

## Association of Intratumoral Vascular Endothelial Growth Factor Expression and Clinical Outcome for Patients with Gastrointestinal Stromal Tumors Treated with Imatinib Mesylate

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**Abstract Purpose:** Imatinib mesylate (imatinib) has revolutionized clinical outcomes of patients with advanced gastrointestinal stromal tumor (GIST). However, the degree of individual benefit varies, and little is known about prognostic factors for these patients. Importantly, selected patients may be treated with an approach to target both Kit and vascular endothelial growth factor receptor (VEGFR) expression.

**Experimental Design:** Using tissue microarray technology, we analyzed 53 imatinib-naive GISTs for vascular endothelial growth factor (VEGF) expression from patients who then received imatinib. In multivariate analyses, we evaluated overall survival (OS) and progression-free survival (PFS) of these patients based on putative prognostic factors, including VEGF expression. In a separate study, 12 matched pre-imatinib and post-imatinib GIST patient specimens and two human GIST cell lines were assessed for VEGF production in response to imatinib.

**Results:** Independent of *kit* genotype, patients with GIST expressing high VEGF had inferior median PFS (7.1 months versus 29 months,  $P = 0.42$ ) and median OS (20 months versus not reached at >50 months;  $P = 0.02$ ) compared with weak or nonexpressers of VEGF. Non-exon 11 *kit* mutation predicted inferior PFS but not OS. High mitotic rate was marginally predictive of improved OS. Imatinib resulted in decreased production of VEGF in only a subset of GIST patients (2 of 12) and both cell lines.

**Conclusions:** We present a study to address the prognostic factors for patients with GIST in the imatinib era. We present a rationale to consider exploration of a front-line therapy of GIST with a regimen targeting both Kit and VEGFR based on the presence of tumor VEGF levels.

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal malignancy in the gastrointestinal tract. GISTs may arise anywhere in the alimentary tract and rarely in extraintestinal sites (1). GISTs are distinct from smooth muscle tumors and share features with the interstitial cells of Cajal, the putative progenitor cell of GIST (2).

Immunostaining of most GISTs shows expression of the receptor tyrosine kinase Kit for which the majority of GISTs

encode activating mutations (2, 3). Most mutations in GIST are deletions or insertions residing in exon 11 of the *kit* gene. Less frequently, mutations can be found in exons 9, 13, or 17 (4). Alternatively, a minority of GISTs encode activating mutations in exons 12 or 18 of the platelet-derived growth factor receptor- $\alpha$  (PDGFR- $\alpha$ ) gene (5).

Before the utilization of imatinib mesylate (imatinib, Gleevec; STI571; Novartis) for the treatment of GIST, patients with advanced disease had limited therapeutic options. GISTs are notoriously chemoresistant and radioresistant, with overall responses of <10% to these modalities (6). Likewise, in the era before imatinib, tumor size ( $\geq 10$  cm), mitotic count ( $\geq 10$  of 50 high power field), small bowel disease, mixed morphology, metastases, BCL-2 expression, exon 11 mutation, and vascular endothelial growth factor (VEGF) expression predicted aggressive disease and poor outcome (4, 7–10).

Imatinib is a small molecule inhibitor whose target in GIST is the protein product of the mutated *kit* or PDGFR- $\alpha$  gene (11). Imatinib has efficacy in the treatment of locally advanced or metastatic GIST with patients realizing a median overall survival (OS) of  $\geq 30$  months compared with 9 months for historical controls (12). However, whereas  $\sim 80\%$  of patients have clinical benefit from imatinib, long-term outcomes are of variable duration (12). Other than limited data on the site of mutation within the *kit* gene, prognostic features of GIST in the imatinib era remain largely undefined.

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**Grant support:** Institutional Physician-Scientist awards (J.C. Trent, A.J.F. Lazar), grant 1K23CA109060-03 (J.C. Trent), Amschwand Sarcoma Cancer Foundation (J.C. Trent), University of Texas Health Science Center at Houston M.D., Ph.D. Program (J.C. McAuliffe) and NIH Center for Clinical and Translational Science T32 Training Program grant no. TL1 RR024147 (J.C. McAuliffe).

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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doi:10.1158/1078-0432.CCR-07-0895

Recent clinical development of sunitinib malate (sunitinib, SUTENT; SU11248; Pfizer) for the therapy of GIST has shown efficacy in some patients refractory or intolerant to imatinib (13). Therefore, with potential alternative therapy, understanding prognostic factors may direct the selection of appropriate targeted therapies. Importantly, although *kit* exon 11 mutation was observed to be an adverse prognostic feature before the widespread use of imatinib, contemporary studies have found that imatinib-treated patients whose tumors expressed *kit* exon 11 mutation have superior progression-free survival (PFS) compared with those patients with other genotypes (14). Thus, the reliability of current prognostic markers remains unknown in the era of relatively universal imatinib therapy. Moreover, the effects of imatinib on the tumor, including vascular cytokines, are not fully understood.

VEGF is a potent angiogenic factor (15). However, to our knowledge, none have determined whether GIST patients whose tumors express VEGF achieve the same clinical benefit on imatinib therapy as those whose tumors do not express this factor. This may be important because sunitinib, an inhibitor of both Kit and VEGF receptor signaling, has proved efficacious in malignancies including GIST (13, 16).

Therefore, we evaluated the predictive value of VEGF expression in GIST patients treated with imatinib. Improved understanding of the VEGF receptor signaling pathway may help optimize clinical management of GIST, because these patients have sunitinib available as a therapeutic option.

Herein, we show that VEGF expression is a predictive factor for early treatment failure and poor survival of GIST patients on imatinib therapy, independent of *KIT* genotype. Additionally, we show that imatinib variably effects VEGF expression in patients and cell lines. Thus, patients whose GIST produces VEGF may benefit from front-line anti-VEGF receptor signaling therapy.

## Patients and Methods

**Patients.** Two separate patient cohorts were used for this study. Imatinib-naive surgical tissue was collected from 53 patients diagnosed with Kit-expressing GIST from 1998 to 2004, who were then treated with imatinib for recurrent, advanced, and/or metastatic disease. This group was used for multivariate analyses of OS and PFS and tissue microarray (TMA) analysis.

Separately, pre-imatinib and post-imatinib tumor specimens were acquired from 12 patients enrolled on a prospective, randomized phase II study of neoadjuvant and adjuvant 600-mg daily imatinib for primary, recurrent, or metastatic resectable, Kit-expressing GIST undergoing planned surgical resection of their tumor (MDACC ID03-0023).

With approval from the institutional review board, we acquired documented informed consent from all patients to use their clinical data and tumor tissue.

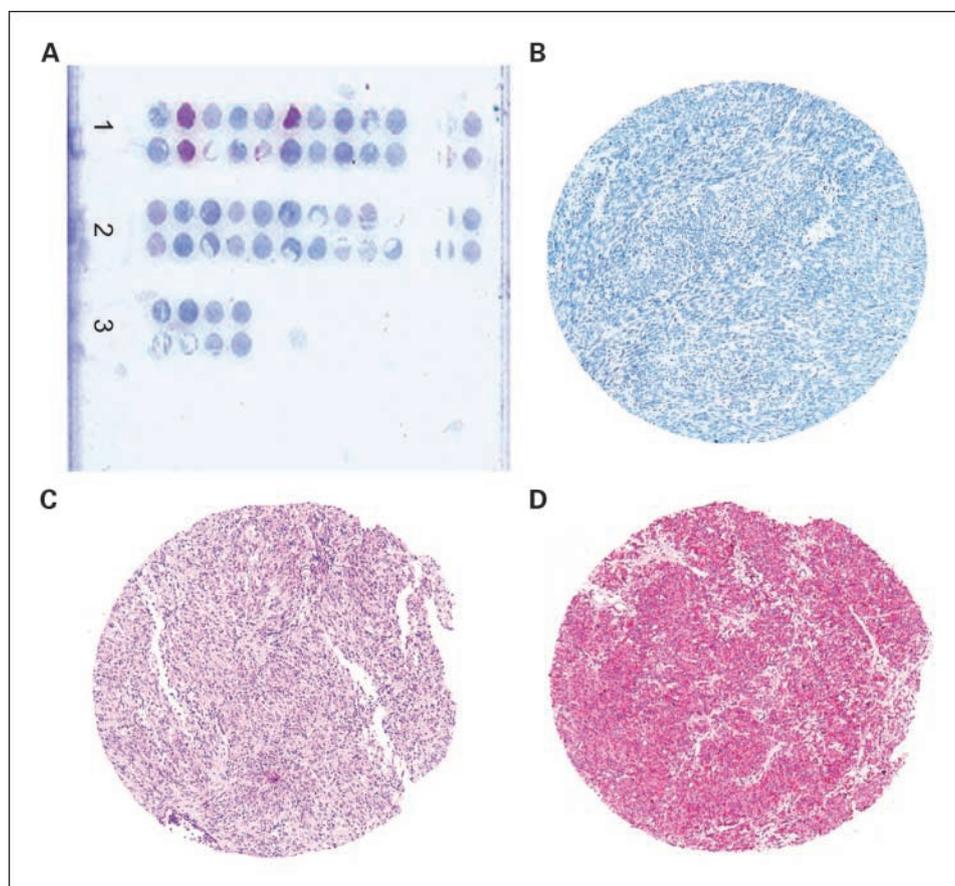
**Tissue array construction and tissue procurement.** We constructed a GIST TMA of surgical specimens from 53 imatinib-naive tumors as previously described (17, 18). Briefly, we collected formalin-fixed, paraffin-embedded archival GIST tissue blocks and their matching H&E-stained slides. These were reviewed and screened by two sarcoma pathologists (A.K.R., A.J.F.L.). The TMAs were constructed using an automated tissue microarrayer (Beecher Instruments). Each tumor was sampled in duplicate from representative areas using a 1.0-mm punch. The tissue cores from each tumor were then incorporated into three TMA blocks. Blocks were cut and placed on slides. Microsections

**Table 1.** Patient characteristics

<b>A. TMA Cohort (n = 53)</b>		
<b>Variable</b>	<b>n (%)</b>	<b>Median (range)</b>
Age (y)		55 (25-75)
Tumor size (cm)		10 (2-35)
Gender		
Male	26 (49)	
Female	27 (51)	
Ethnicity		
Caucasian	42 (79)	
Black	5 (9)	
Hispanic	4 (8)	
Asian	2 (4)	
Imatinib dose		
400 mg	29 (57)	
600 mg	3 (6)	
800 mg	19 (37)	
Primary tumor site		
Stomach	23 (43)	
Small bowel	18 (34)	
Colon	5 (9)	
Esophagus	2 (4)	
Mesenteric	2 (4)	
Liver	2 (4)	
Retroperitoneal	1 (2)	
No. metastatic sites per patient*		
0	1 (2)	
1	24 (45)	
2	25 (47)	
3	2 (4)	
4	1 (2)	
Location of metastatic disease in patient cohort		
Liver	37 (70)	
Peritoneum	37 (70)	
Lung	7 (13)	
Chest wall	1 (2)	
Lymph node	1 (2)	
<b>B. Pre-imatinib and post-imatinib clinical trial cohort (n = 12)</b>		
<b>Variable</b>	<b>No. (%)</b>	<b>Median (range)</b>
Age (y)		56 (31-87)
Tumor size (cm)		9 (1-22.5)
Gender		
Male	6 (50)	
Female	6 (50)	
Ethnicity		
Caucasian	6 (50)	
Black	4 (34)	
Hispanic	1 (8)	
Asian	1 (8)	
Site of disease		
Stomach	6 (50)	
Small bowel	4 (33)	
Colon	0 (0)	
Mesentery	1 (8)	
Liver	1 (8)	
Metastasis	4 (33)	
Days of neoadjuvant imatinib therapy		
3 d	5 (42)	
5 d	3 (25)	
7 d	4 (33)	

\*Many patients had multiple organ sites of metastasis.

**Fig. 1.** TMA immunohistochemistry for VEGF. **A**, representative tissue array slide stained for VEGF. Each tumor was analyzed in duplicate. Rows of microsections are numbered. **B**, representative tumor microsection scored as negative for VEGF expression. **C**, representative tumor microsection scored as weakly positive for VEGF expression. **D**, representative tumor microsection scored as strongly positive for VEGF expression.



were deparaffinized and rehydrated, and high pH antigen retrieval was done.

Pre-imatinib biopsies and post-imatinib surgical tumor specimens were collected from the same location as defined by CT guidance. Specimens were cut, placed on slides, and fixed in 10% formalin followed by 100% cold methanol.

**Immunohistochemistry.** All slides were blocked with universal blocking solution (Biogenex) and incubated with anti-human VEGF antibody (Santa Cruz). Secondary antibody (Vector Labs) with 3-amino-9-ethyl-carbamazole reagent was used to visualize VEGF protein expression.

TMA microsections were scored as negative (0), weakly positive (1), or strongly positive (2) for VEGF expression. Frozen samples were scored positive or negative for expression. Two independent observers, including an experienced sarcoma pathologist (A.J.F.L.) blinded to clinical data, scored all tumor sections.

**Mutational analysis.** Tumor tissue was assayed for kit and/or PDGFR- $\alpha$  mutation. Genomic DNA samples were isolated from paraffin-embedded GIST slides using a QIAamp DNA minikit (Qiagen) according to the manufacturer's instructions. For PCR, we designed primer sets for exons 9, 11, 13, and 17 of the kit gene, as well as exons 12 and 18 of the PDGFR- $\alpha$  gene, as previously described (5). PCR was carried out in a total volume of 25  $\mu$ L containing 50 to 100 ng of genomic DNA and 0.25  $\mu$ L DNA polymerase (Bioline). Mutations in these genes were identified by sequencing the PCR products on a 3,730  $\times$  1 DNA analyzer (Applied Biosystems) at M.D. Anderson Cancer Center Nucleic Acid Core Facility.

**Cell lines and ELISA.** Two cell lines were used in this study. GIST882 (a gift from Jonathan Fletcher, M.D., Dana-Farber Cancer Institute) expresses a kit exon 13 mutation (19). GIST-T1 (a gift from Andrew Godwin, M.D., Fox Chase Cancer Center) expresses a kit exon 11 (V560-Y579del) mutation. Cell lines were grown in 1 $\times$  DMEM

supplemented with 10% fetal bovine serum with 100 units/mL and 0.1 mg/mL penicillin/streptomycin. Cell lines were treated with vehicle (DMSO) or 1  $\mu$ mol/L imatinib in serum-free growth media. After 24 to 72 h, conditioned media was collected and assayed in triplicate for VEGF production by ELISA (R&D Systems).

**Statistical analysis.** Associations between exon mutation and VEGF status were evaluated using a generalized Fisher exact test (20). Comparisons of covariate distributions between exon mutation subgroups and VEGF expression were done using generalized Fisher exact tests for categorical variables and Wilcoxon tests for numerical valued variables (20). Kaplan-Meier product limit survival probability estimates of PFS and OS were calculated, and log-rank tests were done to compare PFS and OS among different exon mutation, VEGF status, and mitotic count subgroups (21, 22). For the multivariate analysis, the log-logistic regression model was used for PFS, and the Cox proportional hazards model was used for OS. Goodness-of-fit for survival regression models for PFS and OS models were assessed using Grambsch-Therneau test, martingale residual plots, and Bayesian information criteria (23). Because the Cox model fit the PFS data very poorly, the Weibull, exponential, log-logistic, and log-normal models were considered. The log-logistic model provided the best fit to the data based on Bayesian information criteria. For fitted PFS and OS regression models, nonsignificant variables were eliminated in a step-down fashion using a *P*-value cutoff of 0.10. For cell line data, a two-tailed paired Student's *t* test was used to compare VEGF production from cells treated with or without imatinib.

## Results

**Patient characteristics.** The 53 GIST patients whose tumors were analyzed by TMA were treated at M.D. Anderson Cancer Center between 1998 and 2004. Patient characteristics are

**Table 2.** VEGF, mutational, and multivariate analysis**A. VEGF expression status versus primary tumor location and *kit* exon 11 or non-exon 11 mutation status versus VEGF expression status**

	VEGF Status		Total (%)
	Negative-weakly positive	Strongly positive	
Primary tumor site			
Stomach (%)	21 (91)	2 (9)	23 (44)
Small bowel (%)	14 (82)	3 (18)	17 (32)
Colon (%)	5 (100)	0 (0)	5 (9)
Other (%)*	4 (50)	4 (50)	8 (15)
Total (%)	44 (83)	9 (17)	53
<i>kit</i> genotype			
Exon 11 (%)	28 (85)	5 (15)	33 (62)
Non-exon 11 (%)	16 (80)	4 (20)	20 (38)
Total (%)	44 (83)	9 (17)	53

**B. Multivariate analysis by log-logistic model for PFS and Cox proportional model for OS<sup>†</sup> are displayed**

	Variable	Coefficient	SD	P
Log-logistic model for PFS	Exon 11 versus non-exon 11	0.86	0.3	0.004
	Weak VEGF versus no VEGF	-0.09	0.31	0.76
	Strong VEGF versus no VEGF	-0.96	0.42	0.02
Cox proportional model for OS	Exon 11 versus non-exon 11	-0.41	0.45	0.36
	Weak VEGF versus No VEGF	0.12	0.05	0.81
	Strong VEGF versus No VEGF	1.49	0.6	0.03
	Log (mitosis + 1)	1.55	0.76	0.04
	Log (mitosis + 1) <sup>2</sup>	-0.4	0.21	0.06

\*Other = mesenteric, esophageal, liver, or peritoneal lesion.

<sup>†</sup> Significant prognostic factors affecting PFS and OS are shown.

summarized in Table 1A. After surgical resection of their primary tumor, patients were subsequently treated with 400-mg to 800-mg daily adjuvant imatinib for recurrent, advanced, and/or metastatic disease. The most common primary site was the stomach followed by the small bowel. The most common metastatic sites were the liver and peritoneum.

Twelve of seventeen patients recruited for the clinical trial were evaluable in this study. Five patients were excluded from study. Four were excluded due to lack of viable tumor tissue in pre-imatinib biopsies. One patient was excluded due to imatinib toxicity and delayed surgery. Baseline characteristics of patients used for this study are summarized in Table 1B. Eight patients presented with primary GIST. Six patients presented with gastric primary disease. Five patients were treated with 3 days of imatinib before surgical resection of their disease, three patients for 5 days, and four patients for 7 days.

**GIST VEGF expression.** We stained the TMA for VEGF expression. Tumors staining positive for VEGF had heterogeneous cytoplasmic and membranous distribution (Fig. 1). Twenty-six (49%), 18 (34%), and 9 (17%) of 53 sections were stained negative, weakly positive, or strongly positive for VEGF, respectively (Table 2A). Most tumors of the stomach, small intestine, and colon were stained negative or weakly for VEGF. The two mesenteric tumors and two esophageal tumors expressed VEGF strongly. VEGF expression seemed to have an equal distribution among primary and metastatic tissue (data not shown). Therefore, high VEGF expression did not associate with metastatic disease compared with primary disease.

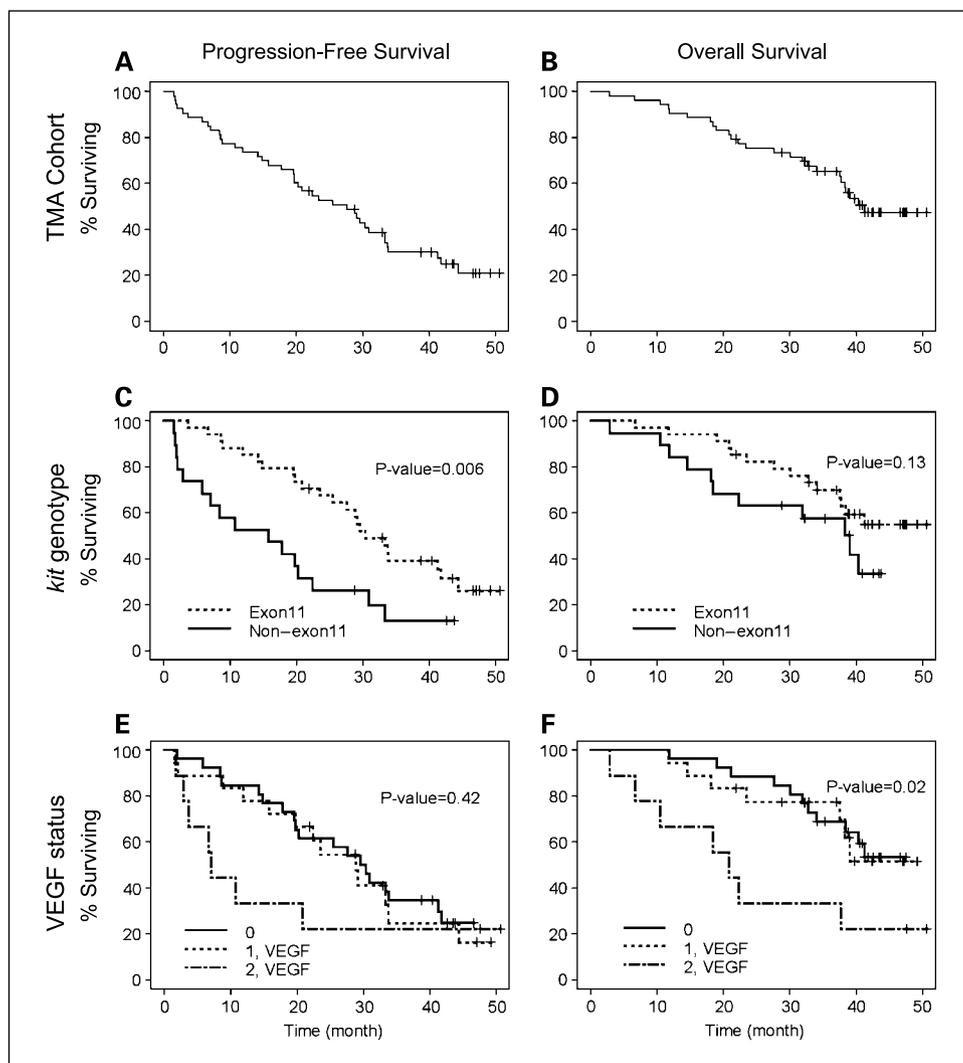
**Mutation status.** Mutational analysis was carried out on all tumors included in the tissue array. Mutations were divided into exon 11 and non-exon 11 (exons 9, 13, 17, wild-type, and PDGFR- $\alpha$ ). Table 2A compares tumor mutation status versus VEGF expression. Thirty-three (62%) of 53 tumors had exon 11 mutations, whereas 20 (38%) had mutations outside of exon 11 (we did not determine whether these tumors expressed mutations other than *kit* exon 11). No significant association between VEGF expression and mutation status was seen ( $P = 0.72$ ).

**Survival analysis.** Characteristics of patients and their tumors included in the TMA were analyzed to determine potential prognostic factors for patients treated with imatinib. Patient's sex, tumor size, primary tumor site, mitotic count, number of metastatic sites, *kit* exon 11 mutation status, imatinib dose, and VEGF expression levels were included as potential predictors in the regression analyses of OS and PFS.

Table 2B summarizes the fitted models for PFS and OS obtained after eliminating nonsignificant predictors. Of the factors analyzed, site of mutation, VEGF expression, and mitotic count were at least marginally predictive ( $P < 0.10$ ) of patient outcome. Interestingly, the risk of death was lower in patients with either comparatively low or high mitotic counts (Supplementary data). Five mitoses per 50 HPF seemed to be the apex or reflection point of the curve associated with highest risk of death.

African-Americans and Caucasians were found to more likely have tumors with exon 11 mutation compared with Asians and Hispanics (data not shown). A slightly larger proportion of patients with no expression of VEGF received 400 mg/day imatinib, whereas a slightly larger portion of patients with

**Fig. 2.** Kaplan-Meier survival plots for patients in TMA cohort. *A-B*, complete cohort's PFS and OS. *C-D*, PFS and OS for patients segregated by *kit* exon 11 or non-exon 11 – expressing GIST. *E-F*, PFS and OS for patients segregated by VEGF expression status of their GIST (markers on survival plots designate patients censored).



strongly positive VEGF received 800 mg/day imatinib (data not shown).

Figure 2A and B displays the Kaplan-Meier survival plots for the tissue array patient cohort. The overall median PFS was 27 months, and the overall median OS was 41.2 months. We segregated the patients based on *kit* mutation status and intratumoral VEGF expression (Fig. 2C-F). Patients with non-exon 11 mutation-expressing tumors had an inferior PFS (median, 17.5 months versus 30 months;  $P = 0.006$ ). However, they did not have a significantly different OS (median, 39 months versus median not reached at >50 months;  $P > 0.10$ ).

Although strong expression of VEGF seemed to be an infrequent event in GIST by immunohistochemistry, it had a significant effect on patient survival. Patients with tumors expressing VEGF strongly had an inferior PFS (median, 7.07 months) in contrast to patients with tumors scoring negative or weakly positive for VEGF (median, 29.6 and 28.8 months, respectively,  $P = 0.42$ ). Likewise, patients with tumors expressing VEGF strongly had a median OS of 20.8 months compared with not reached at >50 months for patients with tumors expressing no or weak VEGF ( $P = 0.02$ ). Survival

curves for patients with tumors expressing no or weak VEGF were virtually identical.

**Imatinib seems to abrogate VEGF production.** To determine imatinib's effect on VEGF production in GIST, we stained frozen core needle biopsies and surgical specimens from clinical trial for VEGF. The summaries of GIST genotype, length of neoadjuvant imatinib, VEGF expression status, and progression are given in Table 3. As expected from the TMA study, a minority of patients (33%) had VEGF-positive tumors. Of the four VEGF-positive GISTs, two were found to no longer express VEGF after a short course of imatinib. Of note, two tumors that continued to express VEGF after neoadjuvant imatinib encoded non-exon 11 *kit* mutant genotypes. Moreover, three of the four patients that presented with VEGF-expressing GIST have experienced progression of disease since resection of their disease.

To further investigate the effects of imatinib on VEGF production in GIST, we treated two separate cell lines for 24 to 72 h with or without imatinib and assayed conditioned media for VEGF. As shown in Fig. 3, GIST T1 and GIST882 produced VEGF. Imatinib significantly reduced VEGF production by GIST T1 (by ~100% at 24, 48, and 72 h;  $P \ll 0.05$ ) and

**Table 3.** GIST VEGF expression and regulation by imatinib

Patient no.	GIST genotype	Neoadjuvant imatinib (d)	Baseline VEGF status	Surgical VEGF status	Progressed
1	WT	5	+	+	Yes
2	PDGFR Asp583 Glu del 2143-2154	7	-	-	No
3	Kit exon 11 del 555 and 556 (V555_Q556del)	3	-	-	No
4	Kit exon 11 del 560 (V560del)	7	-	-	No
5	Kit exon 9 6 bp duplication 507 and 503 (A502_Y503dup)	3	+	+	Yes
6	Kit exon 11 12 bp duplication of codons 577 to 58 (P577_H580dup)	3	+	-	No
7	KIT exon 11 48 bp insertion and duplication of 16 amino acids	3	+	-	Yes
8	Kit exon 11 36 bp deletion codons 559 to 570 (V559_Y570del) and a K55BN change at the junctional site	7	-	-	Yes
9	Kit exon 11 point mutation (TGG to CGG) Trp-Arg (W557R)	5	-	-	No
10	Kit exon 11 6 bp deletion codons 557 to 558 (W557_K558del)	5	-	-	No
11	Kit exon 11 6 bp deletion codons 558 to 559 (K558_K559del)	7	-	-	No
12	KIT exon 11 15 bp deletion 554 to 558 (E554_K558del)	3	-	-	No
Total positive (%)			4 (33)	2 (16)	4 (33)

NOTE: Genotype, pre-imatinib and post-imatinib VEGF protein expression by immunohistochemistry, and progression status from patients on MDACC ID03-0023.

Abbreviations: Baseline, VEGF expression in pre-imatinib core needle biopsy of GIST; surgical, VEGF expression in post-imatinib surgical specimen of GIST; +, positive for VEGF expression; -, negative for VEGF expression; yes, patient did progress after resection of tumor; no, patient has not progressed after resection of tumor.

GIST882 (31% at 24 h, 32% at 48 h, and 39% at 72 h;  $P \ll 0.05$ ) compared with untreated controls (Fig. 3A and B). Thus, imatinib altered VEGF production variably in patient tumors and cell lines.

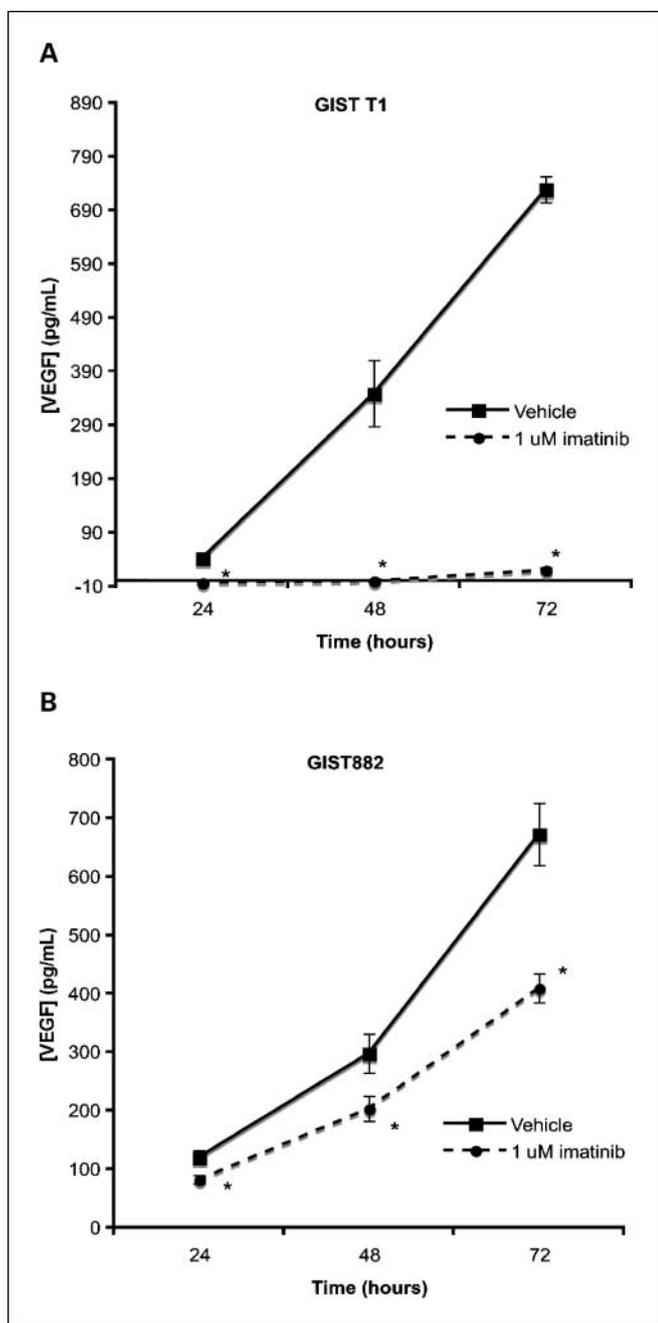
## Discussion

Before the utilization of imatinib in the management of GIST, patients with advanced disease had limited therapeutic options. Patients with large, high-grade, BCL-2-expressing, VEGF-expressing, or *kit* exon 11 mutant tumors had an inferior prognosis. Although surgery remains the stalwart for managing resectable disease, imatinib has greatly improved the clinical outcomes for patients with unresectable or metastatic GIST (24). Yet, prediction of early failure to therapy or long-term outcome is a conundrum. With the recent clinical trial results and Food and Drug Administration approval of sunitinib, physicians and patients may have a choice between therapies to maximize clinical outcomes (13, 25). Therefore, elucidation and understanding of adverse prognostic factors in the imatinib era is necessary to better predict response to therapy and perhaps stratify patients to optimal therapy between imatinib and sunitinib or other regimens that allow concomitant inhibition of KIT and VEGFR signaling. Tumoral production of VEGF may be one such biomarker.

Herein, we have presented data suggesting that VEGF expression is a *kit* genotype-independent adverse prognostic indicator for survival on imatinib therapy. Debiec-Rychter et al. presented a patient population whose median PFS and OS were 24 and 42 months, respectively (14). Our tissue array

patient cohort has a similar survival pattern. However, by stratifying patients based on putative adverse prognostic indicators, we found that patients with tumors expressing high levels of VEGF realized a significantly inferior OS compared with all other variables. Relatively low levels of VEGF expression did not alter survival on imatinib as our data shows. Most interestingly, only 51% of tumors expressed VEGF and only 17% expressed high levels of VEGF. This implies that other vascular cytokines may play a role in this disease. Recently, most GISTs have been shown to express the ligand for Kit, stem cell factor (26). Stem cell factor may play an important role in GIST progression and imatinib efficacy in lieu or in combination with VEGF.

Our cohort includes 62% of patients whose tumors express *kit* exon 11 mutation, which is similar to the 65.8% previously published (14). We divided our cohort into patients expressing *kit* exon 11 mutations and non-exon 11 mutations. Interestingly, after 8 months, the slope of the PFS survival curves seemed to be parallel. This suggests that after rapid progression of the first five patients, the remaining patients whose GISTs encoded a non-exon 11 mutation progressed at a rate similar to that of patients with *kit* exon 11 mutation. This finding is similar to other published cohorts (14). This suggests that a subset of non-exon 11 patients may realize clinical benefit from alternative therapies or increased doses of imatinib. Moreover, other factors besides GIST genotype, such as VEGF expression, may play a role in determining what treatment is optimal for each patient. Thorough study and an increased number of non-exon 11 patients are necessary to elucidate significant differences in tumor biology and survival.



**Fig. 3.** VEGF production by GIST cell lines. Points, ELISA data of VEGF production from human GIST cell lines treated with or without 1  $\mu\text{mol/L}$  imatinib for 24 to 72 h; bars, SD. pg/mL, picograms VEGF per milliliter conditioned media; \*,  $P < 0.05$ ; vehicle, DMSO.

Analysis of mitotic counts presented interesting results. Low and high mitotic counts were associated with decreased risk of death. However, intermediate counts (around five mitoses per 50 HFP) were associated with increased risk of death. This analysis was marginally statistically significant ( $P = 0.04-0.06$ ). Therefore, these data may be a statistical artifact. Alternatively, this may indicate that highly proliferative GISTs are more sensitive to imatinib therapy and more dependent on Kit signaling. This may imply that highly proliferative GISTs have a better prognosis with imatinib therapy. Increased patient number would increase the power of our mitotic count analysis, and our data suggests that further study is required to better understand this phenomenon.

Nevertheless, with controlling for confounding variables, our multivariate and survival analyses show that VEGF is a strong adverse prognostic factor for OS in the imatinib era. From our prospective study of patients treated with imatinib, we show that pretherapeutic tumor VEGF expression can be assessed using core needle biopsy and immunohistochemistry. Whereas the question remains whether VEGF expression is a critical factor for GIST progression, we present a rationale to assess the benefit of stratifying patients based on pretherapeutic tumor VEGF expression to receive imatinib alone or a regimen that targets both Kit and VEGFR.

Of note, we show that mutation status does not seem to correlate with VEGF expression. Moreover, intratumoral VEGF expression is regulated in a subset of VEGF-positive tumors from study of patients on clinical trial. Also, our tested GIST cell lines did have reduction of VEGF production, albeit variably, after imatinib therapy. These data suggest that VEGF production may have variable dependence on Kit signaling. Thus, a subset of VEGF-positive tumors regulate VEGF expression through signaling pathways targeted by imatinib, whereas others may not. Previous studies have shown that Kit signaling modulates VEGF expression in a GIST cell line (27). Yet these data should be taken with caution. VEGF expression seems to be a relatively infrequent event in GIST, and two VEGF-positive tumors did not show abrogation of VEGF expression whereas our cell lines showed variable reduction of VEGF after imatinib treatment. Deusing et al. showed that varying mutation types and site of mutation in *kit* of GIST lead to differences in downstream intracellular signaling (28). Thus, it remains unlikely that VEGF expression is consistently down-regulated by imatinib therapy.

Collectively, these data suggest that better understanding of signaling and vascular biology in GIST is required to provide rational therapeutic interventions to optimize patient care. Likewise, these studies suggest that patients should be considered for pretreatment analysis of intratumoral VEGF expression and *kit* genotype to better aid in prognosis and appropriate selection of therapy.

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