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Aurora Kinase A Inhibition and Paclitaxel as Targeted Combination Therapy for Head and Neck Squamous Cell Carcinoma

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Abstract

Background—Aurora kinase A (AURKA) is **amplified with varying incidence** in multiple human cancers including head and neck squamous cell carcinoma (HNSCC). We investigated whether AURKA is a potential therapeutic target in HNSCC.

Methods—We conducted an immunohistochemical analysis of AURKA expression in paired normal and tumor samples ($n = 63$). HNSCC cells treated with siRNA specific for AURKA were assessed for AURKA mRNA and protein expression levels by RT-PCR and Western blot analysis. Tumor cells treated with siRNA and paclitaxel were assessed for cell proliferation by MTT assay and for cell cycle distribution by flow cytometry.

Results—AURKA expression was higher in tumor than in adjacent normal in most (85%) of the samples analyzed. HNSCC cells and primary tumors revealed high expression levels of AURKA. Most primary tumors also showed high kinase activity of the enzyme. Targeted AURKA inhibition increased the sub-G1 cell fraction, with a concomitant reduction in the G1 cell population, indicating induction of apoptosis and thus markedly suppressed proliferation of HNSCC cells. Combining siRNA-induced AURKA inhibition with 5-10 nM paclitaxel synergistically enhanced apoptosis induction.

Conclusions—AURKA is a potential therapeutic target for HNSCC. Further investigation of small-molecule AURKA inhibitors as therapeutic agents is warranted.

Keywords

HNSCC; AURKA; paclitaxel; combination therapy; anti-proliferation

INTRODUCTION

Approximately 500,000 new cases of HNSCC are diagnosed worldwide each year, 1 including approximately 40,000 in the United States.² HNSCC is the sixth leading cause of cancer-related death worldwide.³ Despite advances in treatment, including the improvement of surgical techniques, the evolution of nonsurgical organ-sparing approaches, and the advent of concomitant chemo-radiotherapy, the overall 5-year disease-specific mortality rate for patients with HNSCC still remains 50%.⁴ The most common cause of death among patients with HNSCC is failed local and regional control. ⁵ The morbidity associated with recurrence at head and neck sites is tremendous. Clearly, better therapeutic approaches for HNSCC and a clearer understanding of HNSCC development and progression at the cellular and molecular levels are needed.

AURKA, a member of the conserved Serine/Threonine protein kinase family represented by the prototypic *Ipl1* kinase in yeast, is an essential mitosis regulatory protein encoded on human chromosome 20q13.2 that induces oncogenic transformation accompanied with centrosome amplification and aneuploidy when over expressed in rodent cells *in vitro* and *in vivo* (6-8). Aurora Kinase-A gene is **amplified** and overexpressed in many human cancers, including colorectal, breast, ovarian, bladder, gastric and pancreatic cancers. (6-9-13) In addition, AURKA overexpression overrides the mitotic spindle checkpoint and promotes resistance to paclitaxel Taxol. (14-15) DNA gain on chromosome 20q is frequently observed in HNSCC (16-17) and associated with node metastasis. (18) One report to date suggested a correlation between AURKA mRNA overexpression and tumor progression and shortened survival in patients with HNSCC. (19)

In the present study, we investigated whether AURKA is a potential therapeutic target in HNSCC. To this end, we evaluated (a) AURKA expression in HNSCC biopsy specimens and cells in vitro, (b) the phenotypic changes in HNSCC cells following small interfering RNA (siRNA)-induced knockdown of AURKA expression, and (c) the synergistic cytotoxic potential of paclitaxel combined with siRNA targeted against AURKA. The rationale for adding paclitaxel was our belief that inhibition of AURKA would **affect activation** of sustainable spindle checkpoints in the treated cells and thus synergistically induce the cytotoxic effects of paclitaxel. Our results suggest that AURKA inhibitors might be effectively utilized as a paclitaxel adjuvant in the systemic HNSCC treatment approaches.

MATERIALS AND METHODS

HNSCC Cell Lines and Materials

Tu138, UMSCC1, Tu167, OSC19, Tu177, and JMAR cell lines were maintained in Dulbecco's modified Eagle medium (DMEM)-F12 high glucose containing 10% fetal bovine serum (FBS) in an atmosphere containing 5% CO₂ at 37°C. NHEK-cells were grown in keratinocyte-SFKM with supplements (serum free keratinocyte medium; Cascade Biologics, Portland, OR). Trypsin-ethylenediaminetetraacetic acid, L-glutamine (200 mM), and penicillin-streptomycin solution were purchased from Invitrogen (Carlsbad, CA). We obtained rabbit polyclonal anti-AURKA and anti-poly (ADP-ribose) polymerase (PARP) antibodies from Cell Signaling Technology (Danvers, MA) for Western blot analyses, antirabbit polyclonal antibody from Bethyl Laboratories (Montgomery, TX) for immunohistochemical analyses, and agarose-tagged anti-AURKA rabbit polyclonal antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) for kinase assays. Myelin basic protein, dithiothreitol, MgCl₂, MnCl₂, propidium iodide, and anti-β-actin antibody were obtained from Sigma (St. Louis, MO).

Immunohistochemical Analysis of Tumor Specimens

All tumor tissue specimens **with** adjacent normal mucosa were obtained from 63 patients at The University of Texas M. D. Anderson Cancer Center who had received a diagnosis of primary HNSCC and undergone surgical resection. We retrieved clinical data from the patients' medical records, and we analyzed all tissue specimens in accordance with a protocol approved by the institutional review board of M. D. Anderson Cancer Center and with the informed consent of all patients whose tissue specimens were used. Briefly, we sectioned the frozen tissue samples, stained them with hematoxylin and eosin, and evaluated them microscopically. We used pathologically confirmed nondysplastic epithelium from the resection margins as a control reference in each case.

Sections were deparaffinized and rehydrated with successive washes of xylene and decreasing concentrations of ethanol in water, steamed in citrate solution to retrieve antigens, and then placed in 5% goat serum to block endogenous peroxidase and protein. Next, we incubated the sections with the primary anti-AURKA antibody or control rabbit immunoglobulin G at a 1:500 dilution in phosphate-buffered saline with Tween at 4°C overnight in a humid chamber. Then, we subjected the sections to secondary antibody staining with horseradish peroxidase-linked streptavidin followed by 3, 3'-diaminobenzidine (Vector Laboratories, Burlingame, CA). Finally, we counterstained the specimens with hematoxylin. Slides containing the specimens were placed under a light microscope to visualize staining and to record digital images of the stained specimens with a polychromatic camera (Leica Microsystems, Inc., Bannockburn, IL). In each case, we compared the tumor specimens with corresponding adjacent normal tissue specimens. An experienced head and neck pathologist (A.E.N.) semiquantitatively evaluated AURKA expression. We scored the intensity of AURKA staining as no detectable expression, weak-to-moderate expression, or strong expression

Protein Extraction, Western Blot Analysis, and Kinase Assay

Tumor lysates were prepared in RIPA buffer and whole-cell extracts in NP40 lysis buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1% NP40 containing protease inhibitors, and sodium orthovanadate). Unless otherwise noted, lysates were resolved and then analyzed by subjecting protein (50 µg per lane) to electrophoresis through 10% sodium dodecyl sulfate (SDS) polyacrylamide gels and then by Western blotting. For PARP cleavage analysis, we loaded 125 µg protein per lane. For the in vitro kinase assay, we incubated cell lysates immunoprecipitated with agarose-tagged anti-AURKA for 4 hours at 4°C were incubated in kinase buffer (20 mM HEPES [pH 7.4], 10 mM MgCl₂, 2 mM MnCl₂, and 1 mM dithiothreitol) containing 20 mM cold ATP and 10 µCi [γ -³²P]ATP and MYELIN BASIC PROTEIN as a substrate. (20-22) Each reaction was performed in a volume of 40 µL at 30°C for 30 minutes. We analyzed the samples by 10% SDS-polyacrylamide gel electrophoresis (PAGE), transferred them to nitrocellulose, and quantified them using a phosphor imager (GE Health Care, Piscataway, NJ).

Transfection of AURKA-Targeted siRNA

We obtained nonspecific scrambled siRNA (control) and siRNA duplexes targeting AURKA from Ambion (Austin, TX). The sense primer sequence was 5'-GGC AAC CAG UGU ACC UCA Utt-3'; the antisense primer sequence was AUG AGG UAC ACU GGU UGC Ctg. We plated HNSCC cells (2×10^5) in antibiotic-free DMEM-F12 medium containing 10% FBS for 16 hours before transfection. Transfections were performed according to the manufacturer's suggested protocol. We harvested the cells after 72 hours and assayed for AURKA knockdown by Western blot analysis.

Cell Proliferation Assays

Sixty hours after transfection with siRNA targeted to AURKA or scrambled (control) siRNA, we replated the cells (5×10^3) in 24-well plates containing paclitaxel or dimethyl sulfoxide (DMSO). Cell proliferation was assayed by the MTT method on days 1-5. The doses of AURKA siRNA (1-2 nM) and paclitaxel (5-10 nM) were based on the results of previous experiments (data not shown). Note that, in those previous experiments, the half maximal paclitaxel inhibitory concentrations (IC_{50}) for Tu138 and UMCC1 cells were 30 nM and 41 nM, respectively.

Cell Cycle Analysis

Sixty hours after cells were transfected with siRNA or scrambled siRNA, we replated cells in 10-cm plates and then incubated the cells with either paclitaxel (10 nM) or DMSO for 48. Next, we collected and analyzed all of the cells in the plates, including cells floating in the medium. Adherent cells were released from the plates by trypsinization and added to the collection tubes. We washed the cells in PBS and fixed them with 5 mL 95% ethanol at 4°C overnight. Next, the cells were centrifuged to remove ethanol, resuspended in PBS containing propidium iodide (50 µg/mL) and RNase (1 mg/mL), and then incubated at 37°C for 30 minutes. Finally, we analyzed the samples by flow cytometry. (BD Biosciences, San Jose, CA).

Real-Time Reverse Transcriptase Polymerase Chain Reaction

To investigate the status of AURKA and its role in HNSCC progression, we compared AURKA expression in HNSCC cell lines (Tu138, UMSCC1, Tu167, Tu177, and OSC19) with AURKA expression in a normal human epithelial keratinocyte (NHEK) line by quantitative real-time polymerase chain reaction (PCR) analysis. We prepared total RNA from cells using TriZol reagent (Invitrogen) according to the manufacturer's instructions. Two micrograms of total RNA was reverse transcribed using Superscript II (Invitrogen) in a 25-µL total reaction volume containing reverse transcriptase (RT) buffer, random hexamers, deoxyribonucleoside triphosphate (dNTP), and RNase inhibitor (Roche Applied Science, Indianapolis, IN). We purchased specific primers for the AURKA gene, including two unlabeled PCR primers and one FAM dye-labeled TaqMan minor groove binder probe (Applied Biosystems, Foster City, CA) as Assays-on-Demand gene expression products. Real-time PCRs were performed on an ABI Prism 7900HT. We performed each reaction in a 25-µL total reaction volume containing 1 µL cDNA (1:10 dilution) obtained from the RT reaction, 12.5 µL TaqMan Universal PCR Master Mix without AmpErase uracil-N-glycosylase, and 1.25 µL specific primers for each gene. We used 18S primers (1:500) as a control and serial dilutions of the standard templates for parallel amplifications. We calculated the threshold cycles using ABI Prism 7900HT SDS software (Applied Biosystems). We then plotted standard curves with threshold cycles on one axis and log template quantities on the other. We determined the quantities of the samples from the standard curves. In each PCR sample, AURKA mRNA levels were normalized to those of 18S.

RESULTS

AURKA Expression in HNSCC Cell Lines

AURKA expression was markedly (approximately 10- to 20-fold) higher in all HNSCC cell lines tested than in NHEK (Figure 1A). Furthermore, AURKA mRNA expression varied among HNSCC cell lines, suggesting that the AURKA gene was being regulated at the transcriptional level. Similarly, on Western blot analysis, AURKA protein expression was markedly higher in the HNSCC cell lines than in NHEK (Figure 1B) and varied among cell

lines, suggesting possible involvement of posttranscriptional and posttranslational regulatory mechanisms.

Immunohistochemical Analysis of AURKA Protein Expression

To determine whether AURKA expression was elevated in HNSCC biopsy specimens, we conducted an immunohistochemical analysis of HNSCC sections from 63 patients (Figure 2). Tumors were strongly positive for AURKA protein in 65% ($n = 41$) of cases, weakly to moderately positive in 19% ($n = 12$), and negative in 15% ($n = 10$). Adjacent normal tissue showed significantly less positivity for AURKA protein. In general, positive nuclear staining was seen only in the suprabasal cells (differentiated cells) in nondysplastic epithelium. In contrast, positive nuclear staining was seen basal and suprabasal cells in dysplastic and carcinoma epithelium.

AURKA Expression in Tumor Tissues

We assessed AURKA protein expression in eight pairs of tumor tissue and adjacent normal tissue by Western blot analysis (Figure 3A). AURKA expression was markedly higher in the tumor tissues than in the normal tissues in five cases and only slightly higher than normal in the other three cases. In our assessment of AURKA activity, the kinase activity in six cases was markedly higher in tumor tissues than in normal tissues but unaltered in the rest (Figure 3B). Thus, in five cases, there was a direct relationship between the levels of AURKA kinase activity and AURKA protein expression.

AURKA Suppression and Its Inhibitory Effect on HNSCC Cell Proliferation

To determine whether AURKA is a therapeutic target in HNSCC, we employed the siRNA knockdown method to deplete the expression of AURKA in cultured HNSCC cells. Because Tu138 and UMSCC1 cells express markedly higher than NHEK levels of AURKA, we transfected scrambled AURKA siRNA into these two cell lines to see the effects of AURKA silencing, which were verified by SDS-PAGE analysis. Our Western blot results showed that AURKA siRNA at a 75 nM concentration was able to knock down AURKA protein levels by 80%-90% (Figure 4A, upper panel). AURKA siRNA did not induce nonspecific inhibition of gene expression as shown by unaltered expression of β -actin (Figure 4A, lower panel). We also investigated the effects of AURKA siRNA on in vitro growth of HNSCC cells. We analyzed cell proliferation by MTT assay for 3-5 days our results showed that suppression of cell proliferation correlated with the concentration of AURKA siRNA in Tu138 cells (Figure 4B). AURKA siRNA at a 1 nM concentration did not have any effect on growth, whereas an AURKA siRNA concentration of 10nM suppressed tumor cell growth by approximately 50%. Similar dose dependent inhibition by AURKA siRNA was observed in UMSCC1 (data not shown). Almost complete inhibition of cell proliferation was achieved at an AURKA siRNA concentration of 75 nM, which can effectively knock down AURKA protein levels (Figure 4C). Our results suggest that AURKA plays an important role in cell proliferation and that inhibition of AURKA might be a therapeutic target in HNSCC.

Cytotoxic Effects of AURKA siRNA plus Paclitaxel

By stabilizing the microtubules, paclitaxel impairs the spindle function and segregation of chromosomes during mitosis. Because AURKA is required for proper spindle assembly, (23, 24) we hypothesized that inhibition of AURKA may synergistically induce the effect of paclitaxel. We chose a siRNA concentration that would have a minimal effect on cell proliferation. From our experiments, we knew that 1-2 nM AURKA siRNA had minimal effects on HNSCC cell proliferation and that the IC_{50} values of paclitaxel in Tu138 and UMSCC1 cells were 30 nM and 41 nM, respectively (data not shown). One of our objectives for the combination therapy experiment was to utilize reduced concentrations of

chemotherapeutic agents that would elicit less-toxic therapeutic effects. We therefore chose 5-10 nM paclitaxel for our investigation. In the MTT assay, we found that at 5-10 nM, paclitaxel had very little effect on HNSCC cell proliferation when combined with scrambled siRNA (Figure 5A). However, combining AURKA siRNA with identical doses of paclitaxel resulted in marked inhibition of proliferation (Figure 5A-C). Thus, we were able to enhance the cytotoxic effects of paclitaxel by inhibiting AURKA activity in HNSCC.

Cell Cycle Disruption and Apoptosis Induction Caused by AURKA Knockdown

To determine whether tumor cell proliferation was inhibited by a combination of siRNA-induced cell cycle disruption and apoptosis induction, changes in DNA content were assayed in cells treated with AURKA siRNA with or without paclitaxel. As shown in Figure 6A, control siRNA alone or in combination with 10 nM paclitaxel did not alter cell cycle distribution. In contrast, AURKA siRNA alone caused a marked decrease in the fraction of cells in the G1 phase of the cell cycle (from 57% to 35%) and a concomitant increase in the sub-G1 or apoptotic cell fraction (from 5% to 30%). AURKA siRNA combined with paclitaxel caused a similar decrease in the G1 fraction (from 54% to 25%) and a similar increase in the sub-G1 population (from 7% to 45%) (Figure 6B). These changes suggested apoptotic cell, a hypothesis we confirmed by Western blot analysis of PARP cleavage in proteins from cells that had undergone both AURKA inhibition and paclitaxel treatment. We found that scrambled siRNA did not induce PARP cleavage but that AURKA siRNA alone or in combination with 10 nM paclitaxel induced marked PARP cleavage (Figure 6C).

DISCUSSION

HNSCC is the sixth leading cause of cancer death in United States. In addition to high mortality rates, there is tremendous morbidity associated with the recurrence of disease in head and neck sites. Therefore, the discovery of new targets is critically important, for both prevention and treatment of this disease. AURKA mRNA expression was 10-30 folds more in all HNSCC cell lines compare to NHEK. In this study we have shown that HNSCC cell line expresses 6-15 folds more AURKA protein than NHEK. Similarly AURKA kinase activity of the tumor samples was ranging from 2.5 to 14 folds. Immunohistochemical analyses showed strong AURKA expression in most (65%) of the primary tumor samples and weak to moderate expression among a notable minority (19%). To our knowledge, this is the first comprehensive analysis of AURKA protein expression in a large number of HNSCC specimens to be reported.

Given the established role of anomalous AURKA expression in aberrant mitosis, one might expect to see a correlation between AURKA overexpression and more aggressive clinical outcomes in HNSCC as observed in several cancer types. (11, 13, 25-27) Indeed, a recent study assessing AURKA mRNA expression in primary HNSCCs (19) found a strong correlation between the overexpression of AURKA mRNA and tumor progression, metastasis, and shortened overall and disease-free survival. Our results corroborated those findings by demonstrating that AURKA expression and activity are markedly elevated in most HNSCCs. These findings provide compelling evidence that AURKA is an attractive target for HNSCC treatment.

AURKA knockdown inhibited HNSCC cells proliferation in vitro, markedly reducing the proportion of G1- cells and increasing the proportion of sub-G1 (and ultimately apoptotic) cells. These findings echo recent studies in pancreatic cancer by Hata et al. (28) and Rojónala et al., (29) who observed similar AURKA inhibition by treatment with siRNA and antisense molecules. Our results also show that inhibiting AURKA markedly enhances the cytotoxicity of paclitaxel. Together, these findings make a strong case for targeting AURKA

in HNSCC since doing so would not only inhibit the proliferation of HNSCC cells but also sensitize them to chemotherapy.

As expected from its documented role in mitosis, AURKA is essential for bipolar spindle assembly and proliferation of somatic cells and thus a good target for halting cell growth and inducing apoptosis. It is conceivable that selective inhibition of AURKA results in activation of the spindle assembly checkpoint and prolonged mitotic arrest, leading to apoptosis, in much the same way as microtubule toxins or kinesin spindle protein inhibitors.³⁰ This effect is likely to be exacerbated by the synergistic cytotoxic activity of paclitaxel, which stabilizes microtubules by binding tubulin and interferes with microtubule disassembly, causing cells to accumulate at the transition between metaphase and anaphase and ultimately causing apoptotic death. Such robust antiproliferative effect of AURKA inhibition in combination with paclitaxel makes this an attractive therapeutic strategy for HNSCC. It is noteworthy in this context that a selective small-molecule inhibitor of AURKA has recently been shown to inhibit growth of human tumor xenografts and in mice.³¹ It would be interesting to investigate the effect of such selective AURKA inhibitors alone and combined with paclitaxel as therapeutic agents in HNSCC and elucidate the mechanism by which these treatments promote initiation of apoptosis.

In conclusion, our results suggest that many HNSCCs significantly overexpress AURKA and that AURKA inhibition alone or combined with paclitaxel may be a potentially useful and effective therapeutic approach to treating HNSCC. Further investigations into small-molecule inhibitors of AURKA either alone or combined with chemotherapeutic agents are warranted.

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FIGURE 1.

Expression of aurora kinase A (AURKA) in head and neck squamous cell carcinoma (HNSCC) cell lines and normal human epithelial keratinocytes (NHEK) (control). (A) Real-time polymerase chain reaction analysis of AURKA total mRNA revealed overexpression in all HNSCC cell lines (upper panel) and fold induction when compared with NHEK (lower panel) (B) Western blot analysis showed overexpression of AURKA protein in HNSCC cells. Lower bar diagram shows AURKA expression over β -actin.

FIGURE 2.

Aurora kinase A (AURKA) expression in head and neck squamous cell carcinoma (HNSCC) and adjacent normal tissues as detected immunohistochemically. Nuclear staining for AURKA was weak or nonexistent in normal tissue (A and C) but strong in tumor tissue (B and D).

FIGURE 3.

Overexpression of aurora kinase A (AURKA) in head and neck squamous cell carcinoma (HNSCC) biopsy specimens. (A) Western blot analysis of AURKA expression in paired frozen normal (N) and tumor (T) tissues from 8 representative patients with HNSCC (upper panels). Overexpression of AURKA is seen in most tumors ($n = 5$). Lower panel shows AURKA expression over β -actin in normal versus tumor tissues. (B) Activation of AURKA in human HNSCC. In vitro kinase assay of AURKA from immunoprecipitates of 8 paired normal (N) and tumor (T) specimen's shows increased AURKA activity in tumors (upper panels) Myelin basic protein (MBP) was used as an exogenous substrate. The lower panel shows relative AURKA activity in normal versus tumor tissues.

FIGURE 4.

Specific siRNA-induced inhibition of aurora kinase A (AURKA) expression. (A) AURKA expression was assessed 48 hours after transfection of siRNA (75 nM) directed against AURKA or of scrambled control (Con) siRNA in Tu138 and UMSCC1 tumor cell lines. Western blot analysis shows marked inhibition of AURKA in both cell lines. (B) AURKA siRNA inhibited Tu138 cell proliferation in a dose-dependent manner. (■ Con siRNA, ▲ 1nM, ▼ 2nM, ◆ 5nM, ● 10nM AURKA siRNA. (C) AURKA siRNA at a concentration of 75 nM completely inhibited tumor cell (Tu138 and UMSCC1) proliferation.

FIGURE 5.

Synergistic cytotoxic effect of aurora kinase A (AURKA) siRNA and paclitaxel in head and neck squamous cell carcinoma tumor cells. We quantitated survival of Tu138 (A), UMSCC1 (B), and JMAR (C) tumor cells by MTT assay after their transfection with 1 nM of either AURKA or scrambled control (con) siRNA and incubation for 1-4 days in the presence or absence of 5 or 10 nM paclitaxel (Pac). The assays showed significant synergy between AURKA inhibition and paclitaxel-induced cytotoxicity. All assays were performed 3 times

FIGURE 6.

Aurora kinase A (AURKA) siRNA regulates cell cycle transition. (A) **Tu138** cells transfected with control (Con) or AURKA siRNA (**10nM**) were collected after 48 hours of treatment with paclitaxel (Pac, **10nM**) or dimethyl sulfoxide (control) and assayed for their DNA content by flow cytometry ($n = 3$). Representative results are shown in table (B). (C) After transfection with control (Con) or AURKA siRNA (**10nM**) treatment with or without paclitaxel (Pac, **10nM**), lysates were collected and subjected to Western blot analysis with anti-PARP or anti- β -actin (loading control) antibodies. PARP cleavage was enhanced (lower panel).