Anti-SIP Antibody as a Novel Therapeutic Strategy for VEGFR TKI-Resistant Renal Cancer

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Abstract

Purpose: VEGFR2 tyrosine kinase inhibition (TKI) is a valuable treatment approach for patients with metastatic renal cell carcinoma (RCC). However, resistance to treatment is inevitable. Identification of novel targets could lead to better treatment for patients with TKI-naïve or -resistant RCC.

Experimental Design: In this study, we performed transcriptome analysis of VEGFR TKI-resistant tumors in a murine model and discovered that the SPHK–SIP pathway is upregulated at the time of resistance. We tested spingosine-1-phosphate (S1P) pathway inhibition using an anti-S1P mAb (sphingomab), in two mouse xenograft models of RCC, and assessed tumor SPHK expression and S1P plasma levels in patients with metastatic RCC.

Results: Resistant tumors expressed several hypoxia-regulated genes. The SPHK1 pathway was among the most highly upregulated pathways that accompanied resistance to VEGFR TKI therapy. SPHK1 was expressed in human RCC, and the product of SPHK1 activity, S1P, was elevated in patients with metastatic RCC, suggesting that human RCC behavior could, in part, be due to overproduction of S1P. Sphingomab neutralization of extracellular S1P slowed tumor growth in both mouse models. Mice bearing tumors that had developed resistance to sunitinib treatment also exhibited tumor growth suppression with sphingomab. Sphingomab treatment led to a reduction in tumor blood flow as measured by MRI.

Conclusions: Our findings suggest that S1P inhibition may be a novel therapeutic strategy in patients with treatment-naïve RCC and also in the setting of resistance to VEGFR TKI therapy.

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Introduction

Renal cell carcinoma (RCC) is the seventh most common cancer among U.S. men and ninth among U.S. women (1). Twenty percent of patients present with locally advanced disease and an additional 30% eventually develop distant metastases (2, 3). Traditionally, treatment for metastatic RCC has been limited. Standard chemotherapeutic agents have been largely ineffective and cytokine-based treatments with interleukin-2 (IL2) or interferon-α (IFNα) benefit only a small proportion of patients.

A major advance in the treatment of advanced RCC over the last decade has been the development and approval of several anti-tumor agents that function primarily as inhibitors of vascular endothelial growth factor (VEGF)–driven angiogenesis or the mammalian target of rapamycin (mTOR). The tyrosine kinase inhibitors (TKI), sunitinib, sorafenib, axitinib, and pazopanib, have shown single-agent activity in patients with metastatic RCC, leading to their approval in subsets of patients with advanced RCC (10, 11). Despite these exciting results, there are few if any complete or durable responses to these agents and tumors develop resistance to all of these treatments usually within 4 to 12 months. Thus, there is still a critical need for the development of new treatment strategies for patients with this disease.

Sphingosine-1-phosphate (S1P) is a bioactive lipid with both tumorigenic and angiogenic activity (12). Sphingolipids comprise a group of intracellular and extracellular signaling molecules that have effects on cell proliferation, chemotaxis, and cytoskeletal stabilization. S1P was initially identified for its role in wound healing and inflammation where it acts via interaction with a series of G protein–coupled receptors. Previous studies have shown that S1P is a proangiogenic growth factor responsible for...
Translational Relevance

Vascular endothelial growth factor (VEGF) pathway inhibition can be efficacious in metastatic renal cell carcinoma (RCC), but resistance to therapy is a clinical problem. This study shows that VEGF receptor inhibition in murine models leads to upregulation of sphingosine kinase 1 (SPHK1) and bioinformatics analyses indicate that SPHK1 may be a central signaling pathway in RCC. SPHK1 catalyzes the production of the bioactive sphingolipid, sphingosine-1-phosphate (S1P). SPHK1 is expressed in human RCC, and S1P is present in RCC patient plasma. *In vivo*, S1P inhibition with a neutralizing antibody, sphingomab, slows tumor growth in murine RCC. Efficacy is seen in treatment-naive tumor models and also after VEGF receptor inhibition. This study provides the preclinical evidence for testing of S1P pathway inhibition in metastatic RCC.

Gene expression analysis

The transcriptional profile of tumor response to drugs was characterized by oligonucleotide microarray analysis using the human U133 PLUS 2.0 Affymetrix GeneChip, according to previously described protocols for total RNA extraction and purification, cDNA synthesis, *in vitro* transcription reaction for production of biotin-labeled cRNA, hybridization of cRNA with U133 PLUS 2.0 Affymetrix gene chips, and scanning of image output files. The differentially expressed genes were identified by group-wise comparison of untreated versus VEGFR-TKI-resistant samples after preprocessing and normalization of data. Differentially expressed genes were identified using a linear model for microarray analysis (34). The microarray data were deposited in the NCBI/GEO database (accession number GSE64052). Limma estimates the differences between Control and Resistance by fitting a linear model and using an empirical Bayes method to moderate standard errors of the estimated log-fold changes for expression values from each probe set. The differentially expressed probes were identified on the basis of absolute fold change (fold change >1.5) and *P* value (*P* value < 0.05).

Interactive network analysis

To decipher the interaction among commonly differentially expressed genes, we performed interactive network analysis. The interactive network was generated using known protein–protein, protein–DNA, coexpression, and protein–RNA interactions. The interaction information was obtained using literature search and publicly available databases. The interaction networks were analyzed using the bottleneck algorithm (35) to identify top master regulator genes involved in the stability of the resistance network.

Human plasma analysis

Human plasma was collected from patients under a protocol approved by our Institutional Review Board at Dana-Farber/Harvard Cancer Center (Boston, MA). Plasma was collected from patients with RCC with known metastatic disease or from healthy volunteers. Sodium citrate tubes were used for plasma collection. S1P levels in human plasma samples were determined by a competitive ELISA using a biotinylated anti-S1P antibody and

the development of capillary tube formation *in vitro* as well as vascular networks *in vivo* (13, 14). In addition, S1P has oncogenic functions including tumor cell growth promotion by inhibition of apoptosis and enhancing metastasis of human cancer cell lines by promoting motility and invasion (15–17). Sphingosine kinase 1 (SPHK1) activity catalyzes the production of S1P. A number of studies have shown overexpression of SPHK1 in human solid tumors including breast, colon, lung, ovary, gastric, endometrial, and rectal cancers (18–25). Moreover, increased expression of SPHK1 is correlated with decreased survival in patients with several forms of cancer (18, 20, 21, 23, 26, 27). It has been proposed that upregulation of the oncogene, SPHK1, and the resulting release of S1P into the tumor microenvironment could represent an important way cancer cells become resistant to treatment (28, 29). Because of S1P’s antiapoptotic, prometastatic, and angiogenic properties, the S1P signaling system is the target of anticancer drug discovery efforts, particularly in the setting of treatment resistance (17). One potential therapeutic intervention point is to block extracellular S1P with the use of specific anti-S1P mAbs (12, 14, 30). In this report, we demonstrate that the murine anti-S1P mAb, sphingomab, slows tumor growth in treatment-naive murine RCC xenograft models. In addition, VEGFR TKI treatment leads to upregulation of the SPHK1 pathway and S1P inhibition exhibits activity after VEGF TKI treatment. We also demonstrate for the first time that patients with RCC have elevated S1P levels in plasma and SPHK expression in tumor tissue, suggesting that the behavior of human RCC could, in part, be due to overproduction of S1P.

Materials and Methods

**Tumor xenograft induction**

Female athymic nude/beige mice (Charles River Laboratories) were used for subcutaneous xenograft tumor models as described previously (31). All experiments were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee. To establish RCC xenografts, 1 × 10^7_7_86-0 or A498 tumor cells were prepared and injected subcutaneously into the flanks of 6- to 8-week-old mice. To ensure a consistent size at the outset of treatment, tumors were measured with calipers daily once they reached a diameter of 3 to 5 mm. Sunitinib (additive-free, 53.6 mg/kg) was administered 6 out of 7 days per week by gavage beginning when the tumors had grown to a diameter of 12 mm as previously described (31, 32). Treated and control tumors were again measured daily during therapy. Mice were sacrificed when the tumors reached 20 mm in long axis. Resistance was defined as the time required for tumors to show the smallest measurable increase in diameter (2 mm in long axis). This increase also roughly approximates the clinical definition of resistance [20% increase in long axis of tumors by Response Evaluation Criteria In Solid Tumors (RECIST) criteria; refs. 31, 33].

**Quantitative real-time PCR analysis**

Quantitative real-time PCR (qRT-PCR) analysis was performed by a two-step process, a 15-cycle preamplification step (AmpliTaq DNA Polymerase Kit; Applied Biosystems Inc.) followed by measurement of mRNA with an ABI PRISM 7900HT Sequence Detection System. For the measurement of mRNA levels SPHK1, primers were custom designed and ordered from IDT. The sequences of the primers are: forward 5′-CITGGAGCCCTTCCCGAGTC-3′, reverse 5′-GCCTCGTACGTAGCATCAGCGTG-3′.

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a S1P-coating conjugate covalently linked to bovine serum albumin (BSA; ref. 30). High binding plates were coated with 1 g/mL S1P-BSA conjugate in carbonate buffer for 1 hour at 37°C, and then blocked overnight in 1% fatty acid free BSA/(phosphate-buffered saline) PBS solution. For the primary incubation, S1P standards (1–2 mM) or the human plasma samples were opportune diluted in delipidated human serum (DHS), premixed with 0.8 μg/mL biotinylated anti-S1P antibody, and then added to the S1P-BSA–coated plates for 1 hour at room temperature. Plates were washed four times in 1/2 PBS, and then the biotinylated antibody competing for S1P coated on the plate against the S1P present in the biologic samples was revealed by horseradish peroxidase (HRP)–streptavidin system (final dilution 1:60,000; Jackson ImmunoResearch) secondary detection. Plates were finally developed with TMB (Invitrogen), blocked with 0.1 mol/L H2SO4, and optical densities were read at 450 nm using a Thermo Multiskan (Thermo Scientific). S1P levels in human plasma samples were then extrapolated from S1P standard curves plotted on GraphPad for analysis.

Tumor perfusion imaging
Tumor perfusion imaging with arterial spin-labeled MRI (ASL MRI) was performed as previously described (32, 33, 36). Briefly, mice were anesthetized and imaged with a 3-cm surface coil on a 3.0 T whole-body clinical MRI scanner. A single transverse slice of ASL imaging was carefully positioned at the center of the tumor, which was marked on the skin with a permanent marker pen for follow-up MRI studies. Each ASL image was obtained with a single-shot fast spin echo sequence by using a background suppressed and flow-sensitive alternating inversion-recovery strategy. Using standard methods to quantify tumor perfusion (37), a region of interest was drawn around the peripheral margin of the tumor on the reference image that was then copied to the perfusion image. The mean blood flow for the tumor tissue within the region of interest was derived.

Immunohistochemistry
Formalin-fixed paraffin-embedded (FFPE) tissue blocks from 12 primary clear cell RCC (ccRCC) patients were retrieved. One primary ccRCC was paired with a corresponding lung metastasis. Areas of primary tumors containing low (G1–G2) and high (G3–G4) Fuhrman nuclear grade (FNG) were selected for the analysis. Immunohistochemistry was performed on 4-μm thick, FFPE tumor sections, which were initially deparaffinized, rehydrated, and heated with a pressure cooker to 125°C for 30 seconds in citrate buffer for antigen retrieval and then incubated with peroxidase (Dako; #S2003) and protein-blocking reagents (Dako; #X0909), respectively, for 5 minutes. Sections were then incubated with an anti-SPHK1 antibody (1:500; Abcam; #ab16491) for 1 hour at room temperature followed by incubation with the

Figure 1.
Heatmap depicting transcripts that are differentially expressed in untreated versus sorafenib-resistant murine 786-O tumors is shown. The columns represent the samples, and rows represent the genes. Gene expression is shown with pseudocolor scale (–3 to 3), with red denoting high expression level and green denoting low expression (A). RT-PCR for SPHK1 is shown in B and is elevated in resistant tumors versus untreated tumors (*, P = 0.005).
Dako EnVision+ System HRP-labeled polymer anti-rabbit (Dako; #K4011) for 30 minutes (26, 38). All sections were developed using the DAB chromogen kit (Dako K3468) for 2 minutes and then lightly counterstained with hematoxylin. A case was considered positive if any positivity in tumor cells was detected.

Statistical analysis
Differences in tumor length and volume were analyzed with ANOVA. Significant differences were assessed with the Fisher least significant difference test.

Results
Sphingosine kinase is upregulated at the time of VEGFR TKI resistance
We have previously shown that VEGFR TKI treatment initially induces rapid tumor devascularization. Then, as tumors develop resistance to treatment, we note resumption of blood flow and tumor growth despite continued therapy (31, 36). A murine VHL-deficient RCC xenograft model (786-O) was treated with the VEGFR TKI, sorafenib. Transcriptome analysis of these tumors

Figure 2.
A systems biology–oriented analysis of the 75 commonly differentially expressed genes from 786-O tumors treated with sorafenib or sunitinib and A498 tumors treated with sorafenib is shown. A, the interactive network of the top 25 bottleneck molecules linked to the resistant phenotype. Each node specifies a gene, and the color at the node edges represents the interactions. The inner color of each node indicates the bottleneck rank (red indicates higher ranking, and green indicates lower ranking), which signifies criticality in maintaining the stability of the network. The outer ring of the node indicates the fold change comparing the untreated versus resistant samples. Upregulation and downregulation are shown by red and green, respectively. B–D, heatmaps of the 25 bottleneck molecules linked to resistance in the three treated conditions (786-O sorafenib, 786-O sunitinib, and A498 sorafenib). In the heatmaps, columns represent tumor samples and rows represent genes. The expression is shown using a pseudocolor scale (−1 to +1, with upregulation and downregulation shown by red and green, respectively).
was performed and is shown in Fig. 1. **SPHK1** was among the genes showing significant upregulation in the resistant tumors. In addition to **SPHK1**, which has been shown to be regulated by hypoxia and HIF, several other known HIF-regulated genes were identified in the array analysis. These include angiotensin like 4 (ANGPTL4), HIF2 (HIF2), and C-X-C chemokine receptor type 4 (CXCR4). RT-PCR was also performed to validate the **SPHK1** increase (Fig. 1B; \( P = 0.005 \)).

To further explore the importance of **SPHK1** regulation, we also analyzed transcriptome profiles from another VHL-deficient RCC xenograft model, A498, treated with sorafenib. In addition, the 786-O tumor model transcriptome was also performed after treatment with sunitinib. Seventy-five genes were significantly differentially expressed in all three treatment groups after VEGFR TKI treatment. These 75 genes were then used for systems biology analysis using interactive network analysis to identify the master regulators involved in resistance to VEGFR TKI treatment. We then selected the top ranked 25 genes by a bottleneck algorithm and considered them as critical players in mediating TKI resistance (Fig. 2A). **SPHK1** was among these top molecules and may initiate systemic therapy with a VEGFR TKI. **SPHK1** was found upregulated in resistant tumors. In the case for which primary and metastasis were available, both the primary (Fig. 3C) and the metastasis (Fig. 3D) were positive for **SPHK1** expression.

**SPHK1** expression in tumor cells (Fig. 3B and C). Faint **SPHK1** positivity was seen in the proximal tubules of the normal kidney (Fig. 3A).

**Patients with metastatic RCC have elevated plasma S1P**

**SPHK** catalyzes the production of S1P. Thus, plasma S1P was compared in patients with metastatic RCC who were scheduled to initiate systemic therapy with a VEGFR TKI. S1P was measured in 97 individuals (Fig. 3, bar graph and Supplementary Table S1). S1P levels were significantly higher in patients with RCC (\( F_{1,95} = 44.71; \ P < 0.001 \)). Average S1P concentration was \( 0.689 \pm 0.411 \) mmol/L for patients with RCC versus \( 0.159 \pm 0.127 \) mmol/L for healthy volunteers. S1P was initially measured in 110 individuals. Thirteen individuals in the control group had S1P levels that were too low to be reliably quantified by the assay. To be conservative, these individuals were excluded from the analysis.

**Sphingomab treatment delays RCC tumor growth**

To block S1P signaling in *vivo*, mice bearing 786-O xenografts were treated at two dose levels of sphingomab. As shown in Fig. 4A, all doses tested slowed tumor growth compared with the vehicle-treated tumors. Both long axis and tumor volume were measured and recorded and both means of following tumor growth showed similar results. The number of days to increase by 2 mm in long axis was recorded as the smallest measurable increase in tumor size and is longer in the 10 and 50 mg/kg arms compared with the vehicle-treated tumors. This change is similar to parameters followed in patients by RECIST criteria. Vehicle-treated tumors increased in volume more quickly than those treated with sphingomab (\( F_{2,16} = 4.228; \ P = 0.034 \)). Average time to +75% volume was 8.5 days in the control versus 14.7 and 17.5 days in the 10 and 50 mg/kg groups, respectively. Vehicle-treated tumors also increased in length more quickly than those treated with sphingomab (\( F_{2,16} = 23.437; \ P < 0.001 \)) and all pairwise treatments were significantly different. Average time to
To confirm these findings in another model, mice bearing A498 tumors were treated with 50 mg/kg sphingomab, vehicle, or isotype control antibody. Similar to findings in the 786-O tumor model, and shown in Fig. 4B, sphingomab delays tumor growth in the A498 tumor model as measured by either long axis or volume. Vehicle-treated tumors increased in length more quickly than those treated with sphingomab \((F_{2,12} = 10.513; P = 0.002)\). Average time to \(+2\) mm was 5.0 days in both the vehicle and isotype treatments \((n = 5\) and 3, respectively). Time to \(+2\) mm averaged 15.29 days in the sphingomab group \((n = 7)\).

Sphingomab slows tumor growth of sunitinib-resistant tumors

Sunitinib treatment slows tumor growth but despite continued treatment, tumors eventually grow and develop resistance to therapy. To study S1P inhibition in sunitinib-resistant tumors,
were nearly significant differences in tumor volume between vehicle-treated tumors and those switched to sphingomab, rapamycin, or both sphingomab and rapamycin (A). A 2-day treatment washout was initiated after the tumors developed resistance to sunitinib (growth by +2 mm in long axis), and then the second treatment regimen was started. A, differences in tumor volume between vehicle-treated tumors and those switched to sphingomab, rapamycin, or both sphingomab and rapamycin were nearly significant ($F_{3, 22} = 2.576; P = 0.080$). Average time to +40% was 10.2 days in the control, 17.75 days in the sphingomab, 20.83 days in the rapamycin, and 23.29 days in the combination sphingomab and rapamycin group (A). Vehicle-treated tumors increased in length more quickly than those treated with sphingomab, rapamycin, or both sphingomab and rapamycin ($F_{3, 22} = 4.914; P = 0.009$). B, average time to +2 mm was 6.6 days in the control group, 17.67 days in the sphingomab group ($n = 6; P = 0.057$), and 18.88 days in the rapamycin group ($P = 0.102$). Tumors treated with both sphingomab and rapamycin grew the least, averaging 30.43 days to reach +2 mm ($P = 0.001$ compared with control, and 0.044 and 0.049 compared with sphingomab or rapamycin alone). Letters on the bar chart denote the results of Fisher’s LSD multiple comparisons procedure. Treatments that share a letter are not significantly different from each other.

Discussion
In the setting of RCC, VEGF is one of the most critical survival factors for tumors likely due to defects in the von Hippel Lindau (VHL) tumor suppressor and resultant accumulation of HIF, seen in the majority of patients with clear cell RCC. In line with this, VEGF inhibition with TKI therapy leads to an antiangiogenic effect and improved clinical outcomes. However, there remains a great unmet need in treating patients with RCC as nearly all patients treated with VEGFR TKIs develop resistance to therapy (39, 40). An understanding of the mechanisms responsible for resistance is critical for the design of second- and third-line therapies. We have previously shown that resistance to VEGF inhibition is due, in part, to resumption of angiogenesis, despite continued treatment with TKIs (31, 32, 36). We have also known that the hypoxia that follows initial tumor devascularization produced by anti-VEGF therapy can modulate pathways responsible for acquired resistance (40). Several groups (28, 41, 42) have invoked an association between upregulation of key HIF proteins in the hypoxic tumor microenvironment and S1PR1 expression to explain the contribution of S1P production to tumor growth and treatment resistance (43, 44).

Because of the well-known S1PR1–HIF axis associated with hypoxia and resistance in the context of prostate and other cancers (42, 43, 45), we hypothesized that the resistance to VEGF TKI therapy in mice bearing human RCC tumors and patients with
RCC would show an upregulation of the SPHK1/S1P signaling system. Accordingly, we found that mice bearing human RCC tumors showed an upregulation of several hypoxia-related genes, including SPHK1. Upregulation of SPHK1 and its interactants was seen in three treatment contexts and network analysis identified SPHK as a master regulator molecule. In addition, we demonstrated for the first time that patients with RCC show SPHK1 expression in their tumors and elevated plasma S1P levels. These data support the hypothesis that an anti-S1P therapy would be beneficial after VEGFR TKI failure and that the SPHK1/S1P signaling system could contribute to VEGFR TKI resistance. Sphingosine kinase 1 (SPHK1) is upregulated in many solid tumor types (18–25; ref. 46). For ovarian, glioblastoma, breast, and other cancer types, SPHK1 expression correlated with mortality in patients (18, 20, 21, 23, 26, 27). This is to our knowledge, the first study to demonstrate SPHK1 expression in RCC.

We tested the antitumor activity of murine anti-S1P mAb, sphingomab, as a first-line therapy and second-line therapy in sequence with sunitinib. In both settings, sphingomab inhibited tumor progression. These data place S1P inhibition in two spaces of clinical testing. The effects seen in both the treatment-naïve setting and after VEGFR TKI resistance could be a result of the overall susceptibility of RCC to S1P inhibition in addition to its role in resistance. Importantly, the combination of sphingomab and mTOR inhibition with rapamycin resulted in some additive benefit, and merits further exploration.

To date, multiple experimental findings support the role of S1P as a promoter of tumor growth, angiogenesis, chemoresistance, and metastasis. Sphingomab is the first monoclonal therapeutic antibody that works as a molecular sponge for extracellular S1P, blocking its biologic activities through all S1P receptors on tumor cells and the tumor microenvironment. On the basis of the result presented, the inhibition of S1P signaling using the anti-S1P antibody approach may represent a novel approach to addressing VEGFR TKI failures in the human RCC setting. In our models, sphingomab appears to have an antiangiogenic effect as evidenced by reduced ASL MRI measured tumor blood flow. Our prior studies have shown that treatment with sunitinib results in a 50% to 80% reduction in tumor blood flow from baseline (33, 47). S1P inhibition lowers perfusion to a lesser magnitude. Thus, we hypothesize that anti-S1P exerts either an effect on vessel function beyond flow and/or angiogenic independent effects.

However, in general, the efficacy and antiangiogenic effects of sphingomab in the renal cancer models is in line with effects of anti-S1P therapy shown in other animal models of human tumors (14).

In this study, we identified the S1P pathway as relevant in patients with RCC as plasma levels were elevated in patients with metastatic RCC versus healthy volunteers. S1P may represent a biomarker for selection of patients who might benefit from S1P pathway inhibitors. Assays to detect free and bound S1P are currently being developed for this purpose and may help guide...
future trials. Decrease in lymphocyte counts has also been reported to be a biomarker for S1P pathway inhibition supporting a potential immunomodulatory role for S1P inhibitors (48, 49). Future studies will also help define the specific effects of S1P on tumor immunity. We also detected expression of SPHK1 in most primary RCC tumors and concordant expression in one metastatic lesion. SPHK1 expression may be another marker for response to S1P pathway inhibition and we are conducting further studies to assess SPHK1 expression in relation to tumor grade and clinical outcome in primary tumors and metastases.

Concurrent with our studies, the humanized form of the anti-S1P mAb, sonepcizumab/ASONEP, was developed for clinical testing. A phase I safety study was recently completed for sonepcizumab. The ASONEP trial was a multicenter, open-label, single-arm, dose-escalation study of sonepcizumab administered as a single agent weekly to subjects with refractory advanced solid tumors at doses between 1 and 24 mg/kg (50). Sonepcizumab was well tolerated with no dose-limiting toxicities observed. On the basis of this study and our preclinical studies, a phase II trial is under way in patients with RCC who have progressed following VEGFR TKI treatment.

Future studies will focus on the specific mechanisms by which S1P pathway blockade slows tumor growth. In addition, biomarkers of response and resistance to S1P pathway inhibition may help guide the clinical development of this new therapeutic option for patients with RCC.

Disclosure of Potential Conflicts of Interest
K. Moreno is an employee of Lpath. B. Visentin and R.A. Sabbadini are employees of and have ownership interest (including patents) in Lpath.

D. Deutschman has ownership interest (including patents) in Lpath. R.S. Bhatt reports receiving a commercial research grant from Lpath. No potential conflicts of interest were disclosed by the other authors.

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