

Role of PLK1 signaling pathway genes in gastrointestinal stromal tumors

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Received November 11, 2017; Accepted May 14, 2018

DOI: 10.3892/ol.2018.9003

Abstract. In previous studies by the authors, aurora kinase A (AURKA) was demonstrated as an independent poor prognostic marker for the recurrence of localized gastrointestinal stromal tumors (GISTs) and for the progression of advanced GISTs. In the present study, the prognostic effect of genes involved in cell cycle regulation in GISTs was further examined. Leading edge analysis in gene set enrichment analysis was used to identify the most common genes in the top 10 enriched gene sets of high-risk patients with GISTs in a Japanese study. The obtained gene list was uploaded to the Pathway Interaction Database to search for critical pathways. Selected genes within the pathway were subsequently verified through immunohistochemistry (IHC) in another cohort of patients. A total of 5 genes in 'PLK1 signaling events,' namely AURKA, polo-like kinase 1 (PLK1), cell division cycle 25C (CDC25C), budding uninhibited by benzimidazoles (BUB1), and targeting protein for Xklp2 (TPX2), were identified

for subsequent study. Among the Japanese cohort, all 5 genes, except BUB1, were significant prognostic factors for poor recurrence-free survival (RFS). Among 141 patients enrolled for the IHC study, all 5 genes exhibited variable expression patterns. In the association study, only AURKA exhibited significant overexpression in non-gastric tumors. Although all 5 genes were considered as risk factors for poor RFS based on a univariate analysis, only the mitotic count and expression levels of CDC25C, BUB1, and TPX2 retained prognostic effects in the multivariate analysis. The PLK1 signaling pathway is crucial in the disease progression of GISTs. Genes within this pathway may serve as predictive markers for adjuvant therapy.

Introduction

Gastrointestinal stromal tumors (GISTs) are the most frequently observed intra-abdominal sarcomas and occur primarily from the gastrointestinal tract or less commonly from the mesentery and retroperitoneum (1-4). The majority of cases are characterized by an activated mutation of the receptor tyrosine kinase, KIT (5), or platelet-derived growth factor receptor α (6). Patients with advanced GISTs were successfully treated with imatinib mesylate, a potent tyrosine kinase inhibitor (TKI), with a median overall survival period of 5-6 years. However, acquired resistance mainly resulting from secondary mutation inevitably developed within 2-3 years after treatment (7,8). Salvage TKIs, including sunitinib maleate and regorafenib, yielded less satisfactory results (9,10). Therefore, novel biomarkers or treatment targets for TKI-refractory cases are required.

Genes that regulate cell cycle have a crucial role in predicting a prognosis for soft tissue sarcomas (11,12). By reanalyzing available expression profiling data on GISTs (13), our group has previously identified gene sets, including the cell cycle process or its regulation, that were strongly associated with the risk of recurrence. Aurora kinase A (AURKA) was identified as an independent poor prognostic marker for GIST

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Key words: aurora kinase A, budding uninhibited by benzimidazoles, cell division cycle 25C, gastrointestinal stromal tumor, polo-like kinase 1, targeting protein for Xklp2

recurrence (14). A subsequent study by the authors confirmed that AURKA may also serve as an independent unfavorable marker for predicting disease progression or mortality from advanced GISTs and as a potential treatment target for GISTs (15). The aforementioned studies have implicated the crucial role of genes that regulate cell cycle in sarcomas and GISTs. In the present study, the prognostic effect of genes involved in cell cycle regulation in GISTs was examined.

Patients and methods

Bioinformatic analysis. In a previous study by the authors (14), using gene set enrichment analysis (GSEA; downloaded from <http://www.broadinstitute.org/gsea/index.jsp>), 32 cases in Gene Expression Omnibus dataset GSE8167, previously reported by Yamaguchi *et al* (13), were classified into two risk groups with distinct recurrence-free survival (RFS) and expression profiles according to the modified criteria of Yen *et al* (14) from the Armed Forces Institute of Pathology (AFIP). Of the 715 Gene Ontology (GO) gene sets, which exhibited differential expression between two risk groups, 316 were upregulated in the high-risk phenotype. To identify significant genes and pathways in these gene sets, leading edge analysis (LEA) was used in GSEA to examine genes in the leading edge subsets of the top 10 enriched gene sets (14). It was hypothesized that genes that appear in multiple subsets are more likely to be of interest than those that appear in only one subset. The obtained gene list was uploaded to the Pathway Interaction Database (PID; <https://wiki.nci.nih.gov/pages/viewpage.action?pageId=315491760>) for analyzing the distribution of the molecules within predefined pathways. The query uses a hypergeometric distribution, which models the probability of observing k genes from a cluster of n genes (network frequency) by chance in a pathway or biological process category containing m genes from a total genome size of N genes (genome frequency), to compute the probability that each pathway in the database is hit by molecules in the list. It then returns a list of pathways ordered by P-value, which indicates the probability that the specific pathway is enriched by chance (16). The expression level of each individual probe was obtained using Z-score transformation. For genes encoded by more than one probe, average Z-score values were used for comparison. The differences among the risk groups were subsequently compared using the t-test.

Tumor samples for immunohistochemistry study. This study is a retrospective study. A total of 141 patients who received the diagnosis of GIST between 1989 and 2008 at Chang Gung Memorial Hospital (Taoyuan, Taiwan) were enrolled for immunohistochemistry (IHC) study. These cases were reported previously (14); they were patients with localized GIST who had received surgical excision only with no adjuvant imatinib therapy, had formalin-fixed paraffin-embedded tissues available for IHC study and were regularly followed up with appropriate radiological imaging evaluations at Chang Gung Memorial Hospital. The protocols for tumor sample collection and clinical record review were approved by the Institutional Review Board of Chang Gung Memorial Hospital (IRB no. 98-0352B), and all patients had provided informed consent for the use of their tissues and clinical data

in research. All identifying information of individual patient was removed.

IHC staining of cell cycle regulation genes in GIST. A 4- μ m section of each specimen was stained for selected proteins. Primary antibodies used in the present study were anti-Aurora (catalog no. ANB100-212; 1:1,500) and anti-polo-like kinase 1 (PLK1; catalog no. NBPI-02851; 1:100) antibodies from Novus Biologicals, (LLC, Littleton, CO, USA) as well as anti-cell division cycle 25C (CDC25C; catalog no. ab66235; 1:100), anti-budding uninhibited by benzimidazoles (BUB1; catalog no. ab4636; 1:100), and anti-targeting protein for Xklp2 (TPX2; catalog no. ab32795; 1:1,000) antibodies from Abcam, (Cambridge, UK). The antibodies were diluted as suggested and added to the slides, which were incubated overnight at 4°C. The slides were then washed three times for 5 min each in a mixture of tris-buffered saline and Polysorbate 20 (the mixture is referred to as 'TBST' hereafter) prior to visualization using the LSAB2 system, Peroxidase (K0675; DAKO A/S). The control slides were incubated with the secondary antibody alone [also contained within the LSAB2 system, Peroxidase (K0675; DAKO A/S)]. After 3 TBST washes for 5 min for each wash, the slides were mounted and blindly analyzed under microscopy by the authors. Immunostaining was scored.

For the assessment of IHC staining, the percentage of stained target cells was evaluated in 10 optical microscope fields per tissue section (magnification, x400) and the average staining percentage was calculated. For BUB1 and CDC25C, only nuclear staining was considered positive. Staining intensity was scored as 1 (mild), 2 (moderate), or 3 (intense). H-scores were calculated as the percentage of positive staining (0-100%) x the corresponding staining intensity (0-3) (17). The specimens with low and high H-scores were classified as having low and high IHC expression, respectively (cutoff value of H-scores: AURKA=60, TPX2=40, BUB1=50, CDC25C=50, and PLK1=60; Fig. 1).

Statistical and survival analysis. The associations between clinicopathological variables and IHC staining patterns of cell cycle regulation genes were analyzed using the χ^2 test or Fisher's exact test for univariate analysis and using multinomial logistic regression for multivariate analysis. RFS was measured from the date of surgery to the date of tumor recurrence documentation. Survival analysis was estimated using the Kaplan-Meier method, and the log-rank test was conducted for survival curve comparison. The prognostic effect of clinicopathological factors and the IHC staining of genes that regulate cell cycle was determined by univariate and multivariate (stepwise forward conditional method) Cox regression analysis. In two-sided tests, $P < 0.05$ was considered statistically significant. SPSS software (version 10.0; SPSS, Chicago, IL, USA) was used for all statistical analyses.

Results

Bioinformatic analysis identifies 5 critical genes and their expression levels are associated with risk groups and survival. Among the top 10 GO gene sets (14), 15 genes were found in ≥ 9 of the sets (Table I). These 15 genes were uploaded to the

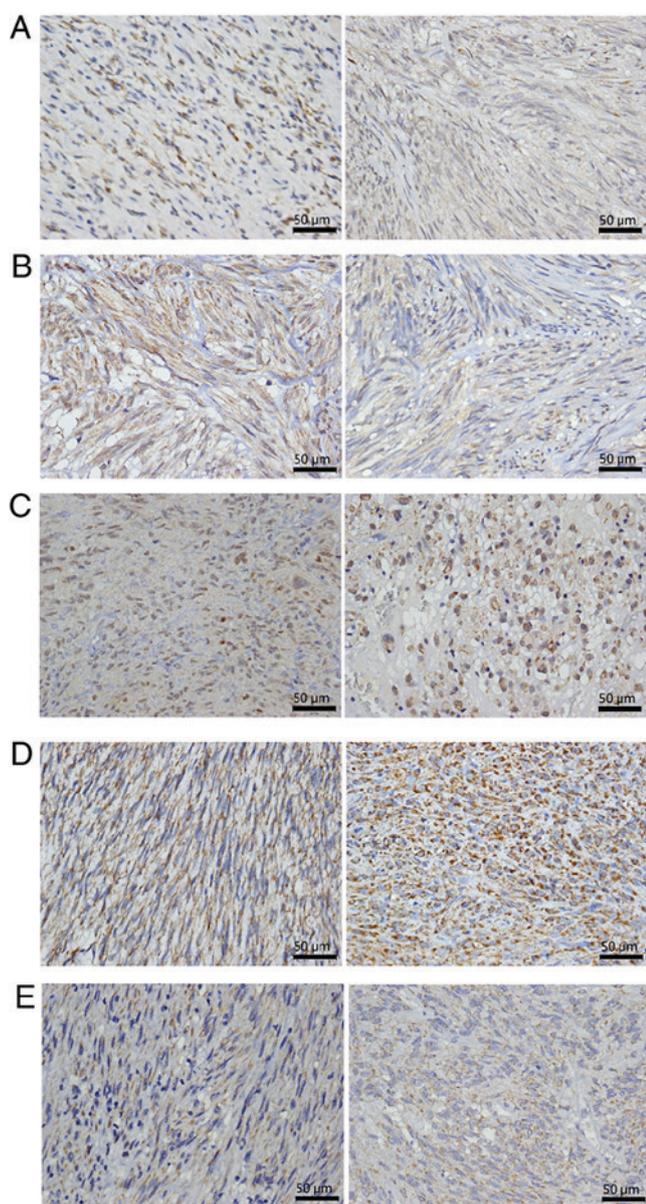


Figure 1. Examples of positive immunostaining of 5 genes (two figures are provided for each gene): (A) AURKA; (B) BUB1; (C) CDC25C; (D) PLK1; (E) TPX2. AURKA, aurora kinase A; BUB1, budding uninhibited by benzimidazoles 1 homolog; CDC25C, cell division cycle 25C; PLK1, polo like kinase 1.

PID, and 6 significant pathways were predicted. Among these pathways, 'PLK1 signaling events' was the most significant. A total of 5 genes-AURKA, PLK1, CDC25C, BUB1, and TPX2-were present in this pathway (Table II). These 5 genes were also identified in ≥8 GO gene sets that were associated with cell cycle regulation identified by gene set enrichment analysis (GSEA) (Table I). Therefore, these 5 genes were selected for further study.

Differences in the expression levels of these 5 genes between the AFIP high-risk group and moderate- and low-risk groups were verified. As indicated in Fig. 2, the expression levels of the 5 genes, except for CDC25C, were significantly higher in the high-risk group compared with the moderate- and low-risk groups. Cox regression analysis revealed that patients with a high expression (Z score >0) of AURKA, PLK1, CDC25C or TPX2 had significantly lower RFS compared with those with

Table I. Identification of 15 genes by GSEA and the presence of these genes in the top 10 enriched gene sets.

	KNTC1	TTK	NEK2	BUB1 ^a	BIRC5	AURKA ^a	MAD2L1	ZWINT	ANLN	PLK1 ^a	KIF2C	KIF11	KIF15	TPX2 ^a	CDC25C ^a
Mitosis	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V
Cell cycle process	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V
Mitotic cell cycle	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V
M phase of mitotic cell cycle	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V
Cell cycle phase	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V
M phase	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V
Microtubule cytoskeleton	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V
Cell cycle GO 0007049	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V
Regulation of mitosis	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V
Non-membrane bound organelle	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V

^aV indicates the presence of gene in the GSEA gene sets. ^aGenes in the 'PLK1 signaling events' identified by Pathway Interaction Database; GSEA, gene set enrichment analysis; ANLN, anillin actin binding protein; AURKA, aurora kinase A; BUB1, budding uninhibited by benzimidazoles 1 homolog; CDC25C, cell division cycle 25C; KIF11, kinesin family member 11; KIF15, kinesin family member 15; KNTC1, kinetochore associated 1; MAD2L1, mitotic arrest deficient 2 like 1; NEK2, NIMA related kinase 2; TPX2, targeting protein for Xklp2; TTK, phosphotyrosine picked threonine kinase; ZWINT, ZW10 interacting kinetochore protein.

Table II. Pathways identified by the Pathway Interaction Database.

Pathway name	Biomolecules in the group	P-value
PLK1 signaling events	AURKA, BUB1, CDC25C, PLK1 and TPX2	2.16x10 ^{-9a}
Aurora B signaling	AURKA, BIRC5, BUB1 and KIF2C	2.05x10 ^{-7a}
Aurora A signaling	AURKA, BIRC5 and TPX2	8.55x10 ^{-6a}
FOXM1 transcription factor network	BIRC5, NEK2 and PLK1	2.16x10 ^{-5a}
ATR signaling pathway	CDC25C and PLK1	0.00109 ^a
p73 transcription factor network	BUB1 and PLK1	0.00474 ^a

^aP<0.05 (hypergeometric distribution). AURKA, aurora kinase A; BUB1, budding uninhibited by benzimidazoles 1 homolog; CDC25C, cell division cycle 25C; TPX2, targeting protein for Xklp2; PLK1, polo like kinase 1.

Table III. Univariate analysis of the prognostic effect of expression level of the 5 genes on recurrence-free survival.

Gene	Median survival, months (95% CI)	HR	Univariate analysis, 95% CI	P-value
AURKA				
Low	Not reached	1		0.013 ^a
High	56.000 (21.635-90.365)	7.242	1.527-34.345	
PLK1				
Low	Not reached	1		0.006 ^a
High	53.000 (10.278-95.722)	8.727	1.847-41.221	
CDC25C				
Low	Not reached	1		0.049 ^a
High	53.000 (6.515-99.485)	3.685	1.008-13.475	
BUB1				
Low	Not reached	1		0.082
High	53.000 (7.269-98.731)	112.965	0.549-23248.895	
TPX2				
Low	Not reached	1		0.001 ^a
High	28.000 (16.500-39.500)	14.204	2.994-67.390	

^aP<0.05, univariate Cox regression analysis. AURKA, aurora kinase A; BUB1, budding uninhibited by benzimidazoles 1 homolog; CDC25C, cell division cycle 25C; CI, confidence interval; HR, hazard ratio; TPX2, targeting protein for Xklp2.

low expression (Z score ≤ 0) of these 4 genes. The differences in survival between patients with different expression levels of BUB1 were of borderline significance (P=0.082) (Table III).

Demographic and clinicopathological characteristics of patients enrolled for IHC study. To validate the data from microarray analysis, the expression of the 5 genes (AURKA, PLK1, CDC25C, BUB1 and TPX2) were examined by using IHC in another group of patients with GIST. The demographic data and clinicopathological features of the 141 patients are listed in Table IV. In total, the group contained 74 men and 67 women with a mean age of 57.9 years (range, 22-89 years). The stomach constituted the most common tumor sites, and the mean tumor size was 7 cm. Under the AFIP criteria, ~35% of the patients were considered high risk.

Association between the clinicopathological features and expression of the 5 genes in the IHC-validated patient cohort.

All 141 patients had IHC staining for AURAK and BUB1. Due to limited availability of tissue slides, IHC staining for TPX2, CDC25C and PLK1 were performed in 95, 121 and 140 patients, respectively. The association between the clinicopathological characteristics and expression pattern of the 5 genes in the IHC-validated patient cohort is illustrated in Tables V and VI. Higher AURKA expression was associated with non-gastric locations and a higher expression of BUB1, CDC25C and PLK1. Higher TPX2 expression was also associated with higher expression of BUB1, CDC25C and PLK1. Higher BUB1 IHC staining was associated with higher expression of 4 other mitotic check proteins similar to CDC25C and PLK1.

Multivariate analysis was performed in the 80 patients who had IHC for all 5 genes analyzed using multinomial logistic regression revealed that higher AURKA expression was independently associated with non-gastric locations and higher PLK1 expression. TPX2 staining was independently associated with higher PLK1 expression. BUB1 expression

Table IV. Clinicopathological characteristics of 141 patients with gastrointestinal stromal tumors.

Clinicopathological features	Patients, n (%)
Mean age (years, mean \pm SD)	57.9 \pm 12.6
Sex (M:F)	74:67
Location	
Gastric	83 (58.9)
Non-gastric	58 (41.1)
Tumor size (cm, mean \pm SD)	7.06 \pm 4.93
Mitotic count, HPF	
<5/50	89 (63.1)
5-10/50	16 (11.3)
>10/50	36 (25.5)
AFIP risk	
None	10 (7.1)
Very low	28 (19.9)
Low	27 (19.1)
Moderate	27 (19.1)
High	49 (34.8)
AURKA immunostaining	
Low	73 (51.8)
High	68 (48.2)
TPX2 immunostaining	
Low	64 (67.4)
High	31 (32.6)
BUB1 nuclear immunostaining	
Low	64 (45.4)
High	77 (54.6)
CDC25C nuclear immunostaining	
Low	55 (45.5)
High	66 (54.5)
PLK1 immunostaining	
Low	79 (56.4)
High	61 (43.6)

AFIP, Armed Forces Institute of Pathology; AURKA, aurora kinase A; BUB1, budding uninhibited by benzimidazoles 1 homolog; CDC25C, cell division cycle 25C; F, female; HPF, high-power fields; M, male; SD, standard deviation.

was independently associated with higher CDC25C and PLK1 expression. Higher CDC25C expression was independently associated with higher BUB1 expression. Higher PLK1 expression was independently associated with higher TPX2 and BUB1 expression (Table VII).

Prognostic effect of clinicopathological factors and expression of 5 genes on RFS. A total of 43 patients experienced recurrence, consisting of 11 locoregional relapses, 22 distant metastases and 10 multiple site recurrences. The univariate analysis indicated that the RFS was significantly affected by the tumor size, mitotic count, AFIP risk group classification, and expression level of the 5 aforementioned genes

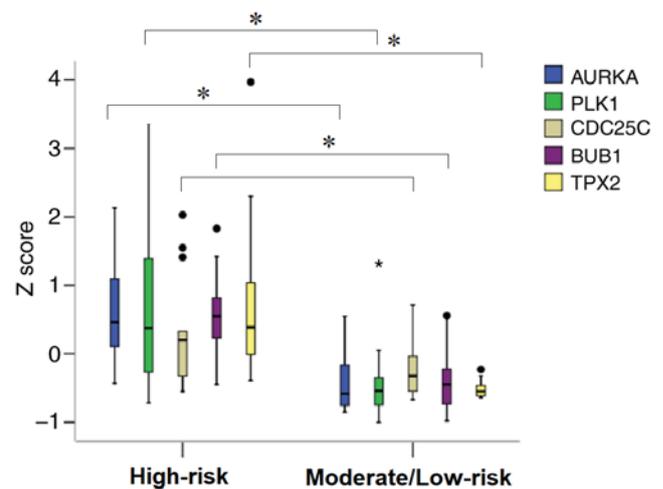


Figure 2. Comparison of expression level (indicated by Z scores) of 5 genes, AURKA, PLK1, CDC25C, BUB1 and TPX2, between the AFIP high- and moderate-/low-risk groups in 32 gastrointestinal stromal tumors (GIST) cases in the Japanese cohort using the t-test. With the exception of CDC25C, the expression level of the other 4 genes were significantly higher in the high-risk group compared with the moderate-/low-risk group. *P<0.05. AFIP, Armed Forces Institute of Pathology; AURKA, aurora kinase A; BUB1, budding uninhibited by benzimidazoles 1 homolog; CDC25C, cell division cycle 25C; PLK1, polo like kinase 1.

(Table VIII). However, only the mitotic count (P<0.001) and expression levels of TPX2 (P=0.008), BUB1 (P=0.023), and CDC25C (P=0.017) were identified as independent unfavorable prognostic factors for RFS through multivariate analysis (Table VIII). The Kaplan-Meier RFS curves for these 4 factors are shown in Fig. 3.

Discussion

In the present analysis, LEA in GSEA was used to examine the genes in the leading edge subsets of the top 10 enriched gene sets and the resultant list of genes was uploaded to PID. A total of 5 genes-AURKA, PLK1, CDC25C, BUB1 and TPX2, were identified. In the patient cohort of dataset GSE8167, all but BUB1 were significant factors for poor RFS. In the IHC-validated patient cohort, only AURKA exhibited significant overexpression in non-gastric tumors when compared with gastric tumors (Table V). Although all 5 genes were considered risk factors for poor RFS based on the univariate analysis, only the expression levels of CDC25C, BUB1 and TPX2 together with mitotic count exhibited prognostic effects in the multivariate analysis.

Genes that are involved in the regulation of cell cycle have a crucial role in sarcomas. A prognostic gene expression signature-complexity index in sarcomas (CINSARC)-established by Chibon *et al* (12) and composed of 67 genes that are associated with chromosome integrity, mitotic control and genome complexity were able to predict metastasis outcomes. Similar findings were reported in studies of individual subtypes of sarcoma. In a study of uterine leiomyosarcomas (ULMSs) and their benign counterparts, Shan *et al* (18) showed that 26 of the 50 most overexpressed genes in ULMSs were involved in the regulation of mitosis and spindle function. On the other hand, our group (14) and Lagarde *et al* (11) have identified that genes

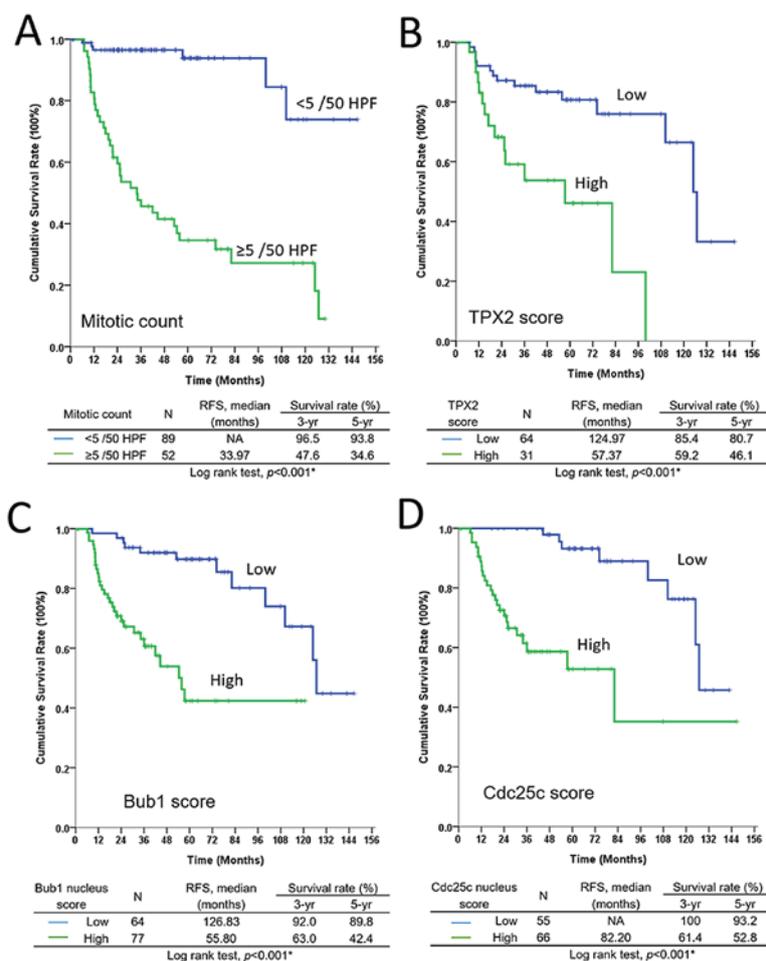


Figure 3. Kaplan-Meier plot of recurrence-free survival of patients with gastrointestinal stromal tumors according to: (A) Mitotic count (n=141); (B) TPX2 expression level (n=95); (C) BUB1 expression level (n=141); and (D) CDC25C expression level (n=121). The P-values for survival comparison, obtained by the log-rank test, were all < 0.05 . RFS, recurrence-free survival; TPX2, targeting protein for Xk; BUB1, budding uninhibited by benzimidazoles 1 homolog 1p2; CDC25C; cell division cycle 25C.

that are involved in the progression or regulation of the cell cycle are strong prognostic factors of GIST recurrence. Most notably, all three studies have revealed that AURKA as one of the most crucial genes associated with ULMS and GIST recurrence (11,14,18).

In the present study, instead of random selection, LEA in GSEA was used in combination with PID to identify biologically relevant genes/pathways in GISTs. LEA in GSEA was performed to find genes that frequently appeared in the top 10 enriched gene sets. PID was used to search genes that are involved in pathways that are important in GISTs. 'PLK1 signaling events' was identified as a crucial pathway in GISTs, and 5 genes-AURKA, PLK1, CDC25, TPX2, and BUB1-in this pathway were examined.

AURKA and PLK1 are the key members of the Aurora kinase family and polo-like kinase family, respectively. Both proteins mainly function in the G₂-M phase of the cell cycle. In the late G₂ phase of the cell cycle, AURKA and PLK1 are recruited to the centrosome. PLK1 promotes the recruitment of AURKA that binds to centrosomin, whereas AURKA is responsible for the initial activation of PLK1 in G₂ (19). TPX2 is a protein that interacts with AURKA and can activate AURKA in the late G₂ and M phases (19). CDC25C is a phosphatase that

is largely inactive in G₂. However, mitotic entry is triggered by a steep increase in cyclin B-cyclin-dependent kinase 1 activity, which is promoted by PLK1-activated CDC25C (19). BUB1 is a critical component of the spindle assembly checkpoint, and the BUB1-PLK1 kinase complex promotes spindle checkpoint signaling through the phosphorylation of cell division cycle protein 20 (20). AURKA and PLK1 are involved in cytokinesis (19,20).

In the study that examined the associations among the expressions of the 5 genes, the expression levels of PLK1, BUB1, and CDC25C were observed to be highly associated. This finding is most likely because of the interaction between PLK1 and CDC25C during mitotic entry and the formation of BUB1-PLK1 complex during spindle assembly. In addition, the expression levels of PLK1 was closely associated with that of AURKA, indicating the interactive function of these proteins in the G₂-M phase (Table VII).

Schaefer *et al* (21) recently identified a tumor suppressor gene called MYC-associated factor X (MAX) in GISTs. MAX is localized on chromosome 14q, one of the most frequently deleted sites in GISTs (22). Inactivated mutations of MAX can be found in sporadic GISTs and patients with GISTs and neurofibromatosis type 1. These inactivated mutations of

Table V. Association of IHC intensity of five genes with clinicopathological characteristics of patients with gastrointestinal stromal tumors.

Parameters	AURKA score, (%)		TPX2 score, (%)		BUBI score, (%)		CDC25C score, (%)		PLK1 score, (%)		
	Low	High	Low	High	Low	High	Low	High	Low	High	
Age											
<60	38 (46.3)	44 (53.7)	34 (63.0)	20 (37.0)	37 (45.1)	45 (54.9)	31 (41.9)	43 (58.1)	48 (58.5)	34 (41.5)	
≥60	35 (59.3)	24 (40.7)	30 (73.2)	11 (26.8)	27 (45.8)	32 (54.2)	24 (51.1)	23 (48.9)	31 (53.4)	27 (46.6)	
P-value	0.128		0.293		0.940		0.323		0.550		
Sex											
Male	35 (47.3)	39 (52.7)	35 (67.3)	17 (32.7)	33 (44.6)	41 (55.4)	23 (39.0)	36 (61.0)	40 (54.1)	34 (45.9)	
Female	38 (56.7)	29 (43.3)	29 (67.4)	14 (32.6)	31 (46.3)	36 (53.7)	32 (51.6)	30 (48.4)	39 (59.1)	27 (40.9)	
P-value	0.264		0.989		0.842		0.163		0.549		
Location											
Gastric	53 (63.9)	30 (36.1)	36 (62.1)	22 (37.9)	36 (43.4)	47 (56.6)	33 (47.8)	36 (52.2)	43 (52.4)	39 (47.6)	
Non-gastric	20 (34.5)	38 (65.5)	28 (75.7)	9 (24.3)	28 (48.3)	30 (51.7)	22 (42.3)	30 (57.7)	36 (62.1)	22 (37.9)	
P-value	0.001 ^a		0.168		0.565		0.546		0.258		
Tumor size, cm											
<5	35 (59.3)	24 (40.7)	32 (76.2)	10 (23.8)	29 (49.2)	30 (50.8)	31 (56.4)	24 (43.6)	37 (63.8)	21 (36.2)	
5-10	25 (48.1)	27 (51.9)	23 (63.9)	13 (36.1)	26 (50.0)	26 (50.0)	17 (38.6)	27 (61.4)	28 (53.8)	24 (46.2)	
>10	13 (43.3)	17 (56.7)	9 (52.9)	8 (47.1)	9 (30.0)	21 (70.0)	7 (31.8)	15 (68.2)	14 (46.7)	16 (53.3)	
P-value	0.288		0.193		0.161		0.077		0.275		
Mitotic count, HPF											
<5/50	45 (50.6)	44 (49.4)	43 (71.7)	17 (28.3)	44 (49.4)	45 (50.6)	39 (49.4)	40 (50.6)	53 (60.2)	35 (39.8)	
≥5/50	28 (53.8)	24 (46.2)	21 (60.0)	14 (40.0)	20 (38.5)	32 (61.5)	16 (38.1)	26 (61.9)	26 (50.0)	26 (50.0)	
P-value	0.706		0.242		0.207		0.236		0.238		
AFIP risk											
Low	38 (58.5)	27 (41.5)	31 (70.5)	13 (29.5)	32 (49.2)	33 (50.8)	32 (56.1)	25 (43.9)	37 (57.8)	27 (42.2)	
Moderate	14 (51.9)	13 (48.1)	13 (72.2)	5 (27.8)	15 (55.6)	12 (44.4)	9 (36.0)	16 (64.0)	17 (63.0)	10 (37.0)	
High	21 (42.9)	28 (57.1)	20 (60.6)	13 (39.4)	17 (34.7)	32 (65.3)	14 (35.9)	25 (64.1)	25 (51.0)	24 (49.0)	
P-value	0.256		0.586		0.152		0.084		0.576		

^aP<0.05 (χ^2 or Fisher's exact test). AFIP, Armed Forces Institute of Pathology; HPF, high-power fields; AURKA, aurora kinase A; BUB1, budding uninhibited by benzimidazoles 1 homolog; CDC25C, cell division cycle 25C; PLK1, polo like kinase 1. All 141 patients have IHC staining of AURAK and BUB1. Due to limited availability of tissue slides, IHC staining for TPX2, CDC25C and PLK1 were performed in 95, 121 and 140 patients, respectively.

Table VI. Association of IHC intensity between five genes.

	AURKA score, (%)		TPX2 score, (%)		BUB1 score, (%)		CDC25C score, (%)		PLK1 score, (%)	
	Low	High	Low	High	Low	High	Low	High	Low	High
AURKA score										
Low	-	-	29 (70.7)	12 (29.3)	41 (56.2)	32 (43.8)	35 (56.5)	27 (43.5)	50 (69.4)	22 (30.6)
High	-	-	35 (64.8)	19 (35.2)	23 (33.8)	45 (66.2)	20 (33.9)	39 (66.1)	29 (42.6)	39 (57.4)
P-value	-	-	0.542		0.008 ^a		0.013 ^a		0.001 ^a	
TPX2 score										
Low	-	-	-	-	34 (53.1)	30 (46.9)	28 (51.9)	26 (48.1)	41 (64.1%)	23 (35.9)
High	-	-	-	-	7 (22.6)	24 (77.4)	6 (23.1)	20 (76.9)	6 (19.4)	25 (80.6)
P-value	-	-	-	-	0.005 ^a		0.015 ^a		<0.001 ^a	
BUB1 score										
Low	-	-	-	-	-	-	37 (66.1)	19 (33.9)	51 (79.7)	13 (20.3)
High	-	-	-	-	-	-	18 (27.7)	47 (72.3)	28 (36.8)	48 (63.2)
P-value	-	-	-	-	-	-	<0.001 ^a		<0.001 ^a	
CDC25C score										
Low	-	-	-	-	-	-	-	-	41 (75.9)	13 (24.1)
High	-	-	-	-	-	-	-	-	26 (39.4)	40 (60.6)
P-value	-	-	-	-	-	-	-	-	<0.001 ^a	

^aP<0.05 (χ^2 or Fisher's exact test). AFIP, Armed Forces Institute of Pathology; HPF, high-power fields; AURKA, aurora kinase A; BUB1, budding uninhibited by benzimidazoles 1 homolog; CDC25C, cell division cycle 25C; PLK1, polo like kinase.

Table VII. Multivariate analysis of association of clinicopathological characteristics with IHC intensity of five genes in 80 GIST patients.

	AURKA score		TPX2 score		BUB1 score		CDC25C score		PLK1 score	
	Odds ratio	P-value	Odds ratio	P-value						
Age (<60/≥60)		NS		NS		NS		NS		NS
Gender (Male/Female)		NS		NS		NS		NS		NS
Location (Gastric/Non-gastric)	4.91 (2.02-11.94)	<0.001		NS		NS		NS		NS
Tumor size (<5 cm/5-10 cm/>10 cm)		NS		NS		NS		NS		NS
Mitotic count (<5/50 HPF/≥5/50 HPF)		NS		NS		NS		NS		NS
AFIP risk (Low/Moderate/High)		NS		NS		NS		NS		NS
AURKA score (Low/High)	-	-	1.73 (0.54-5.58)	0.358	1.29 (0.45-3.73)	0.633	1.67 (0.50-5.56)	0.405		
TPX2 score (Low/High)		NS	2.21 (0.59-8.26)	0.24	1.56 (0.46-5.34)	0.476	3.76 (1.05-13.51)	0.042		
BUB1 score (Low/High)	1.62 (0.63-4.16)	0.317	2.36 (0.62-8.94)	0.206	-	-	7.42 (2.27-24.29)	0.001		
CDC25C score (Low/High)	1.38 (0.57-3.34)	0.481	1.58 (0.46-5.42)	0.463	3.37 (1.05-10.76)	0.041	2.90 (0.88-9.58)	0.081		
PLK1 score (Low/High)	3.79 (1.41-10.18)	0.008	3.92 (1.07-14.35)	0.039	7.41 (2.26-24.34)	0.001	2.84 (0.87-9.30)	0.085		-

Multinomial logistic regression was used for statistical analysis. AFIP, Armed Forces Institute of Pathology; AURKA, aurora kinase A; BUB1, budding uninhibited by benzimidazoles 1 homolog; CDC25C, cell division cycle 25C; HPF, high-power fields; PLK1, polo like kinase 1; NS, not significant.

Table VIII. Univariate and multivariate analyses of the prognostic effects of clinicopathological factors on recurrence-free survival.

Factors	Median survival		Univariate analysis		Multivariate analysis	
	Months (95% CI)	HR	95% CI	P-value	HR	95% CI
Age						
<60 (n=82)	124.97 (88.15-161.78)	1		0.580		
≥60 (n=59)	Not achieved	1.189	0.644-2.194			
Sex						
Female (n=67)	Not achieved	1		0.104		
Male (n=74)	124.97 (52.27-197.66)	1.672	0.900-3.104			
Location						
Gastric (n=83)	Not achieved	1		0.262		
Non-gastric (n=58)	110.27 (66.15-154.39)	1.44	0.762-2.721			
Tumor size, cm						
<5 (n=59)	124.97 (104.1-145.83)	1		<0.001 ^a		>0.05
5-10 (n=52)	99.86 (59.64-140.10)	4.899	1.646-14.583			
>10 (n=30)	21.67 (15.17-28.17)	18.436	6.324-53.740			
Mitotic count. HPF						
<5 /50 (n=89)	Not achieved	1		<0.001 ^a	1	<0.001 ^a
≥5 /50 (n=52)	33.97 (15.05-52.88)	12.086	5.094-28.679		9.207	2.958-28.661
AFIP risk						
Low (n=65)	Not achieved	1		<0.001 ^a		>0.05
Moderate (n=27)	Not achieved	12	1.401-102.793			
High (n=49)	30.80 (17.57-44.04)	56.914	7.791-415.752			
AURKA score						
Low (n=73)	Not achieved	1		0.040 ^a		>0.05
High (n=68)	99.87 (53.50-146.23)	1.904	1.031-3.517			
TPX2 score						
Low (n=64)	124.97 (108.87-141.07)	1		0.001 ^a	1	0.008 ^a
High (n=31)	57.37 (22.95-91.78)	3.895	1.800-8.431		4.016	1.440-11.196
BUB1 score						
Low (n=64)	126.83 (122.04-131.63)	1		<0.001 ^a	1	0.023 ^a
High (n=77)	55.80 (39.99-71.61)	5.038	2.419-10.494		4.979	1.247-19.870
CDC25C score						
Low (n=55)	Not achieved	1		<0.001 ^a	1	0.017 ^a
High (n=66)	82.20 (22.76-141.64)	6.484	2.786-15.091		5.154	1.344-19.762
PLK1 score						
Low (n=79)	126.83 (122.23-131.43)	1		0.001 ^a	1	>0.05
High (n=61)	54.40 (35.35-73.45)	3.132	1.615-6.071			

Univariate and multivariate Cox regression analyses were performed. ^aP<0.05. AFIP, Armed Forces Institute of Pathology; HR, hazard ratio; CI, confidence interval; AURKA, aurora kinase A; BUB1, budding uninhibited by benzimidazoles 1 homolog; CDC25C, cell division cycle 25C; HPF, high-power fields; PLK1, polo like kinase 1.

MAX were also detected in micro-GISTs, indicating that it is an early event (21).

MAX is a binding partner of MYC and has been reported as a tumor suppressor in hereditary pheochromocytomas and small-cell lung cancer (23,24). Previous studies have demonstrated that MAX may impair MYC function by impairing the ability of MYC to bind to DNA (25,26). MYC is a crucial

regulator of cell cycle progression. Schaefer *et al* (21) demonstrated that the inactivation of MAX resulted in the silencing of the p16 gene, possibly via MYC activation (27). In addition, MYC induces cell proliferation that is generally associated with increases in the activity of CDK2, CDK4 and CDK6 to regulate G₁-S phase progression (28). Therefore, it was hypothesized that the inactivation of the MAX tumor suppressor occurs early

in GIST progression and leads to p16 inactivation and increased proliferation by enhancing G₁-S phase progression (21). Further cell cycle dysregulation in high-risk GISTs is most likely due to the overexpression of genes in the PLK1 signaling pathway, which may be a result of mutations of other tumor suppressor genes (29-31) with subsequent increased progression of the G₂-M phase and transition to high-grade cancer (22).

In addition to the expression levels of the aforementioned 5 genes, the clinicopathological factors that associated with recurrence included tumor size, mitotic count and AFIP risk group classification. This finding is reasonable as all these factors are considered risk factors for recurrence. In the multivariate analysis, only mitotic count and the expression levels of TPX2, BUB1 and CDC25C were identified as independent factors for poor RFS. TPX2, BUB1 and CDC25C have been previously reported to have prognostic effects on many types of cancer but not on sarcomas or GISTs (32-38). However, unexpectedly the multivariate analysis in the present study did not reveal AURKA and PLK1 to have statistically significant effects on RFS. In a previous study by the authors, AURKA expression was associated with non-gastric tumor (14). In the present study, after including the expression of 4 other genes in the multivariate analysis, AURKA expression remained independently associated with non-gastric locations. This result indicated that AURKA might be responsible for a distinct and more rapidly deteriorating clinical course of non-gastric GISTs.

Other molecules involved in cell cycle regulation that may be associated with the progression of GISTs have been previously reported. For example, in an European Organization for Research and Treatment of Cancer (EORTC) study, impaired p53, p16, BCL2 and CHK2 expression was commonly detected in advanced GISTs (29). Alteration of genes involved in G₂-M phase of cell cycle, including cyclin A, cyclin B1 and cdc2, were identified to be markers for predicting the aggressive behavior of GISTs in a Japanese study (39). These studies further supported the important roles of cell cycle regulators in GISTs.

There are limitations of statistical analysis in the present study. Due to limited availability of tissue slides, only AURKA and BUB1 IHC staining were done in all 141 patients. IHC staining for TPX2, CDC25C and PLK1 were done in 95, 121 and 140 patients, respectively. This definitely jeopardized the final analysis of this study. A total of 43 patients experienced recurrence. This relatively low number of recurrence may limit the statistical power of the study. Hopefully, there may be a larger cohort of patients through collaboration of multiple hospitals for further validation in the future. Another issue is that the possibility of multicollinearity (40) when using Cox regression model for multivariate analysis cannot be ruled out, since there is a high number of interactions between all the genes in cell cycle regulation. Nonetheless, CDC25C and BUB1, the two genes identified as independent prognostic factors, were also the only two genes that could be found in the GESA GO gene set 'regulation of mitosis' (Table I), indicating their critical roles in the disease. This result demonstrated that the present study was still able to identify important genes through Cox regression analysis.

In conclusion, through bioinformatics analysis and IHC validation, 5 genes-AURKA, PLK1, CDC25C, BUB1 and TPX2-in the PLK1 signaling pathway were identified as risk

factors for poor prognosis of GIST. AURKA was significantly overexpressed in non-gastric GISTs. All 5 genes were considered as risk factors for poor RFS based on the univariate analysis. The mitotic count and expression levels of CDC25C, BUB1 and TPX2 retained prognostic effects in the multivariate analysis. The results of the present study indicated that the PLK1 signaling pathway might be crucial in the disease progression of GISTs. Furthermore, genes in this pathway may serve as predictive markers for adjuvant therapy.

Acknowledgements

Not applicable.

Funding

The present study was supported by grants from the Department of Health in Taiwan (Center of Excellence for Cancer Research, Taipei Veterans General Hospital, grant nos. DOH99-TD-C-111-007 and DOH100-TD-C-111-007 and the National Research Program for Biopharmaceuticals, grant no. DOH100-TD-PB-111-TM026), from the National Science Council (grant nos. NSC 100-2314-B-075-081 and NSC 101-2314-B-075-029), from the Ministry of Science and Technology, (grant nos. MOST 103-2314-B-075-066, MOST 105-2314-B-075-059 and MOST 106-2314-B-075-065), from the Taipei Veterans General Hospital (grant nos. V97ER2-010, V98ER2-004, V102E8-003, V103E8-001, V101C-133, V102C-034, V103C-188, V104C-099, V104E16-001-MY3-1, V104E16-001-MY3-2, V104D16-001-MY3-3, V105C-094 and V106C-160) and from the Yen Tjing Ling Medical Foundation (grant nos. CI-100-19, CI-103-6 and CI-105-4) in Taiwan to Dr CC Yen, as well as by grants from the Bayer and the Chang Gung Medical Research Program XMRPG3D0411, XPRPG3D0412, XPRPG3D0413 and XPRPG3D0414 to Dr CN Yeh. Dr CC Yen and Dr CN Yeh were also partially supported by grants from the Taiwan Clinical Oncology Research Foundation as well as Chong Hin Loon Memorial Cancer and Biotherapy Research Center of National Yang-Ming University, and the Taiwanese Society of Molecular Medicine, respectively.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JC, CNY, CC and CCY were responsible for study design. SH was the pathologist and responsible for the pathology slide review and scoring. YC, KC and TY were members of the GISTs team from Chang Gung Memorial Hospital and responsible for clinical data collection. SC, TC, MY and YC were responsible for data analysis. JC and YC were also responsible for the completion of final manuscript.

Ethics approval and consent to participate

The protocols for tumor sample collection and clinical record review were approved by the Institutional Review Board of

Chang Gung Memorial Hospital (IRB no. 98-0352B), and all patients had provided informed consent for the use of their tissues and clinical data in research. All identifying information of individual patient was removed.

Patient consent for publication

Consent for publication of data and any associated images was received from patients.

Conflict of interest

The authors declare that they have no competing interest.

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