Dsh Homolog DVL3 Mediates Resistance to IGFIR Inhibition by Regulating IGF-RAS Signaling

Shan Gao1, Ilirjana Bajrami2, Clare Verrill3, Asha Kigozi2, Djamila Ouaret1, Tamara Aleksic2, Ruth Asher3, Cheng Han1, Paul Allen4, Deborah Bailey4, Stephan Feller1, Takeshi Kashima5, Jean-Yves Blay6, Sandra Schmitz7, Jean-Pascal Machiels7, Nav Upile8, Terry M. Jones8, George Thalmann9, Shazad Q. Ashraf1, Jennifer L. Wilding1, Walter F. Bodmer1, Mark R. Middleton10, Alan Ashworth2, Christopher J. Lord2, and Valentine M. Macaulay1,10

Abstract

Drugs that inhibit insulin-like growth factor 1 (IGFI) receptor IGFIR were encouraging in early trials, but predictive biomarkers were lacking and the drugs provided insufficient benefit in unselected patients. In this study, we used genetic screening and downstream validation to identify the WNT pathway element DVL3 as a mediator of resistance to IGFIR inhibition. Sensitivity to IGFIR inhibition was enhanced specifically in vitro and in vivo by genetic or pharmacologic blockade of DVL3. In breast and prostate cancer cells, sensitization tracked with enhanced MEK–ERK activation and relied upon MEK activity and DVL3 expression. Mechanistic investigations showed that DVL3 is present in an adaptor complex that links IGFIR to RAS, which includes Shc, growth factor receptor–bound-2 (Grb2), son-of-sevenless (SOS), and the tumor suppressor DAB2. Dual DVL and DAB2 blockade synergized in activating ERKs and sensitizing cells to IGFIR inhibition, suggesting a nonredundant role for DVL3 in the Shc–Grb2–SOS complex. Clinically, tumors that responded to IGFIR inhibition contained relatively lower levels of DVL3 protein than resistant tumors, and DVL3 levels in tumors correlated inversely with progression-free survival in patients treated with IGFIR antibodies. Because IGFIR does not contain activating mutations analogous to EGFR variants associated with response to EGFR inhibitors, we suggest that IGF signaling achieves an equivalent integration at the postreceptor level through adaptor protein complexes, influencing cellular dependence on the IGF axis and identifying a patient population with potential to benefit from IGFIR inhibition.

Cancer Res; 74(20); 1–12. ©2014 AACR.

Introduction

Type 1 insulin-like growth factor 1 receptor (IGFIR) signals via recruitment of adaptors, including insulin receptor substrate-1 (IRS-1) and Shc, to drive proliferation, invasion, and cell survival (1). In the clinical and experimental setting, low IGF bioactivity protects from tumor development and metastasis, suggesting that IGFs provide a potent protumorigenic signal (2, 3). In early clinical trials, IGFIR inhibitory drugs induced objective regressions, some dramatic and durable, as monotherapy in Ewing sarcomas and other uncommon tumors, and with chemotherapy or targeted agents in common cancers (4–9). However, these encouraging early reports have not translated to phase III benefit in unselected patients (10). Clearly, a better understanding of IGFIR biology is required if this therapeutic approach is to be successful. Key priorities are to understand what makes tumors resistant to IGFIR inhibition, use this information to target these drugs to potentially responsive patients, and select rational treatment combinations for clinical testing. To address these issues, we used genetic screening and downstream validation to identify novel determinants of resistance to an IGFIR antagonist.
Materials and Methods

Cell lines and reagents
DU145, PC3, LNCaP, and LNCaP-LN3 prostate cancer and MCF-7, MDA-MB-231, and MDA-MB-468 breast cancer cells were from Cancer Research UK Cell Services (Clare Hall Laboratories), 22Rv1 prostate cancer cells from the ATCC, and BT20 and BT549 from Dr. Anthony Kong, University of Oxford, Oxford, United Kingdom. All cell lines were mycoplasma-free when tested with MycoAlert (Lonza Rockland Inc.). AZ12253801 and gefitinib were provided by AstraZeneca, DVL-PDZ inhibitor II (DVLi) purchased from Calbiochem, and BMS-754807, XAV939, and AZD6244 from Selleck Chemicals. DVL3 cDNA (Addgene) was amplified using primers 5′-GGATATGGAACGCAGGAGGAC-3′ and 5′-CTCGAGTCATCATACATCCAAAGAAC-3′ incorporating BamHI and XhoI sites, respectively (underlined), digested with BamHi and XhoI (New England Biolabs), cloned into BamHI-XhoI digested pHRSIN-CSGW HIV vector, and after verification of the insert by DNA sequencing, used for virus production as described (11). Western blotting, immunoprecipitation, and pulldown assays were performed using reagents described in ref. 12, Supplementary Methods, and Supplementary Table S1.

siRNA screens
Screening was performed as described previously (13 and 14), using kinase siRNA library (siARRAY, targeting 779 known and putative human protein kinases; Dharmacon) containing SMARTPools of four siRNAs targeting each transcript, and human DNA Repair siRNA Set V1.0 siRNA library (Qiagen), together with siPLK1 and Allstars siRNA as positive and negative controls, respectively. Cells were reverse-transfected with 50 nmol/L siRNAs using Dharmafect 1 reagent for DU145 and Dharmafect 3 for MCF-7. Two days later, cells were exposed to vehicle (0.01% DMSO) or AZ12253801 at the GI50 for 5 days, and viability was assessed reagent for DU145 and Dharmafect 3 for MCF-7. Two days after siRNA transfection to allow target depletion, cells were treated with solvent or AZ12253801 at the GI50 and viability was assayed 5 days later. Duplicate DU145 and Dharmafect 3 for MCF-7. Two days later, cells were exposed to vehicle (0.01% DMSO) or AZ12253801 at the GI50 for 5 days, and viability was assessed using CellTiter Glo (CTG) assay (Promega). Duplicate primary screens were analyzed to derive Z’ factors (dynamic range) and Z-scores (effect of siRNA on viability, corrected for within/between plate variation), as described (14–16). Triplicate second-round screens used individual siRNAs and the four siRNAs pooled; these and additional siRNAs are listed in Supplementary Table S2. Data were analyzed to generate log2 surviving fractions as the final score for the effect of IGFIR inhibition on viability; scores ≤−2 were regarded as significant, as described (13, 14). Hits were validated by siRNA transfection as above, analyzing after 48 hours by Western blot or quantitative reverse-transcription PCR (qRT-PCR, with primers listed in Supplementary Table S3), or treating with solvent or AZ12253801 and assaying for viability after 5 days or clonogenic survival after 10 to 20 days. Pooled data from ≥3 independent experiments were curve-fitted using GraphPad Prism v5 to interpolate GI50 and SF50 (concentrations inhibiting 50% of growth or survival).

Immunofluorescence and IHC
Cells underwent immunofluorescent staining using ERK antibody (#4695; Cell Signaling Technology). IHC used DVL3 antibody 4D3 (Santa Cruz Biotechnology; see Supplementary Methods).

Xenografts
As detailed in Supplementary Methods, mice bearing DU145 xenografts were randomly allocated to 14 days treatment with 0.05-mL solvent (DMSO), 25 mg/kg AZ12253801 twice daily, 50 mg/kg DVLi once daily, or combination treatment.

Results and Discussion

siRNA screens for sensitization to IGFIR inhibition
IGFIR overexpression occurs frequently in common cancers, but is a poor predictor of clinical sensitivity to IGFIR inhibition (10). Consistent with this, IGFIR overexpression in PC3 prostate cancer cells did not influence response to IGFIR tyrosine kinase inhibitor (TKI) AZ12253801 that has approximately 10-fold selectivity over the insulin receptor (INSR; Supplementary Fig. S1A; ref. 17). To identify proteins that influence response to AZ12253801, we performed siRNA screens in DU145 prostate cancer and MCF7 breast cancer cells, confirming that AZ12253801 inhibited IGFIR phosphorylation and cell viability (Supplementary Fig. S1B). Primary screens were performed to deplete approximately 1,000 targets; given our interest in the involvement of IGFIR kinase in the DNA damage response (12, 18), we selected siRNA libraries targeting kinase-related and DNA repair–associated proteins, with positive (siPLK1) and negative (Allstars) control siRNAs, as described (13, 14). Forty-eight hours after siRNA transfection to allow target depletion, cells were treated with solvent or AZ12253801 at the GI50 and viability was assayed 5 days later. Duplicate DU145 screens were highly reproducible (R2 values ≥0.8) and sensitive, with Z’-factors of 0.23 to 0.6 (Supplementary Table S4) indicating good discrimination between positive and negative controls (15). We calculated drug sensitization Z-scores (13) for each siRNA, rank-ordered siRNAs by Z-score, and selected 54 genes for validation (Supplementary Table S5). Triplicate second-round screens identified 12 putative resistance mediators, including regulators of the cell cycle and DNA damage response, and proteins with poorly characterized functions (Fig. 1A). Seven hits in DU145 screens (CDKN2C, CNKSR1, DUSP5, HUNK, LMTK3, MMP2, and DVL3) were also candidate hits in MCF7 cells. Validation in low-throughput format confirmed that depletion of each hit enhanced AZ12253801 sensitivity (Supplementary Table S6).

DVL3 mediates resistance to IGFIR inhibition
Known cross-talk between the IGF and WNT axes (19–21) prompted us to investigate one of the most robust hits: Dishevelled homolog 3 (DVL3), a poorly characterized WNT component. DVL3 is one of three mammalian homologs of Drosophila Dishevelled (Dsh), a cytoplasmic protein that is phosphorylated on binding of Wnts to Frizzled (Fz) receptors, blocking the β-catenin destruction complex (22). DVL3 was the dominant isoform in both DU145 and MCF7 cells (Fig. 1B), and depletion of DVL3 but not DVL1 or 2 sensitized to AZ12253801 (Fig. 1C and D). Expression of siRNA-resistant FLAG-DVL3 was able to rescue from AZ12253801 sensitization induced by DVL3 siRNA targeting the 3′-UTR of endogenous DVL3 mRNA (Fig. 1E), suggesting that sensitization by DVL3 depletion was unlikely to be an off-target effect of DVL3 siRNA. As noted above, AZ12253801 can also inhibit the closely related INSR. Although
DVL3 Mediates Resistance to IGFIR Inhibition

A

Log₂ cell surviving fraction

AKAP1 APEG1 CDKN2C CNKSR1 DUSP5 HUNK LMTK3 MPP2 CDKN3 DVL3 INO80C RAD51 AS

B

DU145 MCF7

DVL mRNA/GAPDH

0 20 40 60 80 100

1 2 3 1 2 3

DVL isoforms

C

Relative cell proliferation

DU145 Allstars DVL3_6 DVL3_7

Relative proliferation at 100 nmol/L

DU145 Allstars DVL3_6 DVL3_7

D

DU145

DVL expression (% Allstars)

sRNA AS DVL1 DVL2 DVL3

G0%nt 141 142 126 50

Ratio 1.0 1.0 1.2 2.8

E

AZ12253801 (nmol/L)

Relative cell proliferation

DU145

GFP-AS GFP-sDVL3_6 GFP-DVL3-AS GFP-sDVL3-sDVL3_6

Relative proliferation at 100 nmol/L

DU145 GFP-AS GFP-sDVL3_6 GFP-DVL3-AS GFP-sDVL3-sDVL3_6

F

Relative cell proliferation

sRNA1 AS D3 INS INS

sRNA2 AS AS D3

DVL3 IGF-1R INSR β-Tubulin

G

Relative DVL3 protein

Sensitive Resistant Colorectal cell lines
DVL3-depleted DU145 and MCF7 cells were sensitized to IGFIR depletion, INSR-depleted cells showed no significant reduction in viability upon DVL3 depletion (Fig. 1F and Supplementary Fig. S1C), supporting the contention that functional interaction between DVL3 and AZ12253801 is related to the ability of AZ12253801 to block IGFIR. We next tested...
whether intrinsic sensitivity to IGFIR inhibition is related to endogenous DVL3 expression. In prostate (n = 5) and breast cancer (n = 5) cell lines, there was no apparent relationship between endogenous DVL3 protein and AZ12253801 GI50 (Supplementary Fig. S1D), perhaps reflecting genotypic differences in these small panels. In a larger sample (n = 40) of well-characterized colorectal cancer cell lines (23, 24), mean DVL3 protein levels were significantly lower in cell lines that were sensitive to IGFIR antibody figitumumab (n = 11), compared with cell lines that were moderately (n = 8) or highly (n = 21) figitumumab resistant (Fig. 1G), supporting the hypothesis that DVL3 protein is associated with resistance to IGFIR inhibition. There was no correlation with IGFIR expression (Supplementary Fig. S1E and S1F), consistent with the unchanged response to AZ12253801 in IGFIR-overexpressing PC3 prostate cancer cells (Supplementary Fig. S1A). We also noted lack of correlation between DVL3 protein and mRNA in the colorectal cancer cell lines, likely due to posttranscriptional regulation of DVL3 expression (25), and DVL3 mRNA did not associate with response to figitumumab (Supplementary Fig. S1G).

DVL3-depleted cells show enhanced ERK activation

We speculated that the association between DVL3 and resistance to IGFIR inhibition may reflect cross-talk between the WNT and IGF pathways. Previously, we and others showed that the adaptor protein IRS-1 undergoes IGF-induced interaction with β-catenin, promoting β-catenin stabilization and transcriptional activity (19, 20). However, in DU145 cells, there was no evidence that IGF1 affected levels of active (nonphosphorylated) β-catenin or phosphorylated DVL3 (Fig. 2A). DVL3 depletion suppressed active β-catenin and phosphorylation of mTOR effector S6, consistent with known roles for DVL3 in canonical WNT signaling and mTOR activation (19, 20). DVL3-depletion suppressed active β-catenin and phosphorylation of mTOR effector S6, consistent with known roles for DVL3 in canonical WNT signaling and mTOR activation (19, 20). DVL3-depletion suppressed active β-catenin and phosphorylation of mTOR effector S6, consistent with known roles for DVL3 in canonical WNT signaling and mTOR activation (19, 20). DVL3-depletion suppressed active β-catenin and phosphorylation of mTOR effector S6, consistent with known roles for DVL3 in canonical WNT signaling and mTOR activation (19, 20). DVL3-depletion suppressed active β-catenin and phosphorylation of mTOR effector S6, consistent with known roles for DVL3 in canonical WNT signaling and mTOR activation (19, 20). DVL3-depletion suppressed active β-catenin and phosphorylation of mTOR effector S6, consistent with known roles for DVL3 in canonical WNT signaling and mTOR activation (19, 20).
The IGF-induced component of ERK activation in DVL3-depleted cells was effectively suppressed by AZ12253801 (Fig. 2D). These data suggest that DVL3 depletion enhances both basal and IGF-stimulated ERK activation, analogous to effects of PTEN loss on basal and ligand-induced AKT activation (28).

Of 12 candidate screen hits (Fig. 1A), five have known or putative roles as signaling regulators. In addition to DVL3, these include dual-specificity phosphatase 5 (DUSP5), connector enhancer of kinase suppressor of RAS (CNKSR1), lemur tyrosine kinase 3 (LMTK3), and hormonally upregulated neuro-associated kinase (HUNK), reported to regulate ERKs, AKT, and EGFR, respectively (29–32). Indeed, in DU145 cells, HUNK depletion enhanced IGF signaling to AKT (Supplementary Fig. S2A–S2C), supporting the concept that response to IGFIR inhibition is regulated by factors downstream of IGFIR.

**Proximal WNT inhibition mimics effects of DVL3 depletion**

Aiming to find a drug that recapitulates effects of DVL3 depletion, we tested compounds that block WNT signaling at different levels. Consistent with a role in Axin stabilization (33), the toolbox tankyrase inhibitor XAV939 upregulated Axin1 and inhibited mTOR, but did not activate ERKs or sensitize to AZ12253801 (Supplementary Fig. S3A–S3C). We then tested effects of WNT inhibition at a more proximal step, blocking DVL function directly. The DVL-PDZ domain has a peptide-binding cleft, involved in transducing signals from the membrane receptor Fz to downstream canonical and noncanonical pathways (22). The small-molecule inhibitor DVLi competitively binds to the cleft of the DVL-PDZ domain and blocks WNT signaling in Xenopus embryo and PC3 prostate cancer cells (34). This agent inhibited DVL3 phosphorylation and phenocopied DVL3 depletion, reducing active β-catenin and phospho-S6, activating ERKs in the absence of ligand, and sensitizing to AZ12253801 in viability and clonogenic assays (Fig. 3A–D). DVLi also sensitized to IGFIR TKI BMS-754807 that is being evaluated clinically (35; Supplementary Fig. S3D). We noted evidence for reciprocal sensitization: despite suppressing active β-catenin and mTOR, DVLi alone caused negligible growth inhibition, perhaps related at least in part to ERK activation, but with AZ12253801 caused significant loss of viability (Fig. 3E).

Although the DVLi we used has relatively low potency, the WNT pathway is an intense focus for drug development (34, 36), offering the prospect of more potent inhibitors in future. Therefore, we used this DVLi to perform proof-of-
principle experiments, testing its ability to influence sensitivity to IGFIIR inhibition in the prostate and breast cancer cell lines we characterized previously (Supplementary Fig. S1D). Two prostate cancer cell lines (DU145 and 22Rv1) were sensitized to AZ12253801 by DVLi inhibition; both had detectable basal ERK phosphorylation that was enhanced by DVLi (Fig. 3F). The other three (PC3, LNCaP, and LNCaP-LN3) did not manifest detectable basal or DVLi-induced ERK phosphorylation and were not sensitized to AZ12253801 by DVLi. The five breast cancer cell lines all showed increased ERK activation and enhanced response to AZ12253801 upon DVLi inhibition (Fig. 3G). Thus, 7 of 10 cell lines showed DVLi-induced ERK activation, and in each, DVLi enhanced response to IGFIIR inhibition. Supporting the existence of functional cross-talk between the IGF axis and proximal WNT components, upregulation of IGF-binding protein 5 was shown to mediate growth inhibitory effects of a soluble Wnt inhibitor in murine MMTV-Wnt1-driven tumors (36).

Therefore, to test the potential clinical relevance of our findings, we evaluated IGFIIR and DVLi inhibition in mice bearing DU145 prostate cancer xenografts. AZ12253801 or DVLi alone had no significant effects on tumor growth, but growth in the combination treatment group was significantly retarded compared with control-treated (P < 0.001), AZ12253801-treated (P < 0.001), and DVLi-treated animals (P < 0.01; Fig. 3H).

**DVL3 regulates IGFI-induced ERK activation**

These data indicate that blockade of proximal WNT signaling enhances sensitivity to IGFIIR inhibition, and suggest that this property tracks with regulation of MEK–ERK signaling (Figs. 2A–C and 3F and G). To characterize this effect further, we performed time-course experiments in DVLi-inhibited DU145 cells. IGFIIR induced rapid activation of IGFIIR and AKT that persisted in control cells for at least 60 minutes, whereas ERK activation peaked at 10 minutes and resolved to basal levels by 60 minutes (Fig. 4A). In contrast, there was clear persistence of IGFI-induced ERK phosphorylation at 30 to 60 minutes in cells where DVL3 was inhibited (Fig. 4A) or depleted (Supplementary Fig. S4A). Persistent ERK activation in DVLi-depleted cells was apparent within 4 hours of DVLi treatment, and was strikingly enhanced upon IGFI treatment (Fig. 4B). An increase in IGFI-induced ERK activation was also observed in MCF7 cells (Supplementary Fig. S4B). These results support the existence of a link between the ability of DVL3 to influence sensitivity to IGFIIR inhibition and to attenuate the response of the IGF axis to a mitogenic stimulus via MEK–ERK. Consequently, DVL3 depletion or inhibition creates an environment

**Figure 5.** DVL3 regulates signaling from IGFIIR to RAS. A, DU145 cells were treated with 100 μmol/L DVLi for 16 hours, lysates were incubated with GST or GST-RAS-binding domain (RBD) of RAF, and pull-downs analyzed by Western blotting for activated (RBD-bound) RAS. Right, blot confirms ERK activation in whole-cell extracts. Bottom, graph, RAS activity in three independent assays, expressed relative to control (DVLi-untreated) cells, *P < 0.05, by t test. B, cartoon, IGFIIR activates RAS via a protein complex that includes Shc, Grb2, and SOS. DU145 cells were transfected with Allstars (AS) siRNA or siRNAs to deplete IGFIIR, Shc, Grb2, or SOS. After 48 hours, cells were treated with 100 μmol/L DVLi for 16 hours and analyzed by Western blotting. DVLi-induced ERK activation was abolished by depletion of Shc, SOS, or Grb2 but not IGFIIR. C, DU145 whole-cell extracts were immunoprecipitated with control (IgG) or DVL3 antibodies and analyzed by Western blot in parallel with DVL3 IP supernatant (v/h) to confirm DVL3 immunodepletion. DVL3 IPs contained DAB2 and Grb2 but not IGFIIR. The same result was obtained in two further independent experiments. D, DU145 whole-cell extracts incubated with GST or GST-Grb2, and precipitated proteins analyzed by Western blot, revealing the presence of DVL2, DVL3, Shc, and DAB2 in Grb2 pull-downs. Representative of three independent experiments. E, DU145 cells were transfected with Allstars or DAB2 siRNA and after 48 hours, treated with AZ12253801 (left), and viability assayed after 5 days, showing GI50 values from three independent assays; right, serum-starved overnight and treated with 10 nmol/L IGF II for 10 minutes. DAB2 knockdown sensitized to AZ12253801 and also activated ERKs. F, Allstars-transfected or DAB2-depleted cells were treated with 100 μmol/L DVLi and/or AZ12253801, and cell viability assayed after 5 days. Graph, mean ± SEM viability, pooled data from three independent assays. Legend shows GI50 values and fold sensitization to AZ12253801. Parallel cultures were analyzed by Western blot, shown to right.
that is permissive for signaling, recently characterized as "signalability" (37). In contrast, DVL inhibition did not influence the response to IGF1 in PC3 prostate cancer cells that were not sensitized to AZ12253801 by DVLi (Supplementary Fig. S4C). To test whether ERK activation is required for DVL3 to modify the response to IGFIR inhibition, we abolished ERK activity using MEK inhibitor AZD6244 (Fig. 4C). MEK-inhibited DU145 cells showed almost complete rescue from DVL1-induced sensitization to IGFIR inhibition (Fig. 4D), suggesting that the ability of DVL3 to regulate MEK–ERK contributes to the sensitization effect.

**DVL3 complexes with SHC, Grb2, and SOS to regulate RAS activation**

To investigate how DVL3 regulates ERKs, we performed RAS activation assays and found that RAS was activated in DVL-inhibited cells (Fig. 5A). This contrasts with previously reported WNT:ERK cross-talk, occurring at more distal WNT signaling nodes, that generates positive feedback between the two pathways (38). Given that DVL3 depletion did not activate IGFIR itself (Fig. 2A and Supplementary Fig. S4B), and DVL3-depleted or DVL-inhibited cells showed no change in EGF-induced ERK activation or sensitivity to EGF inhibitor gefitinib (Supplementary Fig. S5A and S5B), we reasoned that RAS activation in DVL-depleted or DVL-inhibited cells was unlikely to be initiated at the level of these RTKs.

It is increasingly recognized that mitogenic signals generated by RTKs are integrated by complexes of adaptor and scaffolding proteins, including IRS-1, a well-recognized focus for feedback signaling via ERKs and mTOR-S6 kinase (39). Indeed, complexes containing Grb2 and DVL3 were detectable in DU145 cells by immunoprecipitation and Grb2 pulldown (Fig. 5C and D). These complexes also contained the putative tumor suppressor Disabled 2 (DAB2), which is permissive for signaling, recently characterized as "signalability" (37). When DVL3 interacts with Grb2 via DAB2, but found that DAB2-depleted cells still contained DVL3:Grb2 complexes (Supplementary Fig. S5F), and could be further sensitized to IGFIR inhibition by DVLi, with 18-fold reduction in AZ12253801 GI50 in DAB2-deleted, DVL-inhibited cells, compared with 2.8- and 4.6-fold sensitization induced separately by DAB2 depletion or DVL inhibition (Fig. 5F). These data suggest that DVL3 has a different role from DAB2 in regulating signal transduction to RAS.

**DVL3 expression in clinical cancers**

To investigate the clinical relevance of these findings, we evaluated DVL3 expression in patient tumors. Given the lack of correlation shown previously between DVL3 mRNA and protein (Supplementary Fig. S1G), we developed an IHC protocol, with controls including DVL3-depleted DU145 cells, to assess DVL3 expression by intensity × percentage score (IPS; Supplementary Methods; Fig. 6A and Supplementary Fig. S6A and S6B). Approximately 50% of breast and prostate cancers contained moderate or heavy cytoplasmic DVL3 (Supplementary Fig. S6C) that did not correlate with stage, grade, or patient survival (Supplementary Table S7). As a first approach to testing for the correlation between DVL3 protein and sensitivity to IGFIR inhibition, we assessed DVL3 in Ewing sarcomas, reported to be responsive to IGFIR inhibitor monotherapy (4, 5, 46, 47), and head and neck squamous cell cancers (HNSCC) that are resistant (48). The results are shown in Fig. 6B and C; all but one of the Ewing sarcomas contained low or no detectable DVL3, and the mean DVL3 staining score was significantly higher in HNSCCs (P = 0.0032). We then assessed DVL3 protein expression in archival tumors from patients recruited to early-phase clinical trials of IGFIR antibodies figitumumab or AVE164 (Supplementary Table S8; refs. 9, 48). Figure 6D shows examples of DVL3 staining in eight of the trial cases, and Fig. 6E shows analysis with respect to progression-free survival (PFS). Although there was overlap in DVL3 expression between patients experiencing early progression versus prolonged control, it was notable that of eight patients achieving prolonged disease control (>84 days, including one partial remission), six of these tumors had low DVL3 expression (IPS <5; Supplementary Table S8). PFS was longer in patients whose tumors showed no/lows DVL3 (n = 9; PFS, 137 ± 28 days) compared with patients whose tumors had moderate or strong DVL3 (n = 11; PFS, 70 ± 13 days; P = 0.031), and DVL3 staining was negatively correlated with PFS (Spearman r = -0.55; P = 0.0125; Fig. 6E). Included here were melanomas, ovarian, gastrointestinal, and HNSCCs (Supplementary Table S8), suggesting that DVL3 expression may have predictive value for response to IGFIR inhibition in a range of tumor types.

These data define new roles for DVL3 in suppressing signal transduction from IGFIR to RAS, and regulating response to IGFIR blockade. The association between RAS...
activation and sensitization to IGFIR inhibition is counter-intuitive, but is consistent with a report that KRAS-mutated non–small cell lung cancer cells show IGF-dependent PI3K activity and are sensitive to IGFIR inhibition (49). The finding that responses to IGFIR inhibition are modified by manipulating DVL3 expression or function suggests that DVL3 contributes to the resistance phenotype. Thus, DVL3 depletion or inhibition leads to amplification of IGF-induced RAS–MEK–ERK activation, and this property is associated with enhanced sensitivity to IGFIR inhibition (Fig. 7). Similar AZ12253801 sensitization and ERK activation were induced by depletion of DAB2 (Fig. 5E), reported to interact with Grb2 and suppress RAS activation (42–44). DAB2-depleted cells were capable of responding to DVL inhibition with enhanced ERK activation and AZ12253801 sensitization (Fig. 5F), suggesting that DVL3 has a nonredundant function in the Shc–Grb2–SOS complex.

Our screen identified additional candidate resistance mediators (DVL3, DUSP5, CNKSR1, LMTK3, and HUNK) with roles in regulating receptor or postreceptor signaling. The ability to influence IGFIR sensitivity to both ligand and receptor inhibition is reminiscent of the functional effects of EGFR kinase mutations, which favor the active conformation of EGFR kinase and render EGFR sensitive to EGFR TKIs (50). Comparable mutations have been sought but not reported in IGFIR; it is plausible that similar tuning of IGF signaling is achieved by changes in signal transduction complexes downstream of IGFIR. In summary, our data identify a role for DVL3 in suppressing signal transduction from IGFIR to RAS and attenuating response to IGFIR blockade. We propose that the

Figure 7. Model for DVL3 as a regulator of RAS activation and mediator of resistance to IGFIR inhibition. DVL3 exists in a complex of adaptor proteins that includes Shc, Grb2, and the tumor suppressor DAB2. Both DVL3 and DAB2 suppress signal transduction from IGFIR to RAS. When DVL3 and/or DAB2 are depleted, or DVLs are inhibited, there is enhanced basal (ligand-unstimulated) RAS–MEK–ERK activation, and in the presence of ligand, selective amplification of IGF signaling via the RAS–MEK–ERK pathway. This state is associated with increased dependency on IGF signaling and enhanced sensitivity to IGFIR inhibition.

Figure 6. DVL3 protein expression is inversely correlated with response to IGFIR antibody. A, DU145 cells were transfected with Allstars or DVL3 siRNA, after 48 hours were formalin-fixed, paraffin-embedded and used as controls for immunostaining, in parallel with sections of a transurethral resection of prostate, in which 100% of chippings were involved by Gleason grade 4+5 prostate cancer from a previously untreated patient. These cell and tissue controls were included in every staining run for quality control purposes. B, TMAs of Ewing sarcoma and HNSCC underwent IHC staining in the same staining run. Representative images of Ewing sarcoma (top), a sample of normal kidney included as a control on the same TMA slide (left), and HNSCC (bottom). Most of the Ewing sarcomas had light or no DVL3 signal; intense focal signal in one tumor was due to staining of macrophages. C, DVL3 IPS scores for nine cases of Ewing sarcoma and 24 cases of HNSCCs, showing significantly higher mean score in HNSCCs. D, examples of DVL3 IHC on tumors from patients on IGFIR antibody trials, showing diagnosis, PFS (days), and DVL3 IPS. E, graph of PFS versus DVL3 IPS in 20 patients treated on IGFIR antibody trials.
proportion of potentially responsive patients in IGFIR trials could be significantly increased by selecting cases with low-DVL3 tumors.

Disclosure of Potential Conflicts of Interest
T.M. Jones received a commercial research grant from GSK. V.M. Macaulay is a consultant/advisory board member for Consultancy. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: S. Gao, S. Feller, A. Ashworth, V.M. Macaulay
Development of methodology: S. Gao, P. Allen, D. Bailey, C.J. Lord, V.M. Macaulay
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.L. Bajrami, A. Kigoi, D. Ouaert, T. Aleksic, J.-Y. Blay, J.-P. Machiels, N. Upile, T.M. Jones, G. Thalmann, M.R. Middleton, C.J. Lord, V.M. Macaulay
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Gao, T. Aleksic, R. Asher, C. Han, S. Feller, J.-Y. Blay, M.B. Mudan-Eun, C.J. Lord, V.M. Macaulay
Writing, review, and/or revision of the manuscript: C. Verrill, J.-Y. Blay, S. Schmitz, J.-P. Machiels, G. Thalmann, J.L. Wilding, W.F. Bodmer, M.R. Middleton, A. Ashworth, C.J. Lord, V.M. Macaulay
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): I. Bajrami, A. Kigoi, D. Ouaert, T. Aleksic, P. Allen, D. Bailey, N. Athanasou, S. Schmitz, G. Thalmann, J.L. Wilding, V.M. Macaulay
Study supervision: C.J. Lord, V.M. Macaulay
Provision of key materials including constructs and cell lines and tissue samples, and patient recruitment: N. Athanasou, S.Q. Ashraf

References

Acknowledgments
The authors thank Elaine Kilgour, Elizabeth Anderson, and Jon Curwen at Astrazeneca for providing AZ12253801 and advice on its use, James Christensen at Pfizer for providing figitumumab, Ludwig Van den Hove at Pfizer and John Dahlquist at ImmunoGen for permission to use clinical data from the GORTEC-2008-2 and TED241 trials, respectively, Divita Jata (Oxford Centre for Histopathology Research) and Olga Perestenko (Department of Oncology, Weatherall Institute of Molecular Medicine) for assistance with DVL3 IHC. Antonette Wetterwald (University of Bern) for assistance with the prostate tissue microarray (TMA), and Anderson Ryan and Bass Hassan for comments on the article. They gratefully acknowledge the contribution of coauthor Takeshi Kashima, who died while this article was under review.

Grant Support
This work was supported by the NIHR Oxford Biomedical Research Centre, Oxford Experimental Cancer Medicine Centre, HEFCE Clinical Senior Lecture- ship (V.M. Macaulay), and research grants from UCARE-Oxford, Breast Cancer Campaign, Molecular and Cellular Medicine Board of MRC and AstraZeneca. Research in the laboratory of W.F. Bodmer is funded by Roche/Glycera. S.Q. Ashraf was supported by an Academy of Medical Sciences Clinical Lecturer Starter grant and the Nuffield Department of Surgical Sciences (Oxford University Hospitals National Health Service Trust, Oxford). J.-Y. Blay was supported by grants Netsarc-LYRIC_4664 and EuroSarc (FP7 278472). V.M. Macaulay and S. Gao were supported by a grant from AstraZeneca.

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 March 20, 2014; revised July 9, 2014; accepted July 27, 2014; published OnlineFirst August 28, 2014.


Dsh Homolog DVL3 Mediates Resistance to IGFIR Inhibition by Regulating IGF-RAS Signaling

Shan Gao, Ilirjana Bajrami, Clare Verrill, et al.

*Cancer Res* Published OnlineFirst August 28, 2014.

<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-14-0806</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplementary Material</td>
<td>Access the most recent supplemental material at: <a href="http://cancerres.aacrjournals.org/content/suppl/2014/08/29/0008-5472.CAN-14-0806.DC1">http://cancerres.aacrjournals.org/content/suppl/2014/08/29/0008-5472.CAN-14-0806.DC1</a></td>
</tr>
</tbody>
</table>

| E-mail alerts | Sign up to receive free email-alerts related to this article or journal. |
| Reprints and Subscriptions | To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org. |
| Permissions | To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org. |