienced patient is currently growing. Based on this data, we are on track to perform the proposed experiments on the schedule described in the statement of work for this proposal.

Finally, based on our extensive reading related to the topic of this proposal, we wrote a review article providing an update on targeting of AURKA in HNSCC. This article is currently in press at The Cancer Journal (Nguyen, Silva, *et al*, 2022 in press), and cites this DOD support grant.

What opportunities for training and professional development has the project provided?

Nothing to report.

How were the results disseminated to communities of interest?

Two articles reporting on topics related to the work were published.

What do you plan to do during the next reporting period to accomplish the goals?

We will continue progressing through the specific aims of the project, as specified in the SOW. We anticipate significant progress on aims assessing the interaction of drug resistant cell lines with treatment with AURKA and WEE1 inhibitors.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

The Clinical Cancer Research paper by Deneka et al published in 2022 was the first detailed analysis of the profile of TP53 mutations in HNSCC, and their relationship to tumor mutation burden and disease subsite, among other parameters. This provides an important baseline of information for studies of HNSCC involving use of agents expected to cause cell cycle abnormalities that can lead to aneuploidy, such as treatment with WEE1 and AURKA inhibitors (the topic of this proposal) – but also other agents.

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

None

Significant changes in use or care of human subjects

Significant changes in use or care of vertebrate animals.

Significant changes in use of biohazards and/or select agents

PRODUCTS:

Publications, conference papers, and presentations

Deneka, A.Y., Baca, Y., Serebriiskii, I.G., Nicolas, Parker, M.I., Nguyen, T.T., Korn, W.M., Demeure M.J., Wise-Draper, T., Sukari, A., E., Burtness, B., and Golemis, E.A. Association of *TP53* and *CDKN2A* mutation profile with tumor mutation burden (TMB) in head and neck cancer. *Clin Cancer Res* 2022 May 2;28(9):1925-1937; PMID: 35491653, PMCID: PMC9186806. Acknowledges Federal Support

Nguyen, T.T.*, Silva, F.N.*, and Golemis, E.A. Aurora kinases in head and neck cancer. *Cancer J. in press*. PMC in progress; NIHMS ID 1821666; *equal contributions. Acknowledges Federal Support

Website(s) or other Internet site(s)

Nothing to report

Technologies or techniques

Nothing to report

Inventions, patent applications, and/or licenses

Nothing to report

Other Products

Nothing to report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	<i>Erica Golemis, Ph.D.</i>
Project Role:	<i>Principal Investigator</i>
Researcher Identifier (e.g. ORCID ID):	<i>0000-0003-3618-3673</i>
Nearest person month worked:	<i>1</i>
Contribution to Project:	<i>Provided direction.</i>

Funding Support:	<i>DOD</i>
Name:	<i>Ilya Serebriiski, Ph.D.</i>
Project Role:	<i>Co-Investigator</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>4</i>
Contribution to Project:	<i>Provided analysis and effort on aims</i>
Funding Support:	<i>DOD</i>

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Please see attached updated Other Support for Drs. Golemis and Serebriiski. Changes are marked with a line in the right hand margin.

What other organizations were involved as partners? .

Provide the following information for each partnership:

Organization Name:

Location of Organization: *(if foreign location list country)*

Partner's contribution to the project *(identify one or more)*

Financial support;

In-kind support *(e.g., partner makes software, computers, equipment, etc., available to project staff);*

Facilities *(e.g., project staff use the partner's facilities for project activities);*

Collaboration *(e.g., partner's staff work with project staff on the project);*

Personnel exchanges *(e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*

Other.

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Not applicable.

QUAD CHARTS:

Not applicable.

APPENDICES:

Publications.

Other Support

Golemis, Erica A.

CURRENT

P50 DE030707 Pilot (PI: Arora)	4/1/2021 - 6/30/2022	NA
NIH	No Salary	calendar

Developing Biomarkers of Response to Chemoradiation Therapy in Head and Neck Cancer: Towards Precision Medicine

This pilot project is in a no-cost extension.

The major goals of this CEP pilot project are: 1) To determine association of germline SNPs in lysine-modifying genes with CRT outcomes in HNSCC; and 2) To Investigate the functional impact of germline SNPs in lysine-modifying genes on DSB repair.

Procuring Contracting/Grants Officer: Beth Kingsley, osp, 25 Science Pk, 3rd Flor, 150 Munson St., New Haven, CT 06520, 203-737-1103

P30 CA006927 (PI: Chernoff)	8/12/2016 - 7/31/2023	6.7%
NIH	No Salary	0.80 calendar

Comprehensive Cancer Center Program at Fox Chase

The major goal of this Cancer Center Support Grant is to provide partial salary support for professional personnel, including senior and program leadership, administration, planning and evaluation, and developmental funds, as well as support for 4 established peer-reviewed Research Programs, 12 Shared Research Resources and 2 Support Elements.

Procuring Contracting/Grants Officer: Sarah Lee, 9609 Medical Center Dr., BG0609 RM 2W552, Rockville MD 20850, 240-276-6280

R50 CA211479 (PI: Einarson)	9/19/2017 - 8/31/2022	NA
NIH	No Salary	calendar

Margret B. Einarson, Ph.D., Manager, High Throughput Screening Facility, Fox Chase Cancer Center

The major goal of this project is to support research through the High Throughput Screening Facility (HTSF). The HTSF supports ongoing studies to find novel therapeutics for cancer patients and advances toward a deeper understanding of the molecular and biochemical underpinnings.

Procuring Contracting/Grants Officer: Christina Williams, 9609 Medical Center Dr., Bethesda, MD 20892, 240-276-6650

P50 DE030707 (PI: Burtness, Yale Univ.)	9/22/2020 - 6/30/2025	10.0%
NIH		1.20 calendar Yale Head

and Neck Cancer SPORE: Overcoming Treatment Resistance in Head and Neck Cancer

This project is a subcontract to Yale University. Fox Chase Cancer Center will participate on this SPORE as a primary site. Investigators at FCCC will co-Lead Project 2, entitled, "Synthetic Lethal Therapy in TP53 Mutated Head and Neck Cancer." Fox Chase Cancer Center investigators will also support the Biostatistics and Bioinformatics Core, Biospecimen Core and the Career Enhancement Program.

Procuring Contracting/Grants Officer: Makawa Kourouma, OSP, 25 Science Park, 34d Floor, 150 Munson St., New Haven, CT 06520, 203-432-3380

R25 CA259244 (PI: Golemis)	3/15/2022 - 2/28/2027	10.0%
NIH		1.20 calendar

Empowering the Next Generation of Cancer Professionals: the Fox Chase Cancer Center-University of Delaware Partnership for Undergraduate Research and Career Development

The major goals of this project are to: 1) Expose 60 students per year to cancer research careers; 2) Provide immersive mentored research opportunities in laboratories at an NCI-designated Comprehensive Cancer Center; and 3) Mentor research fellows to effectively communicate and establish strong professional networks.

Procuring Contracting/Grants Officer: Long Nguyen, 9609 Medical Center Dr., Rm. 2W532, Rockville, MD 20850, 240-276-5807

N/A (PI: Swayam/Golemis)	1/1/2022 - 12/31/2023	5.0%
TEMPLE	No Salary	0.60 calendar
Exploiting Radiotherapy-Induced MSC Tumor		
Homing to Deliver Immunomodulators to Lung Cancers (Multi-PI)		
The major goal of this pilot project is to explore innovative strategies to increase the therapeutic impact of radiation therapy for lung cancer. In addition to providing advice and guidance, Fox Chase Cancer Center will assist with mouse model experiments.		
Procuring Contracting/Grants Officer: Rosemary Dillon, 1801 N. Broad St., Phila., PA 19122, 215-204-7551		
N/A (PI: Astsaturon)	11/15/2021 - 11/14/2022	2.0%
Hirshberg Fdn	No Salary	0.24 calendar
SCAP Regulation of Pancreatic Acinar Cell Differentiation		
The major goals of this project are to: 1) Confirm the SCAP-SREBP signaling axis regulates normal formation of acinar cells in development; and 2) Evaluate the transcriptional profile induced by SCAP deficiency.		
Procuring Contracting/Grants Officer: Agi Hirshberg, 2990 S. Sepulveda Blvd., Ste. 300C, Los Angeles, CA 90064		
W81XWH-21-1-0487 (PI: Golemis)	7/1/2021 - 6/30/2025	10.0%
DOD	(partial salary)	1.20 calendar
Overcoming Resistance to EGFR Inhibitors in Advanced Head and Neck Cancers		
The major goals of this project are to: 1) Determine how TP53 mutation class determines AURKA expression and alters sensitivity to inhibition of AURKA and EGFR; 2) Explore relative efficacy of AURKA monotherapy and an AURKA-WEE1 inhibitor combination in the setting of, and in preventing or reversing adaptive resistance to, EGFR inhibition; and 3) Define the relationship between TP53 genotype, AURKA expression, and response to EGFR inhibition using clinical trial samples for HNSCC.		
Procuring Contracting/Grants Officer: Amanda Carrera, 820 Chandler St., Fort Detrick, MD 21702, 301-619-2108		

OVERLAP

None

CERTIFICATION REGARDING DISCLOSURE OF FUNDING SOURCES:

In accordance with Section 223(a) of the William M. (Mac) Thornberry National Defense Authorization Act for Fiscal Year 2021, this confirms that: 1) The PI's current and pending support provided on the application is current, accurate, and complete; 2) The PI agrees to update such disclosure at the request of the agency prior to the award of support and at any subsequent time the agency determines appropriate during the term of the award; and 3) The PI has been made aware of the requirements under Section 223(a)(1) of this Act.

Signature:  _____

Date: 7/28/2022

Other Support

Serebriiskii, Ilya G.

CURRENT

W81XWH-21-1-0487 (PI: Golemis)

7/1/2021 - 6/30/2025

30.8%

DOD

Salary only

3.70 calendar

3.70 cal mths yrs 1-3

4.60 cal mths yr 4

Overcoming Resistance to EGFR Inhibitors in Advanced Head and Neck Cancers

The major goals of this project are to: 1) Determine how TP53 mutation class determines AURKA expression and alters sensitivity to inhibition of AURKA and EGFR; 2) Explore relative efficacy of AURKA monotherapy and an AURKA-WEE1 inhibitor combination in the setting of, and in preventing or reversing adaptive resistance to, EGFR inhibition; and 3) Define the relationship between TP53 genotype, AURKA expression, and response to EGFR inhibition using clinical trial samples for HNSCC.

Procuring Contracting/Grants Officer: Amanda Carrera, 820 Chandler St., Fort Detrick, MD 21702, 301-619-2108

R03 CA256234 (PI: Serebriiskii)

12/14/2020 - 11/30/2022

25.0%

NIH

3.00 calendar

Comprehensive Identification of RAS Mutations and Allelic Co-Segregation Patterns in Colorectal

The major goals of this project are to: 1) Comprehensively characterize the landscape of RAS mutations in CRC; and 2) Elucidate gene, codon and variant level analysis of co-occurrence of RAS mutations with other driver genes commonly mutated in CRC.

Procuring Contracting/Grants Officer: Sarah Lee, 9609 Medical Center Dr., BG0609 RM 2W552, Rockville MD 20850, 240-276-6280

OVERLAP

NONE

CERTIFICATION REGARDING DISCLOSURE OF FUNDING SOURCES:

In accordance with Section 223(a) of the William M. (Mac) Thornberry National Defense Authorization Act for Fiscal Year 2021, this confirms that: 1) The PI's current and pending support provided on the application is current, accurate, and complete; 2) The PI agrees to update such disclosure at the request of the agency prior to the award of support and at any subsequent time the agency determines appropriate during the term of the award; and 3) The PI has been made aware of the requirements under Section 223(a)(1) of this Act.

Signature: _____

Date: _____

7/29/22

Other Support

Deneka, Alexander

Dr. Deneka is no longer associated with project. His efforts have been replaced by non-key personnel.

Association of *TP53* and *CDKN2A* Mutation Profile with Tumor Mutation Burden in Head and Neck Cancer



Alexander Y. Deneka¹, Yasmine Baca², Ilya G. Serebriiskii^{1,3}, Emmanuelle Nicolas¹, Mitchell I. Parker^{1,4}, Theodore T. Nguyen^{1,4}, Joanne Xiu², W. Michael Korn², Michael J. Demeure⁵, Trisha Wise-Draper⁶, Ammar Sukari⁷, Barbara Burtneiss⁸, and Erica A. Golemis^{1,9}

ABSTRACT

Purpose: Head and neck squamous cell carcinoma (HNSCC) is a frequently devastating cancer that affects more than a half million people annually worldwide. Although some cases arise from infection with human papillomavirus (HPV), HPV-negative HNSCC is more common, and associated with worse outcome. Advanced HPV-negative HNSCC may be treated with surgery, chemoradiation, targeted therapy, or immune checkpoint inhibition (ICI). There is considerable need for predictive biomarkers for these treatments. Defects in DNA repair capacity and loss of cell-cycle checkpoints sensitize tumors to cytotoxic therapies, and can contribute to phenotypes such as elevated tumor mutation burden (TMB), associated with response to ICI. Mutation of the tumor suppressors and checkpoint mediators *TP53* and *CDKN2A* is common in HPV-negative HNSCC.

Experimental Design: To gain insight into the relation of the interaction of *TP53* and *CDKN2A* mutations with TMB in HNSCC, we have analyzed genomic data from 1,669 HPV-negative HNSCC tumors with multiple criteria proposed for assessing the damaging effect of *TP53* mutations.

Results: Data analysis established the *TP53* and *CDKN2A* mutation profiles in specific anatomic subsites and suggested that specific categories of *TP53* mutations are more likely to associate with *CDKN2A* mutation or high TMB based on tumor subsite. Intriguingly, the pattern of hotspot mutations in *TP53* differed depending on the presence or absence of a cooccurring *CDKN2A* mutation.

Conclusions: These data emphasize the role of tumor subsite in evaluation of mutational profiles in HNSCC, and link defects in *TP53* and *CDKN2A* to elevated TMB levels in some tumor subgroups.

Introduction

Head and neck squamous cell carcinoma (HNSCC) affects over 800,000 people annually, including 65,000 in the United States (1, 2). The main epidemiologic factors causing HNSCC are the use of tobacco and alcohol, or infection with a transforming type of human papillomavirus (HPV; refs. 3, 4). HPV-negative HNSCC has a worse prognosis than HPV-associated (HPV+) HNSCC. While HNSCC diagnosed at an early stage is often cured with surgery or radiation, almost all patients with distant metastases require systemic therapy and nonetheless succumb to this cancer (5). The recent advent of immune checkpoint inhibition (ICI) has been transformative for some patients with advanced HPV-

negative HNSCC, but even for patients expressing the favorable biomarker programmed death ligand 1 (PD-L1), the median time to progression is only 5 months and median survival only 13.4 months (6). Better predictive biomarkers to aid in treatment selection for ICI, as well as more effective therapeutic strategies, are required.

Incidence, presentation, and treatment of HPV-negative HNSCC are each strongly influenced by innate capacity for DNA damage response. This disease is typically associated with chronic use of tobacco, which provides a potent mutagenic stimulus. The significant majority of HPV-negative HNSCC tumors accumulate damaging mutations in the tumor suppressor gene *TP53* (7, 8). In turn, abrogation of p53 function impairs initiation of cell-cycle checkpoints and apoptosis in the face of unrepaired DNA damage, contributing to acquisition of additional mutations and allowing tumors to gradually accumulate a significant tumor mutation burden (TMB). Loss of p53 activity also influences survival of tumors after DNA-damaging treatments including radiation or chemotherapy (9, 10), and influences prognosis (11). Across tumor types, elevated TMB (often defined as ≥ 10 mutations/MB or ≥ 15 mutations/MB, but varying among studies; ref. 12) predicts benefit from ICI. The predictive value of high TMB has been demonstrated in HNSCC specifically for the programmed cell death 1 inhibitor pembrolizumab (13), and for the PD-L1 inhibitor durvalumab (14). These relationships suggest *TP53* status may be predictive of response to immunotherapies, but this remains to be further established.

Notably, *TP53* mutations observed in HNSCC differ in their effect on p53 functionality, ranging from benign, to partial or complete loss of function (LOF), while some mutations (e.g., R248W, R273H, R175H) lead to gain of function (GOF; refs. 11, 15, 16). As p53 acts as a tetramer, some mutations can have dominant-negative (DNE) activity, if they result in modification of p53 that disables function but

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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Translational Relevance

Detection of human papillomavirus (HPV)-negative head and neck squamous cell carcinoma (HNSCC) at an advanced stage is associated with poor outcomes. Advanced HNSCC is often treated with DNA-damaging therapies, and immunotherapies are sometimes effective, although in only a small subset of patients, characterized by high tumor mutation burden (TMB): it is important to identify molecular factors that may predict therapeutic response. The tumor suppressors *TP53* and *CDKN2A*, which mediate G₁ cell-cycle checkpoints critical for DNA repair, are commonly mutated or lost in HPV-negative HNSCC. To gain insight into the interaction of *TP53* and *CDKN2A* mutations with TMB, we have analyzed genomic data from 1,669 HPV-negative HNSCC tumors. This analysis suggests that specific categories of *TP53* mutations are more likely to associate with *CDKN2A* mutation or high TMB based on specific tumor subsite, which may be predictive of therapeutic response or prognostic outcome for patients with HNSCC.

maintains protein capacity for oligomerization. Numerous groups have proposed systems for classification of *TP53* mutations, based on the degree to which they disrupt protein sequence and function. For HNSCC, one study has suggested that the rules first defined by Poeta and colleagues to identify highly damaging mutations (11), when supplemented by splice site mutation information, are most prognostic of reduced survival following surgery for HNSCC, relative to numerous other algorithms for classifying these mutations (17). An evolutionary action score, computationally derived to classify mutations in highly conserved residues as high risk, was also prognostic in HNSCC (18). The relationship of specific *TP53* mutation classes to prognosis for patients with HNSCC receiving DNA-damaging therapies or immunotherapies is of high interest, but not yet well understood (10).

In contrast to HPV-negative HNSCC, *TP53* is very infrequently mutated in HPV+ HNSCC (19). However, p53 activity is greatly reduced in these tumors, because p53 is posttranslationally degraded by an HPV-encoded oncogene, the ubiquitin ligase E6. In parallel, the HPV oncoprotein E7 inactivates the pRB tumor suppressor protein, removing a second cell-cycle checkpoint; both E6 and E7 activities are required for cell transformation by HPV, emphasizing the importance of dual disruption of p53 and pRB. Although Rb is rarely mutated in HPV-negative HNSCC, a significant percentage of HNSCC tumors mutate or otherwise inactivate *CDKN2A*, encoding the tumor suppressor p16. p16 is an essential mediator of pRB tumor suppressor activity, so that loss of *CDKN2A* similarly compromises cell-cycle checkpoints important for repair of DNA damage (20). However, whether *CDKN2A* and *TP53* mutations interact in a manner that influences TMB or other properties pertinent to therapeutic response to chemotherapy and ICIs, or overall prognosis of patients with HPV-negative HNSCC is unknown.

In this study, we have leveraged a large dataset of HNSCC genomic data from Caris Life Sciences (CLS), and a comparator dataset compiled from public sources, to explore these relationships. This work reveals a striking profile of interaction between mutations of *TP53* and *CDKN2A*, with each other and with TMB, in overall HNSCC and in specific HNSCC subsites.

Materials and Methods

Genomic profiling of study cohorts, CLS

A total of 1,010 HNSCC tumors were analyzed by CLS (Phoenix, Arizona) as part of routine comprehensive molecular profiling, of which 875 were selected for further analysis, based on their occurrence in specific tumor subsites of interest. This study was conducted in accordance with guidelines of the Declaration of Helsinki, Belmont Report, and U.S. Common Rule. In keeping with 45 CFR 46.101(b; ref. 4), this study was performed using retrospective, deidentified clinical data. Therefore, this study was considered Institutional Review Board-exempt and no patient consent was necessary from the subjects.

Prior to molecular testing by CLS, tumor enrichment was achieved by harvesting targeted tissue from formalin-fixed paraffin-embedded (FFPE) tumor samples using manual microdissection techniques. For a small subset of the tumors arising in the oropharynx, expression of the *CDKN2A*-encoded protein p16 was tested by IHC (using monoclonal antibody clone E6H4) with a standard cutoff of 2+, >70% being considered p16+; for positive specimens, a confirmatory HPV High Risk ISH was carried out using mRNA probes (HPV 16, 18, and 33). Next-generation sequencing was performed on genomic DNA isolated from FFPE tumor samples using the NextSeq platform (Illumina, Inc.). Matched normal tissue was not sequenced. A custom-designed SureSelect XT assay was used to enrich 592 whole-gene targets (Agilent Technologies); a total of 1.4 MB was assessed. All variants were detected with >99% confidence based on allele frequency and amplicon coverage, with an average sequencing depth of coverage of >500 and an analytic sensitivity of 5%. Splice junctions are covered in the sequencing panel with mutations seen at ± 30 nucleotides from the boundaries of *BRCA1/2* genes and ± 10 nucleotides of the other genes. Splicing variants were annotated only for mutations detected in ± 2 nucleotides from the exon boundaries. The copy-number alteration of each exon was determined by calculating the average depth of the sample along with the sequencing depth of each exon and comparing this calculated result to a precalibrated value. TMB was measured by counting all nonsynonymous missense mutations found per tumor that had not been previously described as germline alterations. The threshold to define TMB-high was greater than or equal to 10 or 15 mutations/MB. Values for TMB are aligned with those generated by other sequencing platforms based on the use of common guidelines recently published in (21).

Genomic profiling of study cohorts, public data

Comparison data sets for studies with information on *TP53* and *CDKN2A* mutation status (including copy-number alterations, sex, age, and tumor subsite) were collected from the publicly available datasets (PAD) at cBioPortal (<http://www.cbioportal.org/index.do>) and American Association for Cancer Research (AACR) Project GENIE (<https://genie.cbioportal.org>) as of July 2021. Data selection criteria are shown in Supplementary Fig. S1. The resulting PAD dataset was used in some cases for comparison with the CLS dataset, and in other cases, was merged with the CLS dataset to increase statistical power.

Assessment of *TP53* variant functionality

TP53 variant functional assessment was derived from integration of multiple sources, in addition to mutations explicitly characterized in detail in the scientific literature. Functional annotation for damaging *TP53* mutations is based on American College of Medical Genetics and Genomics (ACMG) designation as “pathogenic” or

“likely pathogenic” (22), or from merged results of three datasets [the consensus between ACMG and two other commonly used resources: InterVar (<http://wintervar.wglab.org/>; ref. 23) and ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>; ref. 24)]. To characterize *TP53* mutations based on impact on p53 protein structure and function (LOF, DNE, GOF), some *TP53* mutations were classified as DNE, resulting in the inhibition of the wild-type (WT) p53 by mutant protein in transactivation or cell growth assays, as listed in the International Agency for Research on Cancer (IARC) database (<http://p53.iarc.fr>), based on two studies (25, 26). DNE *TP53* mutations with loss-of-function effect (DNE LOF) annotation is based on designations in the IARC database (<http://p53.iarc.fr>), where functional classification for loss of growth suppression and DNE activities is based on Z-scores from Giacomelli and colleagues (16). A Poeta disruptive designation reflects the classification system detailed in Poeta and colleagues (11), which is heavily biased towards mutations associated with truncations, and disruption of DNA binding capacity. A GOF designation is based on mutations that have been specifically described in the literature. Known GOF *TP53* mutations are listed in Supplementary Table S1, along with citations describing function. In a few cases, tumors had more than one mutation in *TP53*; these specimens were classified based on the phenotype of the most damaging mutation observed (typically a truncating mutation, leading to a designation as Poeta disruptive).

Identification and 3D representation of hotspots

Hotspot mutations and mutation-enriched stretches along the primary protein sequence were identified using previously described methods (27). Briefly, to determine if a frequently mutated site on the p53 protein constitutes a mutational hotspot, we have used a binominal distribution model with a *P*-value cutoff 0.005. Similarly, to calculate whether non-hotspot mutations are enriched in certain linear stretches along the *TP53* primary sequence, we used sliding window of 5 aa and a binominal distribution model to identify larger regions of the primary structure that were, in sum, more commonly mutated than expected. Two-dimensional representation of *TP53* hotspot mutations was prepared using MutationMapper tool available at <https://www.cbioportal.org/visualize>. For display of the distribution of mutations on the folded protein structure, figures were prepared using the program PyMOL (<https://pymol.org/2/>), based on a p53 structure deposited in the PDB, 1TUP (28).

Statistical analysis

Relationships between mutations and patient characteristics were assessed using Fisher exact tests (including determination of significant difference between the mutational spectra of dichotomized gender or subsite groups), and/or a Wilcoxon test (where indicated in legends). To allow for multiple mutations within a patient, the predictors used in these models were binary indicators of the presence versus absence of particular mutations of interest. To account for multiple comparisons of various types, we have lowered the threshold for statistical significance tenfold, to 0.005. Co-occurrence or mutual exclusion of mutations was calculated using Fisher exact test. TMB comparisons among groups in the CLS cohort were compared using non-parametric Kruskal–Wallis testing. Potential differences in *TP53*-*CDKN2A* co-occurrence across different sites were evaluated using a Breslow-Day homogeneity test. Potential differences in TMB status (as defined by cut-off values) across different *TP53*-*CDKN2A* mutation groups were evaluated using a χ^2 test for homogeneity. A multiple regression model linking TMB as continuous outcome variable to the mutation

status of *TP53* and *CDKN2A*, and tumor subsite, was built using the *lm* function in R.

Data and materials availability

Web resources used in this paper include: The cBioPortal for Cancer Genomics, <https://www.cbioportal.org>; AACR Project GENIE, <https://genie.cbioportal.org>; InterVar, a bioinformatics software tool for clinical interpretation of genetic variants by ACMG/AMP 2015 guidelines, <http://wintervar.wglab.org/>; ClinVar, a public archive reporting the relationships among human variations and phenotypes, <https://www.ncbi.nlm.nih.gov/clinvar/>. Academic researchers can gain access to CLS data in this study by contacting the corresponding author and filling out a study review committee form to CLS. Researchers and their institutions will be required to sign a data transfer agreement.

Results

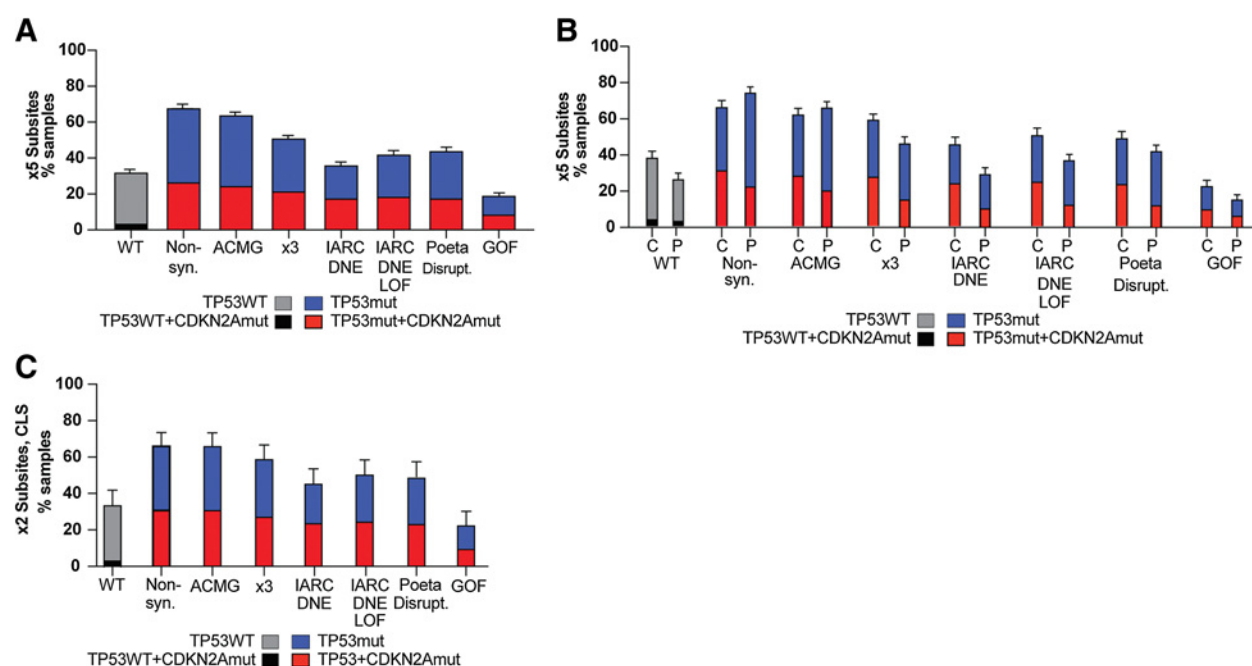
Study cohorts

Two cohorts were analyzed for this study (Supplementary Table S2). The first was a group of 875 patients with HNSCC genomically profiled by CLS. The second was a group of 794 patients with HPV-negative HNSCC for whom data were available in cBioportal from The Cancer Genome Atlas PanCancer and Broad Institute data sets, and from AACR-GENIE public resources (Supplementary Table S2; Supplementary Fig. S1), based on exome sequencing or panel testing. Reflecting the known sex bias of HNSCC (29), the majority of specimens were from males (75.5% for CLS, 70.5% from the PAD). Tumors sequenced by CLS were typically derived from patients with late-stage or metastatic/recurrent cancers (Supplementary Table S2). In contrast, the tumors from the PAD were more diverse in stage, including some earlier-stage tumors.

Tumor subsite has been associated with differences in mutational profile for HNSCC (8). To perform analyses based on tumor subsite, we omitted tumors arising in subsites with very few cases observed and considered separately those tumors where site of origin was unclear (designated “advanced” or “occult primary” in the CLS dataset). We also omitted nasopharyngeal tumors from further analysis because, although many of these tumors arise based on prior infection with Epstein-Barr virus (EBV; ref. 30), analyzed specimens were not tested for EBV. We therefore analyzed in detail the combined set of tumors in five subsites: the hypopharynx, larynx, oral cavity, oropharynx, and sinonasal cavity (733 specimens from CLS and 794 specimens from the PAD), as well as in an additional 142 CLS specimens designated as advanced or occult primary.

TP53 mutational profiles in the overall HNSCC cohort

We analyzed the data using multiple approaches to characterize *TP53* mutations (Supplementary Table S3). First, we evaluated non-synonymous mutations, considering any that altered amino acid sequence. Second, we applied ACMG criteria (31), commonly used by CLS and others to designate *TP53* mutations as pathogenic. Third, we considered mutations that were independently identified by ACMG criteria as well as reported in two other commonly used resources, Clinvar (24) and Intervar (ref. 23; designated x3, based on three sources of input). Fourth and fifth, we considered *TP53* mutations designated as DNE or DNE plus LOF (DNE-LOF) based on criteria of the IARC (32). Sixth, we considered *TP53* mutations viewed as damaging based on the Poeta algorithm, which selects for highly disruptive mutations (including those involving stop codons, or missense mutations severely compromising the integrity of the DNA binding domain; ref. 11), supplemented with mutations disrupting splice sites; this

**Figure 1.**

Frequency of *TP53* mutations, and co-segregation of *TP53* and *CDKN2A* mutations, in CLS and PAD cohorts. **A**, Percent of 1,527 HNSCC tumors from merged CLS and PAD datasets with WT *TP53*, or with mutations in *TP53* in five tumor subsites (hypopharynx, larynx, oral cavity, oropharynx, and sinonasal cavity), based on the algorithms noted, with or without mutations in *CDKN2A*. **B**, Same as **A**, but contrasting the frequency of mutations in the CLS versus PAD datasets, for five tumor subsites. **C**, As for **B**, but data are for 142 CLS specimens designated as advanced or occult primary.

approach was previously demonstrated to have superior prognostic capacity (17). Finally, we additionally considered mutations, which had been described in various publications as GOF, a group typically representing relatively conservative missense modifications (ref. 15; Supplementary Table S1).

In the overall cohort (combined CLS and PAD for five subsites, advanced primary, and occult), 68% of tumors bore a non-synonymous mutation in *TP53* (Fig. 1A), most of which were considered pathogenic by ACMG criteria. Significantly, fewer mutations were called using the x3, DNE, DNE-LOF, and Poeta approaches, with frequencies ranging from 36% to 50%. Relatively few mutations (19%) were identified as GOF. A higher frequency of *TP53* mutations occurs in males versus females for a number of classes of solid tumors (32, 33). However, comparison of mutation profile in males versus females specifically in oral and laryngeal tumors, where sample size allowed testing for statistical significance, indicated no difference by sex (Supplementary Fig. S2).

We then performed detailed comparison of the frequency of *TP53* mutations in the five anatomic subsites in the independent CLS and PAD cohorts (Fig. 1B). Notably, while the frequency of non-synonymous and ACMG mutations was comparable in both cohorts, the fractions of highly damaging mutations (x3, DNE, DNE-LOF, and Poeta) in *TP53* were lower in the PAD datasets than in the CLS datasets. In addition, CLS tumors diagnosed as advanced or with unknown primary (often reflecting larger, advanced tumors) had a similar frequency of *TP53* mutations as did tumors from the five merged sites (Fig. 1C).

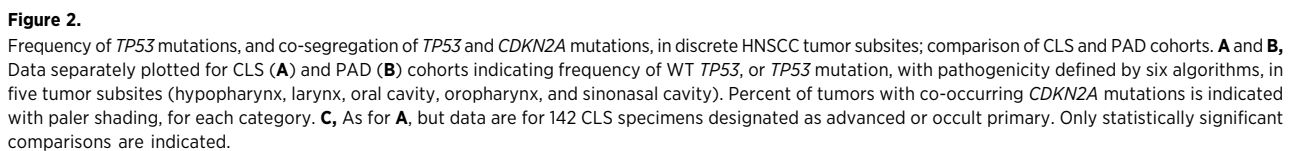
Frequency of damaging *TP53* mutations reflects tumor subsite

One potential explanation for the incomplete agreement of datasets is that *TP53* mutational frequency reflected differing proportions of

tumors originating from specific HNSCC subsites in the CLS versus PAD cohorts (for instance, with CLS having a higher proportion of tumors from the oropharynx). Although HPV-negative HNSCC is often considered as a single disease, a growing number of publications have identified differences between tumors arising in distinct anatomic subsites. For instance, in oral cavity tumors, the composition of the microbiome is associated with disease pathogenesis (34), whereas laryngeal tumors have a greater mutational burden (35). In addition, the tissues that collectively form the structures of the head and neck arise from distinct developmental primordia (36), which may affect gene expression profile and dependence on specific pathways.

For both the CLS and PAD cohorts, the highest frequency of *TP53* mutations, for all classes of mutation, was observed in the hypopharynx, larynx, and oral cavity, and the lowest in tumors of the sinonasal cavity (Fig. 2A and B). Overall, in the PAD, differences in frequency of *TP53* mutation were less marked between tumor subsites, and for each subsite, there were fewer damaging mutations than observed for the same subsites in the CLS. For the PAD, data on deep deletions (loss of both copies) for *TP53* was available; inclusion of this data did not affect estimate of gene LOF, as there were few cases of *TP53* loss (Supplementary Fig. S3A). For oropharyngeal tumors, the CLS dataset suggest a lower frequency of *TP53* mutation than observed in the PAD, although the relatively small total number of oropharyngeal tumors in the PAD limits statistically significant conclusions. This difference most likely reflects lack of testing for HPV in the majority of the CLS specimens, given the common occurrence of HPV+ disease in this specific tumor subsite, and the documented low frequency of *TP53* and *CDKN2A* mutations in HPV+ tumors.

Using the PAD, where information was available, we then compared the frequency of mutations called by the ACMG criteria in the oral and laryngeal subsites, based on tumor stage (Supplementary Fig. S3B).



This indicated no significant differences in the frequency of ACMG-called mutations based on tumor stage, in either the oral or laryngeal subsite, in accord with the established idea that *TP53* mutation occurs early in tumor formation (37). This interpretation is supported by comparison of CLS and PAD data for individual tumor sites (Fig. 2A and B) versus CLS data for advanced and occult primary tumors (Fig. 2C), reflecting large and extremely advanced tumors, as the latter do not have a higher frequency of *TP53* mutation than observed in the subsites.

Segregation of mutations in *TP53* and *CDKN2A*

We separately analyzed mutations in *CDKN2A*. These occurred at a lower frequency than mutations in *TP53* (Fig. 1B and C), but almost all mutations were predicted to significantly impair or eliminate *CDKN2A* protein function, with 64% representing frameshifts or nonsense mutations. Overall, 35.7% of tumors bore a non-synonymous mutation in *CDKN2A* (32.2% in the CLS, and 39.54% in the PAD). Of *TP53* WT tumors, the great majority were also WT for *CDKN2A*, a higher frequency than would be expected based on random assortment ($P < 0.0001$). Among *TP53*-mutated tumors of the five merged subsites, between 37% and 48% also bore *CDKN2A* mutations, across the various *TP53* mutation categories; this frequency was also observed in HNSCC diagnosed as advanced or with occult primary (Fig. 1B and C; Supplementary Table S4). Based on analysis of the PAD, the frequency of *CDKN2A* mutations did not vary dependent on tumor stage (Supplementary Fig. S3B), implying mutation occurred early in tumorigenesis.

Distribution of *TP53* and *CDKN2A* mutations by HNSCC tumor subsite

We also considered the relationship of *TP53* and *CDKN2A* mutations in the context of individual disease subsites (Fig. 2A–C; Supplementary Fig. S3A; Supplementary Table S4). Here, although some consistent features of mutation profile were identified, significant differences emerged discriminating subsite profiles and datasets.

Employing the ACMG criteria for both the CLS (Fig. 2A) and PAD (Fig. 2B) cohorts across five tumor subsites, the highest proportion of *TP53* mutations were called as damaging and the lowest as GOF. For both datasets, there was a very low frequency of tumors with a *CDKN2A* mutation in the absence of an accompanying *TP53* mutation. Among tumors with a *TP53* mutation, the likelihood of an accompanying *CDKN2A* mutation was typically lower in the PAD than in CLS cohort, even though pre-screening (Supplementary Fig. S1) had eliminated AACR-GENIE datasets with exceptionally low reported *CDKN2A* mutation frequency. The greatest likelihood of co-occurring *CDKN2A* and *TP53* mutations was typically in laryngeal and oral cavity tumors. In the CLS dataset (Fig. 2A), the likelihood of co-mutation was most notable in laryngeal tumors bearing moderately to severely damaging classes of *TP53* mutations. For the PAD, inclusion of available deep deletion information (Supplementary Fig. S3A) slightly increased the number of tumors with loss of *CDKN2A*, particularly in the oral and laryngeal subsites. Otherwise, the relative likelihood of co-occurring mutations was not affected by mutation calling method. Both the CLS and PAD cohorts had lower frequency of co-occurrence in oropharyngeal tumors, although the differed regard to observed frequency (PAD, 3.6% of *TP53*-mutated tumors, versus CLS, 17.9% of *TP53*-mutated tumors), with the higher value in the CLS dataset reflecting the likely inclusion of some HPV+ tumors in the dataset. Based on rigorous statistical testing of homogeneity, levels of mutational co-occurrence of *CDKN2A* and *TP53* (pathogenicity annotated according to ACMG criteria) in PAD dataset (Supplemen-

tary Fig. S3C) did not significantly differ between tumor subsites. We also performed similar analysis for the CLS dataset of advanced and occult-primary tumors (Fig. 2C). The patterns of co-occurrence of *TP53* and *CDKN2A* mutations in each of these groups and based on each calling method were similar to those found in the laryngeal and oral cavity cancers.

Patterns of association of *TP53* and *CDKN2A* mutation or comutation with elevated TMB

For the CLS dataset, TMB data were available. Using these data (Figs. 3 and 4), we evaluated whether *TP53* and *CDKN2A* mutation status correlated with TMB. In the merged CLS cohort (five subsites), tumors bearing mutations in *TP53* had significantly higher average TMB than did tumors lacking *TP53* mutations; however, in the case of GOF *TP53* mutations, the increase in TMB was not significant at a threshold of $P < 0.005$ (Fig. 3A). In this analysis, the presence or absence of a *CDKN2A* mutation did not correlate with average TMB (Fig. 3A). We assessed if a co-occurring *CDKN2A* mutation increased the proportion of tumors with a TMB above cutpoints of 10 (Fig. 3B) or 15 (Fig. 3C) mutations/Mb (14). Tumors with ACMG, x3, and IARC-specified mutations in *TP53* were more likely to exceed those cutpoints if they bore a simultaneous mutation in *CDKN2A*, although this did not retain significance upon multiple comparison correction. In contrast, no evidence of a contribution of a *CDKN2A* mutation was observed in tumors bearing Poeta+splicing mutations. We note this observation of differential effect of *CDKN2A* mutation on correlation of distinct classes of *TP53* mutation with elevated TMB would be in line with interpretation of a driver effect of the damaging *TP53* mutation on the higher TMB; otherwise, we would have anticipated comparable results for all *TP53* class combinations with *CDKN2A*. Interestingly, in tumors with no *TP53* mutation, the presence of a *CDKN2A* mutation alone was associated with a significantly elevated TMB average, and increased frequency of tumors with a TMB in excess of 10 (Fig. 3A and B); however, almost no tumors lacking a *TP53* mutation exceeded a TMB of 15, regardless of *CDKN2A* mutational status (Fig. 3C).

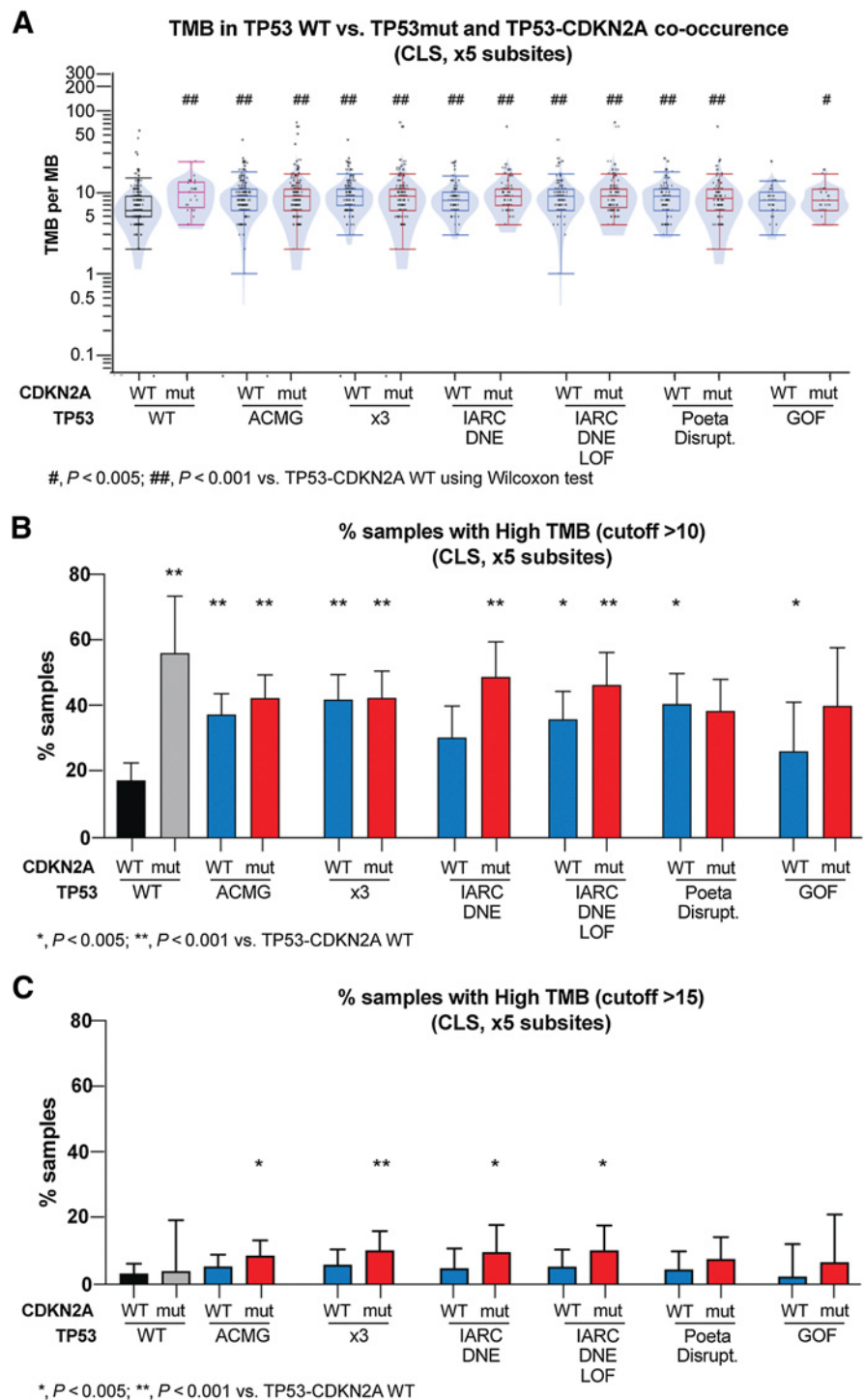
The ACMG classification system is standardly used for clinical reports indicating a pathogenic *TP53* mutation. Therefore, using this *TP53* mutation classification as basis for further analysis, we then examined the correlation of *TP53* mutation, *CDKN2A* mutation, and TMB separately in each of the tumor subsites (Fig. 4). This revealed striking differences. For tumors of the oropharynx, advanced tumors, and those arising from an occult primary, co-occurrence of *CDKN2A* and ACMG-defined *TP53* mutations correlated with a higher average TMB than *TP53* or *CDKN2A* mutation alone, or no mutation (Fig. 4A). Focusing on TMB cutpoints, a significant elevation in the frequency of tumors exceeding TMB 15 was observed in advanced or occult primary tumors bearing mutations in *CDKN2A*, or *CDKN2A* plus ACMG-designated *TP53* mutation; however, *TP53* mutation alone did not correlate with higher TMB (Fig. 4B). No statistically significant correlation was observed between *TP53* mutation and a greater frequency of tumors with high TMB was seen in hypopharyngeal and laryngeal tumors.

Hotspot mutational profiles in *TP53* in HNSCC

Previous studies have identified *TP53* mutational hotspots, including in HNSCC (38, 39). The large size of the combined cohort analyzed in this study, coupled with the questions of the relation between HNSCC mutation class, co-occurrence of a *CDKN2A* mutation, and TMB, motivated analysis of specific *TP53* mutational loci (Figs. 5 and 6; Supplementary Fig. S4). For this analysis, we only included oropharyngeal tumors that were confirmed to be HPV negative based

Figure 3.

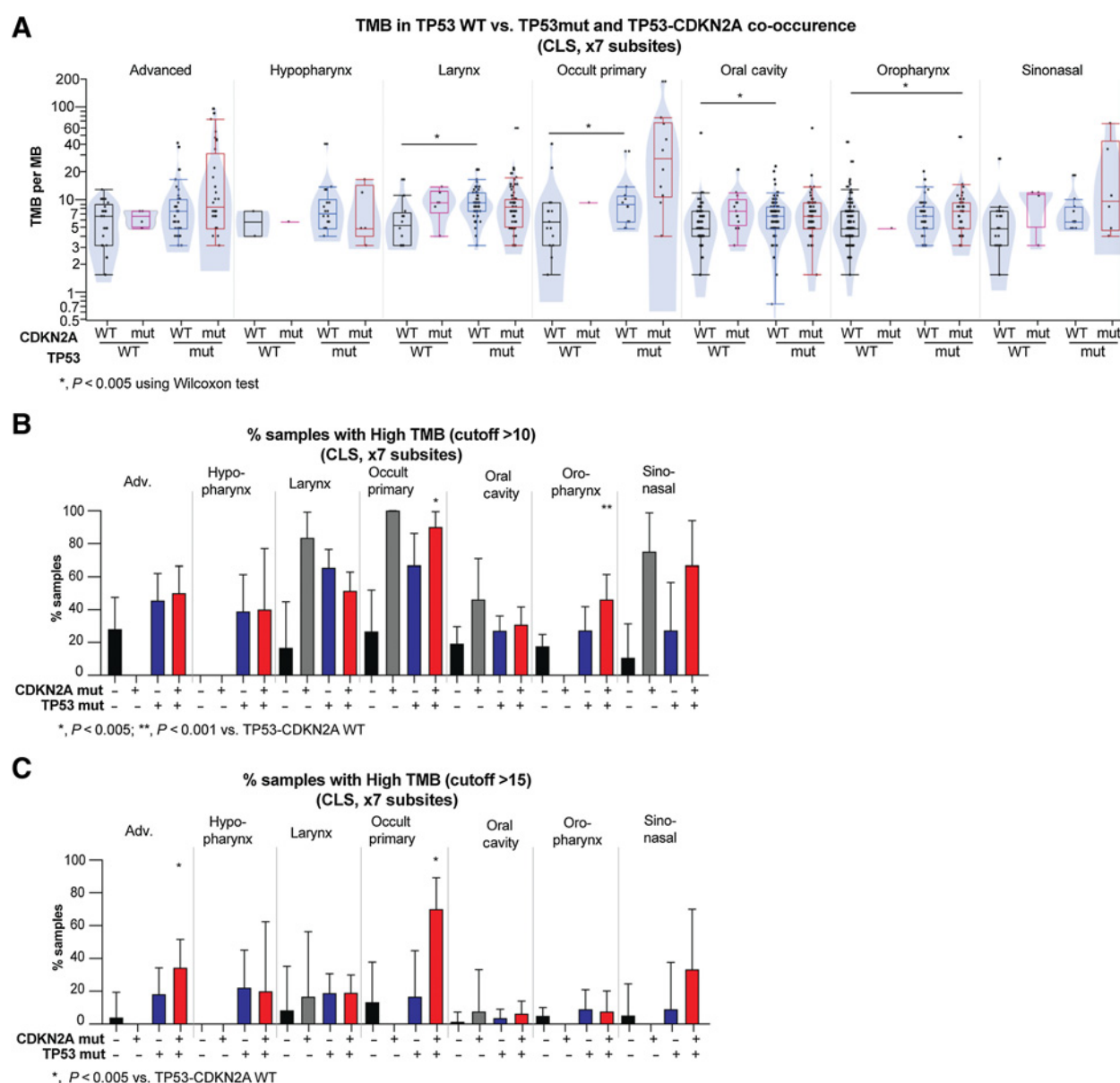
Relationship of *TP53* and *CDKN2A* mutation status with TMB, in the CLS cohort. **A**, Dot plot, indicating values for TMB in tumors bearing *TP53* and/or *CDKN2A* mutations, as indicated, for 728 tumors arising in the hypopharynx, larynx, oral cavity, oropharynx, and sinonasal cavity, based on the indicated algorithms for assessing *TP53* mutation category. **B** and **C**, From the same cohort as **A**, percent of tumors with TMB calculated as above 10 (**B**) or 15 (**C**). χ^2 analysis was of the relationships between mutational profile groups using the ACMG criteria for *TP53* and TMB found *P* values 8.6e-11 for TMB 10 and 2.3e-05 for TMB 15, respectively. Only statistically significant comparisons are indicated.



on direct assessment (21 specimens). Cumulatively, 1,015 missense mutations, 37 in-frame deletions, and 158 truncating mutations were observed in 1,465 samples. Over the complete cohort, missense mutations were clustered within the sequences encoding the DNA binding domain (Fig. 5A; Supplementary Fig. S4). From this analysis, we identified 55 residues (Supplementary Table S5) mutated at higher than expected frequency (≥ 7 alterations, given the length of the *TP53* coding sequence versus the total number of mutations). On the basis of

analysis of tumor subsites (Fig. 5B), there was a marked difference in *TP53* hotspot profile at distinct tumor subsites, likely arising at least in part from the distinct mutagenic environments characterizing oral cavity, larynx, and other regions.

In sum, a comparison of the complete set of *TP53* mutations found with or without accompanying *CDKN2A* mutations (Fig. 5C and D; Supplementary Table S5) indicated that the overall pattern of mutation did not significantly differ between the two groups. However, a focus

**Figure 4.**

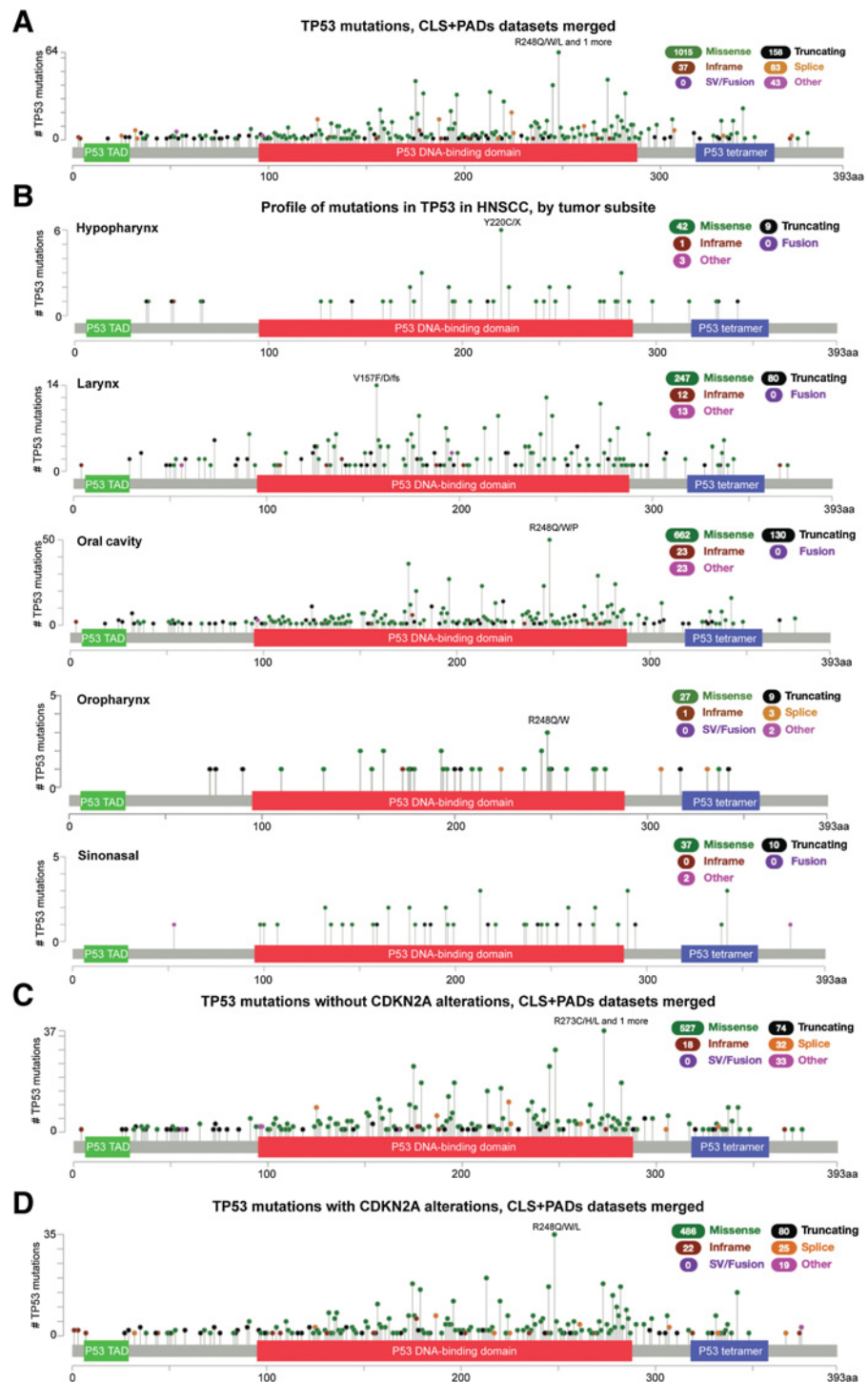
Relationship of *TP53* and *CDKN2A* mutation status with TMB, by tumor subsite, in the CLS cohort. **A**, Dot plot indicates median values for TMB in tumors bearing *TP53* and/or *CDKN2A* mutations, as indicated, for 728 tumors arising in the hypopharynx, larynx, oral cavity, oropharynx, and sinonasal cavity, and 142 specimens designated as advanced or arising from an occult primary, based on the indicated algorithms for assessing *TP53* mutation category. On the basis of a multiple regression model (adjusted $R^2 = 0.11$, P value $< 2.2 \times 10^{-16}$), and partial F test for variable selection, data had significance for *CDKN2A* status ($P = 2.5 \times 10^{-5}$), *TP53* status ($P = 0.0056$), and subsite ($P = 1.3 \times 10^{-11}$). **B** and **C**, From the same cohort as **A**, percent of tumors with TMB calculated as above 10 (**B**) or 15 (**C**). Only statistically significant comparisons are indicated.

on the profile of the most common hotspots for *TP53* missense mutations designated as pathogenic by ACMG criteria suggested some notable differences based on presence or absence of *CDKN2A* mutation, including mutations at hotspot areas near codon 277, more common in the absence of *CDKN2A* co-mutation, and near codons 192 and 157, more common when *CDKN2A* mutations co-occur (Fig. 6A). Interestingly, in tumors without *CDKN2A* mutation, there was higher frequency of *TP53* truncating mutations within the first 150 amino acids of coding sequence (24.4% vs. 15.5% of total mutations; Fig. 6B). These would be associated with total loss of

p53 function. In other analysis, focusing on the most commonly mutated residues (occurring at a frequency of $>1\%$ of the total mutations) observed in tumors with *TP53* alone mutated, versus *TP53* plus *CDKN2A*, we plotted those which occurred at a frequency >1.5 -fold higher in one or the other genotype on the solved structure of the p53 DNA binding domain (Fig. 6C: 3D model). Intriguingly, this revealed a tendency for the most common *TP53* hotspots co-occurring with *CDKN2A* mutations biased towards occurrence at sequences of p53 that directly contact DNA, whereas *TP53* hotspots occurring in the absence of *CDKN2A* mutation were more likely to localize away from

Figure 5.

Distribution of *TP53* mutations in HNSCC tumors from merged PAD and CLS cohorts. **A** and **B**, Location of all *TP53* mutations affecting p53 protein structure identified in the complete HNSCC cohort (**A**), and in each tumor subsite (**B**). **C** and **D**, Dataset from **A**, divided into those occurring without (**C**) or with (**D**) a *CDKN2A* mutation. The height of each lollipop indicates the count of the corresponding mutation in the dataset (left y-axis).

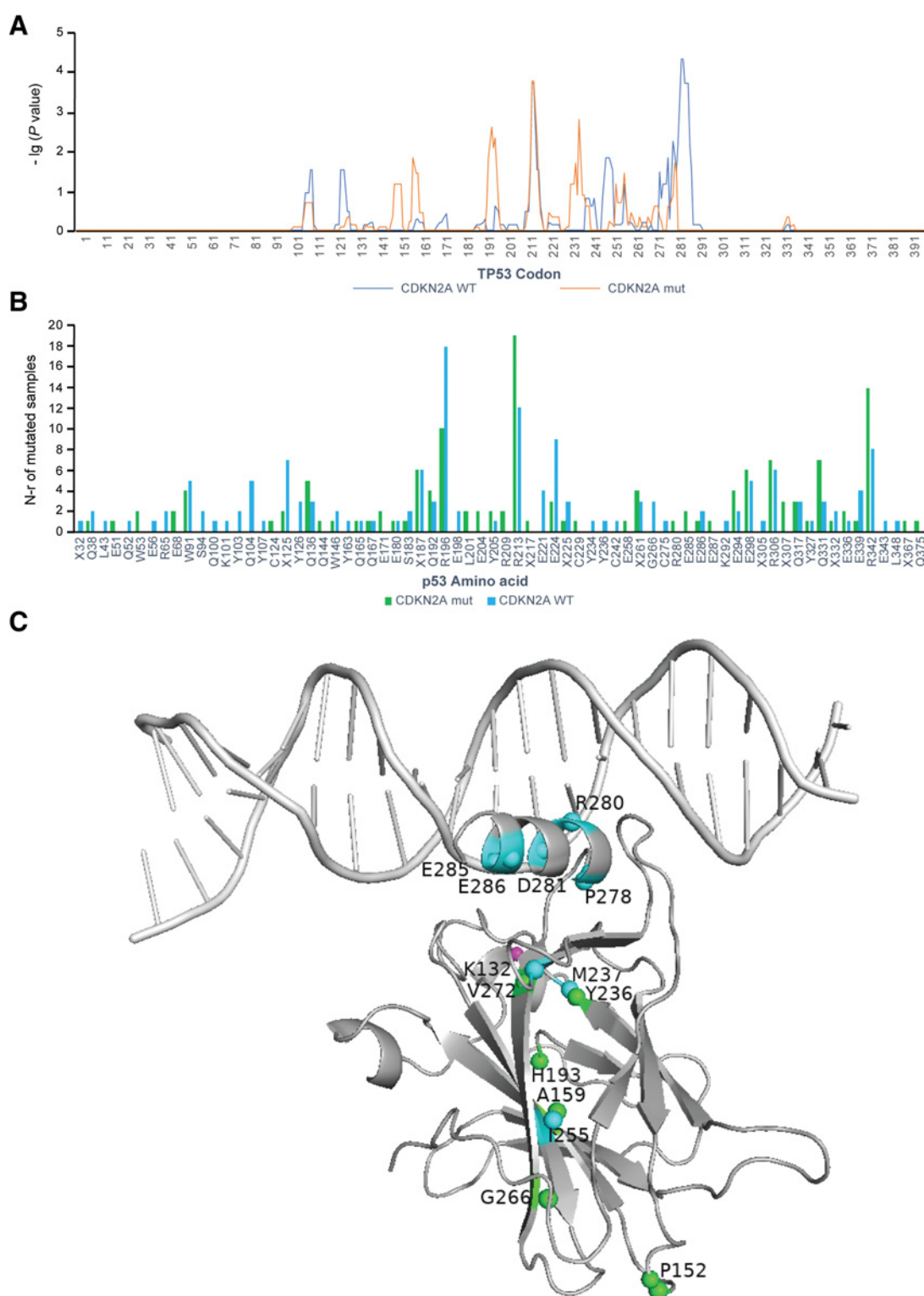


the DNA, in regions associated with control of p53 protein stability (Supplementary Table S5).

Discussion

Although previous studies have investigated profiles of *TP53* mutation in HNSCC and other tumor types, this is the first to analyze the interaction pattern of *TP53* mutations with *CDKN2A* mutational

profile, and with TMB. The data support several conclusions. *First*, considering HPV-negative HNSCC overall, the quantitative likelihood of a *CDKN2A* mutation accompanying a *TP53* mutation is not affected by the degree of impairment of *TP53* as predicted by functional class. *Second*, the frequency of co-occurrence of a *CDKN2A* mutation with a *TP53* mutation varies by HNSCC tumor subsite, being highest in laryngeal and oral tumors. *Third*, the frequency of *TP53* and *CDKN2A* mutations, and the likelihood of their co-occurrence, is not elevated in

**Figure 6.**

Regions of p53 hotspot mutation, in HNSCC with or without *CDKN2A* mutations. **A**, Comparison of regions of elevated mutation in *TP53*-mutated HNSCC (based on ACMG criteria) in tumors with or without accompanying *CDKN2A* mutation. **B**, Location of truncating mutations in the N-terminus of *TP53* in tumors with or without *CDKN2A* mutations. **C**, Location on the 3D structure of the p53 DNA binding domain of amino acids most commonly targeted by missense mutation, occurring with (blue) or without (green) a simultaneous *CDKN2A* mutation.

advanced versus early tumors, confirming occurrence of these mutational patterns as early tumor events. *Fourth*, across a combined set of HPV-negative HNSCC, all classes of *TP53* mutation except GOF are associated with higher TMB versus *TP53* WT tumors. In addition, *CDKN2A* mutation is associated with higher TMB, either occurring as a single mutation or in combination with a *TP53* mutation. *Fifth*, detailed analysis by tumor subsite based on ACMG mutations indicates that the presence either of a *CDKN2A* mutation or a *TP53* mutation is associated with a TMB >10 in laryngeal or oral cavity tumors. *Sixth*, there is a nonequivalent pattern of *TP53* mutations in tumors with versus without a co-occurring *CDKN2A* mutation, and alignment of observed missense mutations with the *TP53* protein structure suggests that the missense mutations may associate with distinct domains, dependent on the presence or absence of *CDKN2A* mutation. Finally, available sets of public data considered for inclusion in this study report different, and in some cases significantly lower levels of *CDKN2A* mutations than does at least one optimized clinical platform (CLS).

Masica and colleagues first reported that the combined set of tumors with *TP53* mutations defined by the Poeta algorithm, or with *TP53* splicing mutations, had worse outcome after margin-negative surgery and risk-based (predominantly radiation) postoperative therapy (17). Because the recognition that these most function-damaging *TP53* mutations were associated with therapeutic response and prognosis, it has been of considerable interest to determine whether these mutations have a driver or passenger function, and might be relevant to other therapies. Given the reported relationship between high TMB and response to ICI, and the central role of p53 in mediating response to DNA damage, defining the relationship of *TP53* mutation and TMB was a priority. Overall, our data are compatible with the idea that highly damaging *TP53* mutations are not primary drivers of the highest TMB levels, as similar TMB ranges are observed across all classes of disruptive *TP53* mutation (Fig. 3). Rather, higher TMB appears to be associated with large, later-stage tumors (such as those annotated as occult primary or advanced; ref. 40), and the presence of both *CDKN2A* and *TP53* mutations (Fig. 4). The association of *CDKN2A* mutation with higher TMB—as an independent factor, as well as in combination with *TP53*—was surprising, as there is no specific precedent for a driver function (Figs. 3 and 4). Interestingly, *CDKN2A* mutation status was also correlated with a difference in location of *TP53* missense mutations, localizing more to *TP53* DNA-binding sequences, rather than those associated with protein stability (Fig. 6).

Although further work is required to determine if the relationship between *CDKN2A* mutation, *TP53* mutation spectrum, and TMB reflects a functional role of *CDKN2A*-encoded proteins in governing mutation status, the observed correlation has interesting implications if a functional role is confirmed. Notably, *CDKN2A* encodes two distinct coding proteins: p16(INK4a) and p14(ARF). p16 is heavily studied in HNSCC based on its role in inhibiting the Rb pathway and its overexpression in the face of Rb inactivation in HPV+ disease; loss of *CDKN2A* is targetable with CDK4/6 inhibitors such as palbociclib (41, 42). Some recent studies have suggested that mutation of *CDKN2A* is associated with resistance to ICIs in solid tumors (43, 44), which is surprising given the correlation we identified with higher TMB; however, no mechanism connecting *CDKN2A* to ICI resistance has yet been identified. *CDKN2A* also encodes p14(ARF), which compared with p16 has attracted little study in HNSCC. p14(ARF) regulates p53 function (45), contributing to p53 stabilization by inhibiting MDM2; *CDKN2A* mutations leading to loss of p14(ARF) function would also have the effect of p53 destabilization. In one early study, an active role of exogenously expressed p14(ARF) in suppress-

ing the growth of HNSCC tumors retaining *TP53* was identified (46). More investigation of the interaction of p14(ARF) and p53 is merited. In future studies, it would also be of interest to incorporate information about copy-number loss of *CDKN2A* and *TP53* (here available only for the PAD), and about tumors with hypermethylation and silencing of *CDKN2A*, given this is a frequent mechanism of inactivation of this tumor suppressor (47).

On the basis of these data, it would also be of interest to evaluate whether a combination signature of *TP53* and *CDKN2A* mutation may be predictive of therapeutic response or prognostic of outcome for HNSCC, given results in this study and identification of such a role in other cancer types (48). The relation of dual *CDKN2A*/*TP53* mutations to higher TMB may also be of interest in the context of gaining insight into which tumors are likely to respond to immunotherapies. However, response to immune checkpoint inhibitors depends on additional factors beyond TMB, including among others an “immune hot” tumor microenvironment, and levels of expression of PD-L1 on individual tumor cells; moreover, this study identifies more HNSCC tumors as having elevated TMB than the typical response rate to ICIs in this disease (49, 50). More work on this relationship is merited.

Finally, this work emphasizes the importance of considering HPV-negative HNSCC tumors based on subsite and clinical designation, rather than as a merged group. Recent studies have detected significant differences between tumor mutational profiles in oral, laryngeal, and other tumor subsites (35, 51–53), associated with differences in the prognostic potential for distinct mutations. Although the relatively large cohort analyzed here for the first time allows us to identify a number of intriguing trends for HNSCC tumors, the need to consider individual tumor subsites still restricts statistical power for some analyses. Notably, this work suggests the need for caution in basing assessments of mutational frequency on PAD data, at least for the genes studied here. Given evidence of consistent mutation frequency of *TP53* and *CDKN2A* across tumor stages (Supplementary Fig. S2), we view the most likely factor contributing to the higher frequency of *CDKN2A* mutation frequency in the oral and laryngeal tumors in the CLS dataset as increases in detection efficiency with more recent technological advances. Conversely, a potential limitation of the current study is the incomplete characterization of HPV status in the CLS dataset; although analyses omitting oropharynx and sinonasal tumors were performed to reduce this concern, availability of large cohorts of tumors from these subsites with confirmed HPV-negative status will be a valuable addition. As the number of sequenced HNSCC tumors continues to increase, and technologies continue to improve, we anticipate the next few years will yield many further insights into the unique biology of distinct HNSCC subgroups.

Authors' Disclosures

No disclosures were reported.

Authors' Contributions

A.Y. Deneka: Conceptualization, data curation, investigation, visualization, methodology, writing—original draft, writing—review and editing. **Y. Baca:** Conceptualization, data curation, software, investigation, visualization, writing—original draft. **I.G. Serebriiskii:** Conceptualization, data curation, software, formal analysis, investigation, visualization, writing—original draft. **E. Nicolas:** Data curation, visualization, methodology. **M.I. Parker:** Data curation, software, investigation. **T.T. Nguyen:** Investigation, writing—original draft, writing—review and editing. **J. Xiu:** Conceptualization, resources, formal analysis, supervision. **W.M. Korn:** Conceptualization, resources, formal analysis, writing—review and editing. **M.J. Demeure:** Validation, writing—original draft, writing—review and editing. **T. Wise-Draper:** Formal analysis, validation, writing—review and editing. **A. Sukari:** Formal analysis, validation, writing—review and editing.

B. Burtress: Conceptualization, resources, supervision, funding acquisition, writing–review and editing. **E.A. Golemis:** Conceptualization, resources, supervision, funding acquisition, project administration, writing–review and editing.

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Aurora kinases in Head and Neck Cancer

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Abstract:	The Aurora kinases (AURKA and AURKB) have attracted attention as therapeutic targets in head and neck squamous cell carcinomas (HNSCC). Aurora kinases were first defined as regulators of mitosis that localization to the centrosome (AURKA) and centromere (AURKB), governing formation of the mitotic spindle, chromatin condensation, activation of the core mitotic kinase CDK1, alignment of chromosomes at metaphase, and other processes. Subsequently, additional roles for Aurora kinases have been defined in other phases of cell cycle, including regulation of ciliary disassembly and DNA replication. In cancer, elevated expression and activity of Aurora kinases results in enhanced or neomorphic locations and functions that promote aggressive disease, including promotion of MYC expression, oncogenic signaling, stem cell identity, epithelial-mesenchymal transition, and drug resistance. Numerous Aurora-targeted inhibitors have been developed, and are being assessed in preclinical and clinical trials, with the goal of improving HNSCC treatment.

Aurora kinases in Head and Neck Cancer

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Abstract.

The Aurora kinases (AURKA and AURKB) have attracted attention as therapeutic targets in head and neck squamous cell carcinomas (HNSCC). Aurora kinases were first defined as regulators of mitosis that localization to the centrosome (AURKA) and centromere (AURKB), governing formation of the mitotic spindle, chromatin condensation, activation of the core mitotic kinase CDK1, alignment of chromosomes at metaphase, and other processes. Subsequently, additional roles for Aurora kinases have been defined in other phases of cell cycle, including regulation of ciliary disassembly and DNA replication. In cancer, elevated expression and activity of Aurora kinases results in enhanced or neomorphic locations and functions that promote aggressive disease, including promotion of MYC expression, oncogenic signaling, stem cell identity, epithelial-mesenchymal transition, and drug resistance. Numerous Aurora-targeted inhibitors have been developed, and are being assessed in preclinical and clinical trials, with the goal of improving HNSCC treatment.

Keywords. Oral cancer, laryngeal cancer, EGFR, alisertib, AURKA, TP53, FBXW7, TPX2, NEDD9

Introduction

There were 878,000 new cases of head and neck squamous cell carcinomas (HNSCCs) worldwide in 2020¹, and approximately 54,000 new cases in the US in 2021, representing 2.8% of all new US cancer cases². These tumors most commonly occur in the oral cavity, pharynx, and larynx. Human papillomavirus (HPV) infection and integration of HPV into the genome is a significant cause of HPV+ HNSCCs arising from the oropharynx³, where carcinogenesis occurs due to p53 and Rb degradation caused by the virally-encoded E6 and E7 oncogenic proteins^{4,5}. HPV- tumors more commonly develop from the oral cavity and larynx as a result of chronic exposure to carcinogens, such as alcohol and tobacco^{6,7}. The typical 5-year overall survival of HNSCC patients is 68% and patients are frequently diagnosed at median age of 64 years²; HPV+ tumors have a better prognosis than HPV- cancers^{8,9}, causing an emphasis in developing therapies on HPV- HNSCC, the primary focus of this article.

HPV- HNSCCs are marked by loss of tumor suppressor genes. *TP53* is commonly mutated (72% of cases); mutation or loss of *CDKN2A* is also frequent (60% of cases)^{10,11}. During disease progression, additional common alterations include *CCND1* amplification¹² and *PTEN* loss^{13,14}. Epidermal growth factor receptor (EGFR) overexpression is frequent in HNSCC and associated with poor prognosis and low response to standard therapies¹⁵⁻¹⁷. Treatment depends on the tumor site and staging, but standard therapy for advanced tumors includes chemotherapy and targeted therapy¹⁸. For targeted therapy, the EGFR inhibitor cetuximab is beneficial, and sensitizes patients to chemoradiation; however, these patients often develop resistance by activation of alternative receptor tyrosine kinases (RTKs)¹⁹ and other mechanisms that compensate for EGFR inhibition. Immune checkpoint inhibitors are being actively explored for HNSCC patients with advanced disease, although response is limited^{20,21}.

The Aurora kinases have attracted attention as therapeutic targets in HNSCC. In humans, the Aurora kinase family has three members - AURKA, AURKB, and AURKC. In normal cells, spatially and temporally limited activation of AURKA and AURKB controls mitosis and other cell cycle related processes. However, elevated expression and activity of AURKA and AURKB often occurs in HNSCC and other forms of cancer, leading to temporally deregulated anomalous activity in multiple cellular compartments that contributes to pathogenicity, and is linked to poor prognosis. AURKC is predominantly expressed in meiotic cells²²; although it is sometimes overexpressed in cancer cells, and some data suggests overexpressed AURKC in transformed cells may enhance cancer cell migration²³, overall AURKC has attracted little study. This article will focus on

summarizing biology of AURKA and AURKB kinases relevant to therapy of HNSCC and discuss recent developments in agents targeting these kinases.

Structure and cell cycle regulation of Aurora kinases

Aurora kinases have a highly conserved kinase domain located at the C-terminus (**Figure 1**) but diverge at the N-terminus²⁴. In contrast to other family members, AURKA has an extended N-terminal domain that is intrinsically disordered²⁵, but is the site of interaction for a number of AURKA partner proteins, such as TPX2 and NEDD9²⁶ (**Figure 1**). The N terminal domain of AURKB is also crucial for partner protein binding, allowing for the recruitment of the Inner centromere protein (INCENP) (**Figure 1**)²⁷.

Aurora kinase activation is usually accompanied by auto-phosphorylation of a residue in the activation loop of the kinase domain: Thr288 for AURKA, and Thr232 for AURKB. For AURKA, this autophosphorylation is induced by complex interactions between AURKA and partner proteins²⁸ which include TPX2, BORA, NEDD9, PAK1, and Calmodulin²⁹⁻³². TPX2 also helps to enhance AURKA signaling by binding to AURKA, inducing AURKA phosphorylation and shielding the phosphorylated T288 residue, thus locking AURKA in the active conformation³³. In addition, some protein interactions that induce allosteric changes in the protein can partially activate AURKA without triggering Thr288 phosphorylation³⁴. For AURKB, autophosphorylation is induced upon interaction with INCENP³⁵, BOP1³⁶ and a small number of other proteins.

The best-studied functions of AURKA and AURKB in untransformed cells involve mitotic control (summarized in **Figure 2**). In non-transformed cells, AURKA predominantly concentrates at the centrosome, the mitotic spindle, and the basal body of cilia³⁷. AURKA contributes to centrosome maturation in G2 phase of the cell cycle, then interacts with a series of partner proteins to mediate formation of the mitotic spindle, and to activate the core cell cycle CDK1-cyclin B complex. AURKB localizes on chromatin and concentrates at the kinetochore, causing chromatin condensation and facilitating the alignment of sister chromatids during mitosis³⁸. The activities of both AURKA and AURKB are crucial for mitosis to initiate, and progress to cytokinesis without chromosomal segregation defects that would lead to aneuploidy. At the end of mitosis, Aurora kinases are degraded to allow cytokinesis.

In normal cells, AURKA also has important non-mitotic roles. For example, in quiescent cells, AURKA localizes to the basal body of the primary cilium, an antenna-like structure protruding from the cell surface that serves as a receptor for soluble growth-regulating factors and mechanical cues. Transient activation of AURKA in G0/G1 cells in response to external cues

leads to resorption of cilia³⁹, altering cellular response to these factors. A number of activators of AURKA during ciliary resorption have been described; these differ from AURKA activators that function during mitosis⁴⁰. Limited activation of AURKB has also been described in normal cells, with some activity in S phase that is important for subsequent accurate chromosome segregation and telomere maintenance^{41,42}.

Overexpression of Aurora kinases in HNSCC and other cancers

The *AURKA* gene is located on chromosome 20q13, a region amplified in a number of cancers⁴³⁻⁴⁵, and a small number of HNSCCs^{46,47}. In contrast, chromosome 17p13, the site of AURKB, is not commonly amplified in tumors. Analysis of TCGA data indicates limited copy number variation (CNV) of AURKA and AURKB in HNSCC (**Figure 3A, B**), although low level gain occurs in a subset of tumors (**Figure 3C**). TCGA analysis also shows that, in contrast to other pro-oncogenic kinases such as EGFR⁴⁸, activating AURKA or AURKB mutations rarely if ever occur in cancer (**Figure 3A, B**). However, mRNAs for both Aurora kinases are overexpressed in many tumors^{43,44,49-52}, including HNSCC (**Figure 3C**), as are the proteins, allowing the overexpressed proteins to have both expanded localization, and greater activity throughout cell cycle. Interestingly, increased copy number for the *AURKA* and *AURKB* genes significantly co-occurs with alterations commonly found in head and neck neoplasms, with co-occurrence for EGFR amplification and gain (q value = <0.001 for AURKA and 0.008 for AURKB); for PIK3CA amplification and gain (q value = <0.001 for both); for PTEN loss (q value = 0.018 for AURKB); and for FBXW7 loss (q value = 0.021 for AURKB). This may reflect important functional interactions.

AURKA overexpression is associated with highly invasive tumors, as well as decreased metastasis-free survival and overall survival⁵³⁻⁵⁵. HNSCC patients with overexpression of AURKA presented increased incidence of lymph node and distant metastasis, advanced disease stage, shorter disease-free survival, and overall survival^{56,57,58}. Increased AURKB expression is also linked to multinuclearity and increased ploidy⁵⁹. For HNSCC, increased AURKB expression and activity has been demonstrated in oral cell lines and tumor samples in comparison with normal epithelium, and correlated with increased cell proliferation, poor tumor differentiation, shorter disease-free survival, and increased disease aggressiveness^{60,61}.

Transcriptional upregulation of AURKA and AURKB in cancer is promoted both by oncogenic activation and tumor suppressor loss (**Figure 4A**). For example, MYC directly binds to *AURKA* gene enhancer regions to promote its transcription⁶², and downregulation of Myc alleviates the oncogenic effects mediated by AURKA⁶³. Conversely, overexpression of AURKA

has been shown to mediate the tumor promoting effects caused by Myc⁶⁴, and increased Myc protein levels⁶⁵, in positive feedback. Enhanced signaling by EGFR also upregulates AURKA expression; in one study, EGFR was defined as a direct transcriptional activator of AURKA, translocating to the nucleus and interacting with STAT5 to bind the AURKA promoter and initiate transcription⁶⁶. EGFR overexpression and activation are well known characteristics of most head and neck neoplasms, where EGFR amplification occurs in approximately 30% of all cases^{17,67,68}. Mutational inactivation or loss of *TP53* - the most mutated gene in HPV- HNSCC¹¹ – also influences AURKA expression. Intact p53 directly binds to the *AURKA* promoter to repress its transcription⁶⁹; p53 can also cause indirect transcription repression of *AURKA* through the cyclin-dependent kinase (CDK) inhibitor 1A mediated inhibition of E2F3, an *AURKA* transcriptional activator; in addition, p53 can directly interact with the AURKA protein to inhibit its kinase activity⁷⁰. Loss of p53 in HNSCC impairs these processes, elevating AURKA expression. Interestingly, it has been reported that distinct *TP53* genotypes can have distinct effects on AURKA levels⁷¹; this topic requires more investigation.

At the protein level, AURKA can be upregulated by post-translational modifications and protein interactions which enhance AURKA protein stability or prevent its degradation (**Figure 4A**). A study in HNSCC showed phosphorylation of AURKA on Ser51 increases its protein levels by inhibiting its APC-mediated degradation⁷²; this post-translational modification is mediated by Ca²⁺/Calmodulin (CaM) binding to AURKA protein, which induces its activation and consequent auto-phosphorylation³². The tumor suppressor and E3 ubiquitin ligase FBXW7 is commonly downregulated in many types of cancer⁷³; as FBXW7 normally promotes proteasomal degradation of AURKA⁷⁴, loss of FBXW7 is typically associated with increased levels of AURKA⁷⁵. Importantly, FBXW7 is frequently inactivated by mutation in HNSCC, and was identified in a 2020 study as the fourth (7.7%) most commonly mutated gene in a panel of HPV+ HNSCC tumors⁷⁶. In addition to frequent loss-of-function (LoF) mutations, transcriptional downregulation of FBXW7 in cancer also is common due to loss of p53, an activator of FBXW7 transcription^{75,77}.

Activation of PI3K/AKT signaling either by activating mutation of PI3K or loss of the PTEN tumor suppressor indirectly regulates AURKA levels through FBXW7 (**Figure 4A**). AKT-dependent inhibitory phosphorylation of GSK3 β blocks its interaction and phosphorylation of AURKA (S245 and S387); this impairs the ability of FBXW7 to bind to AURKA and promote its degradation⁷⁴. In HNSCC, *PTEN* is commonly mutated or lost (27% based on TCGA, **Figure 3C**), with reports as early as 1998 demonstrating loss of heterozygosity (LOH) at the *PTEN* locus in 41% of HNSCC samples, as well as presence of inactivating mutations^{14,78,79}. Amplifications and

activating mutations in *PIK3CA* are also frequent in HNSCC, a genomic characterization study with 279 HNSCCs samples showed that 34% of HPV- tumors had amplification and/or driver mutations on this gene¹¹. Hence, there is a common post-translational upregulation of AURKA in cancers with elevated PI3K/Akt signaling⁷⁹.

An alternative way of increasing AURKA activity in HNSCC is through elevated expression of partner proteins that interact with AURKA to stabilize the protein and support its catalytic activity (**Figure 4A**). The AURKA activator TPX2, is overexpressed in many cancers, and through AURKA binding, allosteric modulation, and protection of the T288 phosphorylation site from dephosphorylation, TPX2 increases AURKA levels and maintains its active state, augmenting AURKA oncogenic effects in the cell^{80,81}. In a study in HNSCC, TPX2 mRNA levels were upregulated in 43 tumor samples in comparison with normal paired samples⁸². Notably, the chromosomal location of TPX2 is very close to that of AURKA, and the two proteins are co-amplified in some tumor types⁸⁰. TPX2 is upregulated in 46% of HPV- HNSCCs in a cohort of 415 samples from TCGA database (**Figure 3C**), with most of the samples with *TPX2* gain co-occurring with gain in *AURKA* gene, reflecting their close chromosomal location (**Figure 4A**). In addition, TPX2 and AURKA mRNA levels strongly correlate (Spearman correlation coefficient = 0.802, q value <0.0001). Similarly, NEDD9 overexpression increases AURKA levels. NEDD9 also stabilizes AURKA by blocking the binding of the APC complex, preventing its degradation^{29,83}; NEDD9 expression is commonly elevated in advanced HNSCC⁸⁴. Both TPX2 and NEDD9 levels have been implicated in sensitivity to AURKA inhibition by small molecule agents^{83,85}.

Cancer-promoting function of overexpressed AURKA

AURKA overexpression frequently occurs as an early event in cell transformation^{44,86}, and promotes tumor initiation and progression in several ways (**Figure 4B**). Mechanistically, AURKA amplification and overexpression in HNSCC and other tumor types causes an increase in centrosome number, and an inability to resolve M-phase, associated with failure of cytokinesis^{44,87,56}. Abnormal centrosome organization and number induced by AURKA upregulation causes formation of multipolar mitotic spindles, which leads to inappropriate segregation of chromosomes, chromosomal instability, and aneuploidy in HNSCC⁸⁸ and other cancers. Factors that lead to AURKA overexpression or anomalous activation, such as overexpression of the AURKA scaffolds TPX2⁸¹ and NEDD9²⁹, produce similar phenotypes.

Besides directly acting on the mitotic apparatus, overexpressed AURKA drives early cellular transformation by inducing G1 checkpoint loss through inducing downregulation of p53 (**Figure 4B**). AURKA phosphorylation of p53 on S215 impairs its DNA binding and transactivation

function, resulting in the downregulation of some p53 target genes that act as tumor suppressors, such as *PTEN* and *CDKN1A*⁸⁹. AURKA also phosphorylates p53 on S315, which enhances p53 binding by the E3 ligase MDM2, promoting the ubiquitination-mediated degradation of p53⁹⁰. Loss of p53 and impairment of p53-mediated cell cycle checkpoints promotes cell survival in the context of increasing DNA abnormalities. In addition, AURKA-dependent loss of p53 may also contribute to the persistence of cancer stem cells in HNSCC, based on findings that downregulation of p53 by AURKA supports pluripotency of embryonic stem cells (ESCs)⁹¹. AURKA also promotes stem cell pluripotency through direct phosphorylation of SOX2, influencing its transcription factor activity⁹². As maintenance of stem cells is a major determinant of therapeutic resistance and tumor recurrence, these AURKA activities likely contribute to the poor prognosis of HNSCC with high AURKA expression.

Several important AURKA oncogenic effects arise due to the expanded localization of the overexpressed protein in cancer⁹³. Many studies of tumors have reported AURKA in the nucleus, where the degree of nuclear expression correlates with disease aggressiveness⁹⁴⁻⁹⁶. One study found AURKA abundant in the nucleus of HNSCCs cells, and directly correlated this location with oncogenic activity⁹⁵; AURKA nuclear localization has also been associated with worse survival in HNSCC patients⁹⁴. At least some nuclear AURKA functions in a kinase-independent manner, by binding to oncogenic proteins to promote their stabilization, and acting as a transcriptional activator to enhance transcription of oncogenic genes, such as MYC and FOXM1^{96,97} – another way in which AURKA increases a stem cell phenotype⁹⁷. Mechanistically, nuclear AURKA can bind to the heterogeneous nuclear ribonucleoprotein K (hnRNPK) to transactivate MYC transcription, with this function retained by a kinase dead AURKA mutant⁹⁶ (**Figure 4B**). In neuroblastoma, AURKA binds and stabilizes N-Myc, preventing its proteasomal degradation⁹⁸; pharmacological inhibition of AURKA triggers conformational change that disrupts its binding to N-MYC, causing a consequent decrease in N-MYC protein levels^{99,100}. Several studies suggest that AURKA inhibition decreases C-MYC protein levels in a similar manner^{101,102}. By supporting MYC levels, AURKA promotes MYC-dependent transcription that enhances cell cycle progression and cell survival in tumors. MYC also promotes transcription of many glycolytic enzymes and represses genes involved in oxidative phosphorylation and fatty acid oxidation; AURKA-mediated Myc induction was shown to contribute to converting tumor metabolism towards a glycolytic state¹⁰². Separately, AURKA has also been found to promotes glycolysis by direct phosphorylation of lactate dehydrogenase B (LDHB), which increases NAD⁺ and lactate formation¹⁰³.

Many studies have found that AURKA overexpression promotes epithelial-mesenchymal transition (EMT), a change in differentiation status linked to enhanced tumor migration and invasion in many tumor types¹⁰⁴⁻¹⁰⁶. In a study in HNSCC, AURKA promotion of migration and invasion involves activation of focal adhesion kinase (FAK)¹⁰⁷, a partner of the AURKA activator NEDD9 (also known as HEF1)^{108,109}. HNSCC tumors typically contain areas with differing degrees of oxygenation, influencing response to radiation and other therapies, and tumor invasion, which is induced by hypoxia¹¹⁰. Although hypoxia-induced transcription factors such as the HIF proteins are normally inactive under conditions of oxygenation, one study has found that nuclear AURKA binds and activates HIF1B under normal oxygen conditions, inducing transcription of hypoxia-response genes that promote migration and stemness (**Figure 4B**). In this work, knockdown of HIF1A/B decreased the epithelial-mesenchymal transition (EMT) effect driven by nuclear AURKA, indicating some dependency of AURKA to HIF1A/B to induce EMT¹¹¹. Moreover, AURKA activates the small GTPase RALA through phosphorylation of the serine 194, increasing cell motility and migration (**Figure 4B**)^{112,113}. The role of increased AURKA in promoting EMT emphasize its involvement in disease progression, especially as a metastatic driver.

Notably, upregulation of AURKA has been implicated in resistance to chemotherapeutic agents, such as taxanes¹¹⁴. AURKA inhibition and paclitaxel have synergistic cytotoxic effects in HNSCC cell lines¹¹⁵ *in vitro*, and *in vivo* for other types of solid tumor¹⁰⁴. In EGFR-mutant lung adenocarcinoma, prior treatment with EGFR inhibitors increased levels of TPX2 and activated AURKA, driving resistance to the inhibitors. Mechanistically, TPX2/AURKA replace the inhibited activity of inhibited EGFR in phosphorylating the pro-apoptotic protein BIM to promote its degradation (**Figure 4B**), thereby contributing to ability of these tumors to survive treatment with EGFR inhibitors; this study showed that AURKA inhibitors synergize with EGFR inhibitors to eliminate EGFR resistant cells *in vitro* and *in vivo*⁸⁵. Aurora inhibitors have been identified as cytotoxic in patient-derived HNSCC cell lines that were both sensitive and resistant to the EGFR inhibitor gefitinib; in this study, resistant cells had wildtype EGFR with increased levels of phosphorylated AURKA¹¹⁶. Such results suggest means to apply AURKA inhibitors in combination therapies, as discussed below.

Cancer-promoting function of overexpressed AURKB

Similar to AURKA, AURKB promotes Myc stabilization through phosphorylation of serine 67 on Myc, which prevents its interaction with the E3 ubiquitin ligase FBXW7 and its consequent mediated proteasomal degradation⁶⁵. AURKB also phosphorylates p53 on residues S183, T211, and S215 to promote its degradation, repressing important p53 functions¹¹⁷ such as transcriptional

induction of its downstream target genes, such as *CDKN1A*¹¹⁸. Knockdown of AURKB can repress AKT and mTOR activity, with co-inhibition of AURKB and use of a histone deacetylase (HDAC) inhibitor further intensifying this repression and inducing apoptosis, although the mechanism remains to be elucidated¹¹⁹. Interestingly, in cells with active AKT signaling due to mutation of PTEN or PI3K, the observed inhibition of tumor cell proliferation and *in vivo* tumor growth caused by the novel AKT degrader, MS21, was linked to destabilization of AURKB¹²⁰. Some studies have also implicated AURKB as a mediator of drug resistance in HNSCC. For example, increased mRNA levels of AURKB were observed in HNSCC cell lines with intrinsic EGFR resistance to cetuximab, and these cells were sensitive to the AURKB inhibitor barasertib; again, more work is required to define the resistance mechanism¹²¹.

Inhibitors of Aurora Kinases: preclinical studies.

Because of the clear contribution of elevated AURKA and AURKB activity to multiple aspects of tumorigenesis, kinase inhibitors have been developed targeting AURKA, AURKB, or both (**Table 1**). While the value of AURKA and AURKB inhibitors is well documented in many tumor types in preclinical studies, investigation of these inhibitors in the setting of HNSCC is limited. Overall, preclinical studies have found that cells with mutant p53 are sensitive to Aurora kinase inhibition monotherapy^{122,123}. AURKA-AURKB dual inhibition is useful in some cancer cells that are resistant to other anti-mitotic therapeutic agents¹²⁴. Adavosertib, a highly selective inhibitor of WEE1 (which mediates checkpoints for mitotic entry), synergizes with AURKA inhibitors, effectively reducing overall tumor size in HNSCC xenograft experiments compared to vehicle and single drug treatment¹²⁵. Disruption of signaling by Rb, which regulates G1/S checkpoints, was also shown to be synthetically lethal with AURKA inhibition¹²⁶.

Importantly, pan-Aurora kinase inhibitors synergize with the EGFR inhibitor cetuximab – a critical first line agent used for HNSCC - and also overcome cetuximab resistance^{121,121,12}. In EGFR-mutated lung cancer cell lines, dual inhibition of EGFR and AURKB is a stronger inducer of apoptosis than EGFR inhibition alone, based on induction of BIM and PUMA caused by AURKB inhibition¹²⁷. Dual inhibition of AURKA and PI3K has been shown to greatly reduce mTOR signaling in breast cancer models, resulting in apoptosis and reduction in tumor size *in vivo*¹²⁸. Dual inhibition of AURKB and AURKA in early-stage lung adenocarcinoma downregulated AKT/mTOR signaling, and also reduced expression of EMT markers such as N-Cadherin and vimentin; these effects were not observed with single inhibition of either kinase¹²⁹. AURKA and AURKB inhibition may be an effective neoadjuvant for subsequent treatments such as immune checkpoint inhibition and radiation. For example, Aurora kinase inhibition in melanoma

potentiated antitumor immunity by sensitizing tumor cells to T-cell mediated cytotoxicity¹³⁰. AURKA inhibition also sensitized xenografted tumors to radiation therapy¹³¹.

While most inhibitors of Aurora kinases are small molecules, PROTACs (Proteolysis Targeting Chimeras) are being developed to AURKA, such as the agents JB170 and JB158¹³². PROTACs contain two binding moieties that connect the endogenous ubiquitin ligase pathway to targeted proteins to cause their degradation, and sometimes have greater activity than agents such as kinase inhibitors, particularly if a kinase has non-catalytic roles (e.g. scaffolding complexes). Among the limited tests performed to date, one group has found that an AURKA-targeting PROTAC preferentially binds and degrades spindle associated AURKA at a higher rate than centrosomal AURKA, suggesting that PROTACs could be fine-tuned to target a specific pool of AURKA¹³³. To date, there are no studies that investigate the usage of an AURKA targeting PROTAC in a clinical trial.

Aurora Kinase Inhibitors in Clinical Trials

While Aurora inhibitors have shown great promise in preclinical studies, usage in the clinic have shown varying degrees of success (**Table 2**). In single agent trials, the AURKA/B inhibitor PF-03814735, one of the first inhibitors to be used in clinical trials as monotherapy for patients with advanced solid tumors (including HNSCC), showed tolerable toxicity and established a recommended phase 2 dose, but only limited antitumor activity was observed (NCT00424632)¹³⁴. The AURKA inhibitor alisertib (also called MLN8237), has been evaluated in multiple clinical trials for many types of cancer. One of the first clinical trials for alisertib included head and neck cancer in a cohort of patients with advanced solid tumors (NCT00500903 and NCT00651664)^{135,136}. This trial established alisertib recommended doses and safety and allowed alisertib to proceed to phase II study in a large cohort of patients with nonhematological malignancies, including 55 patients with head and neck cancer (NCT01045421). However, only 4 head and neck patients attained a partial response, with median duration of 2.6 months (95% CI 1.5 – 3.1); more promising results were observed for breast cancer and small-cell lung cancer patients¹³⁷. Overall, alisertib has tolerable side effects, but some common adverse effects including neutropenia, anemia, nausea, and fatigue, which can be problematic at higher dose levels¹³⁶. To date, alisertib is the only AURKA inhibitor that has progressed to phase III clinical trials (e.g. NCT01482962). In patients with advanced solid tumors, monotherapy with a more recently developed AURKA inhibitor with higher tolerability, TAS-119, showed limited antitumor response; no patients showed complete or partial response, with 41% of patients showing stable tumor size (NCT02448589)¹³⁸. While stronger responses are observed in liquid tumors (leukemias and lymphomas), the limited

responses to single agent Aurora inhibitors in solid tumors has focused attention on combination therapies.

The combination of alisertib and paclitaxel in the setting of advanced breast and ovarian cancer increased progression-free survival (PFS) compared to paclitaxel treatment alone (6.7 months, compared to 4.7 months in paclitaxel monotherapy patients)¹³⁹. In patients with advanced solid tumors, combination of the mTORC1/2 inhibitor sapanisertib, also called MLN0128, with alisertib resulted in only 2 patients experiencing dose limiting toxicity at the highest dose level (NCT02719691) and caused 31% of patients to have stable disease¹⁴⁰. Alisertib had also been evaluated with pazopanib, a multi-kinase angiogenesis inhibitor, in patients with solid tumors, including head and neck cancer, showing tolerable safety and tumor inhibition (NCT01639911). Pharmacokinetic analysis indicated that alisertib plasma concentrations were 40% higher with the combination treatment than with alisertib alone, suggesting that pazopanib reduces alisertib clearance, possibly due inhibition of cytochrome P450 isozyme 3A4 (CYP3A4), but further investigation is required^{141,142}. Currently, alisertib is being evaluated with the immune checkpoint inhibitor pembrolizumab in a phase I/II study for patients with recurrent or metastatic HPV+ HNSCCs (NCT04555837), based on a pre-clinical study that showed synthetic lethality of the AURKA inhibitor LY3295668 with an *RB1* loss of function mutation which mimics the Rb deficiency found in HPV+ HNSCCs tumors¹²⁶.

Table 2 also shows the usage of Aurora kinase inhibitors in combination with other agents in completed and ongoing clinical trials for tumor types that have some commonalities with HNSCC. Non-small cell lung carcinoma, for example, often overexpress EGFR and AURKA and have mutated TP53, similar to HNSCC. EGFR-mutated NSCLC can have increased expression of TPX2, which is associated with EGFR inhibitor resistance via upregulation of AURKA⁸⁵. Although these trials do not include HNSCC patients, some pre-clinical studies have shown anti-tumor activity for these combinations in HNSCCs, which may suggest clinical studies in this cohort. The ongoing clinical trials should provide insight into effectiveness of novel AURKA inhibitor combinations, supporting their extension to HNSCC. Drugs being tested in combination with AURKA inhibitors target proteins including mTORC1/2, ACE, and EGFR.

Summary and Future Directions.

Therapy options for patients with advanced HNSCCs are still very limited. Considering the multiple mechanisms upregulating AURKA and AURKB in HNSCC, and their extensive tumor promoting roles, it is likely that ongoing work will reveal synthetic lethal interactions involving Aurora inhibitors and other targets that improve therapeutic windows for HNSCC. Aurora kinase

inhibitors have been showing promising results in combination with a variety of drugs in different types of cancers, although many of these trials do not include head and neck tumors; extension of positive results to evaluation in HNSCC patients should be prioritized. To help guide the usage of Aurora kinase inhibitors in the clinic, it is also important to identify predictive biomarkers and regulators of response to these drugs, to aid in distinguish likely responders and non-responders, and develop useful therapeutic combinations. TP53 status has been of considerable interest as a response biomarker; one interesting study in triple negative breast cancer showed that the administration of alisertib to cells lacking *TP53* results in senescence, rather than the apoptosis as seen in *TP53* wild-type cells, with implications for recurrence¹⁴³. TPX2 expression is associated with resistance to AURKA inhibition, with TPX2 binding to AURKA altering the kinase ATP-binding pocket, reducing binding affinity and efficacy of various inhibitors¹⁴⁴. Similarly, NEDD9 binding of AURKA reduces AURKA inhibitor binding affinity⁸³, and shRNA depletion of NEDD9, combined with AURKA inhibition, greatly reduced xenograft tumor volume⁸³. Incorporation of TPX2, NEDD9, and other biomarkers during design of clinical trials may be successful in increasing rate of therapeutic response of Aurora inhibitors. Finally, understanding of Aurora biology continues to evolve, with new functions being identified (e.g., regulation of cilia-mediated intercellular signaling^{145,146}). Continued investigations in these areas may help nominate ways to better exploit Aurora kinases to improve HNSCC treatment.

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Figure and Table Legends.**Table 1. Preclinical and clinical Aurora kinase inhibitors**

Compound Name	Targets (IC₅₀, <i>in vitro</i> assays)	Results in relation to HNSCC	Reference
MLN8237 (Aisertib)	AURKA (1.2nM)	Extensive preclinical trials using HNSCC xenograft models; Phase I-II in HNSCC patients	[125,147]
VIC-1911 (TAS-119)	AURKA (1.0nM) AURKB (95nM)	Limited <i>in vitro</i> and HNSCC xenograft model	[148]
MLN8054	AURKA (4nM)	Limited <i>in vitro</i> assays in the setting of EGFR mutant HNSCC	[116,149]
PF-03814735	AURKA (0.8nM) AURKB (5nM)	N/A	[150]
BPR1K653	AURKA (124nM) AURKB (45nM)	N/A	[151]
PHA-739358 (Danasertib)	AURKA (13nM) AURKB (25nM)	Extensive preclinical trials using patient derived HNSCC xenograft models	[152,153]
ENMD-2076	FLT-3 (1.86nM) RET (10.4nM) AURKA (14nM)	N/A	[154,155]
AZD1152 (Barasertib)	AURKB (0.37nM)	Limited <i>in vitro</i> assays in HNSCC cell lines	[156,157]
GSK1070916	AURKB (3.5nM) AURKC (6.5nM) FLT1 (42nM) Tie-2 (59nM) SIK (70nM)	N/A	[158]
CYC116	AURKA (44nM) AURKB (19nM) AURKC (65nM) VEGFR2 (69nM)	N/A	[159]
VX-680 (Tozasertib)	AURKA (0.6nM) AURKB (18nM) AURKC (4.6nM)	N/A	[160]

Table 2. Clinical trials using Aurora kinase inhibitors in HNSCC, and other advanced solid tumors.

Clinical Trials for Head and Neck Cancer							
NCT	Therapy	Target	Tumor type	Phase	Trial start year	Status	Study Publication
NCT00424632	PF-03814735	AURKA; AURKB	Advanced Solid Tumors	I	2006	Completed	[¹³⁴]
NCT00560716	CYC116	Pan-aurora; VEGFR2	Advanced Solid Tumors	I	2007	Terminated	N/A
NCT00500903	MLN8237	AURKA	Advanced Solid Tumors	I	2007	Completed	[¹³⁶]
NCT00651664	Alisertib	AURKA	Solid Tumors	I	2007	Completed	[¹³⁵]
NCT01540682	MLN8237; Cetuximab; Radiotherapy	AURKA; EGFR	Head and neck	I	2012	Completed	N/A
NCT01613261	Alisertib; TAK-733	AURKA; MEK1/2	Nonhematological Malignancies	I	2012	Study was withdrawn before participants were enrolled.	N/A
NCT01045421	Alisertib	AURKA	Nonhematological Malignancies	I/II	2010	Completed	[¹³⁷]
NCT01639911	Alisertib; Pazopanib	AURKA; VEGFR; PDGFR	Solid Tumors	I	2013	Completed	[¹⁴¹]
NCT04555837	Alisertib; Pembrolizumab	AURKA; PD-1	Rb-deficient HNSCC	I/II	2020	Recruiting	N/A
NCT02448589	TAS-119	AURKA	Advanced Solid Tumors	I	2014	Terminated	[¹³⁸]
NCT02812056	Alisertib; TAK-228	AURKA; mTORC1/2	Human Papilloma Virus (HPV) Associated Malignancies	I	2016	Study was withdrawn before participants were enrolled.	N/A
NCT01512758	Alisertib	AURKA	East Asian Participants with Advanced Solid Tumors or Lymphomas	I	2012	Completed	[¹⁶¹]
Clinical trials in Selected Tumor Types							
NCT00497731	AZD1152	AURKB	Advanced Solid Tumors	I	2005	Terminated	N/A
NCT00424632	PF-03814735	AURKA; AURKB	Solid Tumors	I	2006	Completed	[¹³⁴]
NCT00858377	AMG 900	Pan-aurora	Advanced Solid Tumors	I	2009	Completed	[¹⁶²]
NCT01118611	GSK1070916A	AURKB; AURKC	Advanced Solid Tumors	I	2010	Completed	N/A
NCT01471964	MLN8237; Erlotinib	AURKA; EGFR	Non-Small Cell Lung Cancer	I/II	2011	Terminated	N/A
NCT01923337	Alisertib; Irinotecan	AURKA; Topoisomerase I	Advanced Solid Tumors or Colorectal Cancer	I	2013	Completed	[¹⁶³]
NCT01924260	Alisertib; Gemcitabine Hydrochloride	AURKA; anti-metabolite for DNA synthesis	Solid Tumors or Pancreatic Cancer	I	2013	Completed	N/A
NCT02134067	TAS-119; Paclitaxel	AURKA; microtubules	Advanced Solid Tumors	I	2014	Terminated	[¹⁶⁴]
NCT02038647	Alisertib; Paclitaxel	AURKA; microtubules	Small Cell Lung Cancer	II	2014	Completed	[¹⁶⁵]
NCT02719691	MLN8237; MLN0128	AURKA; mTORC1/2	Advanced Solid Tumors and	I	2016	Completed	[¹⁴⁰]

			Metastatic Triple-negative Breast Cancer				
NCT03898791	LY3295668; Erbumine	AURKA; ACE	Small-Cell Lung Cancer	I	2019	Completed	N/A
NCT04085315	Alisertib; Osimertinib	AURKA; EGFR	Non-Small Cell Lung Cancer	I	2019	Recruiting	N/A
NCT04479306	Alisertib; Osimertinib; Sapanisertib	AURKA; EGFR; mTORC1/2	Non-Small Cell Lung Cancer	I	2020	Active, not recruiting	N/A
NCT05017025	LY3295668; Osimertinib	AURKA; EGFR	EGFR-Mutant Non-small Cell Lung Cancer	I	2022	Recruiting	N/A

Figure 1. Structure and interactions of AURKA and AURKB. **A., B.** 3D structure of human AURKA in complex with VX-680 (a pan-Aurora kinase inhibitor and non-hydrolyzable analog of ATP) and TPX2 (colored blue), from Protein Data Bank (PDB) entry 3E5A. **A.** Structure of the nucleotide binding pocket of AURKA; the residues that participate in ATP binding are colored red. **B.** AURKA is rotated about the Y axis in A versus B to highlight TPX2 interaction. **C., D.** 3D structure of AURKB in complex with VX-680 and INCENP (colored blue), from PDB entry 4AF3. **C.** AURKB structure with binding pocket, to foreground; in **D.** AURKB is rotated to show INCENP interaction. **E.** Domain organization of AURKA and AURKB. The conserved kinase domain is colored green in both AURKA and AURKB. The domains that are necessary for APC-C mediated proteolysis (D-Box for AURKA; KEN-Box and A-Box for AURKB) are colored orange. The intrinsically disordered regions of AURKA and AURKB contains the binding site for TPX2 and INCENP, respectively. Noted phosphorylation sites are described in the text.

Figure 2. Activity of Aurora kinases in mitosis. In G1, AURKA localizes to the ciliary basal body (a structure derived from the centriole) and is transiently activated to cause resorption of cilia³⁹. As cell cycle progresses, the basal body undergoes changes releasing the centriole to form the centrosome; AURKA accumulates at the centrosomes and contributes to centrosome maturation in G2 phase of the cell cycle. In late G2, AURKA interacts with TACC1 and TACC3, which recruit ch-TOG/XMAP215. Interaction of AURKA with these, TPX2, and additional proteins as AURKA propagates along the spindle asters³⁰ results in the formation and appropriate function of the mitotic spindle, allowing appropriate chromatin segregation¹⁶⁶. AURKA also contributes to mitotic entry by activating core cell cycle machinery. AURKA phosphorylation activates the CDC25 phosphatase, which then removes the inhibitory Tyr15 phosphorylation from CDK1¹⁶⁷; AURKA also phosphorylates PLK1, which inhibits the cell cycle checkpoint phosphatase WEE1¹⁶⁸. Together, these changes activate CDK1 to promote the cell cycle transition from G2 to M phase and sustaining CDK1 activity through M phase.

AURKB primarily functions in association with chromatin. In S phase, AURKB contributes to DNA replication, but has low level activity. In mitosis, the chromosomal passenger complex (CPC), consisting of AURKB, INCENP, Survivin, and Borealin, is crucial for chromosomal condensation. INCENP binding to AURKB induces a conformational change in AURKB, leading to AURKB autophosphorylation on Thr232¹⁶⁹. AURKB remains active through mitosis, mediating the extensive Ser10 phosphorylation of Histone H3 to support chromosome condensation¹⁷⁰. As part of the CPC, AURKB also regulates the activity of mDia3 and Ndc80, ensuring that the chromosomes are properly arranged at the metaphase plate¹⁷¹. Briefly, the CPC accumulates at

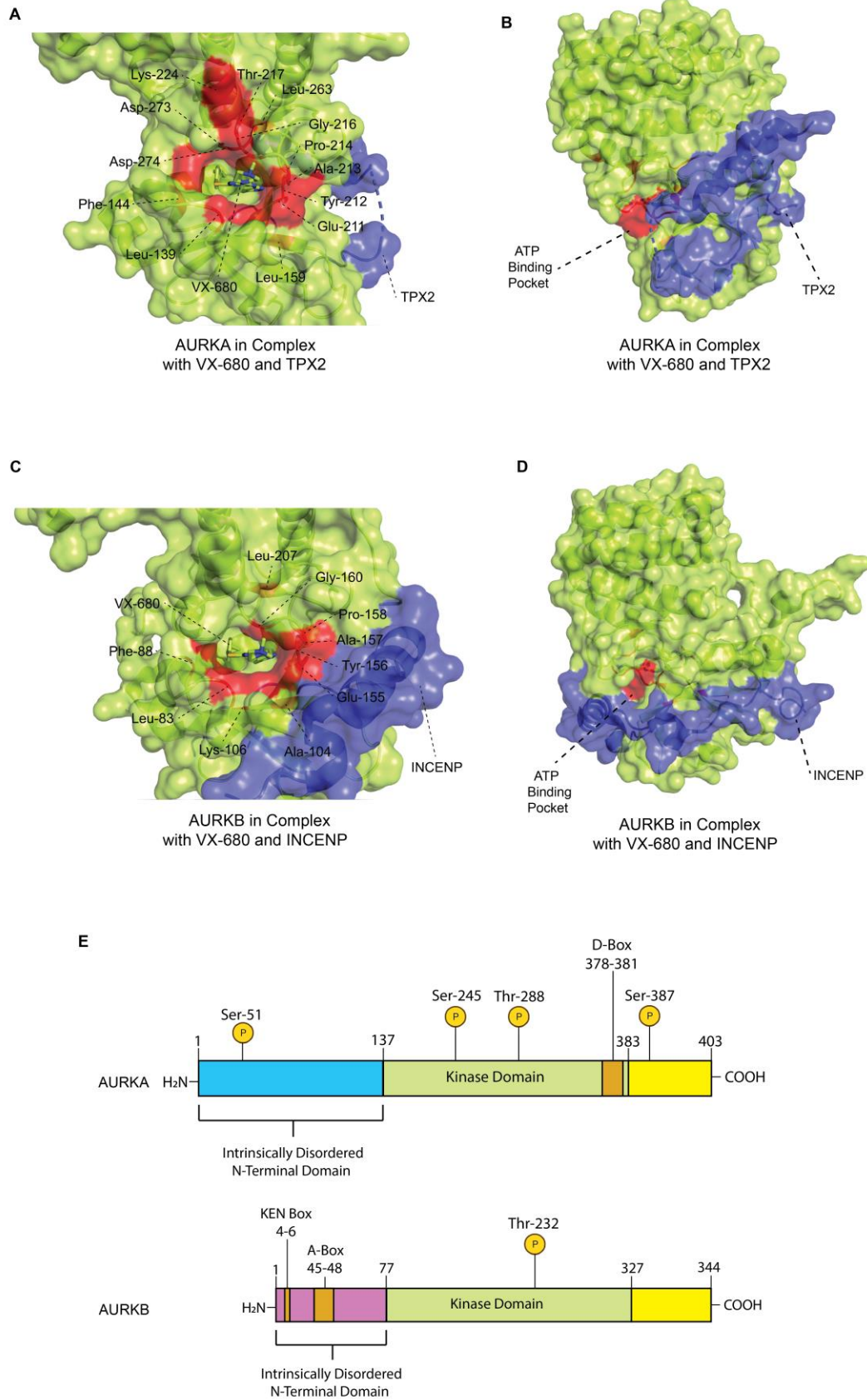
the inner centromere. When the microtubule is tightly pulled, indicating a proper attachment, mDia3 and Ndc80 are distal to the CPC, and cannot be phosphorylated by AURKB^{171,172}; unphosphorylated mDia3 and Ndc80 have high binding affinity for the microtubule and facilitate attachment to the kinetochore. If there is improper attachment of the centromere to the kinetochore, the CPC and AURKB are proximal to mDia3 and Ndc80, and phosphorylates these proteins, causing them to lose affinity for the microtubules. Removal of mDia3 and Ndc80 from microtubules initiates another cycle of attempted microtubule-kinetochore attachment¹⁷¹. This process is crucial for chromosomal alignment during mitosis and ensuring appropriate post-mitotic DNA segregation.

At the end of mitosis, Aurora kinases are degraded to allow cytokinesis. Aurora kinases are subject to numerous mechanisms of negative regulation, that reduce kinase activity or expression. Both AURKA and AURKB interaction with the Anaphase Promoting Complex/Cyclosome (APC/C), which acts as an E3 ubiquitin ligase for the kinases, causing their proteasomal degradation. A number of interacting partners that target AURKA for destruction have been identified, including FBXW7²⁴. The D-Box motif of AURKA is necessary for APC/C recognition and proteasomal degradation¹⁷³. In contrast, the KEN box and A box, rather than the D-box motif, is required for AURKB interaction with APC/C¹⁷⁴.

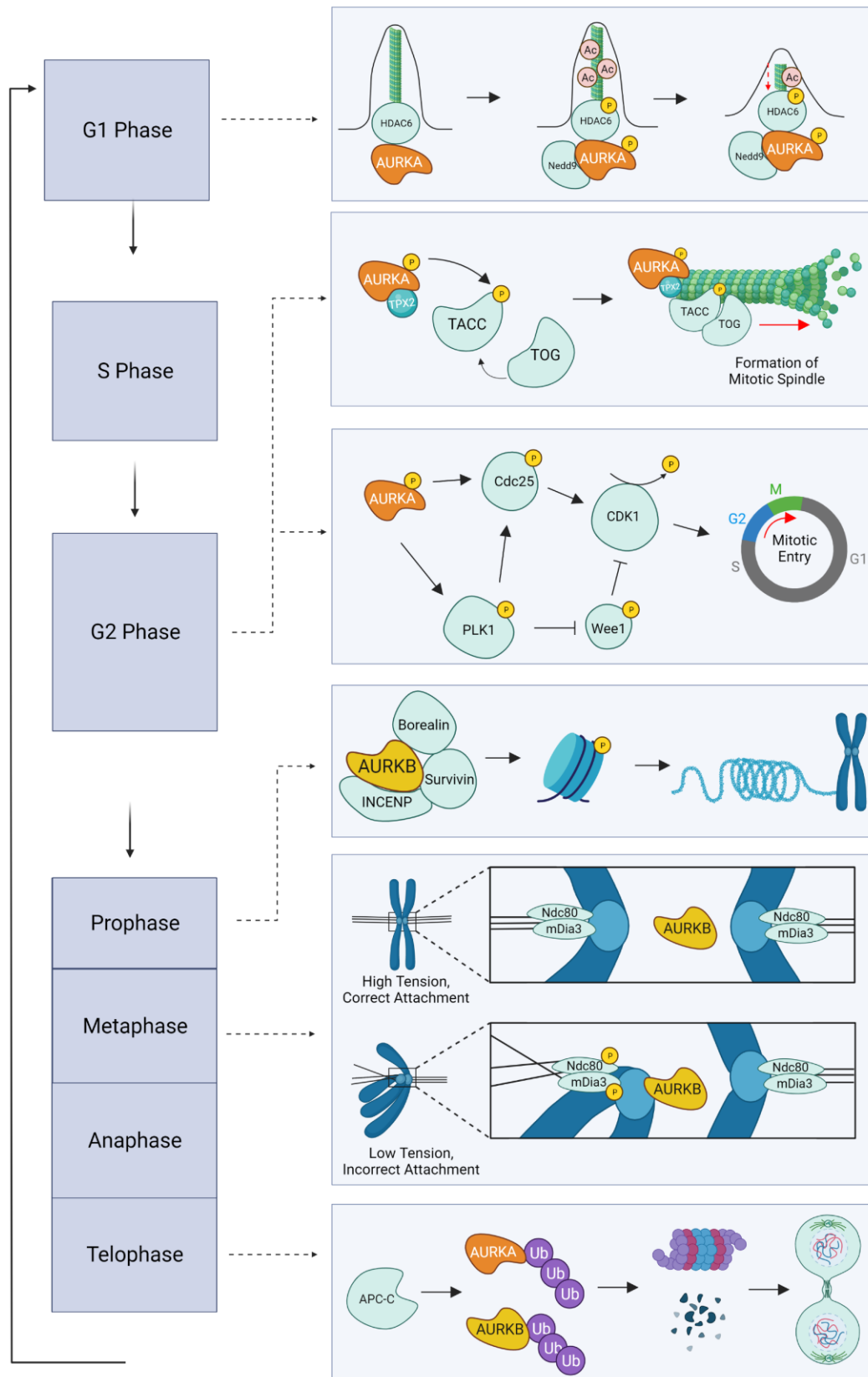
Figure 3. Genomic and transcriptomic alterations in AURKA and AURKB in cancer. A., B. Mutation and amplification data for AURKA (A) and AURKB (B) in HNSCC. Data show reflect information from the MSK-IMPACT Clinical Sequencing Cohort (MSKCC)¹⁷⁵. Data analyzed were accessed through cBioportal^{176,177}. **C.** Analyses were performed with a cohort of 415 HPV- tumor samples from the HNSCC cohort reported in the TCGA PanCancer Atlas¹⁷⁸. The cutoff for high mRNA is z score > 2, for low mRNA is z score < -2. Amplification indicates a number of copies above a limit defined by cBioportal and is often focal; gain indicates a low-level copy number increase, often extending across a significant region of a chromosome.

Figure 4. AURKA upregulation and cancer-promoting activity. A. Mechanisms of upregulation of Aurora kinases include amplification or gain (AURKA and/or TPX2); enhanced transcriptional activation by MYC, STAT5/EGFR, and E2F3 and decreased transcriptional repression by p53; upregulation of AURKA activators (TPX2, NEDD9, etc) and decreased proteolytic degradation. Light pink icon indicates oncogenes, while light blue indicates tumor suppressor genes. Arrow next to the gene names indicates gene up or downregulated in head

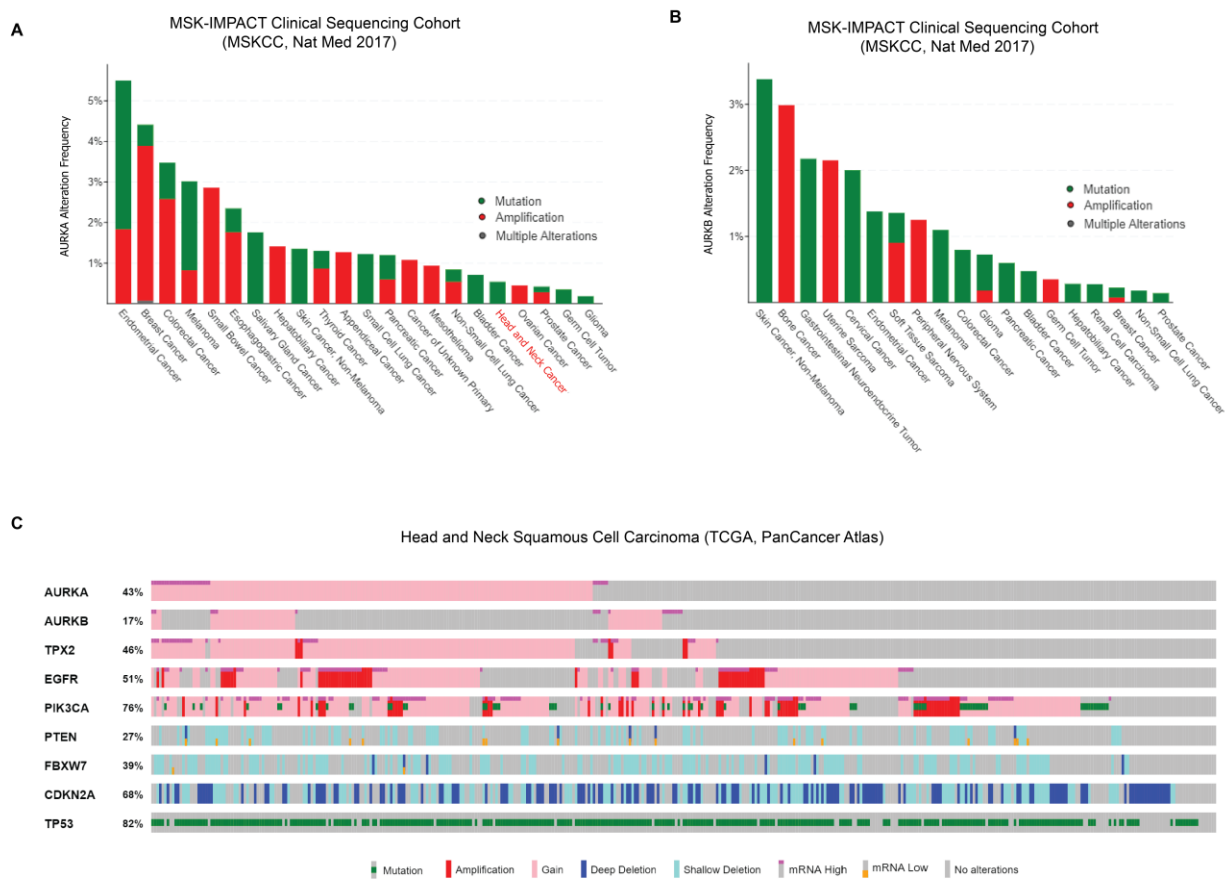
and neck neoplasms. **B.** Cancer promoting activities of upregulated AURKA include induction of p53 degradation or impairment of p53 DNA binding and transactivation functions; induction of signaling by SOX2, NF- κ B, HIF-1b, hnRNPK and MYC; activation of RalA, degradation of BIM, and promotion of aneuploidy. See text for details.



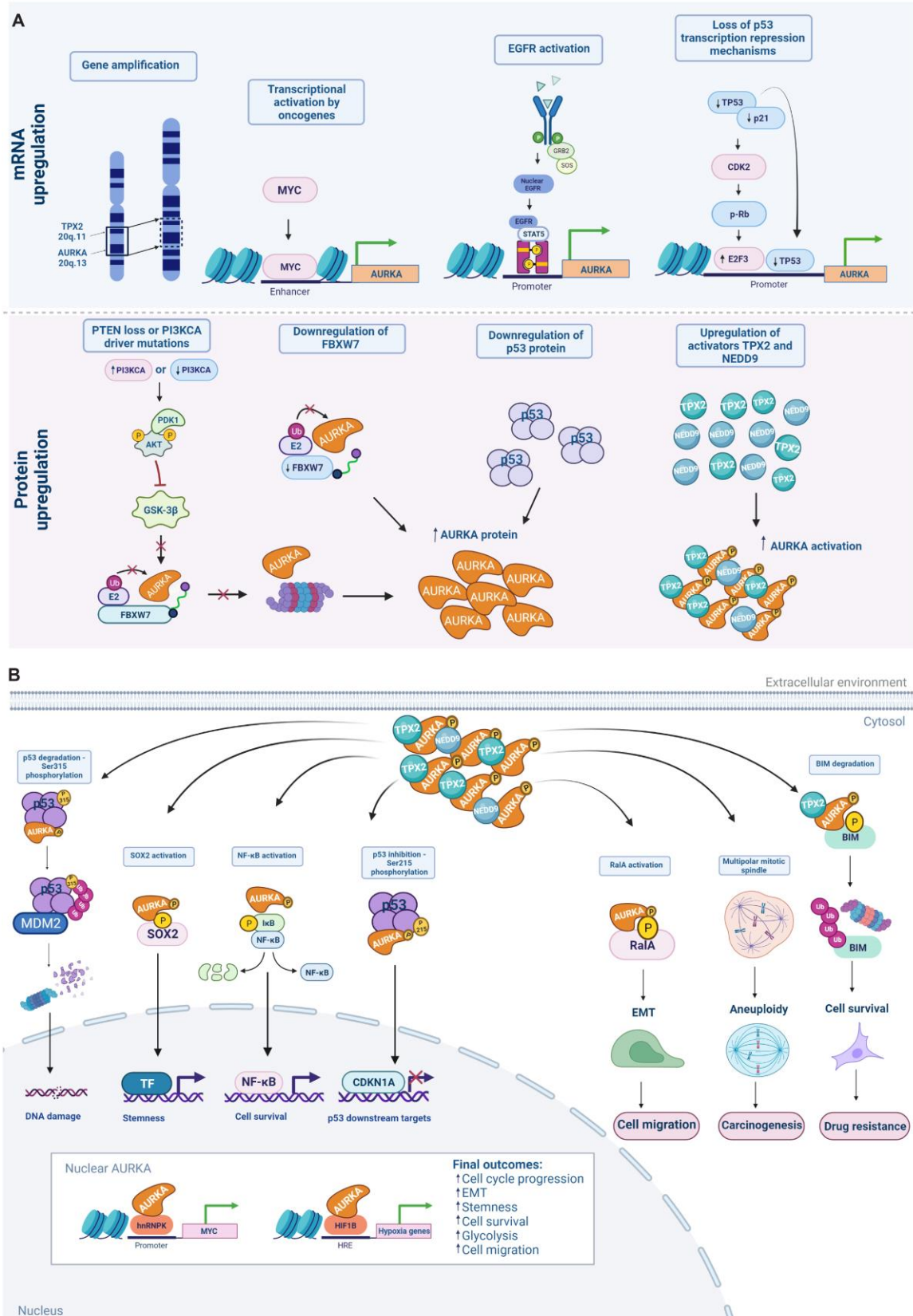
Nguyen, Silva Figure 1



Nguyen, Silva Figure 2



Nguyen, Silva Figure 3



Nguyen, Silva Figure 4