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Integrating Bioinformatics and Clinicopathological Research of Gastrointestinal Stromal Tumors: Identification of Aurora Kinase A as a Poor Risk Marker

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ABSTRACT

Background. For completely resected primary gastrointestinal stromal tumors (GISTs), mitotic rate, tumor size, and tumor location are important risk factors for recurrence. However, molecular markers for recurrence are still lacking.

Methods. We reanalyzed GIST gene expression profile GSE8167 available from the Gene Expression Omnibus (GEO) and confirmed the prognostic influence of one selected gene, aurora kinase A (*AURKA*), in another cohort of 142 patients using immunohistochemistry (IHC).

Results. Thirty-two cases in GSE8167 were classified into two risk groups with distinct recurrence-free survival (RFS) and expression profiles using modified criteria of Miettinen et al. from the Armed Forces Institute of Pathology (AFIP-Miettinen). *AURKA* was among the 19 genes common to the top 50 features of the high-risk phenotype and a 67-gene signature called the complexity index in sarcomas. *AURKA* was significantly overexpressed in the high-risk group, and patients with high *AURKA* expression had significantly worse RFS than those with low expression. In the IHC-validated cohort, *AURKA* expression was significantly higher in nongastric tumors than in gastric tumors and was significantly correlated with AFIP-Miettinen risk group. Univariate analysis showed that RFS was significantly influenced by tumor size, mitotic count, AFIP-Miettinen risk group classification, and *AURKA* expression. However, only tumor size ($P = 0.017$), mitotic count ($P = 0.007$), and *AURKA* expression ($P = 0.039$) were identified as independent unfavorable prognostic factors for RFS in multivariate analysis.

Conclusions. By integrating bioinformatics and clinicopathological studies, *AURKA* was identified as a marker for high-risk GIST.

Gastrointestinal stromal tumors (GISTs) are rare mesenchymal tumors that primarily arise from the gastrointestinal tract and less commonly from the mesentery and retroperitoneum.[1,2] Mutated *KIT* proto-oncogene and platelet-derived

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growth factor receptor alpha (*PDGFRA*) are considered the major driving forces of GIST oncogenesis.[3,4] For the treatment of advanced GIST, imatinib mesylate (Novartis Pharmaceuticals, Basel, Switzerland), a potent tyrosine kinase inhibitor (TKI) of both *KIT* and *PDGFRA*, has greatly improved the median overall survival of patients from less than 1 year in the pre-imatinib era to 5 years.[5,6] However, acquired resistance to imatinib is inevitable and occurs approximately 2 years after treatment.[6] In a randomized, phase III trial, patients with cases refractory to imatinib administered another TKI, sunitinib maleate (Pfizer, New York, NY), had significantly better progression-free survival than those given placebo. Nonetheless, the effect only lasted for approximately 6 months.[7]

For localized GIST, surgical resection is the mainstay of treatment. However, tumor relapse is common, especially in cases with high-risk features, such as large tumor size, high mitotic index, or nongastric site.[8–10] In a randomized trial, compared with placebo, adjuvant imatinib therapy was shown to improve recurrence-free survival (RFS) after resection of primary GIST. However, approximately 8 % of cases in the treatment group developed recurrence, particularly those with large tumors.[11] Therefore, it is important to dissect the molecular mechanism underlying recurrence and identify potential markers/targets in high-risk cases.

Aurora kinases represent a novel family of serine/threonine kinases crucial for cell-cycle regulation. Aurora kinase A (*AURKA*), an important member of this family, localizes to the centrosome and functions primarily in centrosome regulation and mitotic spindle formation.[12] In this study, we analyzed expression profiling data from the National Center for Biotechnology Information (NCBI) and, with further clinical validation, identified *AURKA* as a marker of high-risk GIST.

PATIENTS AND METHODS

Bioinformatics Analysis

The microarray and clinicopathological data from Gene Expression Omnibus (GEO) dataset GSE8167, previously reported by Yamaguchi et al., was obtained from the NCBI website and used for bioinformatics analysis.[13] Gene expression data were normalized using dChip and filtered with a max/min $\geq$8 and then exported for further analysis. Gene set enrichment analysis (GSEA) was performed by using GSEA software downloaded at http://www.broadinstitute.org/gsea/index.jsp. Cases of primary GIST were classified into different groups based on recurrence risk according to the criteria proposed by Miettinen et al. from the Armed Forces Institute of Pathology (the so-called AFIP-Miettinen criteria) with modification (the threshold of mitotic count used in the study by Yamaguchi et al. was 5/10 high-power field [HPF] instead of 5/50 HPF, see supplementary Table S1): low-risk (originally very low- and low-risk), moderate-risk, and high-risk groups.[9,10,13,14] The probe sets correlated with high or moderate/low recurrence risk were determined by using a signal-to-noise statistic and permutation testing. The expression of *AURKA* in GEO GSE8167 samples was obtained using Z-score transformation, and the differences among the risk groups were compared by using the *t* test.

Tumor Samples

A total of 142 patients with localized, pathologically confirmed, c-kit positive GIST, diagnosed at Chang Gung Memorial Hospital between 1989 and 2008 were retrospectively reviewed. All patients underwent curative resection and were regularly followed up by contrast-enhanced computed tomography (CT) every 3–6 months. None of them received adjuvant imatinib after surgery. Formalin-fixed, paraffin-embedded tissue sections were available for immunohistochemical (IHC) staining. The study protocol for the collection of tumor samples and clinical information was approved by the institutional review board, and all patients provided written, informed consent authorizing the collection and use of the tumor samples for research purposes.

IHC staining of AURKA in GIST

A 4-μm section of each specimen was stained for *AURKA*. The primary antibody against aurora kinase A (rabbit anti-Aurora A polyclonal antibody, NB100-212; NOVUS) was diluted 1:1,500 and added to the slides, which were incubated overnight at 4 °C. The slides were then washed three times for 5 min each in TBST before visualization using the LSAB2 System, Peroxidase (K0675; DAKO A/S). Control slides were incubated with the secondary antibody only. After washing three times for 5 min each in TBST, the slides were mounted and blindly analyzed by the authors under microscopy, and GIST immunostaining was scored as low (<60 % staining) or high ($\geq$60 % staining; Supplementary Fig. S1).

Statistical and Survival Analysis

Correlations between clinicopathological variables and *AURKA* IHC staining were analyzed by the χ^2 test or Fisher's exact test. Recurrence-free survival (RFS) was estimated as the time from surgery to the date of tumor recurrence. Survival was estimated by using the Kaplan-Meier method, and the log-rank test was used for comparison of survival curves. Univariate and multivariate (stepwise forward conditional method) Cox regression analysis was used to determine the prognostic influence of

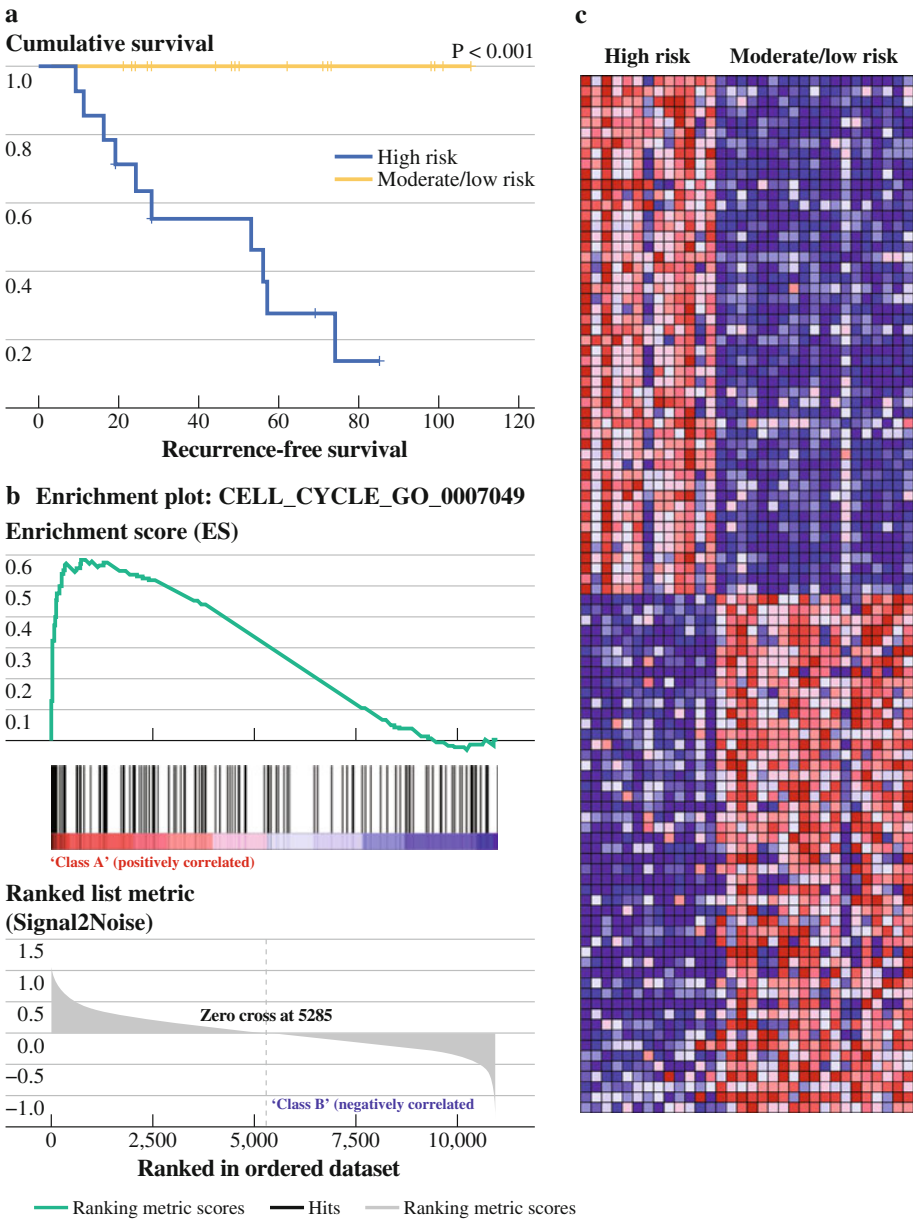

FIG. 1 Analysis of data from Gene Expression Omnibus (GEO) GSE8167. **a** Kaplan–Meier survival plot of recurrence-free survival for high-risk and moderate-/low-risk groups. **b** Representative gene set enrichment analysis (GSEA) results showing significant enrichment of the cell-cycle gene set associated with the high-risk group. **c** Heat map generated by using the top 50 features of each phenotype (High risk vs. Moderate/low risk)

clinicopathological factors and *AURKA* IHC staining. In two-sided tests, $P < 0.05$ was considered statistically significant. SPSS® software (version 10.00; SPSS, Chicago, IL) was used for all statistical analyses.

RESULTS

Distinct Expression Profiles in Different Risk Groups

The clinical information from GEO GSE8167 was analyzed first. Among the 32 cases studied, 13 were reclassified as high risk, 3 as moderate risk, and 16 as low risk for recurrence according to the modified AFIP-Miettinen criteria (Supplementary Table S1). There was a statistically significant difference in survival between the high-risk and moderate-/low-risk groups (Fig. 1a). Microarray data was first normalized by dChip, and a total of 21,005 probes were obtained after filtering with a max/min ≥8 threshold. The expression values were then exported and analyzed using GESA software. Among the 715 gene sets in Gene Ontology, 316 were up-regulated in the high-risk phenotype. Of these 316 gene sets, 79 were significant at a false discovery rate (FDR) <25 %, 75 were significantly enriched at a nominal P value <0.05, and 48 were significantly enriched at a nominal P value <0.01. If these were sorted by normalized enrichment score (NES), gene sets associated with the cell-cycle process or its regulation were among the top 10 (Supplementary Table S2; Fig. 1b). These results indicated the important roles of cell-cycle-related genes in primary GIST recurrence. A heat map was

TABLE 1 Top 50 genes featured high-risk group

Name	Gene titles
UBE2T	Ubiquitin-conjugating enzyme E2T (putative)
NY-SAR-48	–
TIMELESS	Timeless homolog (Drosophila)
CKS2	CDC28 protein kinase regulatory subunit 2
LOC286434	–
MCOLN3	Mucolipin 3
ECT2	Epithelial cell transforming sequence 2 oncogenex
SGOL2	Shugoshin-like 2 (S. pombe)
CENPA	Centromere protein A
FAM83D	Family with sequence similarity 83, member D
SCARF1	Scavenger receptor class F, member 1
C12ORF48	Chromosome 12 open reading frame 48
TSPAN5	Tetraspanin 5
CENPF	Centromere protein F, 350/400 ka (mitosin)
****AURKA***	**Aurora kinase A**
RACGAP1	Rac GTPase activating protein 1
CCNB1	Cyclin B1
MPHOSPH9	M-phase phosphoprotein 9
GMNN	Geminin, DNA replication inhibitor
KIF18A	Kinesin family member 18A
LMNB2	Lamin B2
LOC643509	–
ANLN	Anillin, actin binding protein
CDCA3	Cell division cycle associated 3
ATAD2	ATPase family, AAA domain containing 2
RPP40	Ribonuclease P 40 kDa subunit
HMMR	Hyaluronan-mediated motility receptor (RHAMM)
PTTG3	Pituitary tumor-transforming 3
CDKN3	Cyclin-dependent kinase inhibitor 3
PTTG1	Pituitary tumor-transforming 1
EZH2	Enhancer of zeste homolog 2 (Drosophila)
CDK2	Cyclin-dependent kinase 2
SGOL1	Shugoshin-like 1 (S. pombe)
ZRANB3	Zinc finger, RAN-binding domain containing 3
UBE2S	Ubiquitin-conjugating enzyme E2S
UBE2C	Ubiquitin-conjugating enzyme E2C
UCK2	Uridine-cytidine kinase 2
HMGB3	High-mobility group box 3
FBXO5	F-box protein 5
DHFR	Dihydrofolate reductase
CHEK1	CHK1 checkpoint homolog (S. pombe)
CCNA2	Cyclin A2
BUB1	BUB1 budding uninhibited by benzimidazoles 1 homolog (yeast)
TACC3	Transforming, acidic coiled-coil containing protein 3
BIRC5	Baculoviral IAP repeat-containing 5 (survivin)
PBK	PDZ binding kinase
TRIP13	Thyroid hormone receptor interactor 13
CENPE	Centromere protein E, 312kda
CCNB2	Cyclin B2
C6ORF173	Chromosome 6 Open Reading Frame 173

Nineteen genes, marked as *bold form*, also were identified by Chibon et al. in a 67-gene signature called the complexity index in sarcomas (CINSARC signature).[15]

then generated using the top 50 genes of each phenotype (high risk vs. moderate/low risk; Fig. 1c).

AURKA Overexpression in the High-Risk Group Versus Moderate-/Low-Risk Group

Chibon et al. recently reported that a gene expression signature called complexity index in sarcomas (CINSARC), which is composed of 67 genes and could predict metastasis outcome in sarcoma.[15] By comparing this 67-gene signature with the top 50 genes in our high-risk phenotype, we identified 19 common genes (Table 1). Among them, *AURKA* caught our attention. *AURKA* is an important cell-cycle regulator, and several *AURKA* inhibitors are currently being evaluated in clinical trials.[16] Therefore, it is reasonable to study its role in the progression of primary GIST. We verified the difference in *AURKA* expression between the high- and moderate-/low-risk groups. As shown in Fig. 2, *AURKA* expression level was significantly higher in the high-risk group than in the moderate-/low-risk group (Fig. 2a, b). In addition, patients with high *AURKA* expression (above the 60th percentile of average Z score for all *AURKA* probes, $N = 13, 41$ %) had significantly worse RFS than those with low *AURKA* expression ($N = 19, 59$ %; Fig. 2c).

High AURKA Expression as an Independent Poor Prognostic Factor for GIST Recurrence

We then examined *AURKA* expression using IHC in another set of GIST patients. A total of 142 patients with primary GIST were enrolled, and their clinicopathological characteristics are summarized in Table 2. The mean age of these patients was 58 years, and the gender ratio was similar. Approximately 60 % of the patients had gastric tumors, and more than half of them had large tumors (>5 cm). More than 60 % of these tumors had a low mitotic count (<5/50 HPF). According to the AFIP-Miettinen criteria, 66 (46.5 %) patients were classified as low risk (originally very low and low risk), 27 (19 %) were classified as moderate risk, and 49 (34.5 %) were classified as high risk.[9,10]

The *AURKA* IHC staining pattern showed mainly cytoplasmic expression, which is similar to previous reports.[17–21] Fifty-four (38 %) cases were classified as having high *AURKA* expression based on our definition (Supplementary Fig. S1). *AURKA* expression was significantly higher in nongastric tumors than in gastric tumors and was

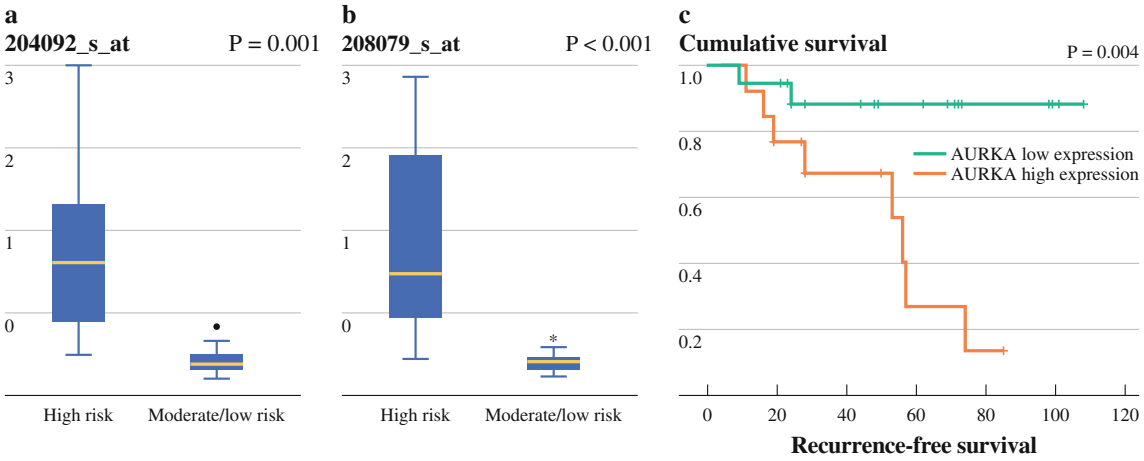

FIG. 2 Expression of aurora kinase A (*AURKA*) in the high- and moderate-/low-risk groups. **a**, **b** The expression of 2 probes coding *AURKA* was significantly higher in the high-risk group than in the moderate-/low-risk group. **c** Patients with high *AURKA* expression of had significantly worse recurrence-free survival (RFS) than those with low *AURKA* expression

TABLE 2 Clinicopathological characteristics of 142 GIST patients and their association with Aurora kinase A (*AURKA*) IHC intensity

Clinicopathological features	No. patients (%)	*AURKA* IHC score		*P* value
		<60	≥60	
Age (yr)				0.182
<60	82 (57.75)	47	35	
≥60	60 (42.25)	41	19	
Gender (M:F)				0.228
Male	75 (52.82)	43	32	
Female	67 (47.18)	45	22	
Location				<0.001*
Gastric	83 (58.45)	65	18	
Nongastric	59 (41.55)	23	36	
Tumor size (cm)				0.113
<5	65 (45.77)	46	19	
5–10	47 (33.1)	27	20	
>10	30 (21.13)	15	15	
Mitotic count (HPF)				0.66
<5/50	90 (63.38)	57	33	
≥5/50	52 (36.62)	31	21	
AFIP risk				0.017*
Low	66 (46.48)	49	17	
Moderate	27 (19.01)	15	12	
High	49 (34.51)	24	25	
Recurrence events				0.007*
Recurrence	44 (30.99)	20	24	
None	98 (69.01)	68	30	
AURKA IHC score				
Low (IHC score <60)	88 (61.97)	–	–	–
High (IHC score ≥60)	64 (45.07)	–	–	

* $P < 0.05$

TABLE 3 Univariate and multivariate analyses of the prognostic influence of clinicopathological factors on recurrence-free survival

Factors	Median survival months (95 % CI)	Univariate analysis			Multivariate analysis		
		HR	95 % CI	*P* value	HR	95 % CI	*P* value
Age (year)							
<60	124.97 (89.79–160.14)	1		0.504			
≥60	Not achieved	1.228	0.672–2.243				
Gender							
Female	Not achieved	1		0.056			0.309
Male	124.97 (46.22–203.71)	1.824	0.984–3.381				
Location							
Gastric	Not achieved	1		0.265			
Nongastric	110.27 (69.72–150.81)	1.433	0.761–2.697				
Tumor size (cm)							
<5	124.97	1		<0.001*	1		0.017*
5–10	99.87 (52.17–147.56)	3.771	1.470–9.673		2.74	0.85–8.87	
>10	21.67 (15.17–28.17)	12.895	5.198–31.987		5.94	1.63–21.57	
Mitotic count (HPF)							
<5/50	Not achieved	1		<0.001*	1		0.007*
≥5/50	33.97 (15.21–52.73)	9.933	4.418–22.333		4.89	1.53–15.63	
AFIP risk							
Low	Not achieved	1		<0.001*			0.557
Moderate	Not achieved	5.794	1.122–29.915				
High	30.8 (17.57–44.04)	26.629	6.389–110.985				
AURKA IHC score							
<60	124.97	1		0.015*	1		0.039*
≥60	82.2 (35.37–129.03)	2.094	1.155–3.796		1.99	1.034–3.81	

HR hazard ratio, *CI* confidence interval

* $P < 0.05$

significantly correlated with AFIP-Miettinen risk groups (Table 2). A total of 44 patients suffered from recurrence, including 11 locoregional relapses, 23 distant metastases, and 10 multiple site recurrences. Patients with high *AURKA* expression had a significantly higher risk of recurrence (Table 2). Univariate analysis showed that the RFS of all 142 cases was significantly influenced by tumor size, mitotic count, AFIP-Miettinen risk group classification, and *AURKA* expression level (Table 3). The Kaplan-Meier RFS curves according to these four factors are shown in Fig. 3. However, only tumor size ($P = 0.017$), mitotic count ($P = 0.007$), and *AURKA* expression ($P = 0.039$) were identified as independent unfavorable prognostic factors for RFS in multivariate analysis (Table 3).

DISCUSSION

In this study, through a reanalysis of clinical and microarray data, we categorized a group of GIST patients into two risk groups with distinct survival and expression profiles.[13] *AURKA* was among the 19 genes common to the top 50 genes in the high-risk phenotype and the CINSARC signature.[15] *AURKA* overexpression was confirmed in the high-risk group, and patients with high *AURKA* expression had significant worse RFS than those with low expression. In the IHC-validated cohort of 142 GIST patients, *AURKA* overexpression was found in 38 % of cases, and both univariate and multivariate analysis confirmed *AURKA* as an independent prognostic factor for GIST recurrence.

In a study by Yamaguchi et al., no distinct expression profiles of different risk groups (based on grade, mitotic count, and size) or risk categories (based on mitotic count and size) were identified.[13,22] However, using modified AFIP-Miettinen criteria, which included mitotic index, tumor size, and location (Supplementary Table S1), we were able to identify two risk groups—high and moderate/low risk—which had significant differences in survival. Moreover, these groups were found to have distinct expression profiles. It is interesting to note that by comparing the top 50 genes of the high-risk phenotype in our study with the CINSARC signature, we identified 19 common genes.[15] Most of these genes are involved in cell-cycle regulation (Table 1). This is not surprising, because the cutoff value of mitotic count used in the study by Yamaguchi et al. was 5 per 10 HPF, rather than 50 HPF, which may enrich the significance of genes associated with the cell-cycle and its regulation.[13] Most of the genes in the CINSARC signature are also related to mitosis and chromosome management.[15]

The prognostic influence of cell-cycle–related genes on GIST has been previously reported. Overexpression of

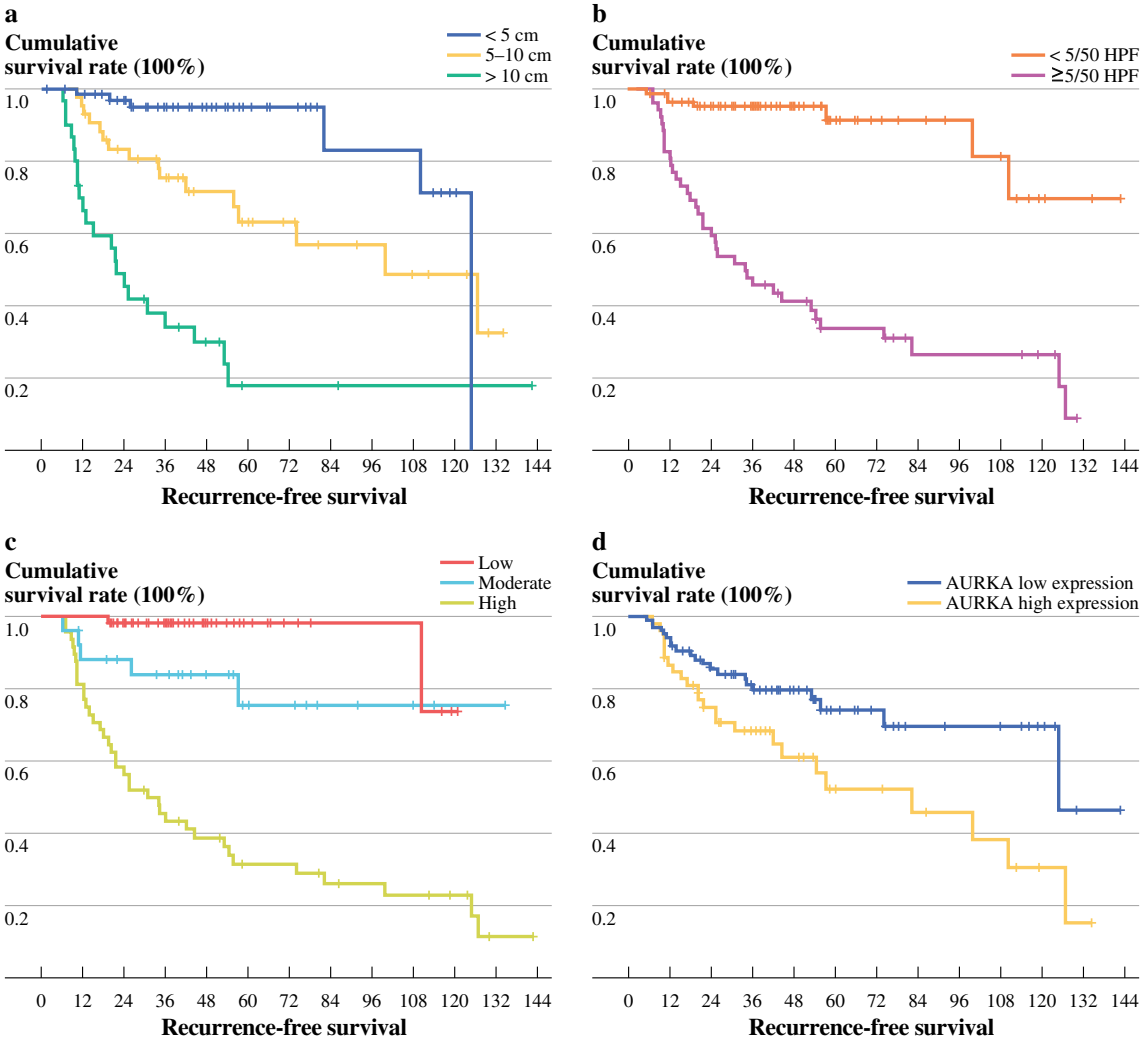

FIG. 3 Kaplan–Meier plot of recurrence-free survival of 142 gastrointestinal stromal tumors (GIST) patients according to **a** tumor size; **b** mitotic index; **c** AFIP-Miettinen risk group; **d** *AURKA* IHC signal intensity. The *P* values for survival comparison, obtained by the log-rank test, were all less than 0.05

CCNA2 and *CDK2* was found more often in cases of the moderate- to high-risk groups, and patients with up-regulation of *CCNA2* and *CDK1* had short progression-free survival.[23,24] *CCNA2* and *CDK2* also were among the top 50 genes in the high-risk phenotype in this study (Table 1). Conversely, impaired *TP53*, *CDKN2A*, *BCL2*, and *CHK2* expression is common in advanced disease.[25] *AURKA*, 1 of the 19 common genes, and the focus of our study, is a key regulator of cell-cycle events from late S phase through M phase, and a bona fide oncogene.[12,26,27] *AURKA* overexpression has been found frequently in a variety of cancers, and often is associated with advanced disease or poor prognosis.[17,21,28–32] Jeng et al. found that *AURKA* overexpression was associated with high-grade and high-stage disease in hepatocellular carcinoma.[29] Comperat et al. showed that *AURKA* was a sensitive marker for predicting tumor recurrence, especially for pTa of urinary bladder cancer.[30] Xu et al. showed that *AURKA* expression was significantly higher in poorly differentiated lung cancers than in well-differentiated or moderately differentiated lung cancers.[32] In the patient cohort from the study by Yamaguchi et al., we found that *AURKA* was significantly overexpressed in high-risk patients.[13] Moreover, patients with high *AURKA* expression had significantly worse RFS than those with low *AURKA* expression.

Among the validated group of 142 GIST cases, 54 (38 %) were classified as having high *AURKA* expression by IHC. The *AURKA* expression level was significantly correlated with AFIP-Miettinen risk group (Table 2), which is consistent with the microarray findings (Fig. 2). Furthermore, we showed that high *AURKA* expression was more frequent in nongastric tumors. This association was not identified in the microarray analysis, probably due to the limited number of cases of nongastric origin in the

microarray patient cohort. These results indicated that *AURKA* expression correlated with the high-risk clinicopathological features of GIST.

High *AURKA* expression was associated with a higher probability of recurrence (Table 2) and is a worse prognostic factor for RFS in univariate analysis (Table 3; Fig. 3d). Tumor size, mitotic count, and AFIP-Miettinen risk group were three other prognostic factors for RFS in univariate analysis. These findings were consistent with previous reports.[8,14] However, tumor location failed to exhibit a prognostic influence. In multivariate analysis, only tumor size, mitotic count, and *AURKA* expression were shown to be independent prognostic factors for RFS in primary GIST cases. This study once again demonstrated the importance of mitotic count and tumor size as prognostic factors for GIST. In addition, *AURKA*, a cell-cycle regulator, was shown to be an important prognostic factor of recurrence for primary GIST.

This study has several limitations. First, the relatively low number of recurrences may lower the probability of identifying significant prognostic factors in multivariate analysis. Second, it was not possible to determine the impact of *AURKA* on overall survival (OS) in this study, because OS would be influenced by postrecurrence imatinib treatment. We are currently planning another study to clarify the impact of *AURKA* expression on the survival of advanced GIST patients receiving imatinib therapy.

In this study, we explored only the association between survival and *AURKA* IHC staining intensity in the validated cohort and did not evaluate *AURKA* expression using other techniques. Recently, Lagarde et al. showed that the CINSARC signature could be used to predict metastasis in a new dataset of 67 primary untreated GISTs. The gene whose expression was most strongly associated with metastasis was *AURKA*. Their result was further confirmed by qRT-PCR.[33] More importantly, both their study and our study showed that the *AURKA* expression level (either by qRT-PCR or IHC) as an independent prognostic factor for GIST recurrence.

In conclusion, by integrating bioinformatics and clinicopathological studies, we identified *AURKA* as a marker for poor-risk GIST. Further studies are needed to explore its role in advanced GIST and as a treatment target.

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CONFLICT OF INTEREST None declared.

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