Abstract

Purpose: ERBB3 is overexpressed in a broad spectrum of human cancers, and its aberrant activation is associated with tumor pathogenesis and therapeutic resistance to various anticancer agents. Neuregulin 1 (NRG1) is the predominant ligand for ERBB3 and can promote the heterodimerization of ERBB3 with other ERBB family members, resulting in activation of multiple intracellular signaling pathways. AV-203 is a humanized IgG1/k ERBB3 inhibitory antibody that completed a first-in-human phase I clinical trial in patients with advanced solid tumors. The purpose of this preclinical study was to identify potential biomarker(s) that may predict response to AV-203 treatment in the clinic.

Experimental Design: We conducted in vivo efficacy studies using a broad panel of xenograft models representing a wide variety of human cancers. To identify biomarkers that can predict response to AV-203, the relationship between tumor growth inhibition (TGI) by AV-203 and the expression levels of ERBB3 and NRG1 were evaluated in these tumor models.

Results: A significant correlation was observed between the levels of NRG1 expression and TGI by AV-203. In contrast, TGI was not correlated with ERBB3 expression. The correlation between the levels of NRG1 expression in tumors and their response to ERBB3 inhibition by AV-203 was further validated using patient-derived tumor explant models.

Conclusions: NRG1 is a promising biomarker that can predict response to ERBB3 inhibition by AV-203 in preclinical human cancer models. NRG1 warrants further clinical evaluation and validation as a potential predictive biomarker of response to AV-203. Clin Cancer Res; 21(5); 1106–14. ©2014 AACR.

Introduction

The v-erb-b2 erythroblastic leukemia viral oncogene homolog (ERBB) receptor tyrosine kinase (RTK) family consists of four cell surface receptors: ERBB1 [also known as epidermal growth factor receptor (EGFR)], ERBB2 [also known as human epidermal growth factor receptor 2 (HER2)], ERBB3 (HER3), and ERBB4 (HER4; refs. 1–4). These receptors play important roles in the regulation of cell proliferation, migration, differentiation, apoptosis, and cell motility and are often overexpressed, amplified, or mutated in human cancers of epithelial origin. The importance of ERBB family receptors in cancers is further highlighted by the successful development of anticancer therapeutics against two of its family members, EGFR and HER2 (5–9). Much of the interest has recently been focused on another ERBB family member, ERBB3, due to its role in the development of resistance to various anticancer agents. A number of inhibitory antibodies targeting ERBB3 are currently in different stages of clinical development (10–15).

ERBB3 lacks significant tyrosine kinase activity (16–18), and activation of ERBB3 requires its heterodimerization with other RTKs such as HER2, EGFR, and MET (19–21). Neuregulin 1 (NRG1), also known as heregulin (HRG), is the predominant ligand for ERBB3 and can promote the heterodimerization of ERBB3 with other ERBB family members, triggering the tyrosine phosphorylation of ERBB3 and activation of diverse intracellular signaling networks (20, 22, 23). In particular, six tyrosine residues, upon phosphorylation, can be used to engage the p85 regulatory subunit of phosphoinositide-3-kinase (PI3K; ref. 24). In contrast, EGFR contains only two binding sites for the p85 subunit of PI3K, whereas HER2 contains none (25). Consequently, within the ERBB family, ERBB3 is the most potent activator of the PI3K/AKT signaling pathway (20, 22, 26, 27).

The ERBB3 receptor is often overexpressed on cancer cells of the head and neck, lung, breast, ovaries, prostate, colon, pancreas, and gastrointestinal tract (28). Its overexpression is often linked to poor prognosis (27, 28). In addition, ERBB3 has been implicated in the development of therapeutic resistance to radiotherapy and various anticancer agents. ERBB3 can serve as a prosurvival signal amplifier of residual RTK activity retained by its heterodimerization partners even in the presence of inhibitors (19, 29–32). For example, ERBB3 is the preferred dimerization partner of HER2, and sustained ERBB3 signaling in HER2-amplified breast cancers may, at least in part, be responsible for the development of resistance to trastuzumab (20, 33).
AV-203 is an ERBB3-targeted antibody that can block both ligand-dependent and ligand-independent ERBB3 signaling (35). AV-203 binds specifically to human and cynomolgus monkey ERBB3 with high affinity and can effectively inhibit the binding of NRG1 to ERBB3, thereby abolishing ERBB3 phosphorylation and its downstream signaling (35). In preclinical studies, AV-203 blocks NRG1-induced cell proliferation and exhibits potent tumor growth inhibitory (TGI) activity as a single agent against human cancer models harboring diverse genetic alterations (35).

The effectiveness of targeted therapies often can be enhanced by the use of predictive biomarker(s) to define patient populations that are most likely to benefit from the therapies. For example, the usefulness of predictive biomarker(s) has recently been demonstrated clinically for crizotinib in NSCLCs with anaplastic lymphoma kinase (ALK) translocation (36) and for vemurafenib in BRAF-V600-mutated advanced melanomas (37, 38). AV-203 has undergone a first-in-human phase 1 clinical study in patients with advanced solid tumors. In an effort to identify biomarker(s) that can predict therapeutic response to AV-203 to guide its clinical development, we investigated the relationship between the expression levels of ERBB3 and NRG1 and the in vivo efficacy of AV-203 in a broad panel of human cancer models representing major human cancer types and subtypes.

### Materials and Methods

Human cancer cell lines

Human cancer cell lines were obtained from the American Tissue Culture Collection (ATCC), German Collection of Microorganisms and Cell Cultures [Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ)], or Korean Cell Line Bank (KCLB) as listed in Table 1. The cell lines were authenticated by short-tandem repeat analysis by the cell banks and the studies were performed within 6 months upon receipt of the cell lines.

### Table 1. Summary of AV-203 in vivo efficacy studies using human cancer xenograft models

<table>
<thead>
<tr>
<th>Xenograft model</th>
<th>Cancer type</th>
<th>NRG1 (×C)</th>
<th>ERBB3 (×C)</th>
<th>TGI (%)</th>
<th>Known mutations</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-453</td>
<td>Breast</td>
<td>16.6</td>
<td>4.3</td>
<td>79.3</td>
<td>CDH1</td>
<td>ATCC</td>
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<tr>
<td>MFM-223</td>
<td>Breast</td>
<td>20.6</td>
<td>4.8</td>
<td>28.6</td>
<td>TP53, PI3K</td>
<td>DSMZ</td>
</tr>
<tr>
<td>H76</td>
<td>Colorectal</td>
<td>19.1</td>
<td>7.4</td>
<td>5.2</td>
<td>MAP2K4, TP53, KRAS</td>
<td>ATCC</td>
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<tr>
<td>LoVo</td>
<td>Colorectal</td>
<td>13.6</td>
<td>3.8</td>
<td>43.7</td>
<td>APC, FBXW7, MSH2, KRAS</td>
<td>ATCC</td>
</tr>
<tr>
<td>AN3 CA</td>
<td>Endometrial</td>
<td>13.3</td>
<td>8.5</td>
<td>10.6</td>
<td>TP53, FGFR2</td>
<td>ATCC</td>
</tr>
<tr>
<td>MFE-296</td>
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<td>TP53, FGFR2</td>
<td>DSMZ</td>
</tr>
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<td>KYSE-150</td>
<td>Esophageal</td>
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<td>6.3</td>
<td>84.6</td>
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<td>DSMZ</td>
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<td>SNU-16</td>
<td>Gastric</td>
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<td>CAL 27</td>
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<td>75.2</td>
<td>CDKN2A, TP53, SMAD4</td>
<td>ATCC</td>
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<td>FaDu</td>
<td>Head and neck</td>
<td>8.0</td>
<td>6.8</td>
<td>75.5</td>
<td>CDKN2A, SMAD4, TP53</td>
<td>ATCC</td>
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<tr>
<td>SCC-9</td>
<td>Head and neck</td>
<td>5.8</td>
<td>6.3</td>
<td>69.0</td>
<td>CDKN2A, TP53</td>
<td>ATCC</td>
</tr>
<tr>
<td>SCC-15</td>
<td>Head and neck</td>
<td>5.0</td>
<td>8.8</td>
<td>40.7</td>
<td>TP53, CDH1</td>
<td>ATCC</td>
</tr>
<tr>
<td>A-498</td>
<td>Kidney</td>
<td>7.9</td>
<td>6.3</td>
<td>67.7</td>
<td>CDKN2A, VHL, SETD2</td>
<td>ATCC</td>
</tr>
<tr>
<td>786-O</td>
<td>Kidney</td>
<td>8.1</td>
<td>11.8</td>
<td>18.1</td>
<td>CDKN2A, VHL, TP53, PTEN</td>
<td>ATCC</td>
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<td>AS49</td>
<td>NSCLC</td>
<td>5.6</td>
<td>5.3</td>
<td>72.4</td>
<td>CDKN2a, STK11, SMARCA4, KRAS</td>
<td>ATCC</td>
</tr>
<tr>
<td>Calu-3</td>
<td>NSCLC</td>
<td>6.3</td>
<td>4.9</td>
<td>69.0</td>
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<td>ATCC</td>
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<tr>
<td>HCC295</td>
<td>NSCLC</td>
<td>1.9</td>
<td>6.4</td>
<td>94.1</td>
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<td>KCLB</td>
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<tr>
<td>HCC327</td>
<td>NSCLC</td>
<td>21.4</td>
<td>7.0</td>
<td>16.5</td>
<td>EGFR</td>
<td>ATCC</td>
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<tr>
<td>NCI-H232</td>
<td>NSCLC</td>
<td>9.7</td>
<td>8.8</td>
<td>0.0</td>
<td>STK11, SMARCA4, TP53, KRAS</td>
<td>ATCC</td>
</tr>
<tr>
<td>NCI-H322</td>
<td>NSCLC</td>
<td>8.0</td>
<td>6.8</td>
<td>62.6</td>
<td>CDKN2A, TP53</td>
<td>ATCC</td>
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<tr>
<td>NCI-H358</td>
<td>NSCLC</td>
<td>6.8</td>
<td>4.9</td>
<td>89.1</td>
<td></td>
<td>ATCC</td>
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<tr>
<td>NCI-H441</td>
<td>NSCLC</td>
<td>17.0</td>
<td>5.1</td>
<td>13.1</td>
<td>TP53</td>
<td>ATCC</td>
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<tr>
<td>NCI-H520</td>
<td>NSCLC</td>
<td>8.3</td>
<td>9.9</td>
<td>0.0</td>
<td></td>
<td>ATCC</td>
</tr>
<tr>
<td>NCI-H522</td>
<td>NSCLC</td>
<td>2.2</td>
<td>8.9</td>
<td>50.3</td>
<td>TP53</td>
<td>ATCC</td>
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<tr>
<td>NCI-H1993</td>
<td>NSCLC</td>
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<td>7.2</td>
<td>39.8</td>
<td>CDKN2a, STK11, TP53</td>
<td>ATCC</td>
</tr>
<tr>
<td>NCI-H1048</td>
<td>Small cell lung</td>
<td>10.0</td>
<td>4.8</td>
<td>31.0</td>
<td>RBL, TP53, PI3K</td>
<td>ATCC</td>
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<tr>
<td>BxPC-3</td>
<td>Pancreatic</td>
<td>9.7</td>
<td>4.8</td>
<td>70.9</td>
<td>CDKN2A, MAP2K4, SMAD4, TP53</td>
<td>ATCC</td>
</tr>
<tr>
<td>Capan-1</td>
<td>Pancreatic</td>
<td>4.1</td>
<td>6.7</td>
<td>37.7</td>
<td>CDKN2A, KRAS</td>
<td>ATCC</td>
</tr>
<tr>
<td>SW 1990</td>
<td>Pancreatic</td>
<td>8.2</td>
<td>5.2</td>
<td>58.5</td>
<td>CDKN2A, MLH1, RBL, STK11, TP53</td>
<td>ATCC</td>
</tr>
<tr>
<td>DU 145</td>
<td>Prostate</td>
<td>6.1</td>
<td>6.1</td>
<td>57.3</td>
<td>CDKN2A, TP53</td>
<td>ATCC</td>
</tr>
<tr>
<td>A-431</td>
<td>Skin</td>
<td>6.1</td>
<td>6.1</td>
<td>57.3</td>
<td></td>
<td>ATCC</td>
</tr>
</tbody>
</table>

NOTE: The cancer type, known mutations of each model, and the supplier of each cell line are indicated. The ΔC values of NRG1 and ERBB3 and TGI are reported.
ERBB3 and NRG1 expression analysis

The expression levels of human ERBB3 and NRG1 were determined using real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Each RNA tumor sample was analyzed in quadruplicate containing total tumor RNA (50 ng) and forward and reverse gene-specific primers as listed in Supplementary Materials and Methods. The average threshold cycle (Ct) values of ERBB3, NRG1, and actin (control) were determined. The Ct value is the cycle number at which the fluorescence generated within a PCR reaction crosses the threshold significantly above the background and is inversely correlated with the expression level of a gene of interest in a sample. ΔCt values were calculated by subtracting the average Ct value of actin (control) from that of ERBB3 or NRG1 of the same sample. Refer Supplementary Materials and Methods for details.

Establishment of patient-derived xenografts

Primary human tumors from surgical resection were obtained through the Cooperative Human Tissue Network (CHTN). See Supplementary Materials and Methods for detailed information on the generation and propagation of patient-derived xenografts (PDX).

AV-203 in vitro efficacy studies

The cells were grown in vitro at 37 °C using media recommended by the suppliers plus 1% penicillin–streptomycin (Invitrogen). All cell lines were harvested, resuspended in serum free media containing 50% Matrigel (BD Biosciences), and inoculated subcutaneously (s.c.) into the right flank of each mouse. Specifically, 6-week-old female NOD/SCID mice (Taconic Labs) were inoculated with 2 × 10^6 MDA-MB-435 cells. Five- to 8-week-old female CB.17 SCID or NCR nude mice (Taconic Labs) were used in all other xenograft models and were inoculated with 10^5 to 10^6 cells, depending on the models. For efficacy studies using PDXs, tumor fragments were implanted s.c. into the right flank of 5- to 8-week-old female NCR nude mice (for the process of generation of PDXs, refer Supplementary Materials and Methods).

Tumor measurements were taken twice weekly using Fisherbrand Traceable Digital Calipers. Tumor volume was calculated with the formula: V = 0.5 × width × depth × length. When tumor volume approached 200 mm^3, mice were randomized into groups of 10 animals each, and dosed twice weekly via intraperitoneal (i.p.) injection with either AV-203 or an IgG1 isotype (control) at 20 mg/kg twice weekly throughout the study. In most studies, phosphate-buffered saline (PBS) was also included as a vehicle control. All statistical analysis was done using a one-way analysis of variance (ANOVA) and Tukey multiple comparison test (GraphPad Prisim).

In vitro proliferation assays

HCC2935 and SKBR3 cells were obtained from the ATCC and seeded at 5,000 cells per well in a 96-well flat-bottomed cell culture plate. After serum starvation, cells were treated with the appropriate concentrations of erlotinib or lapatinib as indicated. AV-203 or mouse IgG (mgG) was added to a final concentration of 10 μg/mL. HCC2935 and SKBR3 cells were then incubated in the absence or presence of 100 or 30 ng/ml NRG1/β1, respectively. The MTT assay was performed after 3 days of incubation at 37 °C as detailed in Supplementary Materials and Methods.

Results

Efficacy of AV-203 in cell line–derived human cancer models

To comprehensively evaluate the antitumor activity of AV-203, we conducted in vivo efficacy studies using a broad panel of xenograft models derived from human lung, colon, skin, breast, pancreatic, gastric, renal, head and neck, esophageal, and endometrial cancer cell lines. A total of 32 tumor models were treated with AV-203 at 20 mg/kg i.p. twice weekly for the duration of the study. AV-203 exhibited a wide range of antitumor activities in the models tested varying from no TGI to tumor regression. Of the 32 tumor models tested, AV-203 treatment resulted in tumor regression in two xenograft models (HCC95 and NCI-H358). Greater than 60% TGI by AV-203 was also observed in 11 additional tumor models (KYSE-150, MDA-MB-453, FaDu, CAL 27, A549, BxPC-3, SCC-9, Calu-3, A-498, Capan-1, and NCI-H322). Modest or insignificant TGI by AV-203 was observed in the rest of the tumor models tested (Table 1 and Supplementary Fig. S1). In total, approximately 41% (13 out of 32) tumor models were considered to be responders to AV-203 treatment (TGI ≥ 60%), whereas 59% (19 out of 32) were considered to be nonresponders (TGI < 60%).

Lack of correlation between efficacy of AV-203 and ERBB3 expression levels in tumors

To understand the molecular basis for the sensitivity of these xenografts to AV-203 treatment and to identify biomarker(s) that can potentially predict response to AV-203, we first investigated the association of the antitumor activity of AV-203 with the expression levels of ERBB3 because ERBB3 is the direct target of AV-203. Quantitative RT-PCR analysis was carried out to determine the Ct values of ERBB3 and a control gene, actin, in pretreatment tumors. The ERBB3 ΔCt values are inversely proportional (log2 scale) to the original expression levels of ERBB3 in tumors, that is, the lower the ΔCt value, the higher the expression. Of the 32 tumor models included in the study, the ΔCt values of ERBB3 varied from 3.6 to 11.8, indicating that these tumor models exhibited a broad range of ERBB3 expression. However, there was no statistically significant correlation between the sensitivity of xenografts to AV-203 and the expression levels of ERBB3 in the tumors (Spearman r = −0.324; P = 0.070; Fig. 1A). The difference in the ERBB3 expression levels in the tumor model with the highest level of ERBB3 expression, LoVo (ERBB3, ΔCt = 3.8), and the lowest level of ERBB3 expression, 786-O (ERBB3, ΔCt = 11.8), was estimated to be more than 200-fold, yet neither model responded significantly to AV-203 treatment (Table 1).

NRG1 expression levels in tumors correlated with AV-203 antitumor activity

Because NRG1 is the predominant ligand that can activate ERBB3, we next investigated the correlation between the expression levels of NRG1 in selected tumor models and their sensitivity to AV-203 treatment. Quantitative RT-PCR analysis revealed that the tumor models exhibited an even broader range of expression for NRG1 than ERBB3, as the ΔCt values of NRG1 varied from 1.9 to 21.4 (Table 1). Interestingly, the xenograft model that had the highest level of NRG1 expression (i.e., with the lowest NRG1 ΔCt value), HCC95, exhibited the greatest sensitivity to AV-203 treatment, whereas the model that had the lowest level of NRG1 expression (i.e., with the highest NRG1 ΔCt value), HCC827, was insensitive to AV-203 treatment (Table 1). Of the 32 xenograft models...
NRG1 as a Biomarker for Response In Vivo to ERBB3 Inhibition

Figure 1.
Correlation analysis between efficacy of AV-203 and the expression levels of ERBB3 (A) or NRG1 (B) in 32 human cancer xenografts included in the study. Quantitative RT-PCR analysis was carried out to determine the \( C_t \) values of ERBB3, NRG1, and actin (control) in tumors. The expression levels of NRG1 and ERBB3 were estimated using \( \Delta C_t \) values by subtracting the average \( C_t \) value of actin control from that of NRG1 or ERBB3 of the same sample. The correlation between NRG1 and ERBB3 \( \Delta C_t \) values and TGI by AV-203 was analyzed by the nonparametric Spearman rank correlation method. The linear regression is shown by a solid line, and the 95% confidence interval is shown by dotted lines. C, ROC analysis was used to determine the optimal NRG1 \( \Delta C_t \) cutoff to predict the response of a tumor to AV-203 treatment. The AUC is indicated.

Because NRG1 \( \Delta C_t \) values are inversely proportional to the expression levels of NRG1 in tumors, tumors with NRG1 \( \Delta C_t \) values below the cutoff were defined as NRG1 positive. Out of 15 NRG1 "positive" models, 11 responded to AV-203 treatment. Among 17 NRG1 "negative" models, only two tumor models, Capan-1 (pancreatic ductal adenocarcinoma) and MDA-MB-453 (HER2-amplified breast cancer), exhibited sensitivity to AV-203 treatment. The Fisher exact test was also conducted to estimate \( P \) value (0.0083) of the enrichment and odds ratio (18.1) for correct prediction of responsiveness (Supplementary Table S1). This analysis indicates that NRG1-positive tumors (with NRG1 \( \Delta C_t \) \( \leq 8.0 \)) are enriched with statistical significance for responders to AV-203 and that the in vivo efficacy of AV-203 can potentially be predicted by NRG1 expression levels in the tumors.

Efficacy of AV-203 in PDXs and validation of NRG1 as a predictive biomarker of response for AV-203

To validate our hypothesis that the NRG1 mRNA expression level within a tumor could predict its sensitivity to AV-203 treatment, we used PDXs representing ovarian, colon, and non-small cell lung cancers (NSCLC) for in vivo efficacy studies. PDX models established directly from primary tumors of patients are thought to closely resemble the original tumors and may serve as clinically predictive models superior to traditional cell line-derived xenograft models (39). The 12 PDXs included in the study were established in house and were determined to carry diverse genetic alterations by mutational analyses (Table 2). RT-PCR analysis revealed that these PDX models expressed varying levels of NRG1 and ERBB3. Using the NRG1 \( \Delta C_t \) value 8.0 as the cutoff, which was established on the basis of 32 xenograft models, five models were predicted to be sensitive to AV-203 treatment and seven models were predicted to be resistant to AV-203 treatment. In vivo efficacy studies showed that tumor growth was significantly inhibited in four out of five models predicted to respond to AV-203 treatment (specificity = 80%), whereas in seven tumor models predicted to be resistant to AV-203 treatment, only one responded to AV-203 (sensitivity = 86%; Table 2; Fig. 2). Similar to what we observed with the cell line xenograft...
models, there was a significant correlation between the ΔCt values of NRG1 and the antitumor activity of AV-203 (Spearman r = −0.678; P = 0.015), whereas there was no significant correlation between the ΔCt values of ERBB3 and the antitumor activity of AV-203 (Spearman r = 0.175; P = 0.587). The tumors that responded to AV-203 appear to have diverse genetic alterations including mutations in KRAS, HRAS, and MET (Table 2). Analysis of NRG1 expression by immunohistochemistry shows that there is a good correlation between the NRG1 mRNA and protein expression in the PDX models tested (Supplementary Fig. S2).

Combining the 32 human cancer cell xenografts and 12 PDX models, ΔCt values of NRG1 within tumors significantly correlated with AV-203 antitumor activity (Spearman r = −0.602; P < 0.001; Fig. 3B). In contrast, ΔCt values of ERBB3 within these same tumors did not correlate with AV-203 antitumor activity (Spearman r = −0.232; P = 0.129; Fig. 3A). There was a significant difference in the ΔCt values of NRG1 between the responders and the nonresponders of all tumor models included in the study (P = 0.0014; Fig. 3D), whereas there was no significant difference in the ΔCt values of ERBB3 between the two groups (P = 0.09; Fig. 3C).

Figure 2.
Effect of AV-203 on selected PDX models in vivo. NCR nude mice were inoculated with PDX tumors as indicated. When tumor volumes reached approximately 200 mm³, mice were randomized, and then dosed twice weekly via i.p. injection with either an IgG (control) or AV-203 at 20 mg/kg.

Table 2. Summary of AV-203 in vivo efficacy studies using PDX models

<table>
<thead>
<tr>
<th>PDX models</th>
<th>Cancer type</th>
<th>Histology</th>
<th>Predicted to respond to AV-203</th>
<th>Response to AV-203</th>
<th>Known mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO-08</td>
<td>Colon</td>
<td>Adenocarcinoma</td>
<td>0.678</td>
<td>No</td>
<td>NRAS, PS3</td>
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<td>CO-66</td>
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<td>0.232</td>
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<td>LU-44</td>
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</table>

NOTE: The cancer type, histology, and known mutations of each PDX model are indicated. The ΔCt values of NRG1 and ERBB3 are reported along with predicted response to AV-203 based on the ΔCt value of NRG1 of each PDX model and the actual in vivo response to AV-203.
NRG1 expression in cancers

The extensive AV-203 in vivo efficacy studies strongly suggest that NRG1 is a predictive biomarker of response to AV-203, and that human cancer patients with high NRG1 expression are likely to benefit from AV-203 treatment. To identify human cancer types/subtypes enriched for high levels of NRG1 expression, we analyzed RNA-Seq expression data that includes approximately 7900 tumor samples across 26 tumor types obtained from The Cancer Genome Atlas (TCGA) established by the National Cancer Institute (Bethesda, MD) and the National Human Genome Research Institute (Bethesda, MD). NRG1 is highly expressed in a wide variety of human cancers: squamous cell carcinoma of head and neck (SCCHN), squamous cell lung carcinoma, renal clear cell carcinoma, and thyroid cancers are especially enriched for high levels of NRG1 expression (Supplementary Fig. S3 and Supplementary Table S2).

AV-203 reverses ERBB3-induced resistance to targeted therapies against other ERBB family members

Activation of ERBB3 has been implicated in the development of therapeutic resistance to various anticancer agents (19, 29–32). To investigate whether NRG1 could contribute to ERBB3-mediated therapeutic resistance to targeted therapies, we first investigated the effect of AV-203 on the sensitivity of HCC2935, a NSCLC cell line bearing a deletion in exon 19 of the EGFR gene, to erlotinib, an EGFR TKI. Erlotinib potently inhibited the proliferation of HCC2935 cells with an IC50 of 0.041 μmol/L as determined by a colorimetric MTT assay (Fig. 4A). In the presence of NRG1, the antiproliferative effect of erlotinib was drastically reduced by about 60-fold to an IC50 of 2.40 μmol/L, suggesting ligand-mediated activation of ERBB3 by NRG1 desensitized the cells to erlotinib treatment. The addition of AV-203, but not mIgG, completely restored the erlotinib-dependent inhibition of HCC2935 cell proliferation, with an IC50 value of 0.040 μmol/L.

We also used a HER2-amplified breast cancer cell line, SKBR3, and investigated the effect of NRG1 on the sensitivity of the cells to lapatinib, a dual EGFR and HER2 TKI. As shown in Fig. 4B, the proliferation of SKBR3 cells was inhibited by lapatinib with an IC50 of 0.027 μmol/L. AV-203 at 10 μg/mL sensitized the SKBR3 cells to lapatinib inhibition by approximately 2.5-fold, with an IC50 of 0.011 μmol/L. In the presence of NRG1, the antiproliferative effect of lapatinib in SKBR3 was reduced by about 50-fold to an IC50 of 1.042 μmol/L. AV-203, but not mIgG, completely restored the sensitivity of SKBR3 cells to lapatinib, with an IC50 of 0.041 μmol/L.

Discussion

Biomarker-driven targeted therapy has transformed the treatment paradigms in oncology. Identification of biomarker(s) that can predict response to a specific therapeutic agent can facilitate the development and approval of a novel therapy. Successful examples of biomarker-based, personalized medicine include HER2-targeted trastuzumab for treating HER2-positive breast and gastric cancers (5, 40), and EGFR-targeted erlotinib for treating lung cancers with EGFR TKI-sensitive mutations (7, 8, 41). Despite the rapid advances in biomarker-guided drug development, only a very small number of drugs have been launched with...
ERBB3 signaling suggests that autocrine activation of the NRG1 appear to be limited to certain cancer types/subtypes, suggesting that AV-203 treatment have diverse genetic alterations and do not further validated in 12 PDX models. The tumors sensitive to NRG1 expression for response to the treatment of AV-203 was ERBB3, the direct target of AV-203. The predictive value of between AV-203 partial response to AV-203 as a single agent (44), further patient with the squamous cell carcinoma subtype exhibited in the phase I study of AV-203, an NRG1-positive NSCLC.

A survey of TCGA datasets across 26 cancer types revealed that NRG1 could be, at least in part, attributed to the upregulation of NRG1 na specimens, suggesting therapy escape and cancer recurrence. Despite the initial impressive response to these targeted therapies, tumors often relapse due to the acquisition of secondary mutations or activation of alternative prosurvival pathways. Data from preclinical and clinical studies suggest that one mechanism to confer resistance to ERBB-targeted therapies is the alternative activation of the PI3K-dependent prosurvival pathway through ERBB3 (48–50). We demonstrate here that NRG1 can indeed confer resistance to erlotinib in a human lung cancer cell line bearing an EGFR TKI-sensitive mutation as well as to lapatinib in a HER2-amplified breast cancer cell line. In both cases, AV-203 completely reversed the NRG1-induced resistance to the TKIs tested. A previous study found that NRG1 expression was higher in recurrent SCCHN in comparison with patient-matched therapy-naive specimens, suggesting therapy escape and cancer recurrence could be, at least in part, attributed to the upregulation of NRG1 (46). These observations suggest that high intratumoral NRG1 expression may confer intrinsic or acquired resistance to EGFR or HER2 inhibitors, and thus combination of AV-203 with EGFR/HER2 targeted therapies could potentially enhance their therapeutic benefit.

A survey of TCGA datasets across 26 cancer types revealed that NRG1 is highly expressed in a wide variety of human cancer types/subtypes. In particular, SCCHN, squamous cell lung carcinoma, renal clear cell carcinoma, and thyroid cancers are especially enriched for high levels of NRG1 expression. A large subset of cancer patients could harbor an activated NRG1–ERBB3 autocrine signaling loop and could potentially benefit from ERBB3-targeted therapy. Consistent with this idea, in the phase I study of AV-203, an NRG1-positive NSCLC patient with the squamous cell carcinoma subtype exhibited partial response to AV-203 as a single agent (44). Further supporting the exploration of NRG1 as a potential predictive biomarker for AV-203. Preclinical studies using an ERBB3-targeted mAb, MM-121, has shown that MM-121 responding xenografts expressed high levels of either NRG1 or β-cellulin (12). An activated NRG1/ERBB3 autocrine signaling loop was found to be critical for the proliferation of ovarian cancer cells in vivo (45). High NRG1 expression was found to be associated with activated ERBB3 in SCCHN and was proposed as an actionable biomarker in patients with SCCHN (46). Recently, a retrospective analysis of a phase II study involving an ERBB3 inhibitory mAb, patritumab, in combination with erlotinib in advanced NSCLCs also identified NRG1 as a potential response biomarker to ERBB3-targeted therapies (47), further validating our preclinical findings.

Activation of ERBB3 signaling has also been implicated in therapeutic resistance of tumors to a variety of anticancer therapies. Over the past decade, a variety of mAbs and TKIs have been successfully developed to target EGFR and HER2 in cancers. Despite the initial impressive response to these targeted therapies, tumors often relapse due to the acquisition of secondary mutations or activation of alternative prosurvival pathways. Data from preclinical and clinical studies suggest that one mechanism to confer resistance to ERBB-targeted therapies is the alternative activation of the PI3K-dependent prosurvival pathway through ERBB3 (48–50). We demonstrate here that NRG1 can indeed confer resistance to erlotinib in a human lung cancer cell line bearing an EGFR TKI-sensitive mutation as well as to lapatinib in a HER2-amplified breast cancer cell line. In both cases, AV-203 completely reversed the NRG1-induced resistance to the TKIs tested. A previous study found that NRG1 expression was higher in recurrent SCCHN in comparison with patient-matched therapy-naive specimens, suggesting therapy escape and cancer recurrence could be, at least in part, attributed to the upregulation of NRG1 (46). These observations suggest that high intratumoral NRG1 expression may confer intrinsic or acquired resistance to EGFR or HER2 inhibitors, and thus combination of AV-203 with EGFR/HER2 targeted therapies could potentially enhance their therapeutic benefit.
Despite the significant correlation of NRG1 expression levels with AV-203 in vivo efficacy, there was a small fraction of tumor models that failed to respond to AV-203 treatment despite the presence of elevated levels of NRG1. One possible molecular mechanism for this intrinsic resistance to AV-203 is that these tumors have acquired mutations in signaling proteins downstream of ERBB3 (such as activating PI3K mutations or PTEN inactivation), which leads to constitutive activation of the PI3K prosurvival pathway. Intriguingly, one of the predicted responder, LU-07, an NRG1-positive NSCLC PDX model that was resistant to AV-203 treatment, contains a mutation in PTEN (data not shown). Further studies are needed to elucidate the molecular mechanism of this intrinsic resistance to ERBB3-targeted therapy. It is also worth noting that out of 22 predicted nonresponders (due to their low levels of NRG1 expression), three responded to AV-203 treatment. It is possible that these tumors were driven by ligand-independent ERBB3 signaling. Indeed, one of these models, MDA-MB-453, is a HER2-amplified breast cancer model in which heterodimerization of ERBB3 with high levels of HER2 can result in constitutive activation of ERBB3 independent of ligands (21). MDA-MB-453 xenografts are likely driven by ligand-independent HER2-ERBB3 signaling and were effectively inhibited by AV-203, which is capable of blocking both ligand-dependent and ligand-independent signaling (35).

In summary, the extensive preclinical data presented here strongly support NRG1 as a predictive response biomarker for AV-203. NRG1 warrants further evaluation and validation clinically as a potential predictive biomarker for response to ERBB3-targeted therapies. We have established an RT-PCR–based assay to detect NRG1 levels in human formalin-fixed, paraffin-embedded tumor samples, and the assay can be used for patient selection in future AV-203 clinical trials.

Disclosure of Potential Conflicts of Interest
R. Nicolleti and J. Gyuris have ownership interest (including patents) in AVEO. No potential conflicts of interest were disclosed by the other authors.

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