

Preclinical Rationale for Combining an EGFR Antibody with Cisplatin/Gemcitabine for the Treatment of NSCLC

SELDA SAMAKOGLU, DHANVANTHRI S. DEEVI, HUILING LI, SU WANG, MARY MURPHY, CHANNA BAO, RAJIV BASSI, MARIE PREWETT and JAMES R. TONRA

ImClone Systems, Eli Lilly and Company, New York, NY, U.S.A.

Abstract. *Background.* Although the addition of epidermal growth factor receptor (EGFR) antibodies to various platinum-based chemotherapy regimens for non-small cell lung cancer (NSCLC) is being actively pursued in the clinic, rationale for the prioritization of specific regimens is lacking. *Materials and Methods:* We evaluated the antitumor effects of necitumumab, a recombinant human IgG1 antibody targeting EGFR, in combination with cisplatin plus gemcitabine, pemetrexed, or paclitaxel in a panel of 9 subcutaneous tumor models of NSCLC established in nu/nu athymic mice. *Results:* Necitumumab in combination with cisplatin/gemcitabine was particularly effective, although interestingly, the mechanisms underlying these benefits were model dependent. For example, increased tumor cell apoptosis contributed towards combination efficacy in the A549 model, in association with increased expression of hsa-miR-29b and reduced expression of antiapoptotic genes including DNA methyltransferase DNMT3B, commonly up-regulated in patients with NSCLC. Such inverse effects of combination therapy on DNMT3B and hsa-miR-29b expression were found in multiple models. Importantly, in the A549 model, hsa-miR-29b down-regulation of DNMT3b reduced promoter methylation of tumor suppressor genes such as Cell adhesion molecule 1 (CADMI), Ras associated (RalGDS/AF-6) domain family member 1 (RASSF1), and Fragile histidine triad gene (FHIT), increasing their expression. *Conclusion:* These results offer a preclinical rationale for combining an EGFR antibody with cisplatin/gemcitabine for patients with NSCLC, and provide potential molecular biomarkers for tailoring therapy.

Non-small cell lung cancer (NSCLC) is the leading cause of cancer mortality in the United States. Approximately 30-40% of patients with NSCLC present with advanced stage

Correspondence to: Selda Samakoglu, MD, Ph.D., ImClone Systems, Eli Lilly and Company, New York, NY, U.S.A. Tel: +1 9176064103, Fax: +1 2126452054, e-mail: selda.samakoglu@imclone.com

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disease (stage IIIb with malignant effusion and stage IV) (1), and although platinum-based combination chemotherapy has positively impacted overall survival and quality of life, it is not curative and fewer than 25% of patients survive two years following diagnosis (1). Although many attempts have been made, changing the cytotoxic therapy paired with the platinum agent has had little impact on this prognosis (1). However more recently, as knowledge of tumor biology has increased, small molecule (2) and monoclonal antibodies (2) specifically targeting proteins thought to be critical to disease progression or treatment resistance have been pursued, alone and in combination with chemotherapy. The positive outcome of the recent randomized, multicenter, phase III FLEX study of cetuximab, epidermal growth factor receptor (EGFR) antibody, in combination with cisplatin/vinorelbine (CV) versus CV alone in the first-line treatment of patients with advanced non-small cell lung cancer (3) confirmed that the use of EGFR antibodies can improve upon the benefits of first-line platinum-based chemotherapy(3). However the survival benefit to patients was only of the order of 1.2 months, and cetuximab did not significantly increase the efficacy of carboplatin plus taxane (4). Preferential platinum-based doublet combination partners for EGFR antibodies therefore remain to be determined, yet a rationale for selecting these partners based on clinical or preclinical experimental data is lacking.

Preclinical evaluation of antitumor efficacy, as well as supportive mechanistic interactions at the molecular level, between EGFR antibodies and cisplatin-based chemotherapies may be of utility in selecting combination partners from among the multiple cisplatin-based doublets utilized in the first-line setting in metastatic NSCLC (5). However, preclinical testing thus far has focused on the demonstration of benefits of adding a chimeric antibody to EGFR, cetuximab (6), or a human IgG2 antibody to EGFR, panitumumab (7), to single chemotherapeutic agents in NSCLC models. Thus additional preclinical data testing the combination benefits of an EGFR antibody with platinum-based chemotherapy doublets is needed.

Profiling of mRNA changes that frequently occur with efficacious therapies, such as EGFR antibodies (8), is a validated quantitative approach that can be utilized on cells or tissue samples for gaining insights into the molecular changes underlying treatment efficacy. This information can then be considered for selecting those patients that will be particularly responsive to the therapy. Real-time polymerase chain reaction (PCR) allows for the evaluation of hundreds of cancer-related mRNAs with high sensitivity and reproducibility. This same methodology can now also be utilized to evaluate effects on mRNA-targeting molecules, microRNAs.

MicroRNAs have recently emerged as key regulators of gene expression during development and frequently have altered expression patterns in human disease states, including cancer. For example, Ragusa *et al.* demonstrated up-regulation of hsa-let-7b and hsa-let-7e (target *KRAS* mRNA) and down-regulation of hsa-miR-17* in colorectal cancer after cetuximab treatment (8), indicating the potential importance of microRNA regulation for achieving the beneficial effects of this EGFR antibody in patients with metastatic colorectal cancer. In NSCLC, overexpression of hsa-miR-200c restored the sensitivity of NSCLC cells to cisplatin plus cetuximab through downstream effects on the methylation of the promoter region of E-cadherin (9). Furthermore, loss of expression of hsa-miR-128b correlates with response to small molecule EGFR-targeted agents in primary NSCLC (10). Thus microRNA effects may be upstream to important changes in mRNA for EGFR-targeted agents, but as with mRNA, effects have not been evaluated with EGFR antibody therapy in combination with platinum doublets in NSCLC models.

Based on the above considerations, the present research utilized a panel of preclinical NSCLC models to compare the efficacy of an EGFR antibody in clinical development for the treatment of NSCLC, necitumumab (11), in combination with three platinum-based chemotherapy doublets utilized in the United States; cisplatin/gemcitabine, cisplatin/pemetrexed, and cisplatin/paclitaxel (12). Necitumumab achieved comparable or better efficacy when combined with cisplatin/gemcitabine in NSCLC models, at the maximum tolerated dose levels tested, so this platinum doublet was selected for further molecular analysis. Profiling of molecular changes in tumor cells growing *in vivo* and *in vitro* allowed for an understanding of the mechanisms underlying the antitumor benefits of this combination.

Materials and Methods

Cell culture and reagents. Human NSCLC cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained at 37°C in a 5% CO₂ incubator. A549 cells were cultured in F12/Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Grand Island, NY); NCI-H1650, NCI-H358,

NCI-H520, NCI-H226, HOP62, NCI-H2405, NCI-H441, HCC827, EKVX-P2 and NCI-H1975 cells were cultured in RPMI-1640 (Invitrogen); Calu-6 cells were cultured in Eagle's minimum essential medium (Invitrogen). All media contained 10% fetal bovine serum (HyClone, South Logan, UT, USA) and 2 mmol/l GlutaMAX (Invitrogen, Grand Island, NY).

In vivo subcutaneous xenograft models in athymic mice. 12 female mice with 5-6 weeks of old per group were supplied by Charles River Laboratories. Subcutaneous xenografts were established as previously published (13) by injecting 2×10⁷ A549 cells in 100% Matrigel (Collaborative Research, Inc, Waltham, MA, USA), or 1×10⁷ NCI-H1650 cells, 5×10⁶ NCI-H358 cells, 2×10⁶ NCI-H520 cells, 3×10⁶ NCI-H441 cells, 2×10⁷ HCC-827 cells, 5×10⁶ Calu-6 cells, 1×10⁷ EKVX-P2 cells, or 3×10⁶ NCI-H1975 cells per mouse in 50% Matrigel with culture medium.

In vivo treatments, tumor collection and immunohistochemical analyses. Mice were treated intraperitoneally with USP saline (0.1 ml per 10 g body weight, twice per week) (Invitrogen Grand Island, NY), necitumumab (60 mg/kg, twice per week) (ImClone Systems, New York, NY, USA), cisplatin (3 mg/kg, *q7d*), gemcitabine (250 or 500 mg/kg, *q7d*), pemetrexed (100 mg/kg, 5 days per week, Monday through Friday for 2 weeks), and/or paclitaxel; (5 mg/kg, *q7d* for 3 weeks). Cisplatin (Sigma-Aldrich, St. Louis, MO, USA) and pemetrexed disodium (Eli Lilly and Company Research Laboratories, Indianapolis, IN, USA) were prepared in 0.9% USP saline; gemcitabine (Syn Chem OHG, Felsberg/Altenburg, Germany) was prepared in 0.9% USP saline containing 12.5 mg/ml mannitol and 0.781 mg/ml sodium acetate. Paclitaxel (Sigma-Aldrich, St. Louis, MO) was formulated in USP saline containing 5% ethanol and 5% cremophore. Chemotherapeutic drug dosing was started one day before the start of necitumumab. Alternative dosing strategies were not tested. For mechanism of action studies, 24 hours after the second weekly chemotherapeutic drug treatment, six tumors per treatment group were excised and half of each tumor was snap frozen in liquid nitrogen for mRNA, microRNA, or gene methylation analyses. The remaining halves of the tumors were formalin fixed and paraffin embedded for immunohistochemistry as previously described (13) and stained for Ki67 (13), Apoptag (6) and cyclin D1 (Thermo Scientific, Rockford, IL, USA). Quantification of immunostaining was performed on images acquired using a Zeiss Axiocam mounted on a Zeiss universal microscope (Carl Zeiss, Chester, VA, USA). Computer-assisted morphological analyses of digital images were performed with ImagePro software (Dallas, TX, USA).

In vitro cell treatment. A549, NCI-H1650, NCI-H1975, HCC827, EKVX-P2, NCI-H358, NCI-H441 and Calu-6 cells were cultured as described above. Following serum starvation overnight, cells were treated with necitumumab (5 µg/ml) for 24 h and subsequently with cisplatin (1 µg/ml) plus gemcitabine (1 µg/ml) for another 24 h.

Transient transfection. A549 cells were plated at a density of 1.6×10⁵ cells/well in 24-well plates (Corning Inc., Corning, NY, USA) as in *Cell culture and reagents*, 24 h before transfection. The cells were transiently transfected in triplicate with an hsa-miR-29b mimic (Mission microRNA Mimic hsa-miR-29b; Sigma-Aldrich) at 5 nM, an hsa-miRNA-29b inhibitor (HmiR-AN0373-SN-10; GeneCopoeia, Rockville, MD, USA) at 50 nM, synthetic scrambled oligonucleotide as a null transfection control (CmiRR-AN0001-SN;

Table I. Statistical comparisons by RM-ANOVA between antitumor efficacy achieved in NSCLC xenograft tumors with necitumumab combined with cisplatin/gemcitabine, cisplatin/pemetrexed and cisplatin/paclitaxel.

Xenograft tumor model	p-Values for necitumumab+ csplatin/gemcitabine versus necitumumab+csplatin/paclitaxel	p-Values for necitumumab+ csplatin/gemcitabine versus necitumumab+csplatin/pemetrexed	p-Values for necitumumab+ csplatin/paclitaxel versus necitumumab+csplatin/pemetrexed
A549	0.077	0.3984	0.8709
NCI-H1650	0.5448	0.3019	0.671
NCI-H358	0.0013	0.002	0.0361
NCI-H520	0.2297	0.0566	0.6697
NCI-H441	0.0001	0.6564	0.68
HCC827	0.058	0.5867	<0.001
EKVX-P2	0.0004	<0.0001	0.0042
Calu6	0.1097	0.0034	0.0164
NCI-H1975	0.3107	0.173	0.4099

GeneCopoeia, Rockville, MD) or transiently co-transfected with the hsa-miR-29b mimic and the hsa-miR-29b inhibitor, utilizing 3 μ l HiPerFect Transfection Reagent (Qiagen, Valencia, CA, USA) per the manufacturer's instructions. Transfected cells were harvested 48 h after transfection to analyze mRNAs, microRNAs and proteins as described below.

Cell protein preparation and western blotting. NSCLC cell lines and A549 tumor cells were harvested to prepare cell lysates that were utilized for western blotting as described elsewhere (13). Blots were probed for DNA (cytosine-5)-methyltransferase 3 beta (DNMT3b) Cell Signaling, Danvers, MA, USA) and actin (Abcam, Cambridge, MA, USA).

RNA isolation. Total RNA from frozen tumors and cell lines were isolated utilizing an RNeasy fibrous kit (Qiagen, Valencia, CA) and QiaCube instrumentation (Qiagen Valencia, CA). The RT² qPCR-Grade miRNA Isolation Kit (SABiosciences, Frederick, MD, USA) was used to isolate small RNAs enriched for microRNA. RNA was quantified by NanoDrop (Thermo Scientific, Rockford, IL). Integrity of RNA was verified on a 2% agarose gel prior to storage at -80°C.

Analysis of gene expression by PCR arrays. RT² First Strand Kit or miRNA First Strand Kit (SABiosciences, Frederick, MD) was used to treat RNA samples for genomic DNA elimination followed by cDNA conversion for mRNA and microRNA analysis. The cDNA samples from each treatment group were evaluated on an Applied Biosystems 7500 Real-time PCR instrument in triplicate to quantify mRNA and microRNA levels, using RT² SYBR Green/Fluorescein PCR master mix and PCR Array (SABiosciences, Frederick, MD). Messenger RNA and microRNA fold changes were calculated versus control treatment utilizing the 2-delta-delta-Cycle threshold values (delta-delta-CTs) method, with the threshold determined by SDS Enterprise Edition software (Life Technologies-Applied Biosystems, Carlsbad, CA, USA).

Messenger RNAs and microRNAs with >2-fold or <2-fold change with a value $p < 0.05$ versus any of the other treatments were selected for further analysis by Ingenuity Pathway Analysis software (Ingenuity Systems, Inc, Redwood City, CA, USA) for pathway and functional analyses.

DNA methylation assay. DNeasy Tissue Kit (Qiagen) was used to isolate genomic DNA from A549 cell line and xenograft tumor tissue as per the manufacturer's instructions (n=3 tumors per group). DNA methylation at the promoter of cell adhesion molecule 1 (CADM1; CpG island location on chr11: 1148783784-114881091), Ras association (RalGDS/AF-6) domain family member 1 (RASSF1; CpG island location on chr3: 50352807-50353544) and fragile histidine triad gene (FHIT; CpG island location on Chr3: 61211643-61212264) was determined using Methyl-Profiler DNA methylation assay (MEA-03; SABiosciences Frederick, MD) as per the manufacturer's instructions. Primers for quantitative real-time PCR for CADM1 (catalog no. MePH28474-1A), RASSF1 (catalog no. MePH28531-1A), and FHIT (catalog no. MePH28495-1A) were from SABiosciences. Data was analyzed using SABiosciences online data analysis software and expressed as the percentage of all tumors for unmethylated or highly methylated DNA of the CpG island of interest.

Statistics. Tumor volumes were analyzed by repeated measures ANOVA utilizing JMP version 5.1 software (SAS Institute Inc, Cary, NC, USA). Regression (final tumor volume <70% initial tumor volume) frequencies were analyzed utilizing a 4-group Chi-squared test. Treatment effects on histological measurements and percentage highly methylated gene promoter were evaluated by one-way ANOVA, followed by Fisher's LSD *post hoc* test (Sigma Stat; Systat Software, Inc., San Jose, CA, USA). Statistical p -values for fold changes of mRNA and microRNA were calculated by two-sided Student's t -test.

Results

Necitumumab inhibits NSCLC tumor growth as a monotherapy, and increases the effects of cisplatin/gemcitabine combination. Necitumumab as a monotherapy had significant antitumor effects in six out of the nine NSCLC models utilized (Figure 1). To compare the benefits, necitumumab was combined with pre-determined maximum tolerated doses (MTDs) of cisplatin/gemcitabine, cisplatin/pemetrexed, and cisplatin/paclitaxel in the same studies. MTDs were established utilizing chemotherapy-associated morbidity and/or weight loss effects

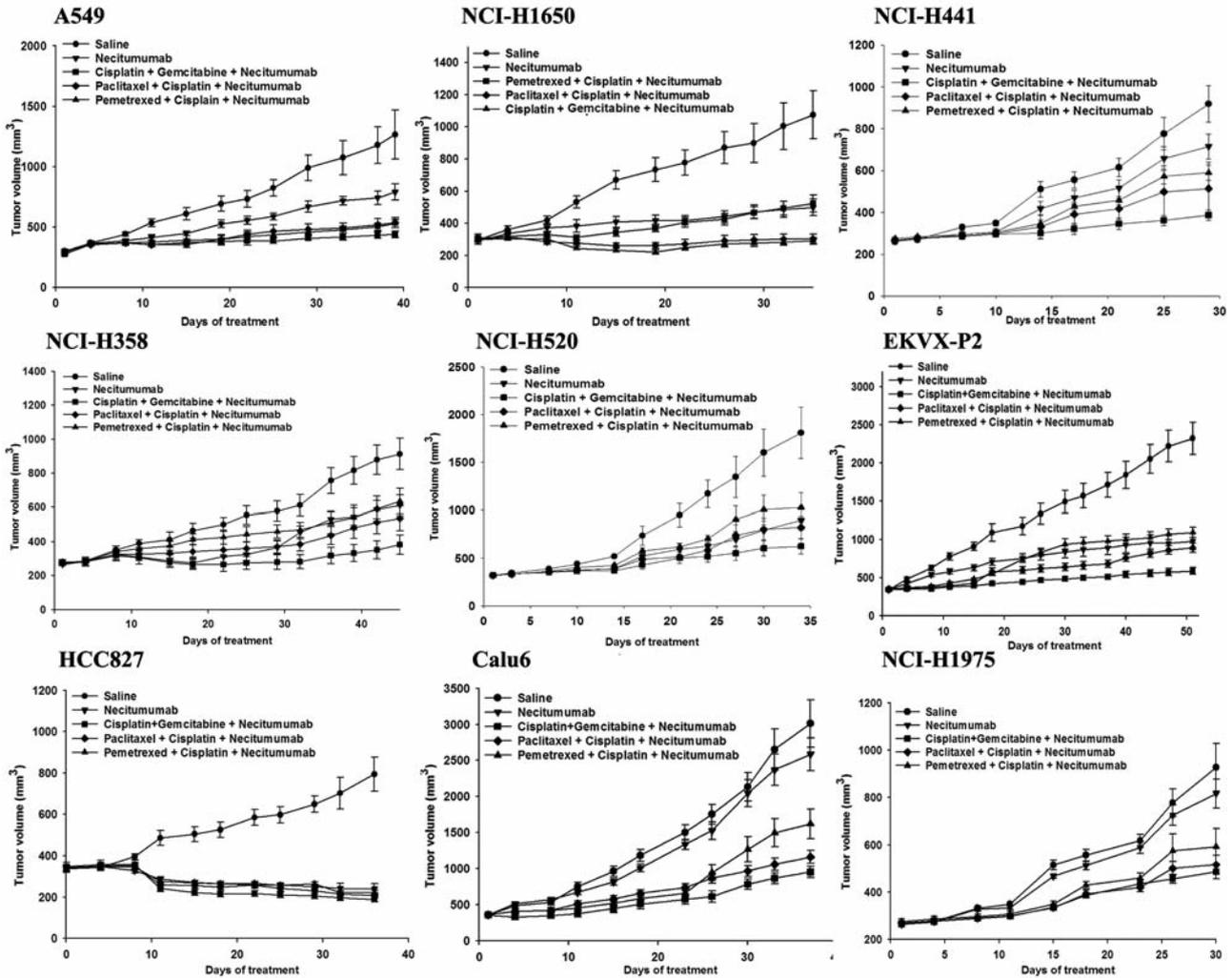


Figure 1. Necitumumab in combination with cisplatin plus gemcitabine, cisplatin plus paclitaxel, or cisplatin plus pemetrexed in the indicated xenograft tumor models. Treatments with necitumumab plus cisplatin doublets were applied as described in the Materials and Methods. The mean±SEM is plotted for n=12 mice per group.

at different dose levels (data not shown). Necitumumab achieved comparable or greater effects on tumor growth in combination with cisplatin/gemcitabine compared to other combinations, reaching statistical significance *versus* both alternatives in the NCI-H358 and EK VX-P2 models (Table I). In the A549 (Figure 2A) and NCI-H1650 (Figure 2B) models, the effects of necitumumab with cisplatin/gemcitabine on tumor growth were further shown to be significantly greater than both necitumumab and cisplatin/gemcitabine alone. In addition, while neither necitumumab nor cisplatin/gemcitabine caused partial tumor regressions (>30% reduction in tumor volume from the start of treatment), the combination of these agents resulted in regressions in 4 out of 12 mice in the A549 model ($p=0.01$). Similarly in the NCI-H1650 xenograft model, while tumor regression occurred in 1 out of 12 mice in both

the necitumumab group and cisplatin/gemcitabine group, the combination treatment resulted in regression in 7 out of 12 mice ($p=0.005$).

Differential effects of treatment on tumor cell proliferation and apoptosis. To gain an understanding of the mechanism of action underlying the benefits of adding necitumumab to cisplatin/gemcitabine, A549 and NCI-H1650 tumors were harvested for analyses after 7 days of therapy, 24 h after the second dose of chemotherapy (Figure 2A and B). Histological analyses of NCI-H1650 (Figure 3A and B) and A549 (Figure 3C and D) tumors demonstrated model-dependent effects on markers of the percentage tumor cells in the cell cycle (Ki-67-positive) and the percentage of apoptotic tumor cells (ApopTag-positive). The percentage of

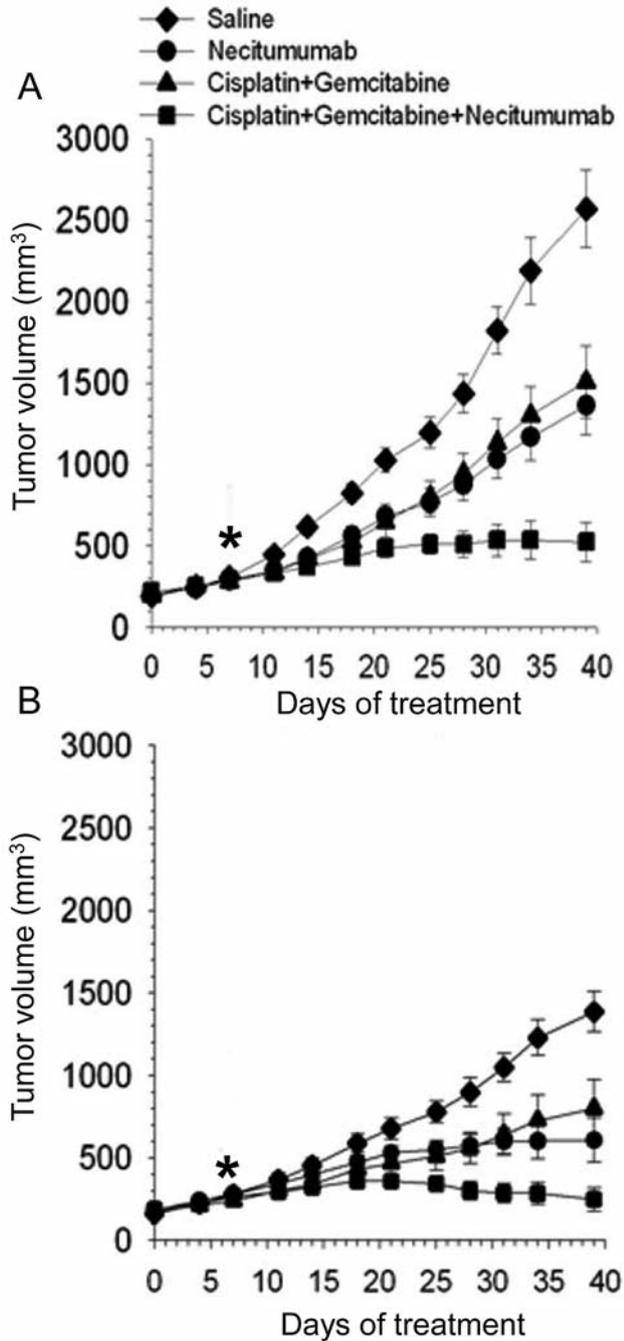


Figure 2. Necitumumab increases the effects of cisplatin/gemcitabine in Non-small cell lung cancer (NSCLC) models. A549 (A) and NCI-H1650 (B) NSCLC xenograft tumor models were established subcutaneously in nu/nu athymic mice and treated with necitumumab, cisplatin/gemcitabine and necitumumab with cisplatin/gemcitabine. *Tumor harvest for histological and molecular analyses of mechanism of action.

Ki-67-positive tumor cells was significantly reduced by necitumumab ($p=0.012$), cisplatin/gemcitabine ($p=0.002$) and the combination of these therapies ($p<0.001$) compared to control treatment in NCI-H1650 tumors (Figure 3A,

Figure 4A). There was also a significant decrease in the percentage of Ki-67-positive tumor cells for the combination therapy compared to monotherapies (Figure 3A, Figure 4A). In contrast in the A549 model, only the combination of necitumumab with cisplatin/gemcitabine reduced Ki-67 expression (Figure 3C, Figure 4B). With regard to apoptosis, no effect of treatment on the percentage of ApopTag-positive tumor cells was detected in the NCI-H1650 model (Figure 3B, Figure 4A). In contrast, in the A549 model, the proapoptotic effect of cisplatin/gemcitabine was significantly augmented by necitumumab ($p\leq 0.001$) (Figure 3D and Figure 4B).

Messenger RNA and microRNA profiling highlights molecular pathways involved in antitumor efficacy. Histological data on tumor cell proliferation and apoptosis after 7 days of therapy were complemented by molecular analysis of 384 cancer-associated mRNAs and microRNAs in A549 and NCI-H1650 tumors, utilizing real-time PCR. Figure 3A and B provide heat maps showing all mRNAs and microRNAs significantly affected by cisplatin/gemcitabine, necitumumab, and the combination therapy *versus* the control treatment (>2 -fold up- or down-regulation with $p<0.05$) in A549 and NCI-H1650 tumors, respectively. Markers are grouped by the pattern of change in the different treatment groups. Given the important role of cyclin D1, the protein product of *CCND1* mRNA, in cell cycle progression (6), the significant down-regulation of *CCND1* mRNA by necitumumab, alone and in combination with cisplatin/gemcitabine in the NCI-H1650 model (Figure 5B) was evaluated further. Histological analyses confirmed that the detected changes in mRNA were consistent with protein changes in NCI-H1650 tumors (Figure 5B, D, and Figure 6B). In fact, as with mRNA analyses, cyclin D1 expression was significantly reduced in tumor cells by necitumumab therapy alone ($p=0.01$) and in combination with cisplatin/gemcitabine ($p=0.01$) in NCI-H1650 tumors (Figure 5B). These effects were not observed in A549 tumors (Figure 5A, C, and Figure 6A). Reduced cyclin D1 expression in NCI-H1650 cells may therefore contribute towards the antiproliferative effects of treatment (Figure 1A and Figure 4A).

Selected mRNA and microRNA were further categorized by their putative function in cancer cells utilizing Ingenuity Software, highlighting those changes with the potential to contribute towards the measured effects, or lack of effect of necitumumab with cisplatin/gemcitabine on tumor cell proliferation and apoptosis (Figure 7A and B). For example, in NCI-H1650 tumors, as with *CCND1*, expression of proliferative Retinoblastoma-associated protein 1 (E2F), cell division cycle 34 homolog (CDC34), forkhead box A2 (FOXA2), v-myc myelocytomatosis viral oncogene homolog (MYC) and v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) mRNAs were reduced by the combination of necitumumab with cisplatin/gemcitabine,

potentially contributing towards the antiproliferative effect of this combination demonstrated histologically (Figure 3A). Figure 7 also highlights mRNA changes, indicating that necitumumab may inhibit mechanisms of resistance to cisplatin/gemcitabine therapy. In particular, up-regulation of Cell division protein kinase 2 (CDK2), Cell division protein kinase 4 (CDK4), Cyclin A2 (CCNA2) and *CCNE1* by cisplatin/gemcitabine alone could result in increased NCI-H1650 cell proliferation, but necitumumab is seen to block this effect when given in combination (Figure 7B).

With regard to microRNA, up-regulation of hsa-miR-15a and hsa-miR-34a microRNA expression may also contribute towards the antiproliferative effects of necitumumab alone and with cisplatin/gemcitabine in the NCI-H1650 model (Figure 7B), given their putative role in the degradation of *CCND1* mRNA (14, 15). Heat maps categorized by antiproliferative and proapoptotic function also highlight that hsa-let-7, a putative tumor suppressor gene in lung cancer (16), was increased by necitumumab and combination therapy in the NCI-H1650 xenograft tumor model (Figure 7B). The induction of *hsa-let-7* has been correlated with the repression of *KRAS* signaling (16) and High mobility group AT-hook (HMGA2), a cell cycle regulator through *CCNA2* (17) (Figure 7B), and therefore could conceivably play a critical role in cell cycle arrest of lung cancer cells in cooperation with oncogenes or tumor suppressor genes (18).

X-linked inhibitor of apoptosis (XIAP), a potent inhibitor of caspases 3, 7 and 9 (19), was increased in NCI-H1650 tumors with treatment *versus* control (Figure 7B). Increased XIAP may increase the resistance of NCI-H1650 cells to apoptosis (19) and contribute towards the lack of apoptosis detected in this model (Figure 3B and Figure 4B). In contrast XIAP mRNA was reduced with necitumumab with cisplatin/gemcitabine in the A549 model, along with the down-regulation of mRNA for other antiapoptotic genes including Wingless-type MMTV integration site family, member 1 (*WNT1*), Baculoviral IAP repeat containing 8 (*BIRC8*), B-cell CLL/lymphoma 2 (*BCL2*), BCL2-associated athanogene 3 (*BAG3*), V-akt murine thymoma viral oncogene homolog 1 (*AKT1*), Baculoviral IAP repeat containing 2 (*BIRC2*), Baculoviral IAP repeat containing 3 (*BIRC3*), TNF receptor-associated factor 2 (*TRAF2*), Interleukin 8 (*IL8*), *CASP8* and FADD-like apoptosis regulator (*CFLAR*), BCL2-associated athanogene 4 (*BAG4*), Nucleolar protein 3 (*NOL3*), BCL2/adenovirus E1B 19kDa interacting protein 1 (*BNIP1*), V-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) (Figure 7A). Down-regulation of the antiapoptotic microRNA hsa-miR-155 (20) was also observed (Figure 7A). Together with the up-regulation of Bcl2-interacting mediator of cell death (*BCL2L11*), BCL2/adenovirus E1B 19kDa interacting protein 3 (*BNIP3*), myeloid cell leukemia sequence 1 (BCL2-related) (*MCL1*), PYD and CARD domain

containing (*PYCARD*), B-cell CLL/lymphoma 10 (*BCL10*), BH3 interacting domain death agonist (*BID*), Harakiri, BCL2 interacting protein (contains only BH3 domain) (*HRK*), BCL2-associated transcription factor 1 (*BCLAF1*) and caspase proapoptotic mRNAs (Figure 7A), these broad changes likely integrate to induce increased apoptosis of A549 cells with combination therapy (Figure 3D).

Necitumumab with cisplatin/gemcitabine increases expression of tumor suppressor gene through up-regulation of microRNA hsa-miR-29b. Combination therapy increased the expression of hsa-miR-29b (14.8-fold, $p=0.018$) (Figure 5A and 8A), which has regions complementary to the 3'-UTRs of *DNMT3B* (*de novo* methyltransferase) (Figure 9). This complementarity suggests that mRNA levels for *DNMT3B*, an important DNA methylation enzyme frequently up-regulated in lung cancer in association with poor prognosis (21), may be targeted by hsa-miR-29b for destruction in A549 tumors. This possibility is highlighted by the finding that hsa-miR-29b levels in A549 tumors were only significantly up-regulated by necitumumab with cisplatin/gemcitabine, with an opposite regulation of *DNMT3B* mRNA (Figure 8A). This finding was not observed in NCI-H1650 tumors (Figure 8B). Reduced DNMT3B may lead to reduced methylation-induced silencing of a number of tumor suppressor genes (22), some of which were included in our mRNA panel. In agreement with re-expression of methylation-silenced tumor suppressor genes induced by therapy with necitumumab with cisplatin/gemcitabine, the following mRNAs were up-regulated in tumors treated with necitumumab with cisplatin/gemcitabine: *APC* (17.3-fold, $p=0.009$), *RARA* (28.7 fold, $p=0.00002$), *CADMI* (27.7 fold $p=0.0004$), *FHIT* (18.68 fold, $p=0.0012$), *RASSF1* (19.36 fold, $p=0.0004$) (Figure 8A). The dramatic up-regulation of these mRNAs, specific to the combination group, was again not observed in the NCI-H1650 tumors (Figure 8B).

The association between increased hsa-miR-29b, reduced *DNMT3B* mRNA, and increased expression of tumor suppressor gene with combination therapy in A549 tumors suggested that up-regulation of hsa-miR-29b by necitumumab with cisplatin/gemcitabine initiates DNA epigenetic modifications that result in increased expression of tumor suppressor genes in some NSCLC cells (23). To further examine this possibility, we first evaluated whether the effects of necitumumab with cisplatin/gemcitabine on DNMT3B could be modeled *in vitro*. Figure 8C illustrates that, as *in vivo*, only the combination of necitumumab with cisplatin/gemcitabine reduced the expression of DNMT3B and up-regulated the expression of hsa-miR-29b *in vitro*. Furthermore, this effect was only observed in A549 cells, and not NCI-H1650 cells (not shown).

To demonstrate that hsa-miR-29b up-regulation can have the impact on A549 cells suggested by the correlative analyses performed with tumor measurements above, A549

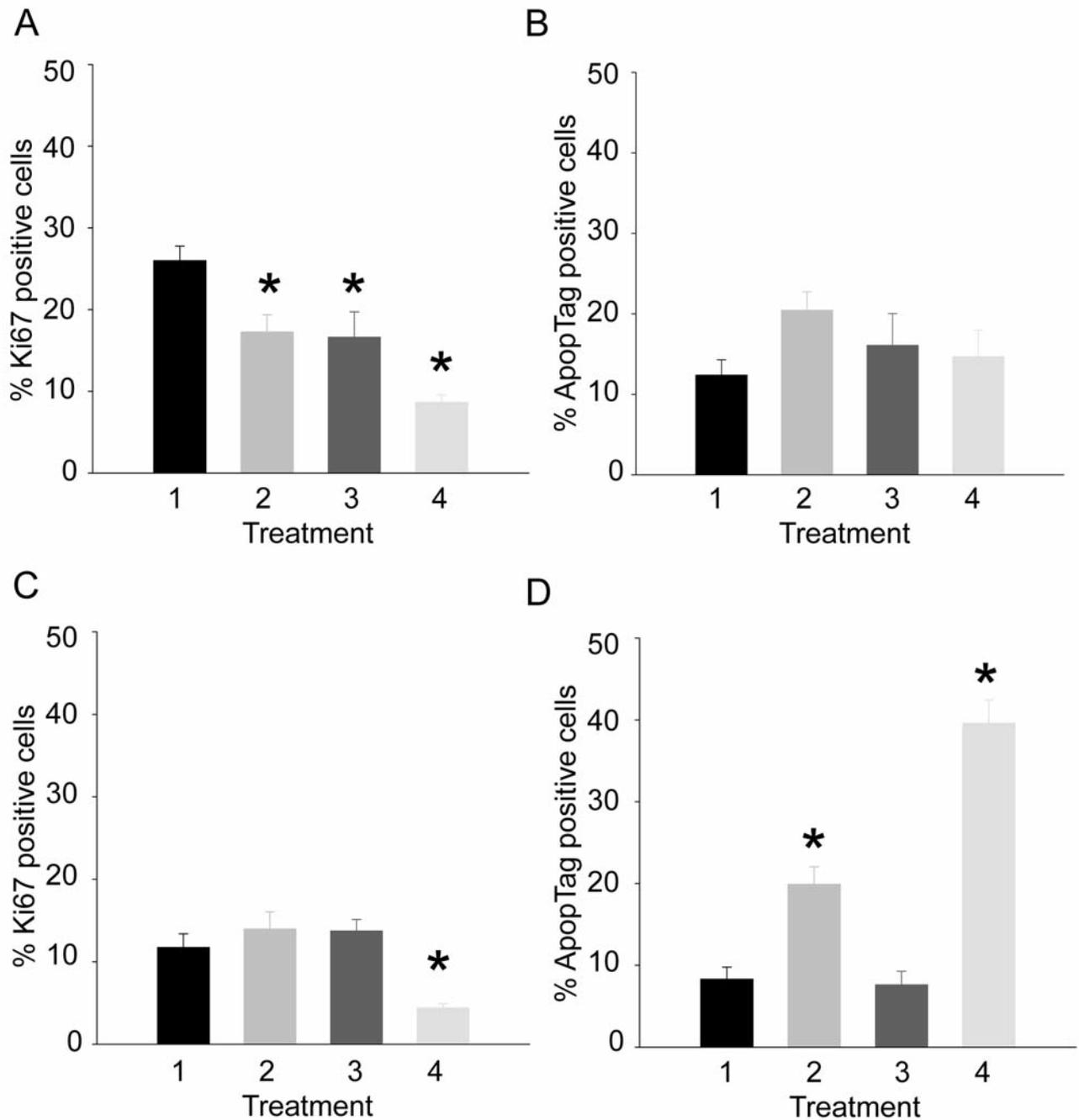


Figure 3. Histological evaluation of A549 and NCI-H1650 xenograft tumors. Percentage of Ki-67-positive (A and C) and Apoptag-positive (B and D) tumor cells were evaluated in NCI-H1650 (A and B) and A549 (C and D) tumors established in nu/nu athymic mice. Tumors were harvested after 7 days treatment. 1, control(saline); 2, cisplatin plus gemcitabine; 3, necitumumab; 4, cisplatin plus gemcitabine plus necitumumab. The mean \pm SE is plotted for n=6 per group.* $p < 0.05$ versus the control.

cells were transiently transfected with hsa-miR-29b precursor or co-transfected with hsa-miR-29b precursor and its specific inhibitor (Figure 8D). Hsa-miR-29b directly targeted *DNMT3B* mRNA in A549 cells, as evidenced by reduced *DNMT3B* protein in precursor transfected A549 cells

compared to untransfected control cells (Figure 8D). *DNMT3B* protein was not down-regulated in A549 cells when hsa-miR-29b precursor was co-transfected with its inhibitor. Thus, the enforced expression of hsa-miR-29b down-regulates *DNMT3B* levels in A549 cells.

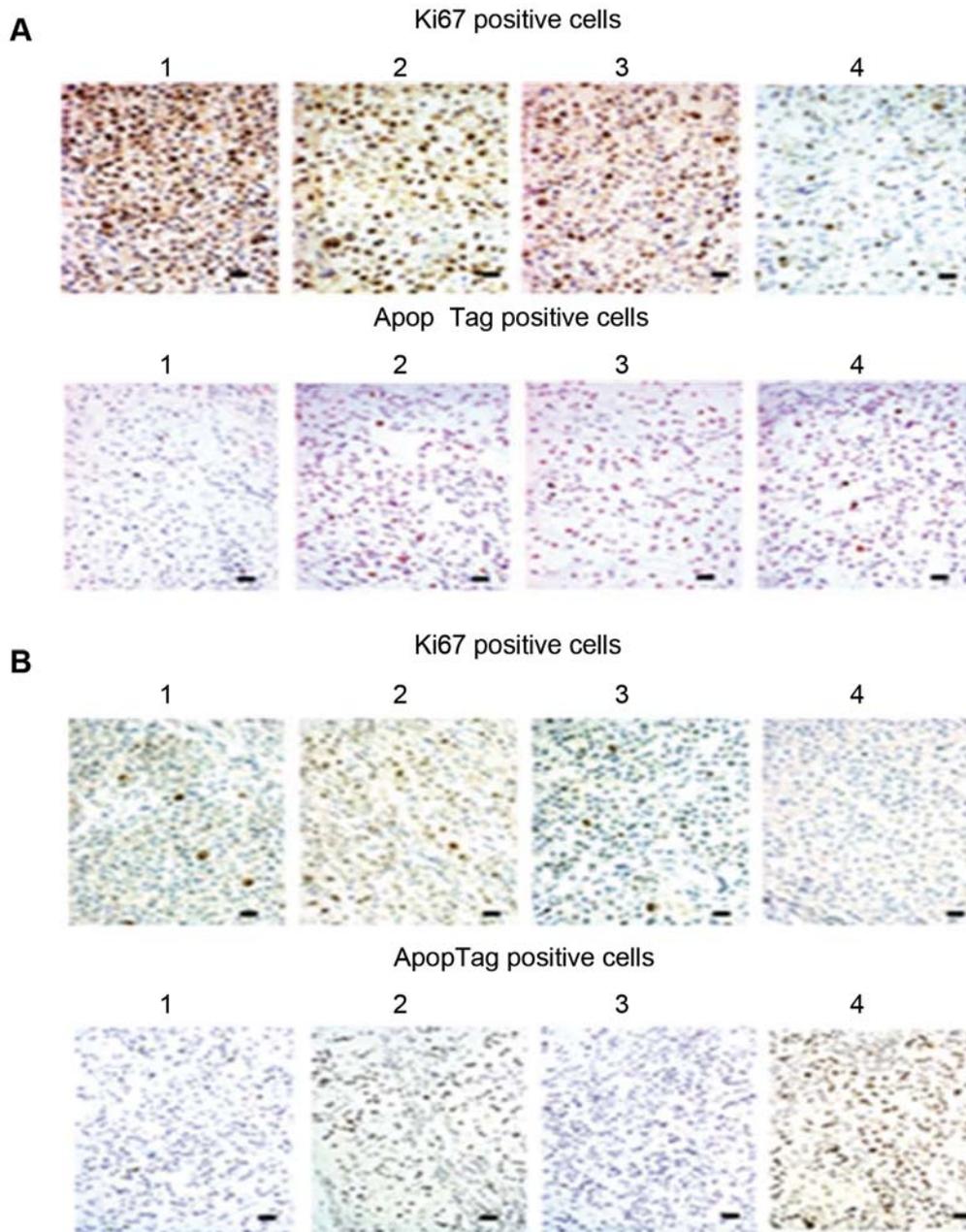


Figure 4. Histological evaluation of NCI-H1650 (A), and A549 (B) xenograft tumors for percentage Ki-67- and Apoptag-positive tumor cells established in nu/nu athymic mice. Tumors were harvested after 7 days' treatment with 1, control(saline); 2, cisplatin plus gemcitabine; 3, necitumumab; 4, cisplatin plus gemcitabine plus necitumumab. Calibration bars=50 μ m.

We further analyzed mRNA expression levels for tumor suppressor genes *RARA*, *RASSF1*, *FHIT*, *CADM1*, *RARB* and *APC* in transfected and control cells. These genes have promoters that are potential targets of *DNMT3B*, and have been reported to be silenced by promoter methylation in lung cancer (24). As shown in Figure 8D, hsa-miR-29b precursor transfection increased *FHIT* mRNA expression 7.2-fold

($p=0.0002$), *CADM1* expression 22.8-fold ($p=0.002$) and *RASSF1* expression 6.7-fold ($p=0.007$). The expression level of *RARA* (3.91-fold, $p=0.07$), *RARB* (-1.17-fold, $p=0.40$) and *APC* (-1.11-fold, $p=0.37$) mRNA were not significantly affected by hsa-miR-29b precursor transfection *in vitro*. The effects of hsa-miR-29b precursor on *FHIT*, *CADM1* and *RASSF1* mRNAs in A549 cells were inhibited by co-

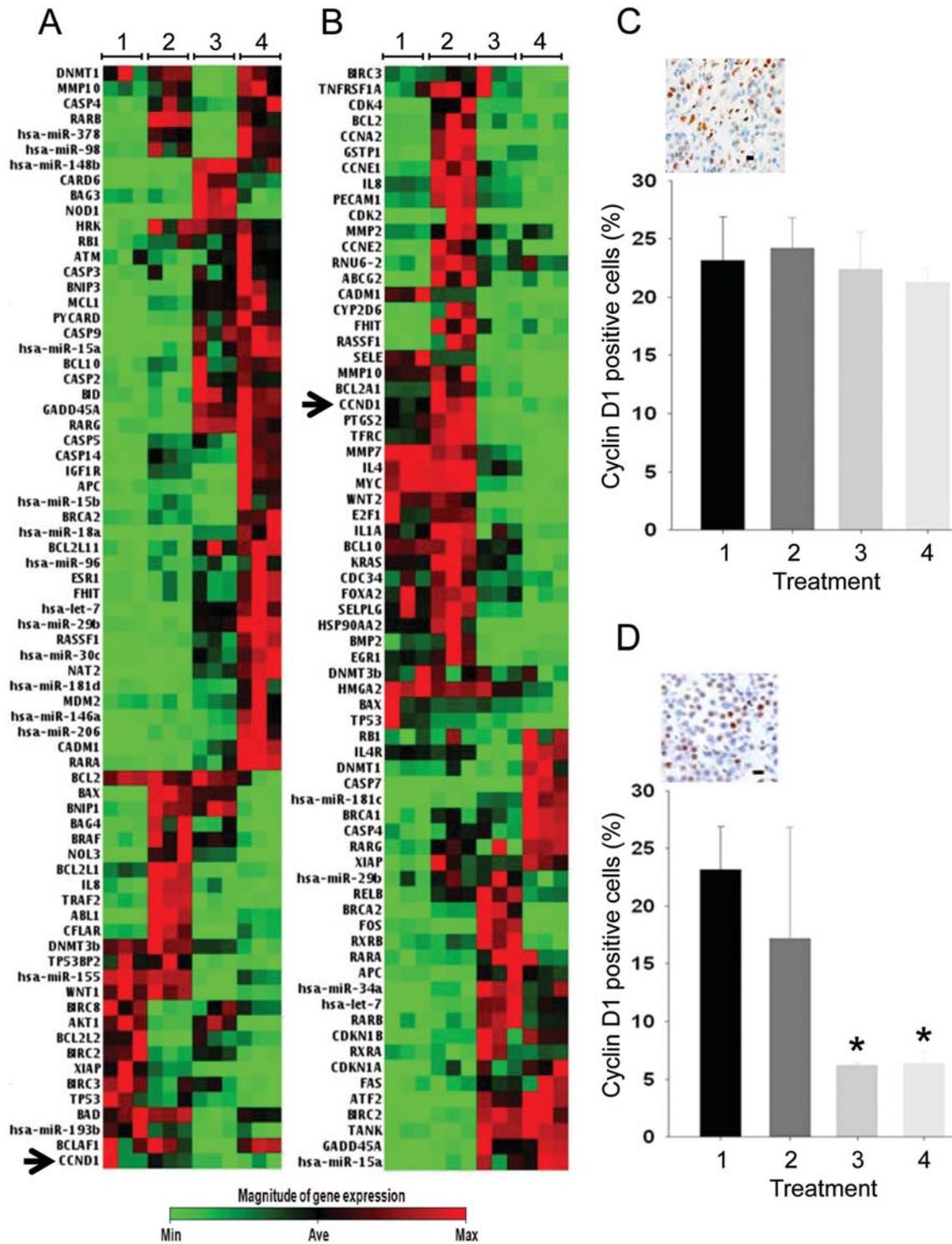


Figure 5. Comparative analysis of mRNAs, microRNAs and protein expression in A549 and NCI-H1650 tumors. Heat-maps showing fold-change patterns of mRNAs and microRNAs in A549 (A) and NCI-H1650 (B) xenograft tumors treated as indicated. Genes shown are those with >2-fold up- or down-modulation in at least one treatment group versus the saline control group, with $p < 0.05$ ($n = 3$ tumors per group). Red, black and green colors indicate high, moderate, and low expression considering levels measured across treatment groups. Arrows indicate cyclin D1. Percentage cyclin D1 positive tumor cells in A549 (C) and NCI-H1650 (D) xenograft tumors were evaluated histologically after 7 days' treatment. Bar charts include photomicrographs of tumor of the saline-treated group as an inset with calibration bar = 25 μm . The mean \pm SE is plotted for $n = 6$ per group. * $p < 0.05$ versus the control. 1, control (saline); 2, cisplatin plus gemcitabine; 3, necitumumab; 4, cisplatin plus gemcitabine plus necitumumab.

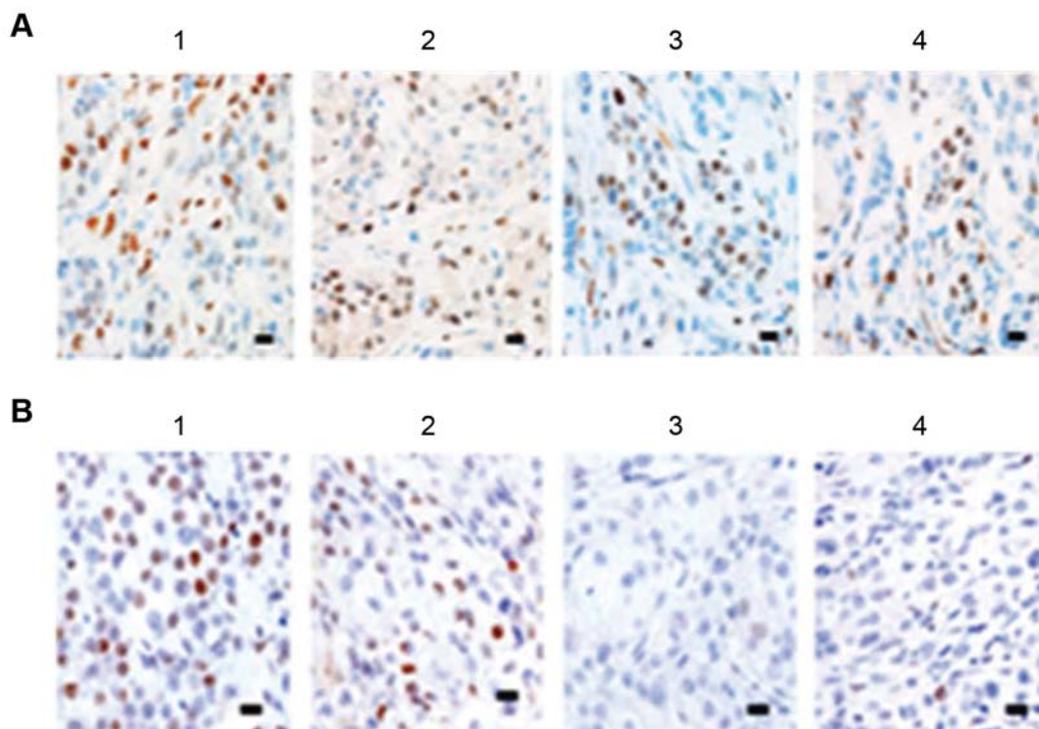


Figure 6. Histological evaluation of cyclin D1-positive tumor cells established in nu/nu athymic mice after 7 days' treatment in A549 (A) and NCI-H1650 (B) xenograft tumors with $n=6$ per treatment group. Calibration bars= $25\ \mu\text{m}$. 1, control (saline); 2, cisplatin plus gemcitabine; 3, necitumumab; 4, cisplatin plus gemcitabine plus necitumumab. Calibration bars= $50\ \mu\text{m}$.

transfection of the specific inhibitor (M versus M+Inh in Figure 8D), demonstrating that these effects were related to hsa-miR-29b up-regulation.

Inverse regulation of DNMT3B and hsa-miR-29b in 5 out of 6 additional NSCLC cell lines. We next examined whether the relationship between DNMT3B and hsa-miR-29b triggered with necitumumab with cisplatin/gemcitabine treatment was exclusive to A549 NSCLC cells by testing treatment effects in additional NSCLC cell lines *in vitro*. Figure 8E illustrates that the inverse nature of the effects of combination therapy on DNMT3B and hsa-miR-29b expression was also observed with HCC827, EKVX-P2, NCI-H358, NCI-H441, Calu6 cells, but not with NCI-H1975 cells *in vitro*. As in A549 cells, this finding was exclusive to the combination group and was not found with necitumumab or cisplatin/gemcitabine alone.

Necitumumab with cisplatin/gemcitabine reduces promoter methylation of tumor suppressor genes in A549 tumors. Hsa-miR-29b is thought to regulate the expression of *CADM1*, *RASSF1* and *FHIT* by altering DNMT3B-mediated promoter methylation of these genes. The methylation status of the regulatory region of *CADM1*,

RASSF1 and *FHIT* was therefore evaluated in A549 tumors treated with saline, cisplatin/gemcitabine, necitumumab or their combination. Methylation status was categorized as highly methylated or unmethylated as per the assay manufacturer's recommendations. Methylation status was significantly shifted by necitumumab with cisplatin/gemcitabine towards the unmethylated state for *RASSF1* ($p<0.0001$) (Figure 10A), *CADM1* ($p=0.0007$) (Figure 10B) and *FHIT* ($p=0.0032$) (Figure 10C), further supporting the involvement of an hsa-miR-29b–DNMT3B–tumor suppressor gene axis in the mechanism of action underlying the benefits of this combination therapy.

Discussion

Preferential platinum-based doublets to be partnered with EGFR antibodies for the treatment of NSCLC remain to be determined. While preclinical testing has demonstrated the benefits of adding cetuximab (6), or another EGFR antibody, panitumumab (25), to single chemotherapeutic agents in NSCLC models, supportive efficacy and mechanistic data in combination with platinum-based doublets is lacking. Here we investigated the efficacy of necitumumab (11), a fully human IgG1 monoclonal antibody directed against EGFR, in

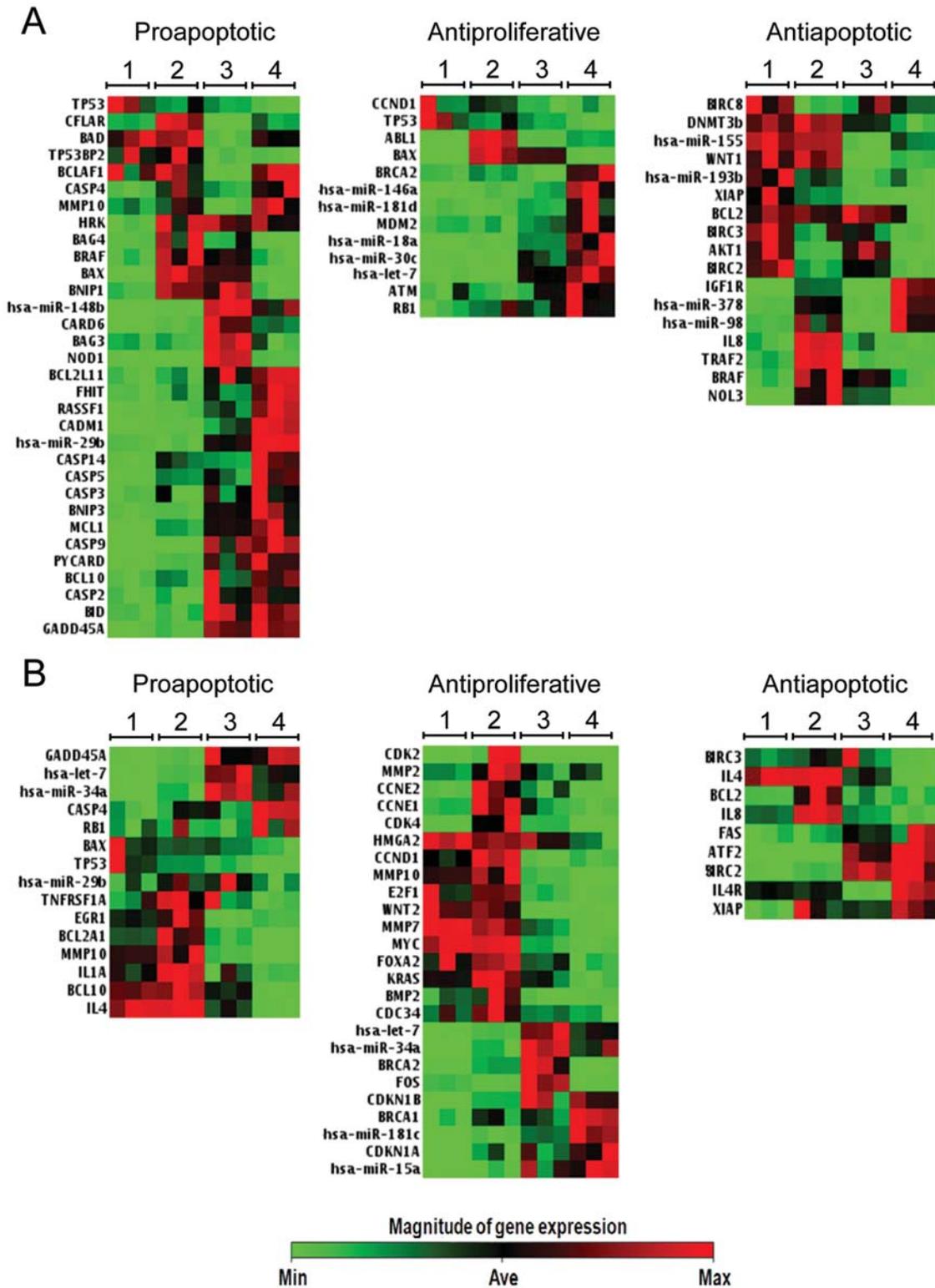


Figure 7. Effects of treatment on microRNAs and mRNAs by function. Messenger RNAs and microRNAs affected by the indicated treatments and potentially contributing towards the proapoptotic; antiproliferative; antiapoptotic function in A549 (A) and NCI-H1650 (B) cells are reported. Genes shown are those with >2-fold up- or down-modulation in at least in treatment group versus the saline-treated control group, with $p < 0.05$ ($n = 3$ tumors per group). Red, black and green colors indicate maximal, average, and minimal expression considering levels measured across treatment groups. 1, control (saline); 2, cisplatin plus gemcitabine; 3, necitumumab; 4, cisplatin plus gemcitabine plus necitumumab.

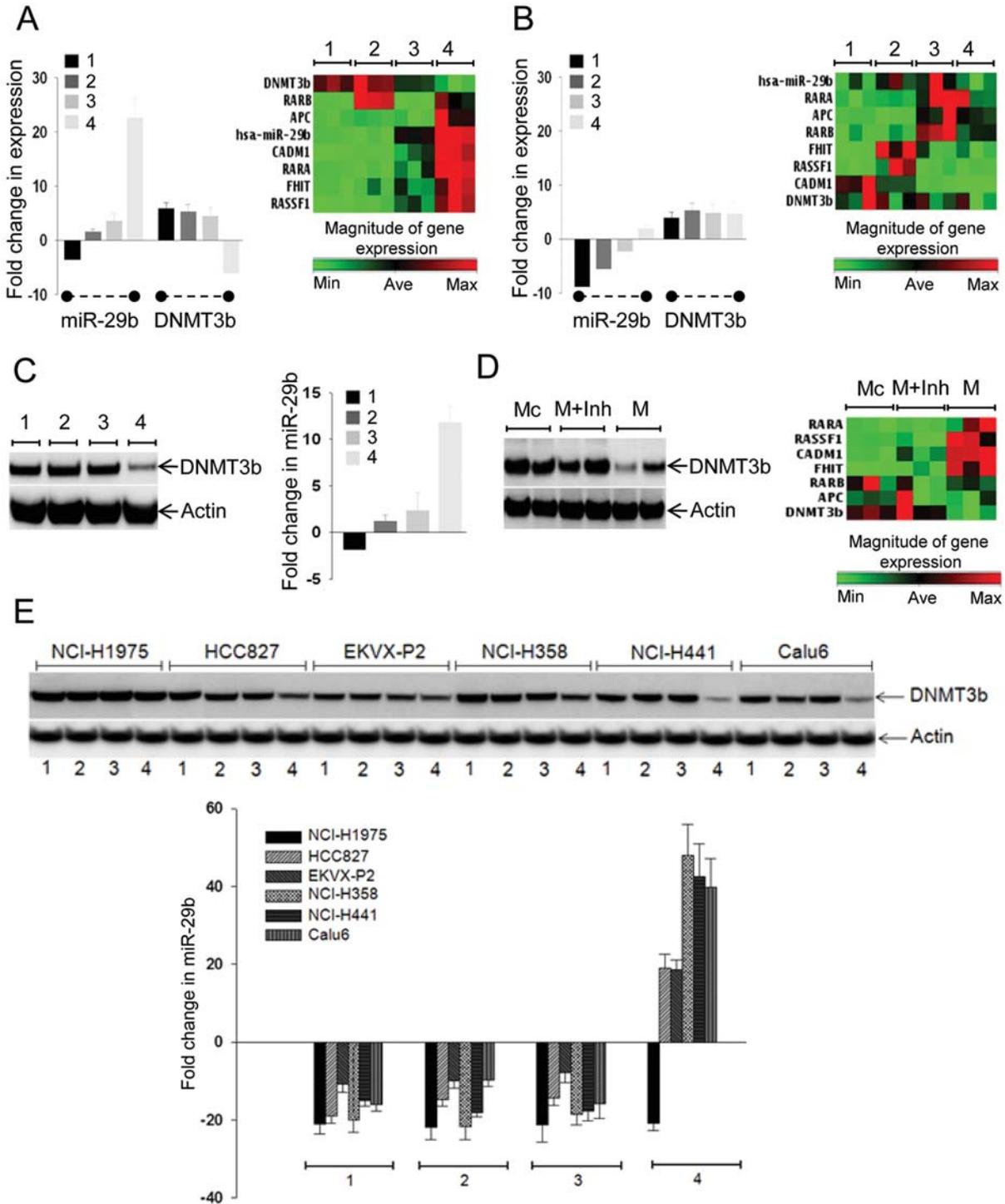


Figure 8. Importance of an hsa-mir-29b-DNMT3b-tumor suppressor gene axis with treatment of necitumumab with cisplatin/gemcitabine. Up-regulation of hsa-miR-29b with necitumumab with cisplatin/gemcitabine was associated with down-regulation of DNMT3b expression in A549 (A) but not NCI-H1650 (B) xenograft tumors. Re-activation of potentially methylation-silenced tumor suppressor genes is caused by necitumumab with cisplatin/gemcitabine in A549 tumors (A), but not in NCI-H1650 tumors (B). Effects of necitumumab with cisplatin/gemcitabine on DNMT3b and hsa-miR-29b expression in A549 (C) cells in vitro were similar to those found in vivo. Transient transfection of hsa-miR-29b mimic (M) in A549 cells (D) was shown to reduce DNMT3b expression and increase tumor suppressor gene expression compared to mock transfected cells (Mc). Co-transfection with an hsa-miR-29b inhibitor (Inh) reduces these effects (n=2-3 replicates). (E) The in vitro effect of necitumumab with cisplatin/gemcitabine is down-regulation of DNMT3b, as shown by western blot, and up-regulation of hsa-miR-29b expression, as shown by real-time PCR, in NCI-H1975, HCC827, EKVX-P2, NCI-H358, NCI-H441 and Calu6 cells. 1, control (saline); 2, cisplatin plus gemcitabine; 3, necitumumab; 4, cisplatin plus gemcitabine plus necitumumab.



Figure 9. Hsa-miR-29b sequence is shown to potentially target DNMT3b through complementarity sites in the 3'UTR region of DNMT3b, where bold letters identify perfect base matches according to TargetScan 4.5 software.

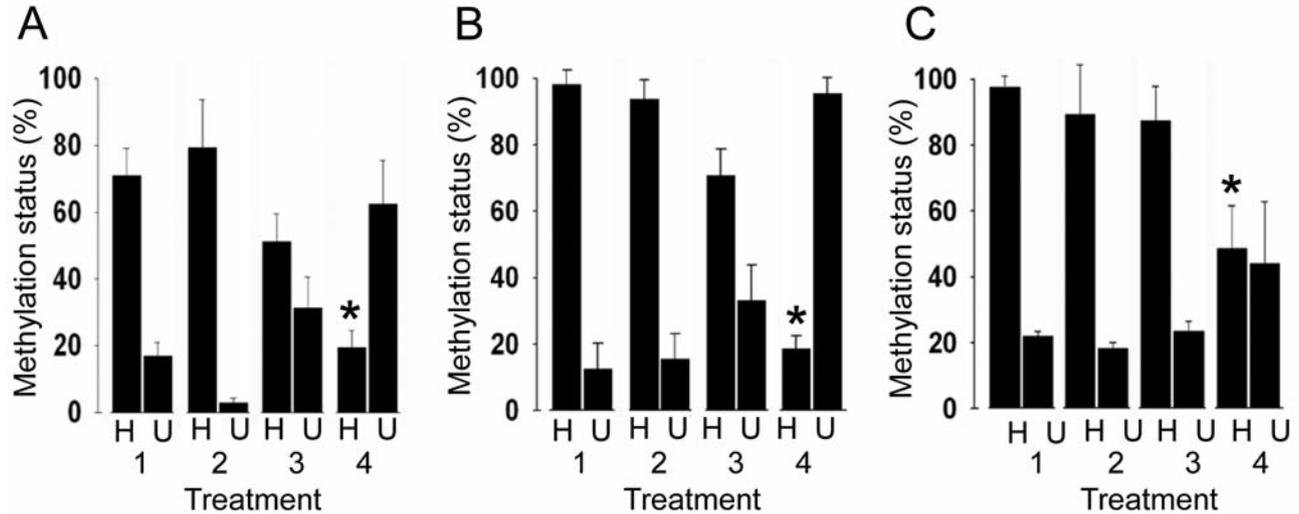


Figure 10. Nectinmumab with cisplatin/gemcitabine significantly reduces promoter methylation of tumor suppressor genes in A549 tumors. Methylation status is displayed as highly methylated (H) or unmethylated (U) for the Ras association (RalGDS/AF-6) domain family member 1 (RASSF1) (A), Cell adhesion molecule 1 (CADM1) (B), fragile histidine triad gene (FHIT) (C) promoter regions in tumors collected after 7 days' therapy with the indicated treatments. The mean \pm SEM is plotted for n=3 tumors per group. *p<0.05 versus control. 1, control (saline); 2, cisplatin plus gemcitabine; 3, nectinmumab; 4, cisplatin plus gemcitabine plus nectinmumab.

combination with three different cisplatin-based chemotherapy doublets in nine different NSCLC xenograft tumor models. Our data support, in particular, the development of an EGFR antibody with cisplatin/gemcitabine, and provide an understanding of the molecular changes underlying the antitumor benefits of this combination.

The antitumor effects of nectinmumab with cisplatin/gemcitabine combination therapy in the NCI-H1650 model were more related to antiproliferative effects than proapoptotic effects *in vivo*. Similar to previous reports demonstrating a role for EGFR in regulating the expression of cyclin D1, a member of the G₁ cyclin family of kinases involved in the regulation of the G₁-S transition of the cell cycle (6), nectinmumab significantly reduced the expression of *CCND1* (cyclin D1) in the NCI-H1650 model. The antiproliferative effects of EGFR targeting with nectinmumab alone and in combination with cisplatin/gemcitabine may therefore be related in part to the down-regulation of cyclin D1. But profiling of many cancer-related pathways utilizing

real-time PCR allowed us to establish that the molecular changes potentially contributing towards the antiproliferative effect were much more complex than a linear signaling pathway. In addition to *CCND1*, mRNAs for pro-proliferative CDK2, CDK4, E2F, CDC34, FOXA2, MYC, Wnt1 and KRAS proteins were also reduced by combination therapy. With regard to the lack of an apoptotic response to nectinmumab with cisplatin/gemcitabine in the NCI-H1650 model, increases in the tumor cell expression of the antiapoptotic genes *XIAP* (19), *IL4R* (26) and *BIRC2* (27) may have inhibited apoptosis, and in this way limited the maximal effect of treatment on tumor burden in this model.

Genes regulating apoptosis and proliferation were also analyzed in A549 xenograft tumors. Interestingly, although the benefits of adding nectinmumab to cisplatin/gemcitabine were similar in the A549 and NCI-H1650 models with regard to tumor volume (Figure 2), the molecular effects associated with these benefits were not consistent. In the A549 model, unlike the NCI-H1650 model, higher expression of pro-apoptotic

genes (CASP3, BCLAF1, BCL2L11, BNIP3, MCL1, PYCARD, CASP9, BID, CASP14, CASP5, HRK, RASSF1, FHIT and CADM1) and lower expression of antiapoptotic genes (*BCL2L1*, *TRAF2*, *CFLAR*, *BAG4*, *NOL3*, *BNIP1*, *BRAF*, *XIAP*, *WNT1*, *hsa-miR-155*, *BAG3*, *TP53BP2*, *DNMT3B*) likely contributed to the increased tumor cell apoptosis in response to necitumumab with cisplatin/gemcitabine. But as noted above for the NCI-H1650 model, the many pathways and proteins implicated in the mechanism underlying the antitumor effects of combination therapy suggest a complex balance of downstream effects ultimately affecting, or not, tumor cell proliferation and apoptosis. The upstream effects responsible for these mRNA changes are likely related to the interactions between alterations in EGFR tyrosine kinase signaling pathways (28) and DNA damage caused by chemotherapy. In some cases this interaction would directly impact the regulation of gene transcription through effects on transcription factors (28). However another recently described means to impact gene expression that may also be important in NSCLC involves microRNA (41).

MicroRNAs suppress gene expression by forming a duplex with a target messenger RNA (mRNA), blocking translation or initiating cleavage (29). To better understand the mRNA changes caused by necitumumab treatments in xenograft tumors, we examined the effects of treatment on tumor microRNAs. Hsa-miR-29b was strikingly up-regulated in A549 tumors with combination therapy, but unchanged in NCI-H1650 tumors with the same therapy. Hsa-miR-29b up-regulation was associated with down-regulation of the mRNA for the predicted target *DNMT3b* (23). Additional data demonstrating inverse regulation of *DNMT3b* mRNA and hsa-miR-29b with combination therapy in five out of six additional NSCLC cell lines examined indicated that the link between these changes has potential relevance in a significant proportion of NSCLC tumors.

Since DNMT3b can alter the methylation status of the promoter region of *RASSF1* (22), *CADMI* (30) and *FHIT* (31) tumor suppressor genes, the reduction in DNMT3b caused by increased hsa-miR-29b with necitumumab with cisplatin/gemcitabine treatment may increase the expression of these genes and in this way reduce the proliferation and increase the apoptosis of cancer cells. These genes were in fact up-regulated by necitumumab with cisplatin/gemcitabine in A549 tumors, supporting the use of a hsa-miR-29b–DNMT3B–tumor suppressor gene axis by combination therapy for achieving at least a portion of the antitumor effects. To more directly demonstrate the use of this axis, *in vivo* effects were modeled by studying A549 cells in culture. Indeed, induced hsa-miR-29b overexpression reduced DNMT3b expression and up-regulated the mRNA levels of tumor suppressors *RASSF1A*, *CADMI* and *FHIT*, in association with down-regulation of the methylation of the promoter region of these genes.

The frequent ability of necitumumab with cisplatin/gemcitabine to reduce *DNMT3b* expression and increase expression of tumor suppressor genes in NSCLC tumor cell lines adds significant support to the testing of the combination in the clinic. Tumor suppressor genes can be methylated in NSCLC patient tumors (32), blocking their effects on cyclin D1 (33), MST1 (32), AKT (34) and caspase activity (34). DNMT3b may be responsible for the silencing of these genes, at times through single nucleotide polymorphisms in the *DNMT3b* promoter, which have even been hypothesized to increase the risk of developing lung cancer (35). Furthermore, DNMT3b expression is up-regulated in human lung cancer cell lines and NSCLC tissue specimens (31), and increased DNMT3b expression can support cancer cell survival through inhibition of tumor cell apoptosis, without effects on normal cell apoptosis (36).

To summarize, we have shown that an antibody to EGFR, necitumumab, significantly increases the antitumor effects of cisplatin/gemcitabine in NSCLC models, with a magnitude that is comparable to or greater than that achieved with other tested cisplatin-based chemotherapy doublets. The interaction between necitumumab and cisplatin/gemcitabine results in complex model-dependent molecular effects that contribute towards increased tumor cell apoptosis and/or decreased tumor cell proliferation. Of particular interest, necitumumab increases hsa-miR-29b expression when given in combination with cisplatin/gemcitabine, affecting the methylation and expression of tumor suppressor genes through effects on DNMT3b expression. The results not only indicate the potential for increasing the efficacy of cisplatin/gemcitabine therapy for patients with NSCLC using EGFR antibodies, but provide potential molecular markers for examination as part of pharmacokinetic/pharmacodynamic studies and therapy tailoring efforts in clinical trials. Our findings may have particular importance in the preoperative treatment of patients with NSCLC prior to radical surgery, given the histology-independent benefits on survival recently demonstrated with cisplatin/gemcitabine in patients with clinical stage IIB/IIIA NSCLC (37).

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