Cancer Therapy: Preclinical

Cross-talk between EphA2 and BRaf/C Raf Is a Key Determinant of Response to Dasatinib

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Abstract

Purpose: EphA2 is an attractive therapeutic target because of its diverse roles in cancer growth and progression. Dasatinib is a multikinase inhibitor that targets EphA2 and other kinases. However, reliable predictive markers and a better understanding of the mechanisms of response to this agent are needed.

Experimental design: The effects of dasatinib on human uterine cancer cell lines were examined using a series of in vitro experiments, including MTT, Western blot analysis, and plasmid transfection. In vivo, an orthotopic mouse model of uterine cancer was utilized to identify the biologic effects of dasatinib. Molecular markers for response prediction and the mechanisms relevant to response to dasatinib were identified by using reverse phase protein array (RPPA), immunoprecipitation, and double immunofluorescence staining.

Results: We show that high levels of CAV-1, EphA2 phosphorylation at S897, and the status of PTEN are key determinants of dasatinib response in uterine carcinoma. As a set of markers essential for dasatinib response was also identified and includes CRaf, pCRafS338, pMAPKT202/Y204 (mitogen-activated protein kinase [MAPK] pathway), pS6S240/244, p70S6kT389 (mTOR pathway), and pAKTS473. A novel mechanism for response was discovered whereby high expression level of CAV-1 at the plasma membrane disrupts the BRaf/C Raf heterodimer and thus inhibits the activation of MAPK pathway during dasatinib treatment.

Conclusions: Our in vitro and in vivo results provide a new understanding of EphA2 targeting by dasatinib and identify key predictors of therapeutic response. These findings have implications for ongoing dasatinib-based clinical trials.

Clin Cancer Res; 20(7); 1846–55. ©2014 AACR.

Introduction

EphA2 is a receptor tyrosine kinase involved in many processes crucial to malignant progression (1–3). Overexpression of EphA2, which has been reported for many human cancers, including breast, melanoma, prostate, lung, ovarian (4, 5) and uterine cancers (6, 7), is often associated with poor prognostic features (8). For these reasons, EphA2 is considered an important therapeutic target. Various therapeutic strategies targeting EphA2 have been developed, including monoclonal antibodies, immunconjugates, small-molecule tyrosine kinase inhibitors, vaccines, and RNA interference (9, 10). Among these, dasatinib is the farthest along with regard to clinical development, even though it was primarily developed as an oral dual inhibitor of Bcr/Abl and Src family kinases. Genomic and proteomic profiling have shown EphA2 to be a direct target of dasatinib (11–13). Dasatinib has shown activity in a fraction of patients with solid tumors (14–17); however, reliable predictors of response to this agent are not known.

Here, we sought to identify a set of markers in uterine carcinoma that are essential for dasatinib response and to investigate the underlying mechanisms of response based on EphA2 function. We discovered that caveolin-1 (CAV-1)- mediated cross-talk between EphA2 and BRaf is a key determinant of dasatinib response in uterine cancer cells. Moreover, EphA2 phosphorylation at S897 and the status of PTEN are important determinants of response to dasatinib.
Translational Relevance
EphA2 is considered an important therapeutic target as it is involved in many processes crucial to malignant progression. Among the various therapeutic strategies targeting EphA2, dasatinib is the farthest along with regard to clinical development. However, reliable predictors of response and the mechanisms relevant to dasatinib response have been largely lacking. We have identified a set of molecular predictors of dasatinib sensitivity in uterine cancer that may have significant implications for ongoing dasatinib-based clinical trials. Our key findings are that CAV-1, EphA2 phosphorylation at S897, and the status of PTEN are key determinants of dasatinib response. Moreover, we have identified a previously unknown mechanism involving the association between CAV-1, EphA2, and the heterodimer BRAF/CRAF, which has implications for designing new therapeutic combinations.

Materials and Methods
Cell cultures
We selected uterine cancer cell lines HEC1-A, Ishikawa, SKUI-2, RL95-2 and KLE (all EphA2 positive) and SPEC-2, AN3CA, and HEC-265 (all EphA2 negative). Cells were purchased from the MD Anderson Characterized Cell Line Core Facility, which supplies authenticated cell lines. The cell lines were routinely tested to confirm the absence of mycoplasma, and all experiments were performed with cell lines at 60% to 80% confluence. Cells were maintained in specific culture medium as described previously (6). The molecular features of cell lines (EphA2 status, CAV-1 status, RAS mutation, and PTEN mutation) are listed in Fig. 1A (left).

MTT assay
Inhibition of cell growth by dasatinib was detected using the MTT cell-proliferation assay, a tetrazolium-based colorimetric assay performed in quadruplicate. Additional details about the treatment and MTT assay are provided in the Supplementary Methods (available online).

Western blot analysis and immunoprecipitation analyses of dasatinib targets in vitro
The expression levels of pEphA2S897, pSrcY416, pFAKY397, pFAKY925, pPaxillinY118, pAKTy473, pp130CasY410, CAV-1, and pS6S240/244 in the uterine cell lines were determined and Santa Cruz Biotechnology), and blots were detected before and after dasatinib treatment were immunoprecipitated with antibodies against EphA2 (Millipore and Santa Cruz Biotechnology), BRAF, or CAV-1 (CST, Danvers, and Santa Cruz Biotechnology), and blots were detected with antibodies against them. Additional details about the immunoprecipitation assays are provided in the Supplementary Methods (available online).

Site-directed mutagenesis of EphA2 at serine 897
We designed 2 types of point mutations at S897 of EphA2, 1 for inactivated pEphA2S897 (serine-897 to alanine: 5’-GGGGGTG-3’ and 5’-CACCCCTCCAGCCGGCTCGTG-3’) and 1 for constitutively activated pEphA2S897 (serine-897 to glutamine: 5’-GGGGGTG-3’ and 5’-CACCCCTCCAGCCGGCTCGTG-3’). The human cDNA open reading frame Myc-DDK-tagged clone of EphA2 (NM_004431; Origene) was amplified using these primers containing the desired mutation by polymerase chain reaction. The product was digested with DPN1 for 1 hour to eliminate methylated DNA. The final mutant EphA2 was confirmed by sequencing (SeqWright).

Double immunofluorescence staining
To test the dynamic changes in the localization of EphA2, CAV-1, and BRAF in SKUT-2 cells treated with dasatinib at 100 nmol/L for 16 hours, double staining was carried out using the rabbit monoclonal antibody against CAV-1 (red) and mouse monoclonal antibody for BRAF (green) or EphA2 (red or green). The stained cells were visualized by confocal microscopy at ×200 magnification.

In situ proximity ligation assay
In situ PLA was performed according to manufacturer’s instructions (Olink Bioscience). Briefly, after incubation with primary antibodies, the cells were incubated with a combination of corresponding PLA probes, secondary antibodies conjugated to oligonucleotides (mouse MINUS and rabbit PLUS). Subsequently, ligase was added forming circular DNA strands when PLA probes were bound in close proximity, along with polymerase and oligonucleotides to allow rolling circle amplification. Fluorescently labeled probes complementary in sequence to the rolling circle amplification product was hybridized to the rolling circle amplification product (Duolink Detection Kit 563; Olink Bioscience). Thus, each individual pair of proteins generated a spot (blob) that could be visualized using fluorescent microscopy at ×200 magnification.

Transfection of siRNA
siRNA was purchased from Sigma-Aldrich. A non-silencing siRNA that did not share sequence homology with any known human mRNA based on a BLAST search was used as control for target siRNA. For in vitro delivery, siRNA (5 µg) was incubated with 30 µL RNAiFect transfection reagent (Qiagen) for 10 minutes at room temperature and added to cells in culture at 80% confluence in 35 mm culture plates. The medium was changed 6 hours later, and cells collected after 48 hours as lysate for Western blot analysis.
SKUT-2, SPEC-2, HEC1-A, and Ishikawa cells were treated with 1 mmol/L dasatinib and 5 nmol/L paclitaxel for 16 hours. Samples were probed with 176 validated primary antibodies by RPPA at the MD Anderson Cancer Center RPPA Core Facility (see Supplementary Methods [available online] for additional details).

Orthotopic in vivo model of uterine cancer and tissue processing

All animal studies were approved and supervised by the MD Anderson Institutional Animal Care and Use Committee. Two uterine cancer cell lines, SPEC-2 (no EphA2 expression) and SKUT-2 (high EphA2 expression) were used for in vivo experiments as previously described (6). For in vivo therapy experiments, 10 mice were randomly allocated into 4 treatment groups: control, dasatinib (15 mg/kg oral, daily), paclitaxel (100 μg in 200 μL of PBS intraperitoneally, weekly), or dasatinib plus paclitaxel. Therapy was initiated 2 weeks after cell injection. Additional details are provided in the Supplementary Methods (available online).

Immunohistochemical staining in the mouse uterine tumor samples

Paraffin-embedded tissues were used to detect cell proliferation (with Ki67) and apoptosis (with cleaved caspase-3). The sections were incubated with the Ki67 antibody (1:400; Dako) and monoclonal mouse antibody against cleaved caspase-3 (1:100; Biocare Medical). CD31 staining with rat monoclonal anti-mouse CD31 (1:800, PharMingen) was used to detect cell proliferation (with Ki67) and apoptosis (with cleaved caspase-3). The sections were incubated with the Ki67 antibody (1:400; Dako) and monoclonal mouse antibody against cleaved caspase-3 (1:100; Biocare Medical). CD31 staining with rat monoclonal anti-mouse CD31 (1:800, PharMingen) was used to detect cell proliferation.
performed on frozen sections. Additional details about immunohistochemistry (IHC) method are provided in the Supplementary Methods (available online).

**Immunohistochemical staining of human uterine cancer specimens**

After approval by the MD Anderson Institutional Review Board, slides from 40 cases of uterine cancer were obtained from the surgical pathology files of MD Anderson. For human uterine cancer samples, immunohistochemical analysis for CAV-1 (1:200 dilution; CST), pAKTS473 (1:100 dilution; CST), and pEphA2S897 and pEphA2Y594 (1:200 dilution; Cell Applications) was performed as described previously (18). See Supplementary Methods (available online) for additional details.

**Statistical analysis**

For in vivo therapy experiments, 10 mice were used in each group, which provided the power to detect a 50% reduction in tumor size ($\beta$ error = 0.2). Continuous variables were compared using Student t test (2 groups) or analysis of variance (all groups) if the data were normally distributed. For nonparametric distributions, the Mann–Whitney U or Kruskal–Wallis test (all groups) was deemed statistically significant.

**Results**

**In vitro effect of dasatinib on EphA2-positive or -negative uterine cancer cell lines**

We first assessed the effect of dasatinib on a panel of 6 uterine cancer cell lines with known EphA2 expression levels (Fig. 1A, left). The median inhibitory concentration (IC50) of these cell lines ranged from 0.03 to 17.9 mol/L (Fig. 1A, middle). Among these, EphA2-positive SKUT-2 cells were the most sensitive to dasatinib, whereas EphA2-negative SPEC-2 cells were the most resistant (Fig. 1A, right), suggesting that EphA2 status is a potential determinant of dasatinib sensitivity. The exception to this pattern was the EphA2-positive HEC1-A cell line (harbors a RAS mutation), with a higher IC50 value than other EphA2-negative cells. These observations were further supported by EphA2-negative HEC-265 and EphA2-positive KLE uterine cancer cells, and by ectopic expression of EphA2 in the EphA2-negative A2780 ovarian cancer cells (Supplementary Fig. S1A and S1B).

**Wild-type PTEN increased sensitivity to dasatinib in EphA2-positive cell lines in vitro**

We found that dasatinib exhibited less growth inhibition in EphA2-positive RL95-2 cells, which harbors high basal level of pAKT$^{S473}$. Therefore, next we addressed whether PTEN, which is frequently altered in uterine carcinoma (19), influences response to dasatinib through its effects on pAKT$^{S473}$, AKT activation and PTEN mutation status in a panel of uterine cancer cell lines is shown in Fig. 1A and B. Next, we transfected wild-type or mutant PTEN into Ishikawa cells carrying mutant PTEN. The dasatinib sensitivity of Ishikawa cells transfected with wild-type PTEN was significantly enhanced by 5-fold, compared with parental cells carrying PTEN mutation (Supplementary Fig. S2), suggesting that wild-type PTEN is also a determinant of dasatinib sensitivity in uterine cancer cells.

**In vitro effects of dasatinib on Src/FAK/EphA2 signaling pathway**

Next, we examined signaling events in the Src/FAK/EphA2 pathway known to be perturbed by dasatinib. The expression level of the traditional targets of dasatinib, including pAKT$^{S473}$, pSrc$^{Y416}$, pFAK$^{Y925}$, pFAK$^{Y397}$, and pFAK$^{Y118}$, were reduced in all 6 uterine cancer cell lines after dasatinib treatment, whereas pFAK$^{Y397}$ expression level was not notably affected in any of the cell lines (Fig. 1B). These results support the known effects of dasatinib, but they do not explain the sensitivity of specific uterine cancer cell lines to it.

**pEphA2S897 status is an important determinant of response to dasatinib in uterine cancer cells**

Given the differential expression of EphA2 in dasatinib-resistant versus dasatinib-resistant cell lines and the role of ligand-independent pEphA2S897 in many oncogenic functions (20), we next examined the effects of dasatinib on pEphA2S897. The expression of pEphA2S897 was significantly decreased in SKUT-2 cells, but moderately so in HEC1-A and Ishikawa cells (Fig. 1B and Supplementary Fig. S4). Furthermore, because the expression levels of pEphA2S897 and pEphA2Y594 are inversely related (20), EphA2 tyrosine phosphorylation was decreased in HEC1-A and Ishikawa cells, but increased in SKUT-2 cells, after dasatinib treatment (Fig. 1C). The extent of inhibition was directly related to dasatinib response. To further test whether phosphorylation at S897 is critical for dasatinib response, we performed site-directed mutagenesis of pEphA2 at S897 and transfected SKUT-2 cells with plasmids for wild-type EphA2, inactivated pEphA2S897, or constitutively activated pEphA2S897. The cells with constitutively activated pEphA2S897 had reduced sensitivity to dasatinib, whereas the cells with inactivated pEphA2S897 remained highly sensitive to dasatinib (Fig. 1D). These results confirmed that pEphA2S897 status is an important determinant of response to dasatinib in uterine cancer cells (Supplementary Table S1).

**Identification of predictive molecular markers**

To explore other potential markers of response to dasatinib, we used RPPA to quantify protein expression of genes involved in cell cycle, apoptosis, angiogenesis, and adhesion that are modulated by dasatinib in the SPEC-2, SKUT-2, HEC1-A, and Ishikawa cell lines (Fig. 2 and Supplementary Figs. S3, S4, and Tables S3–S10). The expression levels of total CRaf, pCRaf$^{S338}$, pS6$^{240/244}$, p70S6K$^{T389}$, pAKTS473, total EphA2, pEphA2S897, pMAPK$^{Y202/204}$, mTOR, and pMEK$^{S172/182}$ were significantly decreased in the dasatinib-sensitive SKUT-2 cells but not in the dasatinib-resistant SPEC-2, HEC1-A, or Ishikawa cells. Moreover, KRAS and PI3K expression levels did not change significantly in...
SKUT-2 cells. Interestingly, a high basal level of CAV-1 was detected in SKUT-2 cells compared with the 3 other cell lines. We next carried out a series of experiments to identify potential mechanistic explanations for response to dasatinib-based therapy. In SKUT-2 cells, Western blot analysis validated the changes in the levels of pS6S240/244 and CRaf following dasatinib therapy; a high basal level of CAV-1 was detected only in SKUT-2 cells and CAV-1 and BRaf had no notable changes (Fig. 3A and B). Next, we carried out immunoprecipitation studies with antibodies against EphA2, CAV-1, and BRaf. Immunoprecipitation analysis showed direct binding between EphA2 and CAV-1, EphA2 and BRaf, and CAV-1 and BRaf in SKUT-2 cells treated with dasatinib (Fig. 3C), but not in HEC1-A and Ishikawa cells (data not shown), or in the absence of dasatinib. Meanwhile, our immunoprecipitation analysis showed that dasatinib induced the heterodimer of BRaf/CRaf in Ishikawa cells, but not in SKUT-2 cells (Fig. 3D), indicating that

Figure 2. Expression of multiple proteins associated with Src/FAK/EphA2 and RAS/RAF/MAPK signaling pathways as detected with reverse-phase protein array. A, heatmap of molecules whose expression significantly (P < 0.05) differed before and after treatment with dasatinib (100 nmol/L), paclitaxel (5 nmol/L), or both in SPEC-2 cells (top) and SKUT-2 cells (bottom) for 16 hours. B and C, normalized expression levels in SPEC-2 cells (B) and SKUT-2 cells (C) treated with (red) or without (blue) dasatinib. Data represent means of triplicate measurements.

Figure 3. Effect of dasatinib treatment on protein expression of pS6S240/244, CAV-1, BRaf, and CRaf in uterine cancer cells. A and B, WB analysis of the expression levels of pS6S240/244 and CAV-1 (A) and BRaf and CRaf (B) in uterine cancer cells treated with (+) or without (−) dasatinib at 100 nmol/L for 16 hours (top). Densitometry was performed to objectively assess potential differences (bottom). C, IP and WB analysis of the interaction of CAV-1, BRaf, and EphA2 in SKUT-2 cells treated with (+) or without (−) dasatinib for 8 hours. D, IP and WB analysis of the interaction of CRaf and BRaf in Ishikawa, SKUT-2, and HEC1-A cells treated with (+) or without (−) dasatinib for 8 hours.
colocalization between BRaf and CAV-1 induced by dasatinib in SKUT-2 cells interferes with the formation of the BRaf/CRAf heterodimer and therefore can inactivate signaling downstream of BRaf/CRAf (mitogen-activated protein kinase [MAPK] pathway). The heterodimer of BRaf/CRAf was found in HEC1-A cells treated with or without dasatinib (Fig. 3D), likely because of the fact that HEC1-A bears a RAS mutation and is more resistant to dasatinib treatment (21). To further explore the association between EphA2, CAV-1, and BRaf, we immunostained SKUT-2 cells for these proteins with or without dasatinib treatment. Immunofluorescence staining analysis showed that BRaf was translocated from the cytoplasm to the plasma membrane and colocalized with CAV-1 after dasatinib treatment (Fig. 4A, left and B, left). EphA2 was also translocated and colocalized with CAV-1 after dasatinib treatment (Fig. 4A, right, top), and EphA2 and BRaf showed colocalization as well (Fig. 4A, right, bottom). No colocalization between BRaf and CAV-1 was observed following dasatinib treatment in SPEC-2 cells (Fig. 4B, right) or in HEC1-A and Ishikawa cells (data not shown). Furthermore, following CAV-1 gene silencing, the growth-inhibitory effects of dasatinib on SKUT-2 cells were attenuated (Fig. 4C). There was no colocalization between BRaf and CAV-1 (Fig. 4B, right) or between BRaf and EphA2 (data not shown) in SKUT-2 cells following CAV-1 silencing, indicating that a high level of CAV-1 is important for the response to dasatinib in tumor cells and required for recruiting BRaf in response to dasatinib therapy. EphA2 silencing resulted in partially increased sensitivity to CAV-1 in SKUT-2 cells, but it did not alter the response to dasatinib in HEC1-A cells (Supplementary Fig. S5), which suggests that EphA2 is also important for the response to dasatinib in tumor cells.

To further investigate whether BRaf or EphA2 have the capability to interact with CAV-1, we used in situ proximity ligation assay (PLA), which is a unique method developed to visualize subcellular localization and protein–protein interactions in situ (22). SKUT-2 and HEC1-A treated with or without dasatinib for 8 hours were incubated overnight with primary antibody pair of different species directed to BRaf or EphA2 (mouse monoclonal antibody) and to CAV-1 (rabbit monoclonal antibody), respectively. The secondary antibodies were modified by addition of complementary oligonucleotides capable of interacting when in close proximity, an event that was detected by PCR amplification using a fluorochrome-based detection method. We observed that the colocalization of CAV-1 with BRaf (Fig. 4D, top) and EphA2 (Fig. 4D, bottom) was increased substantially in SKUT-2 cells after dasatinib treatment, and that the majority of binding interactions were localized on the plasma membrane. No significant increase in the colocalization of CAV-1 with BRaf and EphA2 was observed following dasatinib treatment in HEC1-A cells. Notably, CAV-1 gene silencing by siRNA significantly inhibited the colocalization of CAV-1 with BRaf and EphA2 (Fig. 4D), indicating that high expression of CAV-1 in the cells is required for CAV-1 binding to BRaf or EphA2.

**In vivo effects of dasatinib in orthotopic models of uterine cancers**

Next, we tested the effects of dasatinib-based therapy in orthotopic mouse models of EphA2-positive and -negative uterine carcinoma. In the EphA2-positive SKUT-2 model, dasatinib treatment resulted in significant antitumor activity (79% [P < 0.01] and 63% [P < 0.01], respectively; Fig. 5A). In contrast, in the EphA2-negative SPEC-2 model, dasatinib demonstrated only minimal antitumor activity compared with the untreated (control) group (15% reduction of tumor weight vs. control [P > 0.05] and 10% decrease in number tumor nodules vs. control [P > 0.05]; Fig. 5B). Given the role of paclitaxel in combination with biologically targeted agents, we also tested dasatinib with paclitaxel. The addition of paclitaxel resulted in a large reduction in the SKUT-2 model (95% [P < 0.001] and 68% [P < 0.01], respectively; Fig. 5A), but minimal antitumor activity in the SPEC-2 model (37% [P < 0.05] and 30% [P > 0.05], respectively; Fig. 5B). No obvious toxicity was observed in the various groups in that the mean body weight was similar in all groups (Supplementary Fig. S6A and S6B).

To examine the biologic effects of dasatinib-based therapy, we examined tumors from the SKUT-2 and SPEC-2 models for markers of tumor cell proliferation (Ki67), angiogenesis (CD31), and apoptosis (cleaved caspase-3). Changes in these markers mirrored the antitumor activity in response to dasatinib. Specifically, significant reductions in proliferation and microvessel density and increased apoptosis were noted in the SKUT-2 model (Fig. 5C), whereas more modest changes were noted with the SPEC-2 model (Fig. 5D). In addition, the expression level of pEphA2^S897 was significantly decreased in the SKUT-2 model (79% [P < 0.01]; Fig. 5C, bottom).

**Expression of predictive markers in human samples**

We next examined the expression of the putative dasatinib response markers (CAV-1, pEphA2^Y594, pEphA2^S897, and pAKT^T473) in 30 human uterine cancer samples and 10 normal uterine samples by IHC. Representative pictures of immunohistochemical staining are presented in Fig. 6A. CAV-1 was not highly expressed in the normal uterine samples and was highly expressed in only 6.6% of tumor samples, but varying expression levels of CAV-1 were found in the stroma around the tumor cells (Supplementary Table S2). pEphA2^S897 was overexpressed in none of the normal samples but 70% of tumors, whereas high pEphA2^Y594 expression was detected in 90% of normal samples and 10% of tumor samples.

**Discussion**

The focus of this work is on the multitargeted tyrosine kinase dasatinib, which is used in the clinic for several diseases. Unfortunately, predictive markers guiding its use and subsequent response are largely absent, resulting in “hit or miss” clinical application. Herein, we present evidence from *in vitro* and *in vivo* experiments for a set of markers essential for dasatinib response. Among these, CAV-1, EphA2 phosphorylation at S897 and the status of
PTEN were key determinants of dasatinib response. In addition, CAV-1-mediated cross-talk between EphA2 and BRaf is required for response to dasatinib whereby dasatinib drives BRaf and EphA2 to CAV-1 at the plasma membrane, disrupting the BRaf/CRAf heterodimer and thus downmodulation of the MAPK pathway in dasatinib-sensitive cells, but not in dasatinib-resistant cells (Fig. 6B).
The biologic functions of CAV-1 on cancer cells have been controversial. CAV-1 is known to directly interact via its scaffolding domain with multiple signaling proteins and function as preorganized signalosomes by sequestering and regulating proteins localized in caveolae, including receptor tyrosine kinases and their signaling effectors (23). In general, caveolins bind to and inactivate signaling molecules (24), including those of the MAPK signaling pathway (e.g., Ras, RAF-1, and MAPK; ref. 25). Loss of CAV-1 expression confers a significant growth advantage that is associated with constitutive hyperactivation of the p42/44 MAPK pathway (26). In addition, interaction between Eph families, including EphA2 and CAV-1, has been reported (27). It has been reported that the Eph feedback loop in the activation of the HRAS/RAF/ERK pathway also increases EphA2 expression (28). We found that dasatinib inhibited EphA2 signaling, but unexpectedly stimulated Braf recruitment to the cell membrane, potentially promoting changes in the phosphorylation status and triggering its kinase activity (29). This resulted in increased heterodimerization with CRAF, which resulted in increased MEK/ERK activation (30). Furthermore, we demonstrated the mechanistic basis for high CAV-1 at the plasma membrane holding BRAF, resulting in disruption of the BRAF/CRAF heterodimer and inhibiting MAPK activation following dasatinib treatment. Importantly, this finding could provide new approaches to enhance and extend the activity of dasatinib (e.g., CAV-1 peptide and RAF or MEK inhibitors). The role of estrogen (ER) and progesterone (PR) receptor status could also be relevant for therapeutic considerations.

**Figure 5.** In vivo effects of therapy with dasatinib and paclitaxel in uterine cancer models. A and B, in vivo effect of dasatinib (15 mg/kg oral, daily), paclitaxel (100 µg in 200 µL of PBS intraperitoneally, weekly), or both in an EphA2-positive model (SKUT-2; A) and an EphA2-negative model (SPEC-2; B). Error bars indicate SEM. *, P < 0.05; **, P < 0.01; and †††, P < 0.001. C and D, immunohistochemical staining showing the effect of dasatinib, paclitaxel, or both on uterine cancer cells angiogenesis (CD31), proliferation (K67), and apoptosis (caspase-3) in the SKUT-2 model (C) and the SPEC-2 model (D). Immunohistochemical staining of pEphA2 expression in SKUT-2 cells (C, bottom). *, P < 0.05; **, P < 0.01; and †††, P < 0.001 compared with control group. Original magnification, ×100 or ×200.
previously we have reported that tumors with EphA2 overexpression are more likely to be ER/PR negative. Thus, our findings may have therapeutic implications for patients with poorly differentiated endometrial cancers that are steroid hormone receptor deficient (8).

Although our findings may have clinical implications for ongoing dasatinib-based clinical trials, some potential limitations should be considered. Whether the identified markers are indeed predictive of dasatinib sensitivity in human clinical trials is not known. A pilot and translational study (NSC #732517) of dasatinib, paclitaxel, and carboplatin for women with advanced-stage and recurrent uterine cancer is ongoing. This trial is evaluating pharmacodynamic variances of EphA2 signaling in response to both dasatinib alone (in the lead-in phase) and in combination with chemotherapy. Validation of these biomarkers will be important for the next generation of clinical investigation involving treatment allocation based on specific biomarkers (31). The diversity of biologic characterization that represents "uterine cancer" is only just being described (32) and highlights the need for target prevalence description and prospective determination of expression to effectively test our portfolio of targeted therapeutics (33).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acknowledgments

The authors thank E.L. Hess of the MD Anderson Department of Scientific Publications for helpful editing.

Grant Support

This work was supported by the NIH (P50 CA098258, CA109298, P50 CA083639, U54 CA151668, MD Anderson’s Cancer Center Support Grant CA016672), the Ovarian Cancer Research Fund, Inc. (Program Project Development Grant), the U.S. Department of Defense (OC073399, OC093146), the Ann Rife Cox Chair in Gynecology, the Zarrow Foundation, the Marcus Foundation, the Betty Anne Asche Murray Distinguished Professorship, the RGK Foundation, and the Gilder Foundation. J. Bottsford-Miller and H.J. Dalton are supported by an NCI-DHHS-NIH Program Project Grant (CA16642). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 5, 2013; revised November 27, 2013; accepted December 6, 2013; published OnlineFirst January 31, 2014.

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doi:10.1158/1078-0432.CCR-13-2141

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