Heat Shock Protein 90 Overexpression Independently Predicts Inferior Disease-Free Survival with Differential Expression of the α and β Isoforms in Gastrointestinal Stromal Tumors

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Abstract

Purpose: Most gastrointestinal stromal tumors harbor a mutated KIT or PDGFRA receptor tyrosine kinase (RTK). Heat shock protein 90 (Hsp90) is a chaperone mediating the folding and stabilization of many oncoproteins, including KIT. An Hsp90 inhibitor, 17-AAG, can attenuate KIT activation and proliferation of gastrointestinal stromal tumor cell lines. We further evaluated Hsp90 immunoexpression and the difference between α and β isoforms in gastrointestinal stromal tumor specimens.

Experimental Design: Hsp90 immunostain was assessable in 306 cases on tissue microarrays of primary gastrointestinal stromal tumors and correlated with various variables and disease-free survival (DFS). RTK mutation variants, confirmed in 142 cases by sequencing with or without precedent denaturing high pressure liquid chromatography screening, were dichotomized into two prognostically different groups. Differential expression of transcript and protein isoforms was measured by real-time reverse transcription-PCR and Western blotting in 16 and 6 cases, respectively.

Results: Hsp90 overexpression (55%) significantly correlated with larger size, nongastric location, higher mitotic count and NIH risk level, Ki-67 overexpression (all $P \leq 0.001$), and unfavorable RTK genotypes ($P = 0.020$). It strongly portended inferior DFS univariately ($P < 0.0001$) and remained independent in multivariate analysis ($P = 0.031$; risk ratio, 2.44), along with high-risk category, Ki-67 overexpression, and old age. For both mRNA and protein, Hsp90β was more abundant than Hsp90α, whereas the latter was significantly higher in high-risk cases.

Conclusions: Hsp90 overexpression represents a poor prognosticator that correlates with several adverse parameters, highlighting its role in disease progression and alternative therapy for high-risk, imatinib-resistant gastrointestinal stromal tumors. Hsp90α seems more relevant to the intrinsic aggressiveness of gastrointestinal stromal tumors, albeit less abundant than Hsp90β.

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Gastrointestinal stromal tumors are believed to derive from interstitial Cajal cells or their precursors and constitute the most common mesenchymal tumors of the digestive tract (1, 2). Approximately 75% to 90% of gastrointestinal stromal tumors are characterized by activating mutations of the receptor tyrosine kinase (RTK) proto-oncogenes, mostly affecting *KIT* and rarely involving *PDGFRA*, which confer constitutive kinase activity to drive the development of gastrointestinal stromal tumors (2–4). Despite a better understanding of tumorigenesis, such understanding sometimes remains challenging in the prognostication of gastrointestinal stromal tumor. Although a consensus scheme has been recently proposed by the NIH and proved prognostically useful (1, 3, 5–7), the associations of tumor location and the RTK genotypes with outcomes underscores the need to assess new adjuncts to refine risk stratification and/or treatment strategies of gastrointestinal stromal tumors (3, 8–12). In most patients with advanced gastrointestinal stromal tumors, partial remission or disease stabilization can be achieved by imatinib treatment with a median duration of response of ~1.5 to 2 years (4, 13–15). This targeted therapy, however, is confronted with the

Translational Relevance

The management of imatinib-resistant gastrointestinal stromal tumors remains challenging, because secondary kinase domain mutations of the *KIT* and/or *PDGFRA* gene frequently develop during tumor evolution. Nevertheless, it remains unsatisfactory to use other tyrosine kinase inhibitors for achieving long-term remissions of gastrointestinal stromal tumors. Therefore, it is desirable to develop effective second-line therapies targeting alternative cellular pathways. The chaperone function of Hsp90 has been proven important to the expression and activation of the KIT oncoprotein, and its inhibitor, 17-AAG, can preferentially inhibit the mutant KIT in both imatinib-sensitive and imatinib-resistant gastrointestinal stromal tumor cell lines. The authors provide the first evidence from human gastrointestinal stromal tumor specimens that Hsp90 plays a crucial role in disease progression. Hsp90 expression level correlates with important prognosticators of gastrointestinal stromal tumors, including tumor location, size, mitotic count, NIH risk category, K-67 proliferative index, and mutation types of *KIT* and *PDGFRA* genes. More importantly, Hsp90 overexpression is independently predictive of worse disease-free survival, highlighting its role in disease progression and alternative therapy for high-risk, imatinib-resistant gastrointestinal stromal tumors. In this study, Hsp90β is identified as the more abundant isoform of the Hsp90 protein. However, Hsp90α isoform seems more relevant to the intrinsic aggressiveness of gastrointestinal stromal tumors, because its expression is significantly higher in high-risk cases.

challenge of drug resistance after an initial, short-lived response (4, 13–17). For imatinib-resistant gastrointestinal stromal tumors, sunitinib and several other tyrosine kinase inhibitors are currently undergoing clinical trials, although the median time-to-progression is only 6 months with sunitinib (4, 13, 15). Therefore, it is desirable to develop effective second-line therapies targeting alternative cellular pathways.

Heat shock protein 90 (Hsp90) proteins comprise two distinct isoforms, Hsp90α and Hsp90β, which are encoded by different genes at 14q32-33 and 6p21, respectively (18, 19). Forming homodimers and/or heterodimers in the cytosol, Hsp90 plays a central role in the multiprotein chaperone complex to mediate the correct folding, stabilization, and activation of more than 100 nascent client proteins, including multiple mutated and/or chimeric oncoproteins (19–21). In this manner, various essential processes of carcinogenesis are promoted in cancer cells with up-regulated Hsp90, such as evasion of apoptosis, increased cell proliferation, sustained angiogenesis, and invasive and metastatic properties, etc. (21). Recently, the chaperone function of Hsp90 was proved crucial to the expression and activation of the KIT oncoprotein, mainly by preventing the proteasome-mediated degradation of activated KIT (22). As compared with wild-type KIT, a small-molecule Hsp90 inhibitor, 17-AAG, was found to selectively inhibit the mutant KIT in both imatinib-sensitive and imatinib-resistant gastrointestinal stromal tumor cell lines (22). In this context, the availability of pharmacologic inhibitors may provide an alternative therapeutic opportunity for imatinib-resistant gastrointestinal stromal tumors with high expression of Hsp90.

Although previous studies have identified Hsp90 overexpression as a poor prognosticator in some cancer types (23), the expression status of Hsp90 has never been systematically assessed in human gastrointestinal stromal tumor specimens. By using tissue microarray–based immunohistochemistry, we therefore analyzed (1) the associations of Hsp90 expression with important prognostic variables of gastrointestinal stromal tumor, such as NIH risk category, proliferative index, and RTK genotypes, etc., and (2) whether Hsp90 overexpression confers an independent prognostic implication. Additionally, we also aimed to address the significance of differential expression of Hsp90α and Hsp90β in gastrointestinal stromal tumors. It is technically demanding, however, to attain this end by immunohistochemistry, because to our knowledge, no commercial antibody can reliably distinguish these two isoforms with considerable similarity (93%) in their amino acid sequences (18, 24). Therefore, we took advantage of the greater heterogeneity in transcripts for easier quantitative measurement of both Hsp90α and Hsp90β by real-time reverse transcription-PCR (RT-PCR). Furthermore, we also examined the specificity of proteins and expression ratio of α to β isoform by using isoform-specific antibodies applicable to Western blotting.

Materials and Methods

Patients and tumor materials

This retrospective study was done in accordance with the guidelines of the institutional review board (IRB0910-005). From the archives of two tertiary medical centers in Southern Taiwan (Chi-Mei Foundation Medical Center and Chang Gung Memorial Hospital), we identified primary localized gastrointestinal stromal tumors of 340 consecutive patients who had available tissue blocks, underwent surgical resection between January 1985 and December 2004, and did not receive imatinib treatment before disease relapses. All these 340 cases were confirmed to be gastrointestinal stromal tumors by CD117 and/or protein kinase C-Θ stains, which were then enrolled as previously described to construct tissue microarrays for immunochemical stain of Hsp90 (5). The clinicopathologic variables evaluated were detailed in our earlier studies (5, 25), including age, location, size, histologic type, mitotic count, and risk category of NIH consensus criteria, etc. Clinical information, including therapeutic course, was collected from medical charts and hospital tumor registries. To elucidate the differential expression of α versus β isoform in gastrointestinal stromal tumors, fresh specimens were quantitatively measured for mRNA expression levels of two isoforms. Six cases with more frozen tissues were also assessed by Western blotting with isoform-specific antibodies.

Tissue microarray-based immunohistochemistry for Hsp90

The tissue microarray sections were prepared and heated as previously described for antigen retrieval (5), followed by incubation for 1 h with the primary antibody of Hsp90 (68, 1:300; BD Pharmingen) and detection with ChemMate EnVision kit (K5001; DAKO). This antibody was raised against the COOH-terminal of total Hsp90 protein that could not distinguish between α and β isoforms. One pathologist (CFL) blinded to clinicopathologic, genotyping, and outcome data independently assessed Hsp90 expression of gastrointestinal stromal tumors included in tissue microarray slides. The percentage of tumor cells with moderate or strong cytoplasmic immunoreactivity was recorded; only cases containing two or more preserved tissue cores were scored, and scores of multiple cores from

the same patient were averaged to obtain a mean Hsp90 labeling index (LI). The cutoff of mean LI to define Hsp90 overexpression was cytoplasmic reactivity in ≧50% tumor cells (see statistical methods). Most tissue microarray blocks used for Hsp90 immunohistochemistry had also been previously sectioned and scored for Ki-67 staining in our earlier report, where the mean LI >5% was adopted as the cutoff to define Ki-67 overexpression (5).

Mutation analysis of the RTK genes

DNA extraction, PCR amplification, and direct sequencing of KIT exon 11. After trimming the nonneoplastic part, we adopted the DNAeasy kit (Qiagen) to extract genomic DNA from 8-μm sections of paraffin-embedded tumor tissues. In all samples, 100 ng of genomic DNA were amplified for *KIT* exon 11 using primer sets and thermal conditions as listed in Supplementary Table S1. The final volume of reaction mixture was 50 μL with 2.5 units Platinum Taq DNA Polymerase (Invitrogen), and the PCR products were purified and then bidirectionally sequenced using Big Dye Terminators on an ABI 3100 Prism Genetic Analyzer (Applied Biosystems). Those cases confirmed to carry a mutant *KIT* exon 11 were not studied further.

Denatured high-performance liquid chromatography screening for the remaining hotspot exons of* KIT *and* PDGFRA *genes. For those cases with wild-type *KIT* exon 11, the same amount of DNA was PCR-amplified for exons 9, 13, and 17 of *KIT* and exons 12 and 18 of *PDGFRA* using the primer sets and thermal conditions in Supplementary Table S1. For denaturing high-performance liquid chromatography screening, the purified PCR products were denatured at 95°C for 4 min to create heteroduplex and allowed for reannealing by lowering the temperature at a rate of -0.1°C/min to 25°C. Three to 8 μL of PCR products were then injected on a WAVE DNA Fragment Analysis System (Transgenomic) and eluted at a flow rate of 0.9 mL/min within a linear acetonitrile gradient by mixing buffer A and buffer B as previously described with minor modifications (26, 27). The elution temperatures of denaturing high-performance liquid chromatography used for individual hotspot exons are shown in Supplementary Table S1. The elution of DNA fragments was monitored with UV detector at 260 nm, and samples showing an aberrant elution profile were reamplified, purified, and sequenced. To verify the specificity of the denaturing high-performance liquid chromatography technique, selected cases with a normal elution profile were also sequenced for cross validation.

Real-time quantitative RT-PCR to measure the differential mRNA expression of Hsp90α and Hsp90β isoforms

To circumvent the contamination of the surrounding nonneoplastic cells, we adopted laser capture microdissection technology to isolate pure tumor cells. Three 7-μm sections were serially cut from snap-frozen tumor tissues, placed onto a polyethylene naphthalate-membrane slide, and stained with HistoGene LCM Staining Kit to isolate cells of interest by using the Veritas automated LCM system (Arcturus Engineering). Approximately 2,000 cells were collected onto the Capsure Macro cap, extracted by Picopure RNA isolation kit at 42°C for 30 min with 50 μL of extraction buffer, and then eluted by purification column (Arcturus Bioscience). The adjacent normal gastric muscular tissues from five snap-frozen specimens were also micro-dissected and extracted for RNA to serve as calibrator controls. The amount and concentration of RNA obtained were measured by a nanodrop spectrophotometer (SSP-3000; Infinigen). By using the

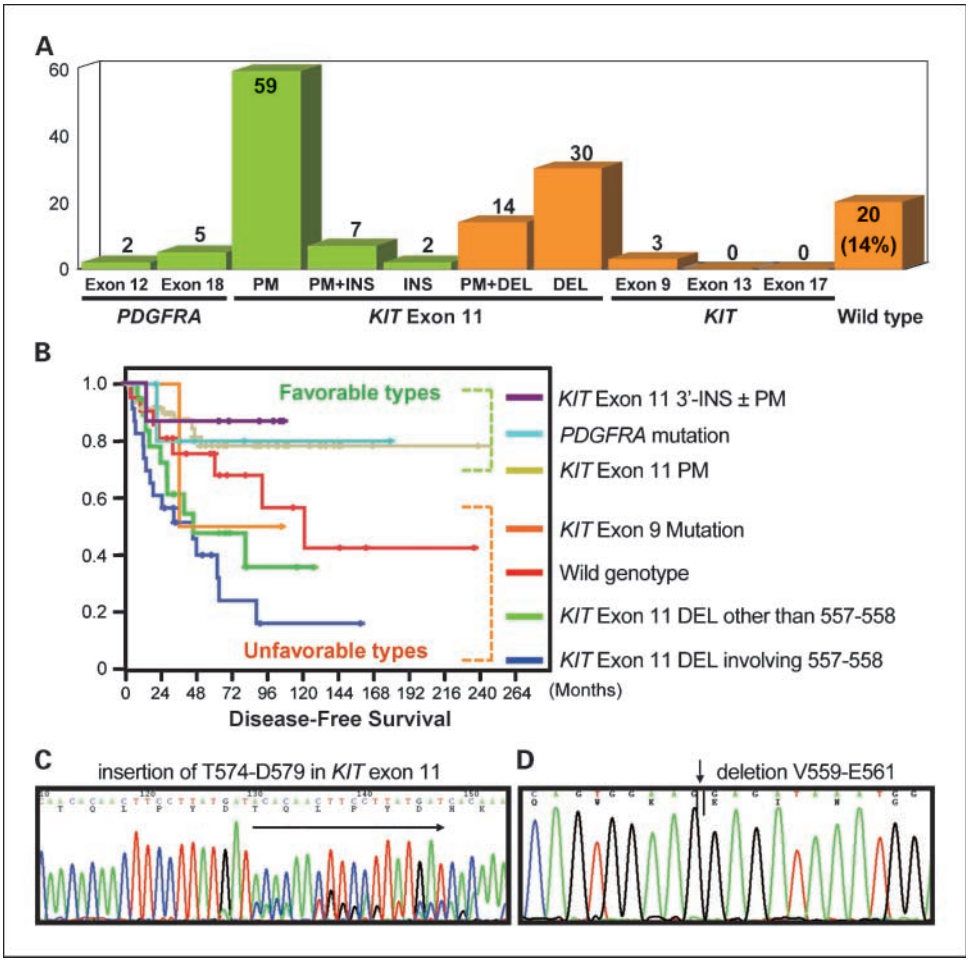


Fig. 1. Mutation analysis and dichotomous classification of receptor tyrosine kinase genotypes in 142 gastrointestinal stromal tumors with informative data. *A,* a histogram showed frequency distribution of various *KIT* or *PDGFRA* mutation types. The most common mutation subtype was single point mutations in *KIT* exon 11, followed by deletions in *KIT* exon 11 with (n = 14) or without (n = 30) point mutations. Note that 20 (14%) cases showed wild genotypes for both *KIT* and *PDGFRA* genes. *B,* based on the preliminary DFS analyses for individual genotypes, two prognostically different groups were identified and referred to as favorable (*green*) and unfavorable (*orange*) genotypes. Sequencing showed nucleotide alterations in representative gastrointestinal stromal tumors with favorable (*C*, insertion of T574-D579 in *KIT* exon 11) and unfavorable (*D*, deletion of V559-E561 in *KIT* exon 11) genotypes of receptor tyrosine kinases. PM, point mutation; DEL, deletion; INS, insertion.

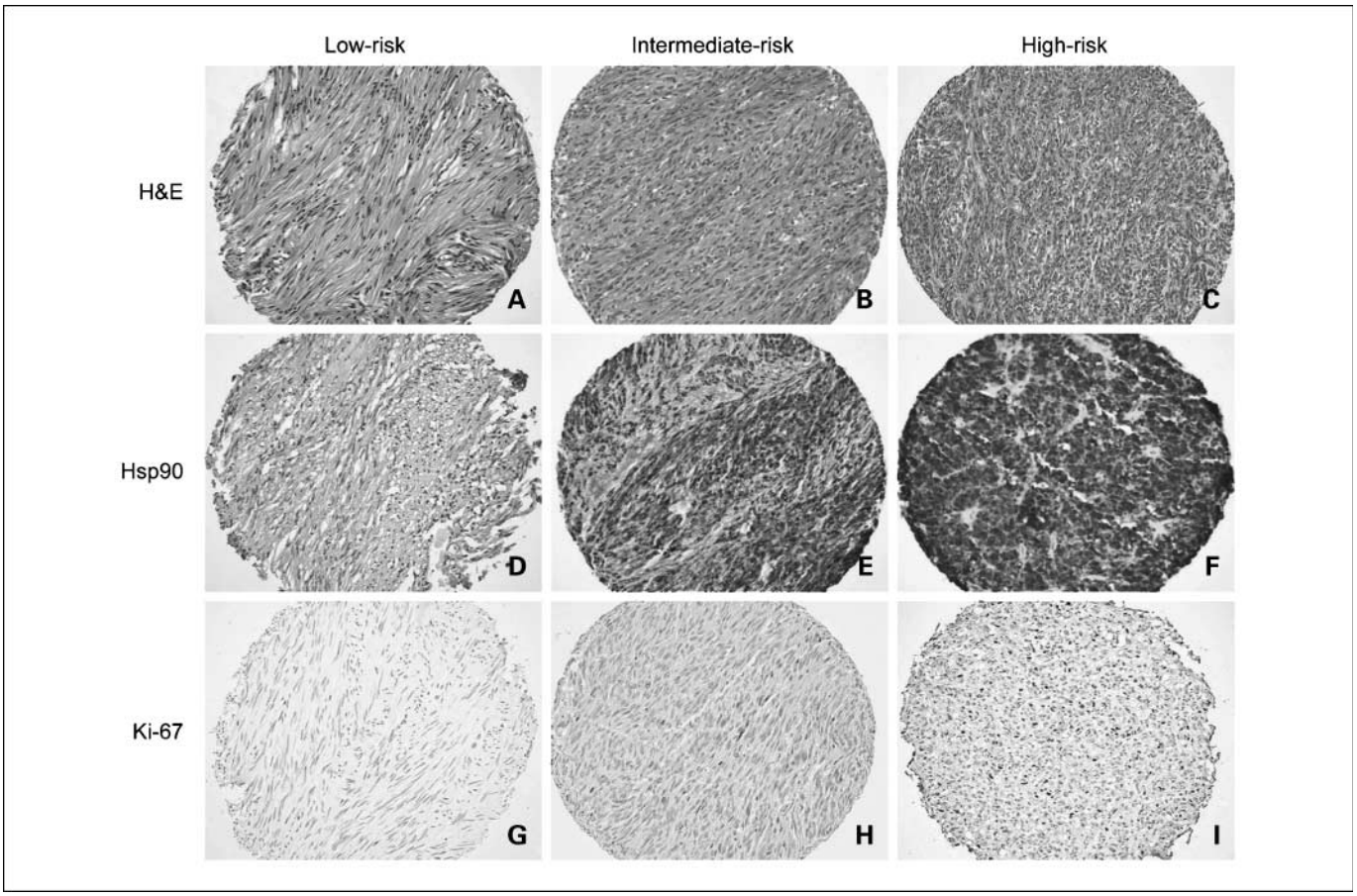


Fig. 2. Representative examples of gastrointestinal stromal tumor tissue microarray, stained with H&E, Hsp90, and Ki-67. Photomicrographs of gastrointestinal stromal tumors classified as low-risk (*A*), intermediate-risk (*B*), and high-risk (*C*) according to NIH consensus criteria with increasing mitoses. Ki-67 LI escalated from the low-risk (*D*) through intermediate-risk (*E*) to high-risk (*F*) cases. Hsp90 showed a gradual increase in both extent and intensity of cytoplasmic staining in the low-risk (*G*), intermediate-risk (*H*), and high-risk (*I*) gastrointestinal stromal tumors.

ImProm-II Reverse transcription system (Promega), 2 μL of total RNA sample was reverse-transcribed in a final volume of 40 μL with the following conditions: 0.5 mmol/L deoxyribonucleotide triphosphates, 25 units of RNase inhibitor, 16 μL of RNA eluant, and 4 μL of random primers. The reactions were done at 42°C for 60 min, followed by inactivation of the enzyme at 70°C for 15 min. Real-time PCR assays to quantify the expression levels of Hsp90α and Hsp90β transcripts were done using the LightCycler instrument 2.0 (Roche Molecular Diagnostics). As listed in Supplementary Table S2, intron-spanning primers and local oxidation nanolithography probes for cDNAs of target and housekeeping (*POLR2A*, a.k.a. RPII) genes were ordered from Universal Probelibrary.[8] Amplification was conducted in duplicate for each reaction with LightCycler TaqMan MasterMix, and the relative expression levels of *Hsp90α* and *Hsp90β* mRNA in individual samples were calculated by the comparative C_T method after normalization to *POLR2A* (Supplementary Table S2). Only samples with C_T value <32 for *POLR2A* were considered to have acceptable RNA quality and included in the analyses.

Cell culture

The gastrointestinal stromal tumor 882 and gastrointestinal stromal tumor 48 cell lines were established and kindly provided by Dr. Fletcher and cultured in the media with supplemented nutrients and antibiotics as previously described (22, 28).

Western blotting assays

Equal amounts of total protein extracted from two gastrointestinal stromal tumor cell lines and six fresh samples were separated on SDS-10% PAGE, transferred to polyvinylidene difluoride membranes (Amersham Biosciences), and then blocked with 5% skimmed milk in TBS Tween 20 buffer at room temperature for 1 h. Afterwards, the membranes were probed with antibodies at room temperature for 2 h against Hsp90α (AB3466, 1:400; Chemicon), Hsp90β (AB3468, 1:400; Chemicon), and GAPDH for a loading control (1:3,000, MAB347; Chemicon), and then incubated with the secondary antibody at room temperature for 1.5 h. Enhanced chemiluminescence reagents (Amersham Biosciences) were used to visualize the targeted proteins, which were then semiquantitatively measured by densitometry.

Follow-up and statistical analyses. Statistical analyses were done using the SPSS 14 software package. Association and comparisons of Hsp90 expression with various parameters were evaluated by χ^2, Student's *t*, or paired *t* tests as appropriate for 306 cases with interpretable Hsp90 LI. In this cohort, 281 patients had available follow-up data with the median duration of 53.7 mo as of August 2007 (mean, 36.6 mo; range, 1-235 mo), forming the basis for univariate prognostic analysis. At last follow-up, 196 patients were alive without relapsed disease; 53 developed tumor relapses, including local recurrences in 25 and hepatic and/or peritoneal dissemination in 38; 33 died of gastrointestinal stromal tumors; and 23 died of unrelated

[8] https://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp is the Web site address of Universal ProbeLibrary that provides the online software to design LON probes for real time RT-PCR assays.

causes. The end point analyzed was disease-free survival (DFS), which would not be confounded by imatinib therapy for patients with disseminated disease as seen in the evaluation of overall or disease-specific survival. A series of cutoff values in 5% increment were tested for continuous variables, such as Hsp90 LI, and those giving the best *P* values were adopted to plot Kaplan-Meier curves and compare prognostic differences by log-rank tests. For the convenience of statistical analysis, RTK genotypes were dichotomized into two prognostically different groups based on the trends of survival curves determined for individual mutation subtypes of *KIT* and *PDGFRA* genes (see Results). In Cox multivariate regression model, all significant parameters identified at univariate level were entered to compare the independent prognostic effect on 142 cases with complete clinicopathologic, immunohistochemical, and genotyping data. However, as component factors of the NIH risk scheme, tumor size and mitotic activity were not introduced in multivariate comparisons (1, 5, 25). Student's *t* test and paired *t* tests were used to analyze the results of real time RT-PCR and Western blotting assays as appropriate. For all analyses, two-sided tests of significance were used with $P < 0.05$ considered significant.

Results

Genotyping of RTK genes. Mutation analysis was successfully done in 142 gastrointestinal stromal tumors with good DNA quality at the end of this study. In the preliminary survival analyses, the trends of survival curves for individual RTK genotypes were generally in keeping with those published recently (6, 8, 9, 26, 29, 30). By grouping mutation subtypes with similar prognostic effect on DFS, these 142 cases were further classified to have favorable ($n = 75$) versus unfavorable ($n = 67$) genotypes (Fig. 1A-D). The former group comprised (*a*) *PDFGRA* mutation involving exons 12 or 18 in 7 cases with 10-year DFS of 87.8%; (*b*) 3′ tandem insertion of *KIT* exon 11 with or without point mutation in 9 cases (Fig. 1C) with 10-year DFS of 87.5 %; and (*c*) single-point mutation of *KIT* exon 11 in 59 cases with 10-year DFS of 78.8%. The group of unfavorable genotypes included (*a*) Ala502-Tyr503 insertion of *KIT* exon 9 in 3 cases with 10-year DFS of 50%; (*b*) wild type for both *KIT* and *PDGFRA* genes in 20 cases with 10-year DFS of 44.9 %; and (*c*) 5′ deletion of *KIT* exon 11 with or without point mutation in 44 cases (Fig. 1D) with 10-year DFS of 23.7%. Of the *KIT* exon 11–deleted subgroup, 23 cases with deletions involving codons 557 and 558 had DFS of 15.1% at 10 years, whereas this DFS rate was 33.7% for 21 cases with deletions involving other codons. No mutation of *KIT* exon 13 or 17 was detected in this series.

Immunohistochemical expression of Hsp90 in gastrointestinal stromal tumors and the correlations with other parameters. Immunohistochemical expression of Hsp90 could be successfully interpreted in 306 cases. This cohort consisted of 152 males and 154 females with a median age of 61 years. A total of 108 cases were classified as very low- or low-risk (Fig. 2A), 99 as intermediate-risk (Fig. 2B), and 96 as high-risk (Fig. 2C) based on the NIH consensus scheme. However, the risk category of three remote cases could not be determined due to the lack of data on tumor size. Hsp90 was overexpressed in 167 cases (55%; Fig. 2D-F) and displayed a wide variation in LI from 0% to 100% (median, 50%). As shown in Table 1, Hsp90 overexpression was significantly more frequent in gastrointestinal stromal tumors that occurred in the nongastric sites ($P = 0.001$) and showed presence of epithelioid histology ($P < 0.001$). As compared with low expressers, gastrointestinal stromal tumors with Hsp90 overexpression had a significantly larger tumor size ($P < 0.001$) and higher mitotic count ($P < 0.001$). Furthermore, Hsp90 overexpression was also

Table 1. The expression status of Hsp90 in localized gastrointestinal stromal tumors and correlations with clinicopathologic features, Ki-67 LI, and mutation types

		No overexpression	Overexpression	*P*
Sex	Number			
Male	152	72	80	0.498
Female	154	67	87	
Age (y), mean ± SD (range, 23-88 y; median, 61 y)		61.2 ± 12.2	58.8 ± 13.3	0.110
Location				
Gastric	181	97	84	0.001*
Nongastric	125	42	83	
Histologic type				
Spindle	234	122	112	<0.001*
Epithelioid and mixed	72	17	55	
Cellularity				
Low/moderate	244	116	128	0.107
High	61	22	39	
Tumor size, cm (mean ± SD)		5.1 ± 3.7	7.4 ± 4.1	<0.001*
Mitotic count, 50HPF (mean ± SD)		3.3 ± 5.7	12.9 ± 27.6	<0.001*
NIH risk				
Low/very low	108	75	33	<0.001*
Intermediate	99	38	61	
High	96	24	72	
Ki-67 LI (mean ± SD)		2.8 ± 3.3	6.5 ± 8.1	<0.001*
Mutation type				
Favorable type	75	36	39	0.020*
Unfavorable type	67	20	47	

Abbreviation: HPF, high-power fields.
*Statistically significant.

Table 2. Univariate analyses for disease-free survival

Parameters	No. cases	DFS	
		No. events	*P*
Sex			
Male	141	43	0.4933
Female	140	42	
Age, y			
<70	203	55	0.0151*
≥70	77	30	
Location			
Gastric	167	41	0.0214*
Nongastric	114	44	
Histologic type			
Spindle	215	52	<0.0001*
Mixed/epithelioid	66	33	
Cellularity			
Mild/moderate	226	58	0.0011*
Hypercellular	54	27	
Tumor size (cm)			
≤5	130	24	<0.0001*
>5; ≤10	99	31	
>10	51	29	
Mitotic count (50HPF)			
0-5	200	39	<0.0001*
6-10	36	15	
>10	44	31	
NIH risk			
Very low/low	101	16	<0.0001*
Intermediate	88	14	
High	89	54	
Mutation type			
Favorable type	75	12	<0.0001*
Unfavorable type	67	36	
Ki-67 LI			
≤5	196	46	<0.0001*
>5	73	36	
HSP90			
No overexpression	125	23	<0.0001*
Overexpression	156	62	

*Statistically significant.

highly related to the increasing risk levels of the NIH consensus scheme ($P < 0.001$) and higher proliferative index ($P < 0.001$). More intriguingly, Hsp90 overexpression was found to preferentially involve gastrointestinal stromal tumors with unfavorable RTK genotypes ($P = 0.02$). The findings of strong associations between Hsp90 overexpression and several adverse prognosticators suggested its crucial role in tumor progression of gastrointestinal stromal tumors.

Survival analyses. Correlations of clinical outcomes with various clinicopathologic, immunohistochemical, and molecular parameters at the univariate level are shown in Table 2 and Fig. 3. Similar to our and others' previous reports, inferior DFS in this series was significantly associated with several clinicopathologic factors, including older age ($P = 0.0151$), nongastric location ($P = 0.0214$), presence of epithelioid histology ($P < 0.0001$), hypercellularity ($P = 0.0011$), larger tumor size ($P < 0.0001$), higher mitotic count ($P < 0.0001$), and increasing NIH risk levels ($P < 0.0001$; refs. 5–8, 11, 12, 25, 31). In addition, high proliferative index ($P < 0.0001$) and unfavorable RTK genotypes ($P < 0.0001$; Fig. 3A) were both highly predictive of adverse outcomes. More importantly, Hsp90 overexpression was also strongly predictive of inferior DFS ($P < 0.0001$; Fig. 3B).

In multivariate analysis (Table 3), Hsp90 overexpression remained prognostically independent ($P = 0.031$), along with high NIH risk level ($P < 0.001$), high Ki-67 index ($P = 0.001$), and older age ($P = 0.027$). Furthermore, Hsp90 overexpression also identified patients at more than 2-fold higher risk of relapsed disease. However, location and genotypes lost statistical significance.

Differential expression of α and β isoforms of Hsp90 in gastrointestinal stromal tumors. Next, we addressed which isoform is more abundant or more relevant to tumor progression. Real-time RT-PCR quantification of Hsp90α and Hsp90β transcripts could be successfully determined with sufficient RNA yields in pure microdissected tumor cells of 16 fresh gastrointestinal stromal tumor samples (Fig. 4A-C), including 8 high-risk, 6 intermediate-risk, and 2 low-risk cases. We found that the β isoform was much more abundant than the α form in mRNA expression ($P < 0.001$; Fig. 4A and B). The expression of the α isoform was significantly higher in the high-risk group than in

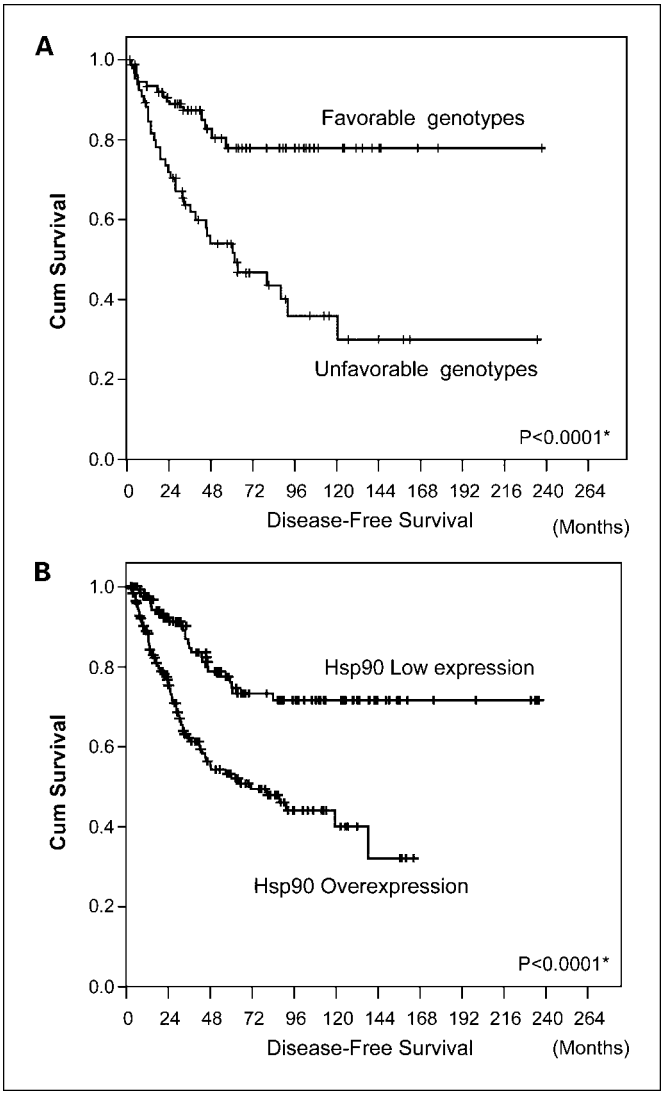


Fig. 3. Kaplan-Meier plots to predict DFS according to genotypes of *KIT* and *PDGFRA* genes (*A*) and Hsp90 expression (*B*).

Table 3. Multivariate analyses for disease-free survival

Parameter	Category	RR (95% CI)	*P*
NIH risk	Very low/low/intermediate	1	<0.001*
	High	3.895 (2.006-7.563)	
Ki-67 LI	≤5	1	0.001*
	>5	3.471 (1.697-7.102)	
Hsp90	Not overexpressed	1	0.031*
	Overexpressed	2.441 (1.084-5.496)	
Age, y	<70	1	0.027*
	≥70	2.152 (1.091-4.244)	
Site	Gastric	1	0.130
	Nongastric	1.808 (0.840-3.891)	
Mutation type	Favorable	1	0.232
	Unfavorable	1.594 (0.759-3.124)	
Cellularity	Low/moderate	1	0.385
	High	1.402 (0.654-3.007)	
Histologic type	Spindle	1	0.561
	Mixed/epithelioid	1.225 (0.617-2.433)	

Abbreviations: RR, relative risk; 95% CI, 95% confidence interval.
*Statistically significant.

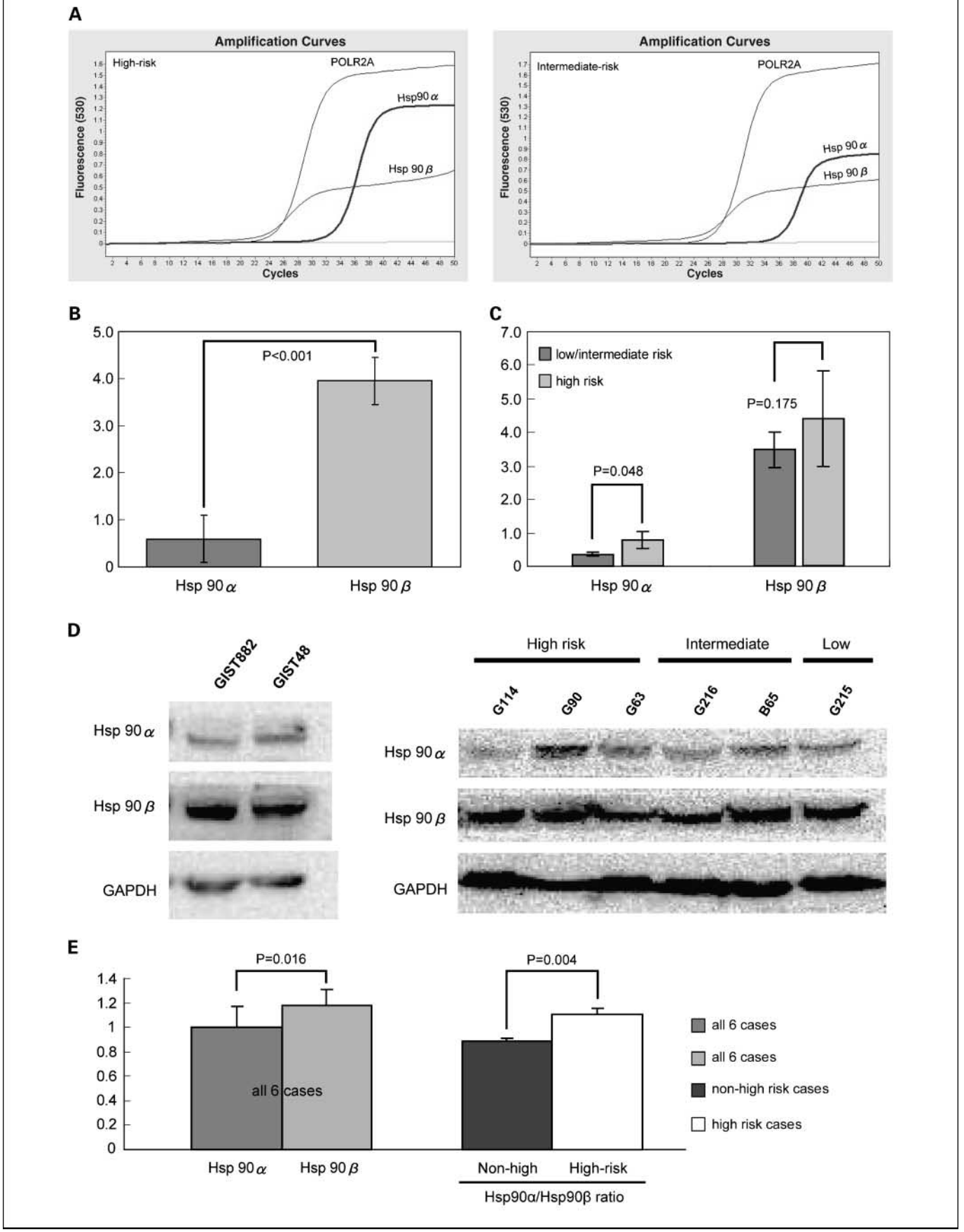
the other cases (P = 0.048; Fig. 4C), whereas this difference between gastrointestinal stromal tumors of various risk categories was not substantiated for the β form (P = 0.175). Consistent with the results of real-time RT-PCR, Western blotting also showed higher protein expression of the β isoform in two gastrointestinal stromal tumor cell lines (Fig. 4D). Despite still being significant in six tumor specimens tested (P = 0.016; Fig. 4D and E), the difference in protein expression between the two isoforms was not as apparent as seen in cell lines. However, we found that the expression ratio of α to β isoform was significantly higher in high-risk cases (P = 0.004, Fig. 4E).

Discussion

In the present study on human gastrointestinal stromal tumor specimens, the results reported herein were the first to show the crucial role of Hsp90 in disease progression of gastrointestinal stromal tumor. This could be exemplified by its frequent overexpression and strong correlations with several adverse prognosticators, including large size, nongastric location, higher risk category, increased mitotic count, and Ki-67 overexpression, etc. Among these, the latter two were indicators of increased cell proliferation, which was conceivably in line with the known function of Hsp90 in stabilizing CDK4, CDK2, and cyclin D to drive cell cycle entry and progression (32–34). Noticeably, Hsp90 overexpression was also substantiated as an independent adverse factor of DFS in our large series of primary resected gastrointestinal stromal tumors without imatinib therapy precedent to disease relapse. Nevertheless, the prognostic utility of Hsp90 expression is considered to be dependent on the unique molecular milieu present in various cancer types, which have different pivotal cancer-associated proteins to be chaperoned (23). In a similar context of transmembrane RTK-mediated oncogenesis, the prognostic value of Hsp90 in gastrointestinal stromal tumors with KIT activation may be casually analogous to those identified in breast cancers with adverse outcomes (21, 23). In the latter setting, human epidermal growth factor receptor 2 hyperactivity is sustained by up-regulated Hsp90 to relay self-sufficient growth signals, thereby contributing to tumor aggressiveness (21, 23).

More intriguingly, we also found that Hsp90 overexpression was associated with unfavorable RTK genotypes. Currently, it has become more explicit that different RTK genotypes may implicate the selective activation of preferential downstream signaling pathways, thereby resulting in variability in transcriptional signatures (35, 36), histomorphology (10, 37), and, consequently, prognosis of gastrointestinal stromal tumors (6, 8, 9, 26, 29). For instance, we and others have corroborated that gastrointestinal stromal tumors with 3′-end insertion of *KIT* exon 11 (9) or with *PDGFRA* mutations (10, 29) usually pursue a relatively indolent clinical course. In contrast, gastrointestinal stromal tumors with *KIT* exon 11 deletions (6, 26, 29), especially when involving codons 557 and 558 (6, 29), were previously reported to be more frequent among high-risk or overtly malignant tumors and predictive of adverse outcomes. This finding was also reaffirmed in our series showing that gastrointestinal stromal tumors with *KIT* exon 11 deletions had the shortest DFS (Fig. 1B) among all informative cases and those with unfavorable RTK genotypes.

Fig. 4. Differential expression of α and β isoforms of Hsp90 in gastrointestinal stromal tumors. *A,* amplification curves of real-time PCR quantification for *Hsp90*α and *Hsp90*β transcripts in one each representative intermediate-risk (*right*) and high-risk (*left*) gastrointestinal stromal tumors, using *POLR2A* as the internal control. *B,* the normalized expression folds of *Hsp90*β mRNA was significantly higher than that of *Hsp90*α (P < 0.001) in 16 cases tested. *C,* the mRNA expression of the α isoform was more abundant in high-risk gastrointestinal stromal tumors (P = 0.048) than in the combined group of low-risk and intermediate-risk cases. However, this difference between cases of various risk categories did not hold true for the β form. *D,* both α and β isoform-specific antibodies were used in Western blotting assays to detect differential protein expression of two Hsp90 isoforms in two cell lines (*left*) and six tumor specimens of gastrointestinal stromal tumor (*right*), using GAPDH protein as the loading control. *E,* although the normalized protein expression level of Hsp90β was more abundant (P = 0.016) than that of Hsp90α in gastrointestinal stromal tumor specimens, this difference was not as apparent as seen in gastrointestinal stromal tumor cell lines. However, the protein expression ratio of Hsp90α to Hsp90β was significantly higher in high-risk cases (P = 0.004).


A
Amplification Curves
High-risk
POLR2A
Hsp90α
Hsp 90β
Fluorescence (530)
Cycles
Amplification Curves
Intermediate-risk
POLR2A
Hsp 90α
Hsp 90β
Fluorescence (530)
Cycles
B
P<0.001
Hsp 90α
Hsp 90β
C
low/intermediate risk
high risk
P=0.048
P=0.175
Hsp 90α
Hsp 90β
D
GIST882
GIST48
Hsp 90α
Hsp 90β
GAPDH
High risk
Intermediate
Low
G114
G90
G63
G216
B65
G215
Hsp 90α
Hsp 90β
GAPDH
E
P=0.016
P=0.004
all 6 cases
Hsp 90α
Hsp 90β
Non-high
High-risk
Hsp90α/Hsp90β ratio
all 6 cases
all 6 cases
non-high risk cases
high risk cases

It is therefore tempting to speculate that a relatively stronger growth-stimulating signal is transduced from unfavorable RTK mutants (e.g., *KIT* exon 11 deletions), compared with other RTK mutations, thereafter generating a greater oncogenic stress to account for Hsp90 up-regulation and more aggressive behavior in gastrointestinal stromal tumors. It must be added, however, that additional oncogenic aberrations, involving events other than RTK mutations, may also significantly contribute to Hsp90 overexpression during tumor progression, given no independent prognostic significance of RTK genotypes in this and one previous studies (29).

Previous studies on gastrointestinal stromal tumors have shown that tumor sites correlate with the variations in phenotypes, RTK genotypes, and clinical aggressiveness (8, 9, 11, 12). Gastrointestinal stromal tumors with 3′ insertion of *KIT* exon 11 or with *PDGFRA* mutations are known to preferentially arise from the stomach (9, 10), whereas *KIT* exon 9 mutations affect the small bowel exclusively (8, 35). At the univariate level, we found the adverse prognostic implication of the nongastric site in gastrointestinal stromal tumors, which also showed significantly higher expression of Hsp90, albeit the underlying causes of such an association remain elusive. Of notice, anatomic sites have recently been reported to specifically distinguish gene expression profiles of gastrointestinal stromal tumors, with different functional genomic clustering between the gastric and small intestinal cases (35). Along these lines, future studies may be warranted to examine whether these site-dependent differentially expressed genes are functionally different in up-regulating Hsp90 expression.

The α isoform of Hsp90 is generally believed to be inducible by various cellular stresses and involved in cell cycle progression or growth factor–mediated signaling. In contrast, the β isoform is usually constitutively expressed and linked with long-term cellular adaptation (18, 24). For the first time, we substantiated this general doctrine about the differential roles of Hsp90 isoforms in gastrointestinal stromal tumors by showing that Hsp90α mRNA expression and the protein expression ratio of Hsp90α versus Hsp90β were both significantly increased in high-risk cases. This suggested the potential relevance of Hsp90α in the tumor progression of gastrointestinal stromal tumor. On the other hand, Hsp90β was more abundant than the α form, although this difference seemed more apparent at the mRNA level than in protein expression, which may be explained by two likely reasons. First, Hsp90β has been reported to display lower translational efficiency of its mRNA in the production of the encoded protein (24), despite having higher mRNA expression compared with Hsp90α. Second, mRNA of Hsp90 isoforms were quantified using pure tumor cells isolated by laser capture microdissection in this study, so as to minimize the dilution artifact from contaminated nonneoplastic cells. However, this method to more precisely measure the actual expression level was not applicable to Western blotting assays using whole-cell lysates from clinical specimens. This possibility could also be partly inferred from Western blots of two gastrointestinal stromal tumor cell lines, which showed results more consistent with those obtained by real time RT-PCR.

The therapeutic management of imatinib-resistant gastrointestinal stromal tumors remains difficult, because secondary kinase domain mutations or, much rarely, genomic amplifications of RTK genes eventually develop during clonal evolution (4, 13–17). These additional genetic aberrations can result in acquired drug resistance in most patients with an initial response (4, 13–17). However, 10% to 20% of gastrointestinal stromal tumors previously untreated with imatinib are even primarily resistant to this first-line therapy because of harboring refractory RTK mutants (e.g., *PDGFRA* D842V; refs. 4, 13, 38). With stronger potency, a new generation of broad-spectrum, small-molecule kinase inhibitors may provide temporary benefit for patients progressing on imatinib therapy (4, 15). Nevertheless, it is still considered insufficient to achieve long-term remissions in gastrointestinal stromal tumors by direct suppression of kinases alone (4). To circumvent these limitations, novel therapeutic strategies are being developed to identify effective agents that depend less on the KIT and/or PDGFRA activation but alternatively blockade the signaling transduced through these RTKs (4, 15, 22). Accordingly, one promising approach is to inhibit Hsp90 by 17-AAG or its analogue, which has recently been proved effective to attenuate the expression and phosphorylation of mutant KIT oncoproteins with imatinib-resistant mutations in both mastocytosis and gastrointestinal stromal tumor cell models (22, 39). This treatment provides antiproliferative and proapoptotic effects and inactivates signaling intermediates, such as protein kinase B and mitogen-activated protein kinase, with the IC_{50}^{S} concentration achievable in the serum of patients. Furthermore, the mutant KIT is much more sensitive to Hsp90 inhibition when compared with the wild-type counterpart (22, 40). Through inhibition of a single molecular target, Hsp90 antagonists are unique in simultaneously decreasing the cellular levels and activities of multiple cancer-associated client proteins depending on Hsp90 for their function (19–21). Moreover, the strong correlations of Hsp90 expression with NIH risk category and DFS may reinforce the rationales to use its inhibitors as an alternative therapeutic strategy for imatinib-resistant gastrointestinal stromal tumors, particularly for those high-risk cases. Furthermore, Hsp90α protein was recently identified by proteomics in the serum of patients with non–small cell lung carcinomas and its expression level detected by ELISA was found to be positively correlated with the clinical stage (41). The finding of this clinical study was consistent with several recent basic investigations, which showed a novel extracellular function of the Hsp90α isoform to facilitate *in vitro* tumor invasiveness (42, 43). When hyperacetylated on its specific residues, Hsp90α, unlike Hsp90β, can be exported to the cell surface of cancer cells to promote the activation of matrix metalloproteinase 2, a protease known to implicate the metastatic phenotype (42, 43). Based on these and our preliminary findings, it seems plausible to further clarify whether Hsp90α in gastrointestinal stromal tumor can be secreted extracellularly and released into the bloodstream for convenient monitoring of Hsp90-targeted treatment response.

In conclusion, Hsp90 is overexpressed in approximately a half of gastrointestinal stromal tumors and correlates with several important prognosticators, such as the tumor sites and sizes, mitotic count, NIH risk category, K-67 proliferative index, and RTK genotypes. More importantly, it is also independently predictive of worse DFS, highlighting its role in disease progression and alternative therapy for high-risk, imatinib-resistant gastrointestinal stromal tumors. Regarding

differential expression of isoforms, Hsp90α seems more relevant to the intrinsic aggressiveness of gastrointestinal stromal tumors, albeit less abundant than Hsp90β. However, this finding is still premature to establish the role of Hsp90α in disease progression, which needs prospective validation in future series using more fresh gastrointestinal stromal tumor specimens.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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