Aurora Kinase A Messenger RNA Overexpression Is Correlated with Tumor Progression and Shortened Survival in Head and Neck Squamous Cell Carcinoma

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Abstract

Purpose: Aurora kinase A (AURKA/STK15/BTAK) encodes a serine/threonine kinase associated with chromosomal distribution and its up-regulation induces chromosomal instability, thereby leading to aneuploidy and cell transformation in several types of cancer. In this study, we investigated the role of AURKA in head and neck squamous cell carcinoma (HNSCC).

Experimental Design: The mRNA expression levels of AURKA were compared in tumor tissues of 66 HNSCC patients with those in corresponding normal squamous epithelium by real-time quantitative reverse transcriptase-PCR. In addition, the association between AURKA mRNA and protein expression, centrosome abnormalities, and aneuploidy was studied in a subset of cases ($n = 34$). All molecular variables were correlated to histomorphologic findings and clinical follow-up data of the patients.

Results: AURKA mRNA up-regulation was significantly associated with tumor stage and the occurrence of regional lymph node, as well as distant metastasis ($P < 0.0001$ for all). Similarly, a correlation was found for protein expression and the occurrence of regional lymph node ($P = 0.0183$) and distant metastasis ($P = 0.03$). The mRNA was positively associated with protein expression ($P = 0.003$) and centrosome abnormalities ($P = 0.03$). Cox regression analysis revealed that AURKA mRNA up-regulation correlated with disease-free survival of the patients ($P = 0.03$) as well as shorter overall survival ($P < 0.001$).

Conclusions: We conclude that the up-regulation of AURKA mRNA may play a critical role in the tumor progression of HNSCC and provides useful information as a prognostic factor for HNSCC patients.

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide and survival rates are not improving (1). Therapeutic decisions are usually based on clinical and histopathologic variables like tumor-node-metastasis stage and tumor grading, which, however, often fail to predict patient outcome. Therefore, there is a need to better understand HNSCC development and progression on the molecular level. This should lead to an improved stratification between higher-risk and lower-risk patients, which can be treated in a more selective and individualized manner.

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DNA gains on chromosome 20q are recurrent findings in HNSCC (2, 3) and are associated with lymph node metastasis, as recently shown by array-based comparative genomic hybridization (4). Aurora kinase A (AURKA/BTAK/AIK1/STK15) maps close to the critical region of this DNA gain and is localized on 20q13.2 (5). AURKA is a member of the Aurora/Ipl1p family of cell cycle–regulating serine/threonine kinases and is localized at interphase and mitotic centrosomes and at the spindle poles where it regulates proper chromosome segregation and cytokinesis (6). Recent studies have shown that the ectopic expression of Aurora-A in mouse NIH 3T3 cells and Rat-1 fibroblasts causes centrosome amplification and transformation *in vitro* as well as tumorigenesis *in vivo* (7, 8). Furthermore, the up-regulation of AURKA leads to abnormal centrosome numbers and the induction of aneuploidy (7, 9), which is a very frequent event in HNSCC, found in up to 90% (10). A correlation between the up-regulation of AURKA and clinical aggressiveness has been described for several cancers (11–19).

In the current study, we determined AURKA mRNA expression by real-time quantitative reverse transcriptase-PCR in 66 primary HNSCC and associated nonneoplastic squamous epithelium. In addition, we examined whether there is an association of AURKA mRNA overexpression with abnormal protein expression, centrosome abnormalities, and chromosomal aneusomy by immunostaining and fluorescence *in situ*

hybridization experiments in HNSCC. All molecular variables were then correlated to histomorphologic findings and clinical follow-up data of the patients to explore the role of AURKA as a prognostic factor in HNSCC.

Materials and Methods

Patient selection and tissue samples. All tissue samples were obtained from patients who were diagnosed with primary HNSCC and underwent a surgical resection in the Department of Head and Neck Surgery, Klinikum Rechts der Isar, Technical University Munich. In total, 66 primary, formalin-fixed and paraffin-embedded HNSCC, 14 oral squamous cell carcinomas, 28 pharyngeal squamous cell carcinomas, and 24 laryngeal squamous cell carcinomas were obtained from the archives of the Institute of Pathology, Technical University Munich. For all tumors, histopathologic and clinical follow-up data were available from follow-up examinations (median follow-up period of 60 months). HNSCC were classified according to the tumor-node-metastasis system and staged according to criteria of the International Union against Cancer (20). Nonneoplastic squamous epithelium from the resection margins was used as reference. Clinical data from the patients were retrieved from medical records. These data then were used to analyze the relation between the molecular and clinical variables such as site of primary tumor, tumor and nodal classification, stage, histologic grade, distant metastasis, disease-free survival, and overall survival. Detailed patient characteristics and histomorphologic features are shown in Table 1. The study was approved by the Medical Ethics Committee of the Technical University of Munich.

RNA extraction and cDNA synthesis. Tissue preparation, microdissection of pure tumor cell populations, and RNA extraction from formalin-fixed tissues were carried out as previously described (21–23). Reverse transcription was carried out with 250 ng of random hexamers (Roche, Basel, Switzerland) and 200 units of SuperScript II reverse transcriptase (Invitrogen Life Technologies, Karlsruhe, Germany) in a final volume of 20 μL, following the directions of the manufacturer.

Real-time quantitative reverse transcriptase-PCR. Target cDNA sequences were amplified by quantitative PCR using a fluorescence-based real-time detection method [ABI Prism 7700 Sequence Detection System (TaqMan), Applied Biosystems, Foster City, CA] as previously described (21–23). Primers and probes for AURKA (24) and the reference gene TATA-box binding protein (*TBP*; refs. 21, 25) were intron-spanning and used according to standard procedures. Sequences were as follows: AURKA, forward 5′-gctggagagcttaaaattgcag, reverse 5′-ttttgtaggtctcttggtatgtg and Probe 5′-ctccatcttccaggaggaccactctctg; *TBP*, forward 5′-gcccgaaacgccgaatat, reverse 5′-ccgtggttcgtggctctct; and probe, 5′-atcccaagcggtttgctgcgg. In brief, the 25-μL PCR reaction mixture contained 600 nmol/L of each primer and probe; 200 nmol/L each of dATP, dCTP, and dGTP; 400 μmol/L dUTP; 5.5 mmol/L $MgCl_2$; and 1× TaqMan buffer A containing a reference dye (all reagents were supplied by Applied Biosystems, Weiterstadt, Germany). PCR conditions were 50°C for 10 seconds and 95°C for 10 minutes followed by 42 cycles at 95°C for 15 seconds and 60°C for 1 minute. For each sample, quantitative reverse transcriptase-PCR data are expressed as a normalized value of AURKA mRNA expression with respect to the mRNA expression of the internal reference gene *TBP*.

Tissue microarrays and* in situ *variable analyses (AURKA protein expression, centrosome abnormalities, and chromosomal aneusomy). For *in situ* variable analyses, a preexisting HNSCC tissue microarray was used, containing tumor tissues and nonneoplastic squamous epithelium from a subset of patients (34 from 66) already analyzed for AURKA mRNA expression. The tissue microarray was sectioned, placed on coated glass slides, and deparaffinized for the subsequent procedures. The thickness of the sections depended on the respective method (7 μm for immunohistochemistry and 15 μm for indirect immunofluorescence and fluorescence *in situ* hybridization).

Table 1. Clinicopathologic characteristics of the patients with HNSCC and AURKA mRNA expression

Patient characteristics	*n*	AURKA (mRNA)	SD	Min	Max	*P*
Sex						
Male	59	3.09	1.86	0.56	8.80	0.56
Female	7	2.67	0.91	1.20	3.80	
All tumor samples	66	3.05	1.78	0.56	8.8	
Tumor localization						
Oral cavity	14	3.00	1.39	1.26	5.78	0.74
Oropharynx	23	2.81	1.48	1.10	6.78	
Hypopharynx	5	3.75	1.63	1.85	5.38	
Larynx	24	3.16	2.27	0.56	8.8	
T category						
T_{is}	4	1.23	0.20	0.98	1.45	<0.0001
T_1	20	2.05	1.50	0.56	6.48	
T_2	26	3.09	1.28	1.20	6.12	
T_3	10	4.53	2.09	2.35	8.80	
T_4	6	4.94	1.13	3.34	6.25	
Lymph node metastasis						
N_0	37	2.20	1.43	0.56	6.78	<0.0001
N_+	29	4.13	1.60	2.09	8.80	
Distant metastasis						
M_0	61	2.80	1.60	0.56	8.80	<0.0001
M_1	5	6.13	0.55	5.38	6.78	
Histologic grade						
1	7	2.22	1.49	0.56	5.17	0.0121
2	31	2.76	1.51	1.06	6.48	
3	24	3.96	1.91	1.25	8.80	
Tumor stage						
I	15	1.35	0.35	0.56	2.10	<0.0001
II	9	1.96	0.47	1.20	2.64	
III	15	3.48	1.06	2.10	5.79	
IV	23	4.61	1.66	2.09	8.80	

NOTE: HNSCC were staged according to the criteria of the International Union Against Cancer (20).

Immunohistochemistry for evaluation of AURKA protein expression. Antigen retrieval was done in a pressure cooker with Tris-EDTA buffer (pH 9.0). All subsequent stainings were done on a Dako Autostainer (DakoCytomation, Glostrup, Denmark). Incubation time with primary antibody (1:50; Aurora kinase 2, clone JLM28, mouse; Novocastra, Newcastle-upon-Tyne, United Kingdom) was 60 minutes, followed by secondary antibody and detection using the ChemMate Alkaline Phosphatase/RED System (DakoCytomation). According to previously published criteria, cytoplasmatic (14, 18, 26) and/or nuclear (11) immunoreactivity of AURKA was evaluated in three tumor areas of each case. Immunoreactivity was scored into four groups according to the percentage and intensity of cytoplasmatic and/or nuclear staining of the positively stained tumor cells. Specimens with >30% of cells stained were scored as strongly positive (3+), those with 10% to 30% of cells stained were scored as moderately positive (2+), and those with <10% cells stained were scored as weakly positive (1+). Specimens with no staining were scored as negative.

Indirect immunofluorescence for evaluation of centrosome abnormalities. Numerical and structural centrosome abnormalities were analyzed as recently described (27, 28). Briefly, the sections were incubated with a primary mouse anti–γ-tubulin antibody (1:100; clone GTU-88, Sigma, Taufkirchen, Germany), followed by incubation with a FITC-labeled goat anti-mouse immunoglobulin G (1:200; Dianova, Hamburg, Germany). Finally, nuclei were counterstained with 4′,6-diamidino-2-phenylindole-2-hydrochloride. For data collection, three microscopic areas were selected and three-dimensional image stacks with a distance of 0.5 μm in the *z* axis were acquired using a confocal laser scanning microscope (Zeiss LSM 510 Meta, PlanNeofluar 63×/1.2

numerical aperture water objective, Carl Zeiss Jena, Jena, Germany). Image stacks were converted into a projection image of all recorded slices and were analyzed semiautomatically with respect to the number of cells, size, and number of γ-tubulin signals as previously published (27). Inclusion of controls in the form of nonneoplastic squamous epithelium and lymphocytes allowed the identification of structural and numerical abnormalities in centrosomes in the cancer tissue specimens. Centrosomes were considered structurally abnormal when they were at least twice the size of those seen in control cells. More than two centrosomes in number were recorded as representing numerical abnormality in the specimens analyzed. A case was classified as abnormal for centrosomes if there were numerical and/or structural abnormalities present. The total number of analyzed cells was between 100 and 150 cells per case.

Fluorescence* in situ *hybridization for evaluation of chromosomal aneusomy. Tissue pretreatment and fluorescence *in situ* hybridization were done with α-satellite repeat sequence DNA probes for chromosomes 9, 15, and 20 (Chrombios GmbH, Raubling, Germany) based on a protocol previously published by our group (22, 29). The selection of chromosomes was based on a recent publication describing chromosomal instability in oral squamous cell carcinoma (30). Fluorescence images of fluorescence *in situ* hybridization–labeled nuclei from tissue cells were acquired with a Zeiss Axioplan2 imaging microscope (Carl Zeiss Jena) equipped with a PlanApochromat 63×/1.4 numerical aperture oil objective lens and appropriate filter settings. For signal evaluation, optical sectioning through the specimen was done by using the recently developed ApoTome (Carl Zeiss Jena), which was implemented into the microscopic setup. To ensure a representative sample, the hybridization signals for chromosomes 9, 15, and 20 were counted in 100 to 150 interphase nuclei from at least three separate areas of the tumor selected for well-preserved nuclear morphology. Only fluorescence signals on complete nuclei were numbered whereas truncated cells were excluded from the count. Aneusomy was defined in the present study by the presence of at least 25% of nuclei having a number of centromeric signals for at least one chromosome different from two signals corresponding to disomy. The threshold value of 25% was in keeping with that of previous publications (28, 31).

Statistical analysis. AURKA expression levels (mRNA and protein), centrosome abnormalities, and chromosomal aneusomy were compared between various groups by ANOVA and in two class cases by *t* test. Survival analysis was done for all patients, except those with carcinoma *in situ* ($n = 4$). Disease-free survival was censored for patients who died without active disease. Overall survival was calculated from the date of surgical resection until the date of death or last follow-up. The significance of AURKA mRNA for predicting disease-free survival or the overall survival time of the patients was determined by stepwise Cox regression analysis (The SAS System, SAS Institute, Inc., Cary, NC). The cutpoints for AURKA mRNA (1.5, 4.1) were chosen in the model 25%, 50%, and 25% of the patients and the cutpoint 2.7 for disease-free survival in the model 50%, 50% to show the results in Kaplan-Meier survival estimates. The significance was set at 95% level.

Results

Patient and tumor characteristics. Demographic details on the 66 patients included in the study and tumor stage are shown in Table 1. The number of cases, mean, SD, and minimum and maximum of AURKA mRNA are given for the different defined classes. The *P* values show the results for the differences of the means of these classes.

AURKA mRNA and protein expression and clinicopathologic characteristics. Reproducible quantitative reverse transcriptase-PCR results were obtained in all samples. The overall frequency and results from univariate statistical analyses are summarized in Table 1. The mean AURKA mRNA expression was 3.05 ± 1.78 (range, 0.56-8.8). AURKA mRNA up-regulation was strongly correlated with tumor and nodal classification, tumor stage, as well as distant metastasis ($P < 0.0001$; Fig. 1). A lower correlation was found for histologic tumor grade ($P = 0.0121$). No statistically significant correlation was found for sex of the patient ($P = 0.56$) and for the site of primary tumor ($P = 0.74$).

The immunohistochemical staining pattern for AURKA was predominantly cytoplasmatic. All tumor samples among the 34 patients were acceptable for analysis. We scored the AURKA immunohistochemical expression as follows: 6 tumors were 3+; 16 tumors were 2+; 9 tumors were 1+; and 3 tumors were negative. Representative staining patterns by AURKA expression level are shown in Fig. 2A and B. AURKA protein expression was significantly associated with the occurrence of regional lymph node ($P = 0.0183$) and distant ($P = 0.03$) metastasis. A trend towards a correlation between tumor stage and protein expression was found ($P = 0.05$).

Centrosome abnormalities. From 34 tumor samples, 29 were analyzable for γ-tubulin. As compared with normal tissues, markedly larger and disoriented centrosomes were observed in HNSCC (Fig. 2C). Moreover, an elevated number of centrosomes was detected (Fig. 2D). As described in Materials and Methods, centrosome abnormalities were detected in 18 samples whereas the remaining tumor samples did not show detectable changes.

Chromosomal aneusomy. From 34 tumor samples, 32 were analyzable for chromosomes 9, 15, and 20. More than two copies for chromosome 9 were found in 21 cases, for

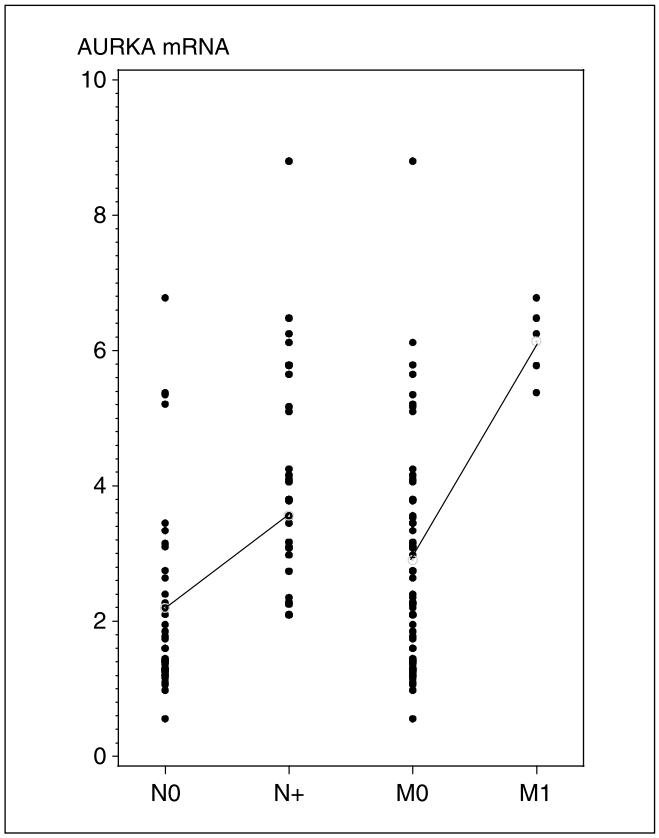

Fig. 1. AURKA mRNA expression in primary HNSCC according to the presence of regional lymph node (pN_0 versus pN_+) or distant metastasis (M_0 versus M1). AURKA mRNA expression values in metastasizing tumors are significantly higher than those in nonmetastasizing tumors ($P < 0.0001$). Mean values of the classes are connected by lines.

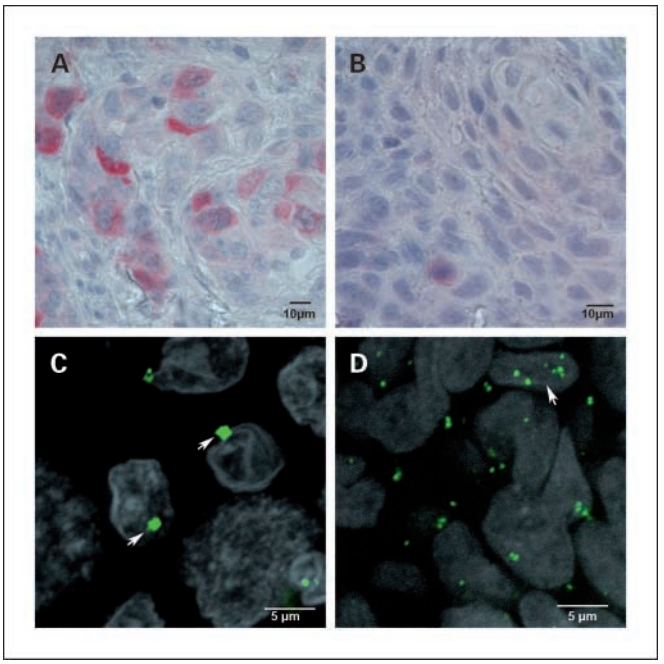

Fig. 2. Immunohistochemical staining for AURKA in HNSCC (*A* and *B*). Representative cases for strongly positive staining with predominant cytoplasmatic staining pattern (*A*) and weakly positive staining (*B*). Immunostained centrosomes (*C* and *D*) detected with an anti –γ-tubulin antibody in HNSCC (*C* and *D*). Representative cases with structural and numerical centrosome aberrations. Note the markedly enlarged (*C, arrows*) and supernumerary centrosomes (*D, arrow*).

chromosome 15 in 28 cases, and for chromosome 20 in 22 cases. Each case showed more than two copies for at least one of the chromosomes investigated. Thus, all samples analyzed showed chromosomal aneusomy.

Relationship between AURKA mRNA expression and protein expression, centrosome abnormalities, and chromosomal aneusomy. There was a correlation between AURKA mRNA and protein expression (P = 0.003). Moreover, we found a correlation between mRNA expression and centrosome abnormalities (P = 0.03). No correlation could be found for AURKA mRNA or protein expression and the presence of chromosomal aneusomy.

Association between disease-free survival, overall survival, and AURKA mRNA expression, protein expression, centrosome abnormalities, and chromosomal aneusomy. The mean overall survival was 53.0 ± 26.7 months with a median follow-up period of 60 months. The follow-up time of the patients ranged from 12 to 84 months. Twenty-six patients died due to this cancer (range, 5-67 months). To test the significance of AURKA mRNA and clinical data for predicting the overall survival time of the patients, stepwise Cox regression analysis was applied. Except for the tumor grading, all available clinicopathologic variables and AURKA mRNA were univariately significant. Stage and AURKA mRNA had comparable χ^2 values with $P < 0.001$. Due to the high correlation of the variables, no second variable could be selected for multiple regressions. In Fig. 3A, three Kaplan-Meier survival curves for AURKA mRNA are plotted to show the result. The cutpoints for AURKA mRNA (1.5, 4.1) were chosen in the model 25%, 50%, and 25% of the patients. AURKA mRNA up-regulation correlated with shorter overall survival of the patients (third curve). Fourteen patients had a recurrence within 3 to 56 months. Two Kaplan-Meier disease-free survival estimates are shown in Fig. 3B with a cutpoint at 2.7 of AURKA mRNA, which are significantly different for P = 0.03. High expression levels of AURKA mRNA were also associated with shorter disease-free survival. There was no correlation between disease-free survival, overall survival, and AURKA protein expression, centrosome abnormalities, or chromosomal aneusomy.

Discussion

In this report, we described the first systematic survey of AURKA expression (mRNA and protein) in head and neck squamous cell cancer (HNSCC). The results showed that mRNA and protein up-regulation of AURKA frequently occur in HNSCC and contribute to a poor prognosis. In particular, AURKA mRNA overexpression is strongly correlated with tumor progression, a metastatic phenotype, and shortened disease-free survival and overall survival. Moreover, we found that AURKA mRNA overexpression is correlated with elevated protein expression and is associated with centrosome abnormalities in HNSCC. Our findings suggest that AURKA up-regulation is a common abnormality in HNSCC and may play an important role in its progression.

Recent studies have described a correlation between the up-regulation of AURKA and tumor progression and clinical

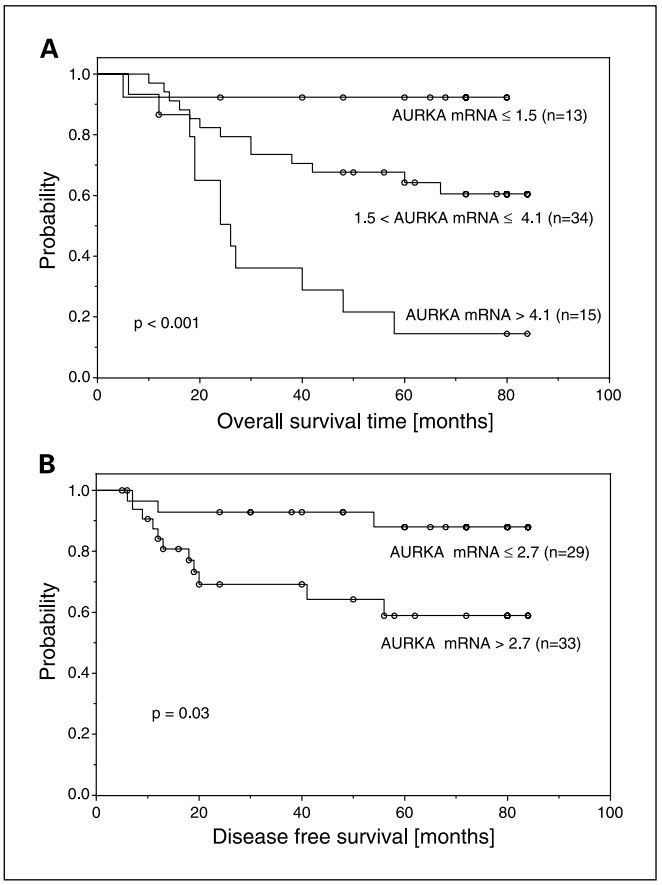

Fig. 3. *A,* effect of AURKA mRNA expression on overall survival in patients with HNSCC (P < 0.001). *B,* effect of AURKA mRNA expression on disease-free survival in patients with HNSCC (P = 0.03).

aggressiveness in several cancer types (11–19), which could be also shown in our series of HNSCC patients. There are a range of mechanisms by which AURKA overexpression in cancers might play a role in promoting tumor progression. Increases in AURKA levels through gene amplification, transcriptional up-regulation, or protein stabilization induce abnormal spindle formation and cytokinesis failure (6). As an important kinase and cell cycle regulator, AURKA has been shown to interact with many important cellular proteins, including p53 and BRCA1 (32, 33), and disrupt or alter their physiologic functions. In cells with impairments in the p53-RB pathway, tetraploid cells tend to have centrosome amplification, which induces chromosome instability (7, 9). Furthermore, the DNA damage–induced G_2 checkpoint is impaired in AURKA-overexpressing cells, which also contributes to genomic instability (6). Nevertheless, the interrelationship between all these pathways as well as a well-defined causative role of AURKA in tumor progression is, however, still unclear.

AURKA up-regulation and a metastatic phenotype were identified as being significantly associated in our study (Fig. 1). In their previous report, Tong et al. (15) examined the association between AURKA expression and cell migration in 12 human esophageal squamous cancer cell lines. They found that disruption of endogenous AURKA using small interfering RNA technique substantially suppressed cell migration ability. Because tumor cell invasion and migration are essential prerequisites for the metastatic process, these findings suggest a role of AURKA in esophageal squamous cancer as well as in squamous cell cancer of head and neck. Interestingly, also a functional AURKA Phe31Ile polymorphism is associated with advanced disease status and metastastic process of esophageal squamous cell carcinoma (34). Thus, not only the up-regulation of AURKA but also the change in AURKA polymorphism may affect the invasiveness and metastatic properties of tumor cells in esophageal squamous cell carcinoma. Moreover, AURKA polymorphisms have been reported in multiple cancer types (35–37). These results further support our conclusion that AURKA abnormalities may contribute to tumor progression and clinical aggressiveness also in HNSCC.

Because of the preponderance of chromosomal abnormalities and aneuploidy in HNSCC (1, 10), the contribution to this phenotype made by genes involved in chromosome segregation and regulation of centrosome function and cytokinesis, such as AURKA, can be postulated. In our series of HNSCC, there was a correlation between abnormal AURKA mRNA expression and centrosome abnormalities, similarly as previously described in breast cancer (18). In contrast to this study, we could not find an association between AURKA expression and chromosomal aneusomy.

With regard to the correlation between AURKA mRNA expression and the prognosis of HNSCC, the disease-free survival and overall survival rates of patients with AURKA-positive tumors were significantly lower than those of patients with AURKA-negative tumors (Fig. 3A and B). Thus, an AURKA-positive status is a prognostic factor, implying that the elevated expression of AURKA mRNA may be an indicator of the patient's prognosis. Recent reports showed that the up-regulation of AURKA results in resistance to apoptosis induced by paclitaxel in a human cancer cell line (38, 39). Hata et al. (40) found that the specific knockdown of AURKA in cultured pancreatic cancer cells strongly suppressed *in vitro* cell growth and *in vivo* tumorigenicity. The knockdown induced the accumulation of cells in the G_2-M phase and eventual apoptosis. Furthermore, these authors observed a synergistic enhancement of the cytotoxicity of taxanes, a group of chemotherapeutic agents impairing G_2-M transition, by the RNA interference–mediated knockdown of AURKA (40). This raises the possibility that AURKA inhibition may provide for a new approach for the treatment of multiple human malignancies (41).

In conclusion, our findings suggest that AURKA up-regulation (mRNA and protein) is a common abnormality in HNSCC and may play a role in its progression. We showed that AURKA mRNA overexpression is correlated with tumor progression, a metastatic phenotype, and shortened survival. Thus, AURKA may be useful as a prognostic factor for HNSCC patients. Future studies on the physiologic targets of AURKA and its potential role in the pathogenesis of HNSCC will be helpful for finding a novel therapeutic strategy for the treatment of HNSCC.

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