Antibody-Mediated Delivery of Anti–KRAS-siRNA
In Vivo Overcomes Therapy Resistance in Colon Cancer

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Abstract

Purpose: KRAS mutations are frequent driver mutations in multiple cancers. KRAS mutations also induce anti-EGFR antibody resistance in adenocarcinoma such as colon cancer. The aim of this study was to overcome anti-EGFR antibody resistance by coupling the antibody to KRAS-specific siRNA.

Experimental Design: The anti-EGFR antibody was chemically coupled to siRNA. The resulting complex was tested for antibody binding efficiency, serum stability and ability to deliver siRNA to EGFR-expressing cells. Western blotting, viability, apoptosis, and colony formation assays were performed for efficacy evaluation in vitro. Furthermore, therapeutic activity of the antibody–KRAS-siRNA complexes was examined in in vivo xenograft mouse tumor models.

Results: Antibody–siRNA complexes were targeted and internalized via the EGFR receptor. Upon internalization, target gene expression was strongly and specifically repressed, followed by a reduced proliferation and viability, and induced apoptosis of the cells in vitro. Clonogenic growth of mutant KRAS-bearing cells was suppressed by KRAS-siRNA–anti-EGFR antibody complexes. In xenograft mouse models, anti-EGFR antibody–KRAS-siRNA complexes significantly slowed tumor growth in anti-EGFR-resistant cells.

Conclusions: The coupling of siRNA against KRAS to anti-EGFR antibodies provides a novel therapy approach for KRAS-mutated EGFR-positive cancer cells in vitro and in vivo. These findings provide an innovative approach for cancer-specific siRNA application and for enhanced therapeutic potential of monoclonal antibody therapy and personalized treatment of cancer entities. Clin Cancer Res; 21(6); 1383–94. ©2015 AACR.

Introduction

Tumors are characterized by a complex molecular landscape, in which several genomic aberrations often coexist within the same sample (1). Targeting a single oncogenic pathway at a time may result in poor efficacy as the presence of other genomic lesions may compensate or bypass single inhibitors. Unfortunately, the majority of these oncogenes are not easily druggable by current therapeutic approaches. KRAS ranks high among the frequently mutated oncogenes in human carcinomas (2, 3). Of relevance to this study, KRAS mutations (either primary or selected after prolonged administration of EGFR inhibitors) prevent the inhibitory effect of cetuximab or panitumumab in EGFR expressing colorectal cancer. One would expect that cotargeting KRAS and EGFR could overcome resistance and provide an effective therapeutic regimen for these cancers. The KRAS molecule is part of the RAS/RAF/MEK/ERK pathway, which links signal transduction from receptor tyrosine kinases to transcription factors by GDP and GTP binding and its intrinsic GTPase function (4). Mutations in the codons 12, 13, and 61 transform the GTPase activity of KRAS to a constitutively active conformation and thus induce a KRAS gain of function. Unfortunately, all attempts to design and test direct pharmacologic inhibitors of the KRAS oncogene have so far failed. Indeed, the GTP-binding pocket of KRAS is considered an undruggable target for a variety of reasons (4). The inability to target KRAS directly has prompted the search for inhibitors of effector kinases in its downstream pathway. These include MEK, PI3K, and AKT inhibitors. Most of these drugs are still in early clinical trials (5–7).

RNAi strategies can offer an alternative to directly target RAS. siRNA constitutes a class of molecules that allows effective and specific targeting the biosynthesis of oncogenic proteins. Indeed, siRNA has emerged as a major tool in molecular biology techniques and an important approach to identify suitable therapy targets in cancer (8). However, siRNA therapy approaches in vivo
Translational Relevance

Anti-EGFR antibody therapy is successful in multiple cancers. Patients with colon cancer harboring KRAS mutations do not benefit from anti-EGFR antibody therapy. This article demonstrates that an anti-EGFR antibody–siRNA complex can partly overcome this resistance of adenocarcinoma cell lines, including colon cancer cells, in vitro and in vivo. Of note, the coupling of specific siRNAs to targeting monoclonal antibodies might enhance the efficacy of antibody therapies in cancer.

Materials and Methods

Coupling of anti-EGFR monoclonal antibody to protaminesulfate

Protamine sulfate (1.67 mmol/L) was amino-terminally coupled to cysteine residues of anti-EGFR monoclonal antibody (mAB; 31 μmol/L stock; cetuximab, Erbitux, Merck-Serono) in a 5:1 molar ratio at 4°C overnight. Nonreacted educts and protamine doublets were separated from the high-molecular weight anti-EGFR mAB–protamine product by gel filtration chromatography in Zeba spin desalting columns (Pierce No. 89891). The anti-EGFR mAB–protamine adduct was stored at 4°C and was stable for several weeks.

siRNAs

For the estimation of siRNA coupling, stability, and internalization efficiency, anti-EGFR mAB–protamine was coupled to Allstars negative control siRNA–Alexa 488 (scrambled, cat. no. 1027284; Qiagen). Treatment experiments were done using esiRNA duplexes against KRAS (KRAS-Mission esiRNA, EHU114431) and as a control anti-GFP esiRNA (EHU-GFP, both Sigma-Aldrich). Allstars negative control siRNA–Alexa 555 was used for in vitro–targeting visualization (cat. no. 1027286, Qiagen).

Coupling of siRNA to anti-EGFR mAB–protamine

siRNA duplexes were bound to anti-EGFR mAB–protamine in a 4- to 10-fold molar excess at 25°C for 3 hours. This complex was prepared freshly before use.

Estimation of siRNA load capacity and serum stability of the complex

Constant concentrations (2.5 μmol/L) of control Allstars siRNA duplexes were preincubated with increasing amounts of anti-EGFR mAB–protamine up to a 40-fold molar excess for 1 hour at 4°C, subjected to agarose gel electrophoresis and stained by ethidium bromide. Anti-EGFR mAB–protamine-complexed siRNA proved to be immobile in 2% agarose, whereas the unbound 25-bp siRNA duplex band traveled at expected size.

For siRNA stability estimation, control Allstars siRNA coupled to anti-EGFR mAB–protamine was exposed to filtered HCT116 cell culture supernatant including FCS for indicated timespans, subjected to 0.4% agarose gel electrophoresis and stained by ethidium bromide. The anti-EGFR mAB–protamine–siRNA adduct was detectable as a barely mobile complex.

Cell culture

MDA-MB-435, HCT116, A549, SW620, and LoVo cells were maintained in DMEM supplemented with 10% FCS, 1% streptomycin and penicillin and 1%glutamine; HT29 was cultivated in IMDM and SW480, HCT15, and DLD1 cells in RPMI medium supplemented as above. MDA cells were KRAS-wild type and proved to be αEGFR-mAB sensitive (23). HCT116, A549, LoVo, and SW620 carry KRAS mutations in codon 12 or 13, respectively, leading to αEGFR-mAB resistance (24, 25). HT29 is BRAF mutated. Cell lines were obtained from ATCC (HT29, LoVo, A549, SW620, HCT-116, ATCC) or ECACC (DLD-1, SW480, HCT-15, ECACC). Identity was confirmed by short tandem repeat profiling before cells were taken for experiments.

Fluorescent microscopy

MDA, LoVo, and HCT116 cells were cultivated on chamber slides (Sigma C7057) and treated with anti-EGFR mAB–protamine or αEGFR-mAB alone incubated with Alexa Fluor 488–labeled Allstars control siRNA (Qiagen 1027284), at 1:10 molar ratio for 3 hours at 37°C and 5% CO2, washed with PBS, methanol-fixed, stained with DAPI, mounted with Dako fluorescent mounting medium and photographed on a Zeiss Axioskop.

are scarce (9, 10). One of the problems that hinder siRNA development as therapeutic tool concerns their delivery, representing a challenge in clinical situations. Methods of siRNA delivery and stabilization include nanoparticles and cationic liposomes (11), cationic peptides such as protamine (12, 13) or poly-arginine (14).

Monoclonal antibodies might be suitable siRNA carriers, but so far have not been thoroughly tested in cancer therapy. Monoclonal antibody-based therapies have significantly improved therapy for multiple cancer types in the last 10 to 15 years (15–17). Prominent examples for antibodies targeting the EGFR family include trastuzumab and pertuzumab for breast cancer therapy, and cetuximab (here referred to also as αEGFR-mAB or anti-EGFR-mAB) or panitumumab for colorectal cancer. Cetuximab is an established and effective therapy in colon cancer (18). Its use in KRAS wild-type colon cancer improves response rates and survival as a monotherapy and particularly in combination with chemotherapy (4, 19). Unfortunately, colon cancer cells that harbor certain RAS mutations are highly resistant toward EGFR-mAB even with a trend for inferior survival upon EGFR-mAB treatment (20–22). Also in patients that develop anti-EGFR antibody resistance over time, the outgrowth of clones with KRAS mutations appears to be the predominant mode of resistance (20).

Because of the high relevance of RAS mutations for colon cancer therapy and the proven role of KRAS mutations for anti-EGFR antibody resistance, we designed an approach to inhibit KRAS with subsequent induction of sensitivity toward anti-EGFR antibody therapy. Using different model cell lines, we demonstrate an effective method to couple specific siRNAs onto therapeutic monoclonal antibodies. An esiRNA against KRAS that was coupled to αEGFR-mAB effectively overcame αEGFR-mAB resistance in vitro and in vivo. These findings suggest a novel approach to treat therapy resistance in cancer.
Flow cytometry

FITC-coupled anti-EGFR antibody (mouse monoclonal antibody No. 528) was purchased from Santa Cruz Biotechnology. For EGF receptor internalization studies cells were first treated with the εEGFR-mAb-based antibody constructs for 1 hour at RT and then stained for EGFR using the FITC-coupled anti-EGFR antibody.

Western blot analyses

Cells were treated with anti-EGFR mAb–protamine (50 nmol/L) coupled to the indicated siRNAs at 1:10 molar ratio once a day for 72 hours, and subjected to Western blot analysis using standard protocols with the following antibodies: anti-KRAS (ab53391, ABCAM), anti-ERK1/2 (4696), anti-phospho-ERK1/2 (4370), anti-c-Myc (9402, Cell Signaling Technology), anti EGFR (sc1005, Santa Cruz Biotechnology), anti-EZH2 (clone AC22, No. 3147 Cell Signaling Technologies), and anti-β-Actin mAb (Clone AC-15, Sigma-Aldrich). Densitometric analysis of gel-electrophoretic bands was carried out using the NIH ImageJ package (http://rsb.info.nih.gov/ij/).

Proliferation assays

Bromodeoxyuridine (BrdUrd) incorporation analysis for cell-cycle analysis was carried out as described in Ji and colleagues (26).

Annexin V

Annexin V-PE/7AAD stainings (#556421, Becton-Dickinson) and 7-AAD staining (#559925 Becton-Dickinson) were carried out following the manufacturer's recommendations.

MTS assays

MTS viability assays (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay, Promega G5421) were carried out following the manufacturer's recommendations.

Clonogenic growth in soft agar

In brief, 5,000 trypsinized cells in 40 μL full medium per sample were incubated with anti-EGFR mAb–protamine coupled to the indicated siRNAs at 50 nmol/L end concentration for one hour at RT, resuspended in 150 μL of 0.5% soft agar in supplemented DMEM and cultivated for colony formation in 96-well format. A second treatment with 50 nm end concentrations was performed after 7 days of culture, fixed with 4% paraformaldehyde (PFA), counterstained with 0.1% crystal violet, photographed, and counted. All treatments were performed in groups of six wells each.

Mouse xenograft tumor model

Female CD1 nude mice (Charles River) were transplanted subcutaneously with 2 x 10^6 HCT116 cells or 1 x 10^6 HT29, DLD1, or SW480, respectively. Mice were randomized into groups of 6 and treated with cetuximab–protamine coupled to KRAS-specific siRNA EH114431, negative control esiRNA EH1-JFP (Sigma) or Alexa 555-labeled control siRNA (1027294324; Qiagen) or uncoupled εEGFR-mAb–protamine at 4 mg/kg twice a week intraperitoneally. For the bioavailability assessment, a single dose was used and tumors prepared 15 hours later. See Fig. 5 for details.

Quantitative real-time RT-PCR analysis

The method is described in the Supplementary Fig. S7.

Ki-67 staining

For Ki-67 staining, the tumors were isolated, washed in PBS, fixed in 4% parafomaldehyde (PFA) in PBS, embedded in paraffin, sectioned, blocked with 3% BSA, and stained with anti-Ki-67 rabbit polyclonal antibody (clone MB-1, DakoCytomation) according to standard methods. Counterstain was performed using hematoxylin.

Statistical analysis

All data are presented as mean ± SD, if not indicated otherwise. The mean values of two groups were compared by the Student t test.

Results

Development of a receptor-specific siRNA carrier system

Cell type specific delivery of siRNA is a major problem for siRNA-based therapies. The coupling of specific siRNAs to therapeutic antibodies against cell surface receptors could be an effective tool for siRNA delivery. In addition, this approach could also enhance the antibody’s efficacy and/or might overcome drug resistance. We tested several methods to couple antibodies with siRNA. The most effective method was chemical coupling using protamine as a siRNA complexing agent. Protamine is a positively charged molecule that is widely used as a heparin-antidote and has been extensively tested in humans in the form of protamine sulfate (28).

We coupled protamine to the monoclonal antibody against the EGF receptor (anti-EGFR mAb) using a sulfo-SMCC linker (Fig. 1A) in a 5:1 molar ratio, enabling the binding of multiple protamine molecules per molecule of mAb. siRNA binds to protamine by electrostatic interactions (13). The specific siRNA-binding capacity of protamine was analyzed by electrophoresis to determine bound versus free siRNA (14). On this low percentage gel, a degradation of siRNA would be seen as a decreased band intensity that represents the stable siRNA–protei conjugate. After ethidium bromide stain, no degradation of the high-molecular weight siRNA–protein complex was observed which indicated an increased stability (Fig. 1B).

Next, we tested protamine dependency of siRNA binding (Fig. 1C and D). A minimum of eight molecules of siRNA were able to bind to one molecule α-EGFR mAb–protamine, indicating a significant siRNA load for the antibody–protamine complex, while a higher molar excess of siRNA leads to an overflow of unbound siRNA.

Internalization of the antibody complex upon binding to the receptor (29) is required for intracellular siRNA activity. Therefore, we first incubated the anti-EGFR antibody either alone or as a complex with protamine and siRNA with HCT116 cells that express EGF on their surface (Fig. 1E). FACS analysis using a FITC-labeled anti-EGFR antibody that binds to a different extracellular epitope of EGF than cetuximab α-EGFR-mAb revealed (Fig. 1E, second panel) that EGF expression on the surface of HCT116 cells was no longer detectable when the cells were preincubated with αEGFR-mAb alone (Fig. 1E, third panel) or anti-EGFR mAb–protamine–siRNA complex (Fig. 1E, fourth panel). These analyses indicated that the εEGFR-mAb–siRNA complex and the chemical modification in combination with the highly anionic siRNA load did not interfere with EGF receptor binding and internalization.
To further verify internalization, we coupled anti-EGFR mAB–protamine with scrambled control siRNA labeled with Alexa Fluor 488. Several carcinoma cell lines (HCT116, LoVo, and MDA cells) were incubated with the conjugate and showed widespread and significant siRNA–antibody internalization as evident by the cytoplasmic localization of the green fluorescence. Detailed results are presented in Supplementary Fig. S1A.
Anti-EGFR mAB-directed siRNA reduces target gene expression in EGFR-expressing carcinoma cell lines

Because the anti-EGFR mAB–protamine–siRNA complexes were effectively taken up by cells, we next checked the intracellular functionality of siRNA. A potential problem with using siRNA as therapeutic agents is off target gene silencing effects. We used enzymatically produced (e)siRNA against KRAS, which consists of a complex pool of active inhibitory RNA fragments and possesses higher specificity and efficiency (30). KRAS in its mutated form in codons 12, 13, and 61 is a strong predictive biomarker for therapy resistance toward cetuximab/EGFR-mAB and panitumumab due to gain of function in its GTPase activity (4, 19). For suppression of KRAS, we made use of enzymatically digested (e) siRNAs, exhibiting maximal RNAi effects with minimal cross-silencing. As a control, esiRNA against xenoprotein GFP was used. The KRAS esiRNA bound to a concentration of 50 nmol/L anti-EGFR mAB–protamine (mAB–P) was sufficient to suppress KRAS protein expression in KRAS-mutant HCT116 and LoVo adenocarcinoma cell lines by more than 80% as analyzed by Western blotting (Fig. 2A). Control esiRNA did not affect KRAS expression level (Fig. 2A). The canonical RAS signaling pathway includes the RAF/MEK/ERK cascade involved in mitogenic signaling. Accordingly, ERK1/2 phosphorylation was markedly diminished in LoVo and HCT116 cells treated with anti-EGFR-mAB-KRAS esiRNA. A known target molecule of the ERK signaling is the c-Myc oncogene (31). Also, c-Myc protein expression was reduced in cells showing antibody-mediated KRAS knockdown and subsequent ERK inactivation (Fig. 2A).

An independent experimental assay to verify the gene-specific effect, we used a specific siRNA against EZH2, a well-known oncogene in multiple solid tumors (32), which is highly expressed in adenocarcinoma cells. The exposure of α-EGFR mAB–P–EZH2–siRNA complex in HCT116 and A549 cells repressed EZH2 expression in an antibody-specific fashion almost completely (Supplementary Fig. S2A). We next tested whether the α-EGFR mAB–siRNA complex inhibited tumor cells in vitro. As a functional assay, we determined anchorage-independent clonogenic growth in semi-solid medium as a readout for tumorigenic potential by self-renewal (33) of the respective cell line (Fig. 2B). As expected, α-EGFR-mAB and α-EGFR-mAB coupled to control esiRNA did not inhibit clonogenic growth in KRAS-mutant cells (Fig. 2B, left and middle), for example, HCT116 (Fig. 2B). LoVo (Fig. 2C), or A549 cells (Supplementary Fig. S4B). However, the α-EGFR-mAB–protamine–KRAS esiRNA complex inhibited clonogenic growth of KRAS-mutant (Fig. 2B–F), but not BRAF-mutant cell lines (Fig. 2G).

As expected, α-EGFR-mAB–protamine alone reduced clonogenic growth in KRAS wild-type MDA cells (Supplementary Fig. S4A) independent of the attached siRNA and targeting with KRAS-siRNA did not enhance therapy response toward anti-EGFR antibody in KRAS wild-type cells.

Overcoming cetuximab/αEGFR-mAB resistance in KRAS-mutated colorectal cell lines in vitro

In a MTS viability-assay, we exposed HCT116, LoVo, DLD1, SW480, and HT29 cell lines to increasing concentrations of αEGFR-mAB–control esiRNA or αEGFR-mAB-KRAS esiRNA ranging from 0 to 500 nmol/L (Fig. 3A). All three cell lines proved to be resistant to αEGFR-mAB–control esiRNA, probably caused by mutation-dependent enhanced and constitutive KRAS/BRAF signaling, but the KRAS-mutant cell lines showed a significant sensitivity to αEGFR-mAB coupled to KRAS esiRNA (Fig. 3A and B), whereas the BRAF-mutant HT29 cell line remained resistant to αEGFR-mAB despite KRAS esiRNA treatment (Fig. 3A–C, right hand). Of note, the EGFR-negative cell line SW620 showed neither reaction upon αEGFR-mAB–control nor αEGFR-mAB–esiKRAS treatment, indicating that the efficacy entirely depended on the expression of EGFR (Supplementary Fig. S3).

Treatment response in LoVo and HCT116 cell lines did not differ in the in vitro applications although LoVo expressed more EGFR than HCT116 (Supplementary Fig. S2B). This finding suggested that αEGFR-mAB-KRAS esiRNA therapy is active in cancer cells with varying levels of EGFR expression, although a threshold expression of EGFR is necessary. On the other hand, the cells lines used here varied in their microsatellite stability index, the majority of the responsive cell lines were stated microsatellite instable, whereas SW480 was MSS without change of responsiveness to the αEGFR-mAB-KRAS esiRNA therapy. HCT116, LoVo, DLD1, SW480, and HT29 cell lines were analyzed for their cell-cycle status using BrdUrd incorporation (Fig. 3B). BrdUrd incorporation was strongly reduced for both cell lines after αEGFR-mAB-KRAS esiRNA exposure (Fig. 3B, first to fourth panel) compared with the control samples. The BRAF-mutant HT29 cells, as expected, did not respond to this treatment (Fig. 3B, right hand).

Last, the same KRAS-mutated cell lines exhibited signs of apoptosis in an Annexin V staining assay when treated with mAB–KRAS-siRNA, but not control samples (Fig. 3C, first to fourth panel). The BRAF-mutant HT29 cells did not undergo apoptosis following treatment with any of the complexes (Fig. 3C, right hand).

Efficacy of KRAS-siRNA–anti-EGFR complexes in mouse tumor xenografts

Next, we analyzed the effect of αEGFR-mAB–esiRNA complexes in vivo. First, we checked for the bioavailability of the antibody–siRNA conjugate in vivo by coupling fluorescence-labeled Alexa 568 siRNA to αEGFR-mAB–protamine in concentrations of 4 mg/kg mouse body weight intraperitoneally or subcutaneously close to a HCT116 tumor. Cryosections from tumors treated with αEGFR-mAB–Alexa 568–control siRNA exhibited accumulations of red fluorescent cellular signals in the rim region of the tumor (Fig. 4A and B), whereas naked Alexa 568–control siRNA administered showed no significant enrichment of fluorescence signals (Fig. 4D and E).

To test the therapeutic effect of the antibody–esiRNA treatment, αEGFR-mAB–resistant SW480, DLD1, HCT116, or HT29 carcinoma cells were subcutaneously injected and mice with acceptable tumor growth were randomized into groups. Mice were treated twice weekly by i.p. injections with anti-EGFR mAB–protamine (mAB–P) alone or with mAB–P–esiRNA complexes and tumor growth was measured (Fig. 5A). Cetuximab-resistant tumors continued to grow despite anti-EGFR mAB–protamine therapy (Fig. 5B–D, blue thomb) and also with control esiRNA coupled to anti-EGFR mAB–protamine (Fig. 5B–D, red squares). In contrast, anti-EGFR mAB–protamine–KRAS esiRNA complexes significantly inhibited tumor growth (Fig. 5B–D, green triangles), including complete loss of tumors in one out of six cases of the mAB–protamine–KRAS esiRNA group in DLD1 and SW480. At the defined end of the therapy, tumor volumes were reduced to about 25% in SW480 and DLD1 compared with the volumes of the fast growing control groups and to less than half...
the size of the anti–EGFR-P mAB only treated HCT116 tumors. BRAF-mutated HT29 tumors did not respond to anti-EGFR mAB–protamine (“mAB-P”) coupled to GFP control esiRNA (“mAB-P-control esiRNA”) or to KRAS esiRNA (“mAB-P-KRAS esiRNA”) for 72 hours at 37°C. Western blot analysis was performed for KRAS, phospho-ERK1/2, total ERK1/2, c-Myc, and actin as loading control. Expression of KRAS and c-Myc and phosphorylation of ERK1/2 were suppressed upon mAB-P-KRAS esiRNA treatment. Shown here is one representative of three independent experiments. B–G, for colony formation assays, cells from different colon carcinoma lines were treated with anti-EGFR mAB–protamine (mAB-P) esiRNA at 50 nmol/L final concentration, resuspended in 96-well plates in soft agar and analyzed for clonogenic growth. After 7 days, a second treatment was performed. Colonies were fixed, stained with crystal violet, photographed, and counted after 3 weeks. Significance: *, P < 0.005, of mean values anti-EGFR mAB–protamine–KRAS esiRNA group versus anti-EGFR mAB–protamine–control group, respectively. Graphs depict mean of three independent experiments ± SD normalized to PBS mean.
Overcoming anti-EGFR antibody resistance in KRAS-mutated adenocarcinoma cell lines in vitro. We exposed cells to αEGFR-mAB in a complex with either control or anti-KRAS siRNA and analyzed the cells for proliferation and apoptosis using MTS assays (A) and the incorporation of BrdUrd (B). We used eight different adenocarcinoma cell lines expressing EGFR, four of them exhibiting the G13D KRAS mutation (HCT116, HT29, LoVo, and SW480), one cell line containing the G12V KRAS mutation (SW480), another the G12S mutation (A549), and finally a KRAS wt cell line with a BRAF V600E mutation (HT29). An overview of the characteristics of cell lines used in this study is given in Supplementary Fig. S2B and S2C. Cells were tested for their EGFR expression status which differed widely among different cell lines, including the G12V KRAS-mutated SW620 colon carcinoma cells, which do not express EGFR. A, MTS viability assay: cells were exposed to increasing concentrations of anti-EGFR mAB-control esiRNA and anti-EGFR mAB-KRAS esiRNA. HCT116 and LoVo, SW480 and DLD1 cells (first to fourth panel) showed significantly decreased viability by increased mAB-KRAS esiRNA, but not mAB-control esiRNA. BRAF-mutant HT29 cells (right) did not differ in viability upon treatment. Significance: *, P < 0.05; **, P < 0.01; ***; P < 0.001. Graphs depict mean of three independent experiments ± SD. B, proliferation/BrdUrd incorporation assay: cells were treated with 50 nmol/L of mAB-KRAS esiRNA, labeled with BrdUrd and analyzed by FACS. HCT116 and LoVo SW480 and DLD1 showed significantly (*, P < 0.05) reduced proliferation when treated with mAB-KRAS esiRNA, but not mAB-control esiRNA (first to fourth panel). BRAF-mutant HT29 cells did not show a decrease in proliferation upon treatment (right). Graphs depict mean of three independent experiments ± SD. *, P < 0.05; **, P < 0.01; ***; P < 0.001. C, apoptosis induction: cells were exposed to 50 nmol/L mAB-esiRNA and stained with anti-annexin V antibody and 7AAD for FACS analysis. Annexin V-positive/7AAD-negative cells represent the apoptotic cell population and were determined by FACS. HCT116 and LoVo SW480 and DLD1 cells treated with mAB-KRAS esiRNA showed increase of cells in early apoptosis (first to fourth panel). BRAF-mutant HT29 cells treated with the same combination did not show an increase in apoptosis (right). Graphs depict mean of three independent experiments ± SD. *, P < 0.05; **, P < 0.01; ***; P < 0.001.

Following up on these results, we also analyzed Ki-67 as proliferation marker in tumors from mice treated in vivo. In the KRAS-mAB tumors, Ki-67 staining was significantly reduced (Fig. 6C: SW480, and 6F: DLD1; Supplementary Fig. S6, bottom left and right hand: HCT116), while Ki-67 positive cells were not reduced in treated HT29 tumors (Fig. 6I, compared with 6G and H). In addition, control esiRNA did not show a significant effect on Ki-67 expression (Supplementary Fig. S6, top).

Interestingly, HCT116 cells contain a PIK3CA activating mutation H1047R besides the KRAS G13D activating mutation (34, 35). We performed colony assays (details presented in Supplementary Fig. S8) with PIK3CA and KRAS esiRNA coupled to αEGFR mAB. Of note, HCT116 cells treated with αEGFRmAB carrying combined KRAS and PIK3CA esiRNAs showed a cooperative effect further reducing colony growth. This clearly indicates that complete molecular characterization of the signaling pathways (see also Supplementary Fig. S8) driving malignant behavior of the respective cell type would enable us to tailor the siRNA delivery system described to optimal activity.

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The specific targeting of oncogenic drivers and resistance mechanisms for conventional therapies offers substantial hope for improved therapies for cancer. However, the therapeutic targeting of many driver mutations in cancer has so far remained elusive. KRAS ranks high among the most frequently mutated genes in carcinomas and as a mediator of therapy resistance against EGFR antibodies such as cetuximab (4, 36). Unfortunately, despite the long available knowledge about mutant KRAS, no successful therapeutic approaches have entered clinical practice. One targetable molecule MEK might help to block signaling downstream of mutated RAS and several inhibitors of MEK are currently in clinical trials (6, 7, 37). Only recently, a new MEK inhibitory substance was reported to rescue cetuximab resistance in KRAS-mutated colorectal cancer cell lines (38). RAS mutations are not only drivers in tumorigenesis, but also mediators of therapy resistance to anti-EGFR antibodies. The frequently encountered RAS mutations in colon carcinoma prelude treatment with anti-EGFR antibodies such as cetuximab and panitumumab for many patients.

In the current study, we demonstrate that a KRAS-specific siRNA can be delivered into tumor cells via cetuximab–protamine and can effectively inhibit tumor growth by downregulation of KRAS and subsequent deactivation of ERK and the MAPK pathway in KRAS-mutated but not BRAF-mutated cell lines. This targeting is dependent of the antibody action of finding, binding, and internalizing EGFR on the tumor cell and as a second step, inhibiting MAPK signaling by KRAS RNAi. One of the possible consequences of ERK signaling activity depicts the regulation of stability of the oncogene c-Myc by its phosphorylation (31, 39). Here, c-Myc expression was diminished upon KRAS knockdown along with the phosphorylation of ERK. Overexpression of MYC proteins in cultured cells and in transgenic animals blocks differentiation and induces neoplastic transformation (40). Accordingly, it is possible that reduced c-Myc activity contributes to the reduction of colony formation, viability, and tumor growth effect of the cetuximab–siKRAS conjugate therapy. Of note, we provide evidence that a threshold expression of EGFR is required to enable cetuximab–KRAS siRNA response, these data are in line with current clinical practice: anti-EGFR antibody therapy is indicated for EGFR-expressing colorectal cancers.

Cetuximab monotherapy delays tumor growth in patients with colorectal carcinoma with less than 10% of patients achieving a partial response according to RECIST criteria (41). Also, approximately 10% of xenografts transplanted from patients-derived, metastatic colorectal cancer samples showed tumor reduction upon cetuximab treatment (42). As a clinical consequence, cetuximab in colorectal cancer is usually used as combination therapy.

Here, we transplanted CD1 nude mice subcutaneously with four colorectal cancer cell lines harboring different cetuximab-resistance connected mutations, for example, KRAS G12V in the SW480 cell line, or the G13D in HCT116 and DLD1, as well as the BRAF V600E-mutant HT29 cell line. Upon EGFR-mAb cetuximab–KRAS-esiRNA treatment, the xenografted KRAS-mutated cell lines reacted with diminished proliferation resulting in considerable reduced growth of treated tumors compared with control groups, including tumor extinction in one out of six tumors in SW480 as well as DLD1 in the cetuximab–KRAS siRNA group. The cell line HCT116 represents one of the most cetuximab-resistant colorectal cancer cell lines harboring additional aberrations that potentially confer therapy resistance such as mutated PIK3CA, low PTEN expression, and mutated β-catenin (24, 35, 43). Therefore, we did not anticipate the conjugate monotherapy against KRAS to induce major remissions in vivo. Instead, the HCT116 xenografts showed a significant delay of tumor growth to about half the size of the controls. Keeping in mind that signaling downstream from EGFR can be mediated by the MAPK as well as PI3K pathway, targeting a crucial checkpoint of PI3K by the cetuximab–siRNA conjugate might be a logical consequence. The conjugation of a PIK3CA esiRNA to cetuximab and consequent treatment of HCT116 cells in colony assays reduced the colony formation in a comparable manner like KRAS esiRNA, whereas the combination of both KRAS and PIK3CA esiRNAs acted cooperatively and reduced colony numbers by 80%, indicating that both aberrant pathways are involved in and are necessary for cetuximab resistance in an cooperative fashion.

**Discussion**

Antibody-dependent siRNA targeting in vivo. A total of 2 × 10^5 cetuximab-resistant HCT116 colon adenocarcinoma cells were subcutaneously implanted in CD1 nude mice. After tumor size reached an average of 200 mm^3, mice were injected with 4 mg/kg anti-EGFR mAb-Alexa 555-labeled Allstars negative control siRNA or with the uncoupled negative control siRNA. Tumors were resected 15 hours after injection. In mice injected intraperitoneally (A) or subcutaneously (B) with mAB-555-siRNA, tumors showed Alexa 555 fluorescent signals in the tumor rim. C and D depict parallel sections of A and B stained hematoxylin and eosin (H&E). In mice injected with uncoupled siRNA-Alexa 555 (D and E), no fluorescent signals were detectable. F and G again show parallel sections of D and E stained H&E.

**Figure 4.** Antibody-dependent siRNA targeting in vivo. A total of 2 × 10^5 cetuximab-resistant HCT116 colon adenocarcinoma cells were subcutaneously implanted in CD1 nude mice. After tumor size reached an average of 200 mm^3, mice were injected with 4 mg/kg anti-EGFR mAb-Alexa 555-labeled Allstars negative control siRNA or with the uncoupled negative control siRNA. Tumors were resected 15 hours after injection. In mice injected intraperitoneally (A) or subcutaneously (B) with mAB-555-siRNA, tumors showed Alexa 555 fluorescent signals in the tumor rim. C and D depict parallel sections of A and B stained hematoxylin and eosin (H&E). In mice injected with uncoupled siRNA-Alexa 555 (D and E), no fluorescent signals were detectable. F and G again show parallel sections of D and E stained H&E.

**Discussion**

The specific targeting of oncogenic drivers and resistance mechanisms for conventional therapies offers substantial hope for improved therapies for cancer. However, the therapeutic targeting of many driver mutations in cancer has so far remained elusive. KRAS ranks high among the most frequently mutated genes in carcinomas and as a mediator of therapy resistance against EGFR antibodies such as cetuximab (4, 36). Unfortunately, despite the long available knowledge about mutant KRAS, no successful therapeutic approaches have entered clinical practice. One targetable molecule MEK might help to block signaling downstream of mutated RAS and several inhibitors of MEK are currently in clinical trials (6, 7, 37). Only recently, a new MEK inhibitory substance was reported to rescue cetuximab resistance in KRAS-mutated colorectal cancer cell lines (38). RAS mutations are not only drivers in tumorigenesis, but also mediators of therapy resistance to anti-EGFR antibodies. The frequently encountered RAS mutations in colon carcinoma prelude treatment with anti-EGFR antibodies such as cetuximab and panitumumab for many patients.

In the current study, we demonstrate that a KRAS-specific siRNA can be delivered into tumor cells via cetuximab–protamine and can effectively inhibit tumor growth by downregulation of KRAS and subsequent deactivation of ERK and the MAPK pathway in KRAS-mutated but not BRAF-mutated cell lines. This targeting is dependent of the antibody action of finding, binding, and internalizing EGFR on the tumor cell and as a second step, inhibiting MAPK signaling by KRAS RNAi. One of the possible consequences of ERK signaling activity depicts the regulation of stability of the oncogene c-Myc by its phosphorylation (31, 39). Here, c-Myc expression was diminished upon KRAS knockdown along with the phosphorylation of ERK. Overexpression of MYC proteins in cultured cells and in transgenic animals blocks differentiation and induces neoplastic transformation (40). Accordingly, it is possible that reduced c-Myc activity contributes to the reduction of colony formation, viability, and tumor growth effect of the cetuximab–siKRAS conjugate therapy. Of note, we provide evidence that a threshold expression of EGFR is required to enable cetuximab–KRAS siRNA response, these data are in line with current clinical practice: anti-EGFR antibody therapy is indicated for EGFR-expressing colorectal cancers.

Cetuximab monotherapy delays tumor growth in patients with colorectal carcinoma with less than 10% of patients achieving a partial response according to RECIST criteria (41). Also, approximately 10% of xenografts transplanted from patients-derived, metastatic colorectal cancer samples showed tumor reduction upon cetuximab treatment (42). As a clinical consequence, cetuximab in colorectal cancer is usually used as combination therapy.

Here, we transplanted CD1 nude mice subcutaneously with four colorectal cancer cell lines harboring different cetuximab-resistance connected mutations, for example, KRAS G12V in the SW480 cell line, or the G13D in HCT116 and DLD1, as well as the BRAF V600E-mutant HT29 cell line. Upon EGFR-mAb cetuximab–KRAS-esiRNA treatment, the xenografted KRAS-mutated cell lines reacted with diminished proliferation resulting in considerable reduced growth of treated tumors compared with control groups, including tumor extinction in one out of six tumors in SW480 as well as DLD1 in the cetuximab–KRAS siRNA group. The cell line HCT116 represents one of the most cetuximab-resistant colorectal cancer cell lines harboring additional aberrations that potentially confer therapy resistance such as mutated PIK3CA, low PTEN expression, and mutated β-catenin (24, 35, 43). Therefore, we did not anticipate the conjugate monotherapy against KRAS to induce major remissions in vivo. Instead, the HCT116 xenografts showed a significant delay of tumor growth to about half the size of the controls. Keeping in mind that signaling downstream from EGFR can be mediated by the MAPK as well as PI3K pathway, targeting a crucial checkpoint of PI3K by the cetuximab–siRNA conjugate might be a logical consequence. The conjugation of a PIK3CA esiRNA to cetuximab and consequent treatment of HCT116 cells in colony assays reduced the colony formation in a comparable manner like KRAS esiRNA, whereas the combination of both KRAS and PIK3CA esiRNAs acted cooperatively and reduced colony numbers by 80%, indicating that both aberrant pathways are involved in and are necessary for cetuximab resistance in an cooperative fashion.
A potential problem with using siRNA as therapeutic agents is off target gene silencing effects. We minimized cross-silencing effects by using enzymatically produced esiRNA rather than chemically synthesized siRNA. It was shown that presenting a multitude of enzymatically produced specific silencing RNA fragments have higher specificity and efficiency. Of note, the eEGFR-mAB cetuximab--KRAS-esiRNA treatment was able to overcome therapy resistance induced by KRAS mutations. Resistance based on absence of EGFR expression or on BRAF mutations was not affected by cetuximab--KRAS-esiRNA. These findings provide further evidence for the dual specificity of the observed therapeutic effects.

The problem to successfully deliver siRNA into cancer cells has remained a major obstacle for the further development of siRNA-based therapies. There are several strategies to deliver siRNA nonspecifically. For example, we have previously shown that injection of naked plasmids that encode shRNAs can inhibit metastatic development in mouse models. Because of the short half-life of siRNA after injection into the blood stream, siRNAs are often directed injected into different organs or tumors. This approach is unlikely to yield major systemic responses and is therefore also unlikely to succeed for metastatic cancer. On the other hand, use of nanotechnology and/or the

Figure 5. Overcoming anti-EGFR antibody resistance in KRAS-mutated adenocarcinoma tumors in vivo in CD1 nude mice. A, outline of the anti-EGFR antibody–siRNA treatment regimen. B–E, a total of 2 × 10^6 to 1 × 10^7 colon adenocarcinoma cells were subcutaneously implanted in CD1 nude mice. After tumors reached an acceptable size, three groups of mice (n = 6 each) were treated with anti-EGFR mAB-protamine (αEGFR mAB-P), anti-EGFR mAB-protamine-KRAS-esiRNA (αEGFR mAB-P-KRAS-esiRNA), or anti-EGFR mAB-P-control esiRNA (αEGFR mAB-P-cntr-esiRNA) at 4 mg/kg twice a week by intraperitoneal injection. For a single dose, 750 pmol of anti-EGFR mAB-protamine was coupled to 3.5 nmol of the respective esiRNA. Tumor growth was followed with standard caliper measurements in a blinded fashion twice a week. Tumor volumes were calculated by the formula length × width^2 × 0.52 (27). At the end of the experiment, animals were euthanized by cervical dislocation in deep CO2 anesthesia. Primary tumors were surgically removed, and tumor weight was determined. Values represent absolute tumor volumes if not stated otherwise. KRAS-mutated colorectal cancer xenograft-tumor-bearing mice treated with anti-EGFR mAB-P–KRAS esiRNA complexes showed a markedly delayed growth compared with anti-EGFR mAB-P and anti-EGFR mAB-P-control esiRNA as detected by caliper measurements. E, in BRAF-mutated HT29 colorectal cancer tumors αEGFR mAB-P–KRAS-esiRNA did not show significant influence on tumor development. Error bars in Fig. 6B–E represent SEM. P values: *, P < 0.05; **, P < 0.01; ***, P < 0.001. F–I, at the end of the experiment, tumors were prepared and photographed and weighted. Photographs show representative examples. Average weight of anti-EGFR mAB-protamine–KRAS esiRNA treated KRAS-mutated tumors was significantly lower than that of the control groups, including complete loss of tumors in 1 out of 6 cases in SW480 as well as DLD1; n = 5/6 (mAB-P–KRAS esiRNA group). BRAF-mutated tumors did not show any influence upon treatment. Note that tumor weight includes a connected section of mouse dermis along with tumor for histologic evaluation leading to elevated weights, especially in small tumors; n = 6 (other groups; contr. = GFP control), P values: *, P < 0.05; **, P < 0.01; ***, P < 0.001. Bar, 1 cm.
complexing of the siRNA with lipophilic agents are strategies that are followed by several academic groups and by biopharmaceutical companies (11). A first in-man study followed this strategy, combining siRNAs against VEGF and KSP in lipid nanoparticles was effective in patients with hepatocarcinoma (9). A recent publication underlines the importance to target KRAS with siRNA: Here, the authors chose to apply KRAS esiRNA especially the local administrations of siRNA would benefit pancreatic tumors (47). All of these therapeutic approaches, miniature polymer capsules locally implanted to xenografted publication underlines the importance to target was effective in patients with hepatocarcinoma (9). A recent combining siRNAs against VEGF and KSP in lipid nanoparticles technical companies (11). A are followed by several academic groups and by biopharmaceuticals as for other cytotoxic drugs. The coupling of siRNAs to conjugates are mainly of cytotoxic or radioactive nature with still antibodies allows cell-specific targeting to tumor cells to receive systemic effects (48).

The most remarkable progress in cancer therapy in the last 15 years is based on the use of monoclonal antibodies. Monoclonal antibodies have entered clinical practice for multiple entities, including hematologic and solid cancers. In many instances, the antibody is not coupled to any drug and the mechanisms of action might depend on direct cytotoxicity or antibody-dependent cellular cytotoxicity (49). In recent years, several drug conjugates have been introduced into clinical practice. First, anti-CD33 antibody coupled to ozogamicin has activity in acute myelogenous leukemia (50). The coupling of trastuzumab to the cytotoxic drug emtansine (TDM1) has shown significant clinical activity even in trastuzumab-resistant breast cancers (51). Drug conjugates, for example, anti-CD30 antibodies (brentuximab) or anti-CD20 (britumomab–tiuxetan) have been approved by the FDA and the EMA for routine clinical use (52). Thus, the coupling of antibodies allows cell-specific delivery of drugs. So far, the drug conjugates are mainly of cytotoxic or radioactive nature with still significant side effects and probably similar resistance mechanisms as for other cytotoxic drugs. The coupling of siRNAs to monoclonal antibodies might thus improve therapeutic efficacy and broaden the therapeutic range. Systemic delivery of antibody–siRNA complexes is feasible as has been demonstrated for an anti-CD7-siRNA construct that was used to treat HIV infection in humanized mice (14). This construct was based on a scFV single chain antibody with a c-terminal histidine that was coupled to a small cationic peptide via a disulfide bond. Ultimately, the positively charged peptide bound the siRNA for delivery by electrostatic charge. Here, we went on to develop a much simpler strategy. Protamine, a positively charged peptide, was covalently linked to the monoclonal antibody. Protamine effectively bound siRNA with a significant excess of siRNA for each molecule of antibody (12, 13).

Protamine is an endogenous protein most highly expressed in sperm. It has long been used to delay the activity of insulin (28). Also, protamine is commonly used to inhibit heparin activity, for example, after overdosing. Thus, there is ample experience with the administration of protamine to patients. Given that protamine is simply used as a linker at low concentration in the antibody–siRNA conjugate, it is likely that the complexes can be used with minimal side effects due to protamine. This approach might allow early proof-of-principle studies in patients with KRAS-mutant cancer. Pharmacokinetic estimates roughly comparing cetuximab doses clinically used with our in vivo protocol hint at the possibility to reach therapeutic activity with our siRNA delivery system with antibody doses at least a log-step lower than the cetuximab dose. However, detailed pharmacokinetic studies have to be performed in the frame of clinical phase 1 studies to define best clinical protocols.

Taken together, chemically coupled antibody–(e)siRNA complexes, designed similar to previously reported immune constructs transporting DNAs (53), are a new and potentially powerful approach for targeted anticancer therapy following a simple building-block strategy. A coupling of anti-KRAS siRNA to cetuximab might overcome the KRAS-mutation mediated primary or secondary resistance toward anti-EGFR antibodies in clinical use. Most important, the combination of multiple siRNAs conjugated to cetuximab targeting independent oncogenes in combination underlines the flexibility and the high potential of this approach. Furthermore, by targeting overexpressed EGFR and mutated KRAS and potentially other
molecules within one molecule, this new treatment represents a dual specificity approach. This therapeutic principle might also be applicable to other cancer-related receptor-targeting monoclonal antibodies and, of course a multitude of siRNA targets. With the high loading capacity of the protamine for siRNA, one even can envisage loading of siRNA molecules targeting different oncogenic molecules tailored to the individual tumor specifications within one antibody construct. In fact, this principle can contribute to a personalized anticancer therapeutic approach where tumors can be treated with specific siRNAs against several driver mutations and genes at the same time. Thereby, antibody–siRNA conjugates can provide a valuable alternative especially in rare cancer entities when the cost-effective design of conventional inhibitory drugs is difficult.

Disclosure of Potential Conflicts of Interest

C. Müller-Tidow reports receiving speakers bureau honoraria from Merck. No potential conflicts of interest were disclosed by the other authors.

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Antibody-Mediated Delivery of Anti–KRAS-siRNA In Vivo Overcomes Therapy Resistance in Colon Cancer

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