

Aurora A is a negative prognostic factor and a new therapeutic target in human neuroblastoma

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Abstract

We studied expression of the *Aurora A* gene and its clinical significance in a cohort of neuroblastoma patients. In addition, we investigated the antitumor activity of MLN8054, a novel small-molecule inhibitor of *Aurora A* kinase, on cultured NB cell lines *in vitro*. *Aurora A* mRNA expression was assessed by quantitative real-time PCR in tumor tissue specimens from 67 patients at diagnosis and in 9 human neuroblastoma cell lines. Western blot assays for *Aurora A* protein were done on tumor tissue of 53 patients. The results were correlated with various prognostic factors of neuroblastoma. *Aurora A* mRNA and protein expression were identified in 9 of 9 neuroblastoma cell lines. Overexpression of *Aurora A* mRNA in neuroblastoma tumor tissue is associated with high risk ($P = 0.019$), high-stage (International Neuroblastoma Staging System III and IV) tumors ($P = 0.007$), unfavorable histology ($P = 0.007$), *MYCN* amplification ($P = 0.017$), disease relapse ($P = 0.019$), and decreased progression-free survival ($P < 0.0001$) but not correlated with the age at diagnosis ($P = 0.877$). Similarly, *Aurora A* protein expression also significantly correlated with high risk ($P = 0.011$), high stage ($P = 0.0028$), unfavorable histology ($P = 0.0006$), *MYCN* amplification ($P = 0.0029$), and disease relapse ($P = 0.044$). Small interfering RNA-

mediated knockdown of the endogenous *Aurora A* gene causes a proliferation defect and enhances chemosensitivity in human neuroblastoma cell lines. In support of these observations, the *Aurora A* kinase inhibitor, MLN8054, markedly inhibited growth of cultured neuroblastoma cell lines through an apoptosis-dependent pathway. Overexpression of *Aurora A* is associated with disease progression in neuroblastoma. Inhibition of this kinase is a promising modality for neuroblastoma treatment. [Mol Cancer Ther 2009;8(8):2461–9]

Introduction

Neuroblastoma is the most common extracranial solid tumor in children and is responsible for 10% to 15% of pediatric cancer deaths (1). Over the past 30 years, much effort has gone into establishing reliable prognostic indicators of high-risk disease. For neuroblastoma, age, stage, and histopathology have consistently correlated well with outcomes. Chromosomal number, or ploidy, and amplification of the *MYCN* oncogene have also proven to be important and are commonly used to stratify patient risk (2–4). Despite recent advances in cancer therapy, the prognosis of patients with high-risk neuroblastoma is still very poor. Currently, much effort is focused on developing novel therapeutic strategies and improving the prognosis of higher-stage neuroblastoma patients. To fulfill this purpose, discovery and characterization of new molecular targets involved in neuroblastoma progression is urgently required.

The *Aurora A* gene (serine/threonine kinase 15, also known as STK15, BTAK, and Aurora 2), encoding a centrosome-associated kinase, is amplified and overexpressed in multiple human adult tumor cell types (5–11) and is involved in the induction of centrosome duplication-distribution abnormalities and aneuploidy in mammalian cells (5). Recent studies have shown that *Aurora A* is overexpressed and/or amplified in breast cancers (26%; refs. 5, 6), hepatocellular carcinoma (61%; refs. 7, 8), laryngeal squamous cell carcinoma (68%; refs. 9–11), and ovarian cancer (67%; ref. 12) as well as in neuroblastoma cell lines (5). The specific knockdown of *Aurora A* strongly suppresses *in vitro* cell growth and *in vivo* tumorigenicity and enhances the taxane or docetaxel chemosensitivity of human cancer cells (13, 14), substantially suppresses cell migration in human esophageal squamous cell carcinoma cells (15), and suppresses cell cycle progression in HeLa cells (16). These findings imply that *Aurora A* is a critical oncogene. Its overexpression in multiple tumor types shows that *Aurora A* may be a potential therapeutic target.

The role of *Aurora A* in neuroblastoma progression has not been well characterized. In the present study, we have examined the expression of *Aurora A* mRNA and protein both in a set of 67 neuroblastoma primary tumor tissue samples and 9

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tumor cell lines and analyzed the clinicopathologic features of *Aurora A* expression in neuroblastoma patients. In addition, the cytotoxic activity and the mechanism of action of the *Aurora A* inhibitor MLN8054 was analyzed in a series of neuroblastoma cell lines. We hypothesize that *Aurora A* could be an important prognostic factor and a new therapeutic target in human neuroblastoma.

Materials and Methods

Patients and Tumor Tissue Collection

The neuroblastoma patients participating in this study were recruited from the Texas Children's Cancer Center at Texas Children's Hospital from 1995 to 2006. All of the procedures were approved by the Baylor College of Medicine Institutional Review Board. Tumor samples were obtained from 67 neuroblastoma patients with International Neuroblastoma Staging System (INSS) stage I to IV or IVS disease in this study. Fresh tumor tissues were collected from patients with pathologically and clinically confirmed neuroblastoma. A portion of tumor specimens were kept in -80°C and sectioned for total RNA and protein extraction. Clinical information was obtained by chart review.

Cell Culture and Compound Treatment

Human neuroblastoma tumor cell lines (IMR-32, SK-N-SH, SH-SY5Y, SK-N-AS, SH-EP, and LAN-1), breast normal epithelial cell line MCF-10A, and human breast cancer cell line MCF-7 were obtained from the American Type Culture Collection, and the neuroblastoma cell line JF was kindly provided by Dr. M. Brenner (Baylor College of Medicine). NB19 and SMS-KCN were kindly provided by Dr. A. Davidoff (St. Jude's Children's Hospital). SH-SY5Y-Luc cells were kindly provided by Dr. Eugene S. Kim (Baylor College of Medicine). Briefly, cell lines were maintained in MEM

(IMR-32, SK-N-SH, NB19, SMS-KCN, and LAN-1), RPMI 1640 (JF, SH-EP, and SH-SY5Y-Luc) and DMEM (SK-N-AS, MCF-10A, and MCF-7). All media were supplemented with 10% heat-inactivated FCS, 2 mmol/L glutamine, 100 units/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin (all from Invitrogen). MLN8054 (Millennium Pharmaceuticals) was diluted in distilled water and added to the cell culture medium at a final concentration of 0.1 to 50 $\mu\text{mol}/\text{L}$. Doxorubicin (Sigma-Aldrich) was diluted in HBSS and added to the cell culture medium at a final concentration of 0.1 to 1 $\mu\text{mol}/\text{L}$.

Quantitative Real-time PCR

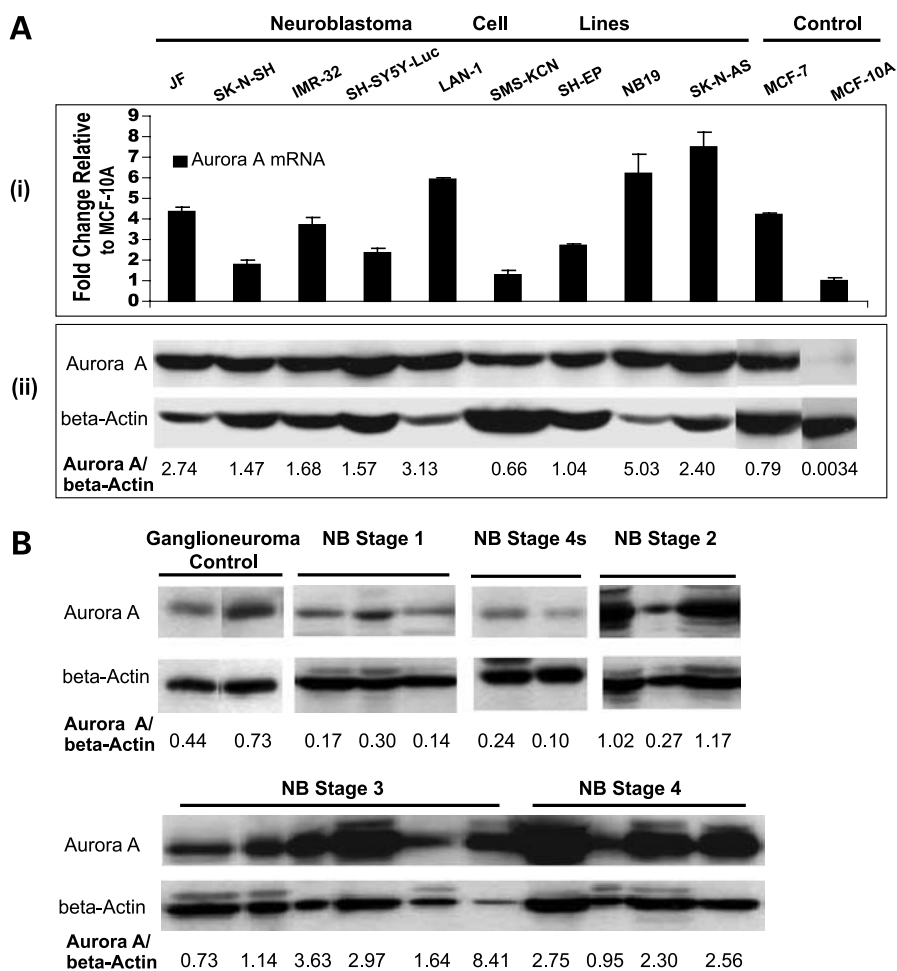
Total RNA was extracted from tumor cell lines and tumor tissues using Trizol reagent (Invitrogen), and the purity of RNA was determined by measuring the absorbance at 260/280 nm (A260/A280) in a spectrophotometer. The following primer pairs were used: *Aurora A* mRNA (forward 5'-TGGAATATGCACCACTTGGGA-3' and reverse 5'-GGCATTGGCCAATTCTGTGA-3'; the product size is 101 bp) and glyceraldehyde-3-phosphate dehydrogenase mRNA (forward 5'-CCACATCGCTCAGACACCAT-3' and reverse 5'-GGCAACAATATCCACTTTACCAGAGT-3'; the product size is 113 bp). Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. One hundred nanograms of RNA were used as the template for a one-step real-time PCR (RT-PCR). QuantiTect SYBR Green (Qiagen) RT-PCR mixes were used for RT-PCR. For each unknown test sample, the amount of *Aurora A* and endogenous reference glyceraldehyde-3-phosphate dehydrogenase was determined from the respective standard curve. Subtracting the glyceraldehyde-3-phosphate dehydrogenase level from the *Aurora A* level resulted in a normalized *Aurora A* value (Table 1). For the cell cultures, the MCF-10A mammary epithelial cell line was considered the normal threshold value,

Table 1. Clinicopathologic characteristics of the patients with neuroblastoma and *Aurora A* mRNA and protein expression

Patient characteristics	mRNA			Protein		
	<i>n</i>	$\bar{X} \pm \text{SD}$	<i>P</i>	<i>n</i>	$\bar{X} \pm \text{SD}$	<i>P</i>
Age at diagnosis (y)						
≤ 1	28	3.10 ± 2.64	0.877	25	1.37 ± 1.67	0.29
> 1	39	2.98 ± 3.30		28	1.82 ± 1.37	
INSS stage						
I + II + IVS	31	1.98 ± 1.34	0.007	26	1.28 ± 1.24	0.0028
III + IV	36	3.94 ± 3.72		27	2.85 ± 2.23	
MYCN status						
Nonamplified	49	2.64 ± 2.66	0.017	36	1.30 ± 1.04	0.0029
Amplified (>10 copies)	14	4.84 ± 3.90		11	2.50 ± 1.31	
Histology						
Favorable	28	2.42 ± 2.00	0.007	23	1.03 ± 0.87	0.0006
Unfavorable	19	4.89 ± 3.94		14	2.26 ± 1.11	
Disease risk						
Low + intermediate	42	2.31 ± 1.80	0.019	35	1.22 ± 0.94	0.011
High	22	4.74 ± 4.18		16	2.11 ± 1.47	
Relapse						
Yes	19	4.59 ± 4.41	0.019	14	2.42 ± 2.07	0.044
No	43	2.63 ± 2.00		35	1.45 ± 1.17	

NOTE: *n*, number of neuroblastoma tumor tissues tested; \bar{X} , mean mRNA and protein expression levels between selected groups and determined by RT-PCR and Western blotting assay.

Figure 1. **A**, *Aurora A* expression in human neuroblastoma cell lines determined by quantitative RT-PCR (i) and Western blot analysis (ii). Western blots were probed with anti-*Aurora A* antibody and reprobed with anti- β -actin antibody to show equal protein loading. Breast tumor cell line MCF-7 and normal breast epithelial cell line MCF-10A were used as positive and negative controls for *Aurora A* expression, respectively. **B**, analysis of *Aurora A* expression in neuroblastoma and ganglioneuroma tumor tissue samples by Western blot. Representative neuroblastoma tumor samples are shown, grouped by INSS stage.



whereas for the neuroblastoma tissue samples the average normalized *Aurora A* level of seven ganglioneuroma samples established the threshold below which the value was considered background (17).

Western Blotting Assay

For *Aurora A* protein extraction, neuroblastoma cells were lysed in prewarmed (95°C) cell lysate buffer [2% SDS/300 mmol/L Tris-HCl (pH 6.8)/10% glycerol]; frozen tissue samples were sectioned into small pieces and proteins were extracted by using Trizol reagent. For poly(ADP-ribose) polymerase (PARP) protein cleavage assay, radioimmunoprecipitation assay buffer was used as described (18). Equal amounts of protein (30 μ g) were separated by SDS-PAGE on 10% gels, transferred to polyvinylidene difluoride membrane (Amersham Pharmacia), and incubated with antibodies specific for *Aurora A* (1:1,000 dilution), PARP (1:1,000 dilution), or the secondary antibodies horseradish peroxidase-linked anti-rabbit immunoglobulin (1:1,000 dilution) and horseradish peroxidase-linked anti-mouse immunoglobulin (1:1,000 dilution; all from Cell Signaling Technology) as well as antibodies specific for β -actin (A2228; 1:5,000 dilution; Sigma-Aldrich). Electrochemiluminescence detection (GE Healthcare) with documentation on X-ray films

was used for the detection of protein, the lysate from NB cell line SK-N-AS was used as an internal control, and the same exposure time (40 s) was set up to standardize the analysis. The net intensity of the immunoreactive bands was quantified by scanning densitometry (Scan Molecular Dynamics; GE Healthcare) using Imaging Analysis software (ImageQuant Tool version 5.2). Specific protein level was expressed as the ratio of the net intensity of the protein band to β -actin band of the same sample [Fig. 1A (ii) and B; Table 1].

Establishment of Stable Short Hairpin RNA *Aurora A* Knockdown Neuroblastoma Cell Lines

Aurora A short hairpin RNA expression was achieved by cloning the following ligated sequences into the pSuper-PURO retrovirus expression vectors per manufacturer's protocol (Oligoengine). The RNA interference target sequences used were as follows: pSuper-Scrambled control (Sh-Control), CGTCTTTTCGGACTTAGAGAG; pSuper-No.1-sh *Aurora A* (Sh-*Aurora A* #1), AACGTGTTCTCGTGACT-CAGC; and pSuper-No.2-sh *Aurora A* (Sh-*Aurora A* #2), AAATGCCCTGTCTTACTGTCA (13). For retroviral transduction, we used a previously published protocol (19). Stable cell lines were established after 10 days of puromycin (2 μ g/mL) selection.

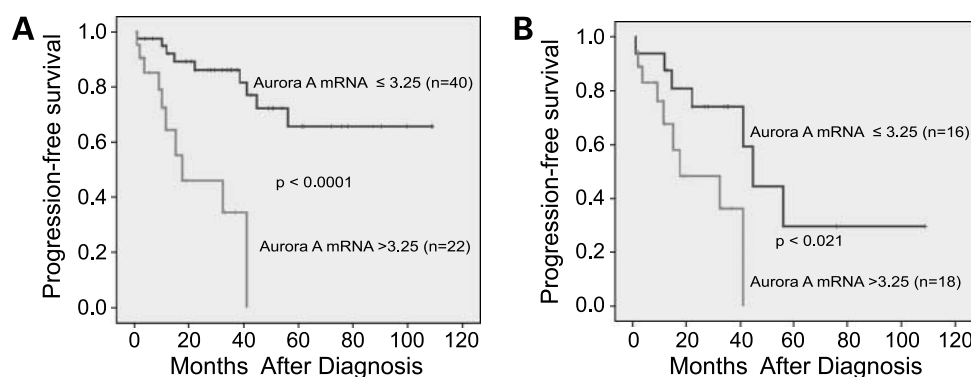


Figure 2. *Aurora A* mRNA expression level is significantly associated with decreased progression-free survival in patients with neuroblastoma. **A**, effect of *Aurora A* mRNA expression on progression-free survival in all patients in this study ($P < 0.0001$). **B**, effect of *Aurora A* mRNA expression on progression-free survival in INSS stage III and IV patients ($P < 0.021$).

Proliferation and Soft-Agar Assays

Cells were plated in 96-well flat-bottomed plates at a concentration of 1×10^3 or 1×10^4 per well. Using the Cell Counting Kit-8 (Dojindo Molecular Technologies), cells were stained at the indicated time point. After a 3 h incubation period, absorbance was measured at 450 nm using a standard plate reader.

For the soft-agar assays, a layer of 0.5% agarose/culture medium was plated and allowed to solidify. Cells (1×10^4 or 5×10^3) were suspended in 0.3% agarose/culture medium and plated on top of the 0.5% layer. Culture medium (500 μ L) was added on top of the agarose to prevent drying of the soft agar. Wells were photographed after 14 days of growth and colonies (>30 cells) were counted. All experiments were carried out in triplicate with the mean \pm SD reported.

Flow Cytometry for Detection of Apoptotic Cells and Cell Cycle Analysis

Neuroblastoma cells were cultured in 6-cm dishes (2×10^5 per dish) for 24 h and then treated for 48 h with MLN8054 at concentrations between 0 and 50 μ mol/L. Subsequently, floating cells in the medium and adherent cells were collected. Using an Annexin V-FITC Apoptosis Detection kit (BD Biosciences), cells were stained with Annexin V-FITC and

propidium iodide according to the manufacturer's instructions. Untreated cells were used as the negative control for double staining. Cells were analyzed immediately after staining by using a FACScan flow cytometer and the CellQuest software. For each measurement, $>10,000$ events were counted. A flow cytometric analysis of DNA content was done to assess the cell cycle phase distribution (20). DNA content was evaluated using a FACScan flow cytometer (Becton Dickinson) and CellQuest software (Becton Dickinson). The experiment was repeated three times.

Statistical Analysis

We computed basic descriptive statistics for age at diagnosis, gender, histology, extent of surgery, radiation therapy, chemotherapy, and level of mRNA and protein expression for *Aurora A*. Two-sample *t* test or ANOVA was used to compare the mean mRNA and protein expression levels (*X* value) between selected groups (Table 1). Kaplan-Meier procedure was used to estimate the progression-free survival (21). The cutoff to discriminate low versus high *Aurora A* mRNA expression (3.25) was selected because it separated the population into two distinct survival groups (Fig 2; Supplementary Table S1). Survival time was calculated in 62 patients for whom the date of diagnosis, date of progression or relapse, death, or date of last follow-up were available. Cox

Table 2. Biological characteristics and sensitivity to MLN8054 in human neuroblastoma cell lines

Cell line	Origin	MYCN	p53 status	Cell type	IC ₅₀ of MLN8054 (μ mol/L)
SK-N-SH	NB tumor	SC	WT	N + S	0.46 \pm 0.01 (2)
SH-SY5Y-Luc	NB tumor	SC	WT	N	0.24 \pm 0.02 (4)
IMR-32	NB tumor	A	WT	N	0.30 \pm 0.08 (2)
LAN-1	NB tumor	A	Null	N	0.67 \pm 0.04 (4)
JF	NB tumor	A	N/D	N	0.26 \pm 0.06 (3)
SH-EP	NB tumor	SC	WT	S	0.92 (1)
NB19	NB tumor	A	WT	S	0.50 (1)
SMS-KCN	NB tumor	A	N/D	S	2.77 \pm 0.18 (2)
SK-N-AS	NB tumor	SC	mt	S	4.11 \pm 1.03 (2)
MCF-10A	Breast normal epithelial		WT		12.92 (1)
MCF-7	Breast tumor		WT		0.44 (1)

NOTE: Numbers represent average \pm SD IC₅₀ derived from a Cell Counting Kit-8 proliferation assay. Values in parentheses represent the number of experiments completed for each cell line.

Abbreviations: NB, neuroblastoma; SC, single copy; A, amplified; WT, wild-type; mt, mutated; N/D, not described; N, neuroblastic; S, substrate adherent.

proportional hazard analysis was used for multivariable analyses adjusting for age at diagnosis, gender, risk group (low-intermediate versus high), stage (stages I, II, and IVS versus stages III and IV), *MYCN* amplification status, and histology. All statistical tests were two-sided and $P < 0.05$ was considered significant. For all of the analyses, we used SPSS (version 15.0) software package.

Results

Aurora A Is Overexpressed in Human Neuroblastoma Cell Lines

To address the possibility of whether *Aurora A* could serve as a therapeutic target for neuroblastoma, we measured the protein and mRNA levels of *Aurora A* in nine cultured neuroblastoma cell lines. Because neuroblastic (N) type and substrate adherent (S) type cells are two distinct cell types in neuroblastoma tumors and tumor cell cultures, we tested the *Aurora A* expression in both four N-type cell lines (JF, SH-SY5Y-Luc, IMR-32, and Lan-1) and four S-type cell lines (SMS-KCN, SH-EP, NB19, and SK-N-AS) as well as one mixed N/S-type cell line (SK-N-SH; Table 2). Total RNA and protein were harvested at the same time from each cell line. As shown in Fig. 1A (i), with the exception of SMS-KCN, the neuroblastoma cell lines had higher levels of *Aurora A* mRNA ranging from 1.7- to 7.5-fold higher than the

control level. Also, compared with the human mammary epithelial cell line MCF-10A (negative control), all nine neuroblastoma cell lines had high levels of *Aurora A* protein expression [Fig. 1A (ii)]. The *Aurora A* mRNA level correlated well with protein expression in the majority of tested neuroblastoma cell lines (Fig. 1A).

High *Aurora A* Expression Is Associated with Poor Clinical Outcome in Human Neuroblastoma

Aurora A mRNA expression was measured in tumor tissue specimens from 67 patients using quantitative RT-PCR. Quantification of the kinase protein was done in a subset of 53 specimens for whom sufficient protein was available. Among the 67 patients, the mean patient age was 18 months (range, 1-75 months), 14 of 63 (22%) patients were *MYCN*-amplified, and 22 of 64 (34%) patients were considered high risk according to the Children's Oncology Group neuroblastoma risk classification system (22). The distribution among the INSS stages was stage I, 18%; stage II, 21%; stage III, 15%; stage IV, 39%; and stage IVS, 7%. Patient characteristics and the relative *Aurora A* expression levels are shown in Table 1.

Aurora A mRNA expression in neuroblastoma tissue samples was compared with the mean of seven ganglioneuroma specimens, with the ratio expressed as fold change. The latter neoplasm was chosen because it is considered a benign cognate of malignant neuroblastoma (23). As shown in

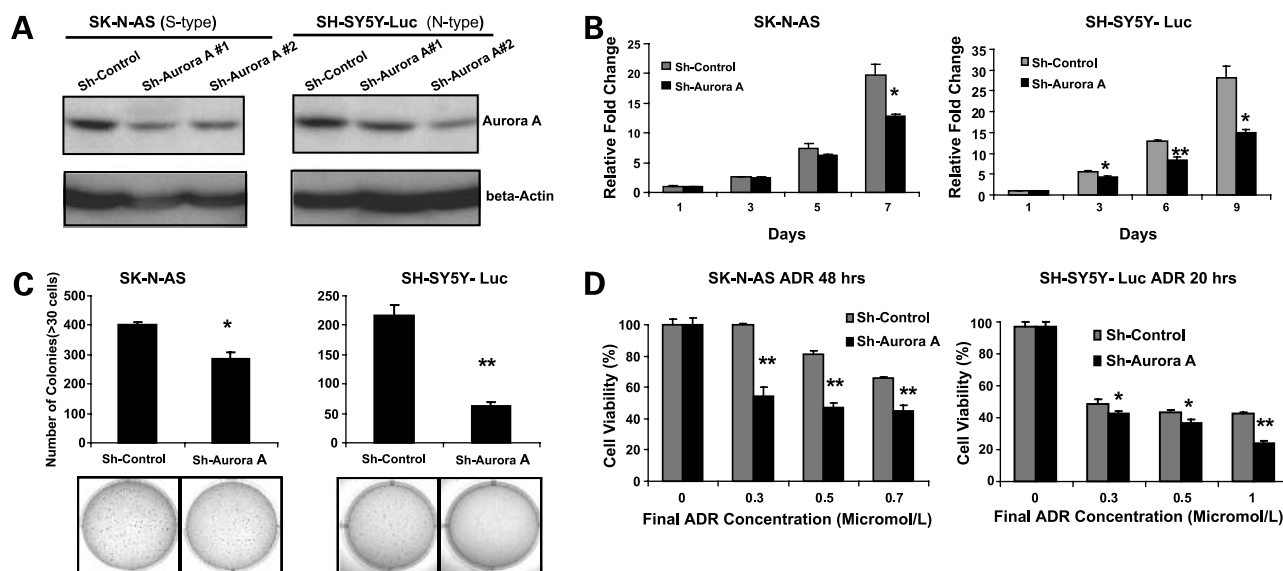


Figure 3. Inhibition of *Aurora A* expression causes a proliferation defect and enhances chemosensitivity in both S-type and N-type human neuroblastoma cell lines. *Aurora A* expression in S-type SK-N-AS and N-type SH-SY5Y-Luc cells stably transduced with pSR-Sh-scramble control and pSR-Sh-*Aurora A* (#1 and #2 sequence). **A**, cells were analyzed by Western blot after 10 d of puromycin (2 $\mu\text{g}/\text{mL}$) selection. **B**, inhibition of *Aurora A* expression causes a proliferation defect. The SK-N-AS and SH-SY5Y-Luc Sh-Control and Sh-*Aurora A* #2 cell lines were plated in 96-well plates at 1×10^3 cells per well. The Cell Counting Kit-8 tetrazolium salt-based proliferation assay was used to quantify cellular proliferation relative to day 1 absorbance measured at 450 nm. These experiments were done in triplicate and reported as mean \pm SD. **C**, SK-N-AS and SH-SY5Y-Luc Sh-Control and Sh-*Aurora A* #2 stably transduced cell lines were plated in 0.3% agarose/DMEM on top of a 0.5% agarose/DMEM layer. After 14 d of growth, colonies were stained with MTT (bottom) and colonies >30 cells were counted (top). A similar result was observed using Sh-*Aurora A* #1 cells (data not shown). **D**, inhibition of *Aurora A* expression increases chemosensitivity. SK-N-AS and SH-SY5Y-Luc Sh-Control and Sh-*Aurora A* #2 cell lines were plated in 96-well plates at 1×10^4 cells per well. After 24 h of growth, all of the cell lines were treated with the indicated micromolar concentration of doxorubicin for the indicated amount of time. Cell viability was determined with the Cell Counting Kit-8 cell viability assay relative to the 0 $\mu\text{mol}/\text{L}$ group. All experiments were done in triplicate and statistical significance was determined by Student's *t* test where $P < 0.05$ was statistically significant. *, $P < 0.05$; **, $P < 0.001$ compared with the Sh-Control group.

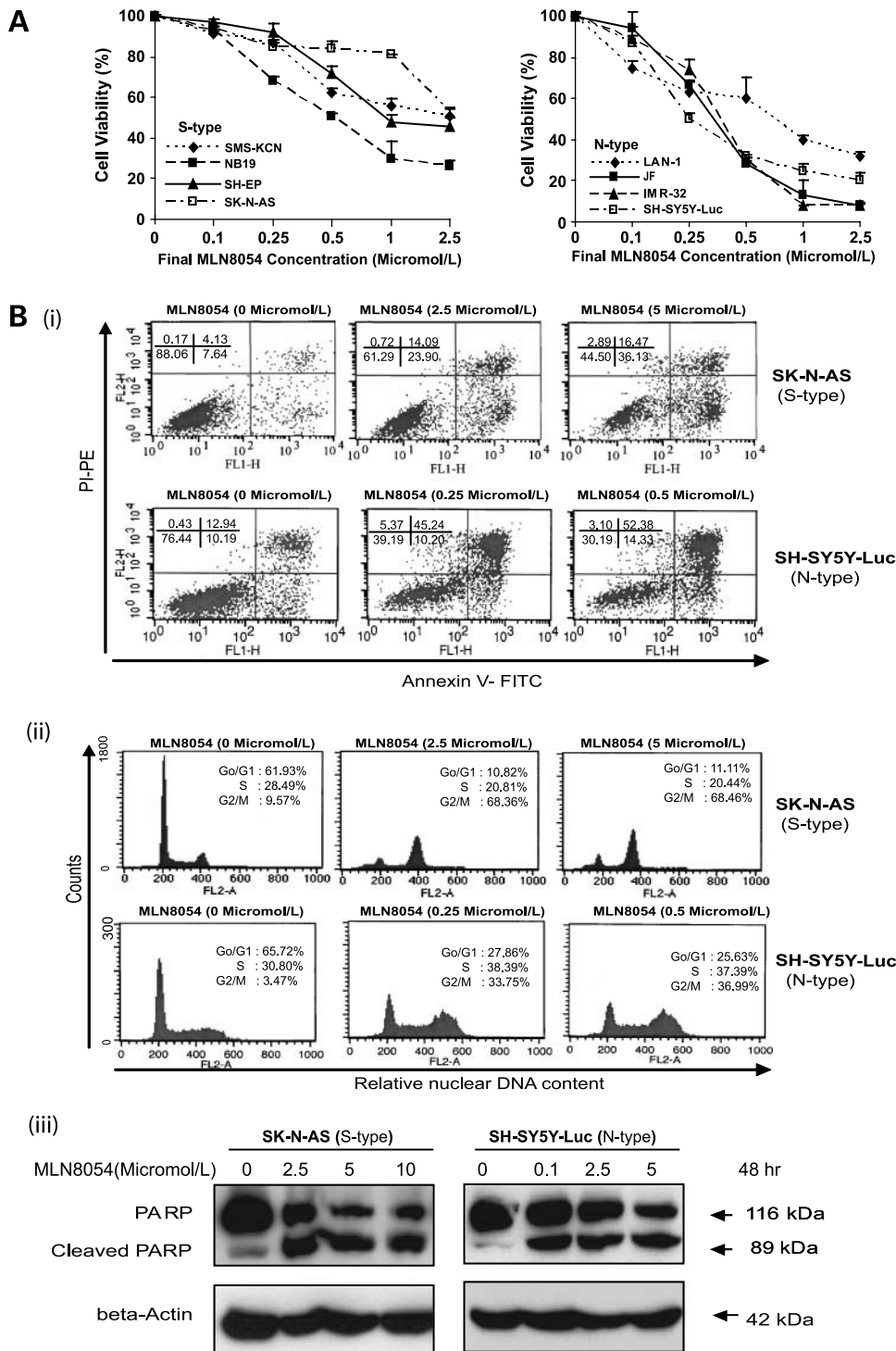


Figure 4. **A**, growth-inhibitory effect of *Aurora A* inhibitor MLN8054 on cultured NB cell lines *in vitro*. N-type and S-type NB cells were plated in 96-well flat-bottomed plates at a concentration of 5×10^3 or 1×10^4 per well and NB cells were treated with increasing concentrations of MLN8054 for 96 h. Cytotoxic activity was determined by the Cell Counting Kit-8 assay. **B**, effect of the *Aurora A* inhibitor MLN8054 on apoptosis and cell cycle distribution in two representative NB cell lines, SK-N-AS and SH-SY5Y-Luc. Cells were preincubated in the absence or presence of different concentrations of MLN8054 for 48 h and stained for Annexin V and propidium iodide [P; (i)], stained with propidium iodide and analyzed for relative DNA content (ii), and harvested and submitted to immunoblotting of full-length (116 kDa) and cleaved (89 kDa) PARP (iii).

Table 1, expression level of *Aurora A* mRNA is associated with high INSS stage (stage III or IV; $P = 0.007$), *MYCN* amplification ($P = 0.017$), unfavorable histology ($P = 0.007$), high Children's Oncology Group risk group ($P = 0.019$), and disease relapse ($P = 0.019$) but did not correlate with the age at diagnosis ($P = 0.877$). As shown in Fig. 1B and Table 1,

Aurora A protein expression also significantly correlated with high INSS stage (stage III or IV; $P = 0.0028$), *MYCN* amplification ($P = 0.0029$), unfavorable histology ($P = 0.0006$), high Children's Oncology Group risk classification ($P = 0.011$), and disease relapse ($P = 0.044$). The kinase protein level did not correlate with age at diagnosis ($P = 0.29$).

Overexpression of *Aurora A* mRNA Is Significantly Associated with Decreased Progression-Free Survival in Patients with Neuroblastoma

For the tumors assessed in this study, 19 of 62 (30.65%) patients had progression of their disease and 9 of 62 (14.52%) patients died. Three-year progression-free survival rate \pm SE was $72 \pm 6.5\%$ with a median follow-up period of 33 months (range, 0.7-122 months) in the survivors. Two Kaplan-Meier progression-free survival analysis curves with stratification by *Aurora A* mRNA expression are shown in Fig. 2. The cut point for *Aurora A* mRNA (3.25) was chosen because it separated the population into two distinct survival groups. In the entire population of patients, survival was significantly worse in those with *Aurora A* expression >3.25 ($P < 0.0001$). In the subgroup of patients with stage III and IV tumors, *Aurora A* expression continued to have a significant association with decreased progression-free survival ($P < 0.021$). In multivariable analysis, patients with high *Aurora A* expression (>3.25) had a 4.8 (95% confidence interval, 1.6-14.4) times higher risk for progression independent of the risk group (Supplementary Table S1).

Knocking Down the *Aurora A* Gene Causes a Proliferation Defect and Enhances Chemosensitivity in Human Neuroblastoma Cell Lines

Given the observed correlation between *Aurora A* expression and clinical outcome in our cohort of neuroblastoma patients, we proposed that inhibition of this kinase might inhibit growth of neuroblastoma tumor cells. The siRNA method was employed to deplete the expression of *Aurora A* in cultured neuroblastoma cell lines, SH-SY5Y-Luc (N-type) and SK-N-AS (S-type). As shown in Fig. 3A, transduction with siRNA targeting *Aurora A* caused a 50% to 60% of decrease in *Aurora A* protein that was not observed with the siRNA scramble control. The effect of *Aurora A* siRNA on the *in vitro* proliferation and colony formation is shown in Fig. 3B and C, respectively. Compared with the scramble control transduced cells, the *Aurora A* siRNA group had much less growth and formed fewer colonies in soft agar. These results indicate the RNA interference-mediated specific knockdown of *Aurora A* induced significant inhibition of neuroblastoma cell growth *in vitro*. In addition, we noted an increase in the relative G₂-M population in the *Aurora A* knockdown cells compared with the scramble controls in these two cell lines (Supplementary Fig. S1).

Doxorubicin, also known as Adriamycin, is an antibiotic of the anthracycline group that has been used for the clinical treatment of neuroblastoma for >30 years. We examined the effect of *Aurora A* siRNA on growth of doxorubicin-treated neuroblastoma cells. As shown in Fig. 3D, there is a significant enhancement of the cytotoxic effect of doxorubicin in the *Aurora A* siRNA-transduced cells that is not observed in the scramble control transduced cells.

Aurora A Inhibitor MLN8054 Inhibits the Growth of Neuroblastoma Cell Lines

Aurora kinases have been proposed as a promising target for cancer therapy (24-26). MLN8054 is a novel small-molecule *Aurora A* inhibitor discovered and developed by scientists at Millennium Pharmaceuticals for the treatment

of human cancers (27). The *Aurora A* inhibitor MLN8054 was tested *in vitro* against a panel of neuroblastoma cell lines as well as the normal and cancer-derived breast cell lines, MCF-10A and MCF-7. MLN8054 exhibited dose-dependent cytotoxicity against in both S-type and N-type cell cultures (Fig. 4A) during 96 h of exposure. Based on the IC₅₀, the compound had less activity against the normal breast cell line, MCF-10A, compared with the cancer cell lines (Table 2). Among the neuroblastoma cell lines tested, SH-SY5Y-Luc, JF, IMR-32, and LAN-1 (N-type) were generally more sensitive than the SK-N-AS, SH-EP, SMS-KCN, and NB19 (S-type) cell lines, although the difference in mean IC₅₀ values between the two groups did not achieve statistical significance.

Aurora A Inhibitor MLN8054 Triggers Apoptosis in NB Cell Lines

To determine the mechanism of action of the *Aurora A* inhibitor MLN8054 in neuroblastoma, Annexin V/propidium iodide staining, cell cycle analysis, and PARP cleavage immunoblotting were done after incubating two representative neuroblastoma cell lines SK-N-AS (S-type) and SH-SY5Y-Luc (N-type) for 48 h with MLN8054. As shown in Fig. 4B (i), apoptosis (Annexin V-positive population) was induced by MLN8054 in a dose-dependent fashion. Drug treatment resulted in a decreased population of cells in the G₀-G₁ phases and increased numbers of cells in the G₂-M phases of the cell cycle in both SK-N-AS and SH-SY5Y-Luc cell lines [Fig. 4B (ii)]. PARP, a 116-kDa nuclear PARP, is involved in DNA repair predominantly in response to environmental stress (28). PARP is important for cells to maintain their viability: cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis (29). As shown in Fig. 4B (iii), the control cells treated with DMSO alone did not manifest appreciable cleavage of the 116 kDa PARP, whereas the cells treated with MLN8054 had significant cleavage of PARP after 48 h incubation. Between the neuroblastoma cell lines tested, SH-SY5Y-Luc is more sensitive than SK-N-AS to MLN8054, resulting in a higher Annexin V-positive cell population and a high percentage of PARP cleavage at lower concentrations of MLN8054. These results suggest that the *Aurora A* inhibitor MLN8054 inhibits the proliferation of neuroblastoma cells by an apoptotic pathway and is associated with mitotic delay.

Discussion

In the current study, we describe the first systematic survey of *Aurora A* expression in human neuroblastoma. We have shown that *Aurora A*, a centrosome-associated oncogene, is overexpressed in human neuroblastoma primary tumor tissue and cell lines at both mRNA and protein levels. Interestingly, overexpression of *Aurora A* is associated with clinically aggressive neuroblastoma and correlates with tumor stage, relapse risk, Children's Oncology Group risk group, and histology. In multivariate analysis, patients with high *Aurora A* expression had a significantly increased risk of disease progression that was independent of the

Children's Oncology Group risk group. Also, we found that small interfering RNA (siRNA)-mediated knockdown of the endogenous *Aurora A* gene causes a proliferation defect and enhances chemosensitivity in human neuroblastoma cell lines. In support with these results, we report significant *in vitro* antitumor activity of the Aurora A inhibitor, MLN8054, against neuroblastoma cell lines.

Although *MYCN* amplification is an important marker to predict poor patient outcome in neuroblastoma, this genetic aberration only occurs in ~20% of primary neuroblastomas (30). Approximately 65% of patients with metastatic disease have tumors that lack amplification of the *MYCN* proto-oncogene. Thus, identification of other oncogenes involved in neuroblastoma progression is needed. Our results provide strong evidence that overexpression of *Aurora A* could be critical in determining the malignant behavior of neuroblastoma tumor cells. Furthermore, Cox proportional hazard analysis shows that *Aurora A* is an independent marker to predict poor patient outcome in neuroblastoma. A recent report has identified Aurora A as a protein that is required for growth of *MYCN*-amplified neuroblastoma cells (31). We found that high *Aurora A* mRNA and protein expression levels correlate with *MYCN* amplification in neuroblastoma tumor tissue samples.

Aurora kinases represent an important therapeutic target for small-molecule inhibitors (5–11). MLN8054 (Millennium Pharmaceuticals) was the first selective small-molecule inhibitor of *Aurora A* to enter clinical trials in adult patients with advanced cancers (32). Because pediatric cancers are extremely rare, it is difficult for pharmaceutical corporations to commit the necessary resources to development of drugs specific for these malignancies. Therefore, we chose to investigate the anti-neuroblastoma activity of MLN8054 because it has been vetted in appropriate preclinical trials with common adult malignancies.

MLN8054 induces apoptosis in human colon cancer cell lines and inhibits tumor growth in xenograft models (19). Prior work has shown that Aurora A inhibition kills tumor cells through the development of deleterious aneuploidy (33). We observe that MLN8054 also inhibits the growth of neuroblastoma tumor cell lines by an apoptotic mechanism. In our studies, MLN 8054 increases the percentage of cells in the G₂ and M phases of the cell cycle, consistent with induction of mitotic delay. We found no direct correlation between the Aurora A expression level and the cytotoxic effect of MLN8054 compound on NB cell lines (Fig. 1A; Table 2).

N-type and S-type cells are two distinct cell types in neuroblastoma tumors and tumor cell cultures. Compared with S-type cells, N-type cells are more tumorigenic in immunodeficient mice and express higher levels of the N-myc and the antiapoptotic protein Bcl-2 (34). The mechanism of doxorubicin-induced death has been reported to be different in these two cell types. Cell death is caspase independent in N-type cells, whereas in S-type cells it does depend on caspase-8 but does not involve death receptors (35). We found high *Aurora A* expression in both N-type and S-type neuroblastoma cells. In our limited sample, we have observed a

somewhat increased sensitivity to MLN8054 in N-type versus S-type cells, although this difference did not achieve statistical significance. This differential effect does not correlate with Aurora A mRNA or protein levels or *MYCN* amplification status. It is interesting to note that p53 status appears to be a determinant of sensitivity to Aurora A inhibition: the two cell lines with null or mutant p53 status, LAN-1 and SK-N-AS, each had the highest MLN8054 IC₅₀ value among the N-type and S-type groups, respectively (Fig. 1A; Table 2). This observation is consistent with a mechanism in which Aurora A and p53 have opposite effects on apoptotic signaling pathways in these tumor cells. In addition, we noted that diminution of Aurora A activity, by either siRNA knockdown or MLN8054 inhibition, results in an increase in sensitivity to doxorubicin, suggesting a potential synergy between Aurora A inhibitors and standard chemotherapy agents.

In summary, our studies show that *Aurora A* may play an important role in tumorigenesis and progression of neuroblastoma. In addition, our work strongly suggests that MLN8054 may be an effective therapeutic agent for treatment of neuroblastoma. Further *in vivo* studies will provide a better assessment of the potential therapeutic utility of Aurora kinase inhibitors in this pediatric tumor.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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