CD44 isoform status predicts response to treatment with anti-CD44 antibody in cancer patients
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Potential conflict of interest: A patent on CD44 isoforms as biomarkers for treatment with RG7356 has been filed.

Statement of Translational Relevance
CD44 has been associated to tumor development, progression, metastasis and drug resistance in several tumor indications by e.g. adhesion of CD44 positive tumor cells to its ligand hyaluronic acid.
(HA) within the extracellular matrix. Thus, blocking CD44 in tumor patients is regarded as a promising approach in anti-cancer therapies. However, when developing anti-CD44-based therapies it needs to be considered the fact that CD44 is indeed a family of proteins originating from alternative splicing and posttranslational modifications with diverse functions on different cell types. In this report we demonstrate how preclinical \textit{in vitro} as well as \textit{in vivo} characterization of CD44 isoform expression resulted in the identification of a potential response prediction marker for patients treated with the CD44 blocking antibody RG7356. Confirming activity with preliminary patient data from a phase I clinical trial, this initial preclinical finding might help enrich responses to RG7356 by cancer patients.

**Abstract**

**Purpose:** CD44, a cell surface glycoprotein, plays important roles in the development, progression and metastasis of various tumor types. The aim of this study was to investigate how the expression of CD44 isoforms influences the interaction with hyaluronic acid and how differential isoform
expression impacts anti-tumoral responses in vivo to treatment with RG7356, a humanized anti-CD44 antibody inhibiting CD44–HA interaction.

**Experimental Design:** CD44 isoform expression on various tumor cell lines was analyzed by RNASeq while data on patients with different tumor types was obtained from the publicly available TCGA RNASeq dataset as well as a Phase 1 clinical study (NCT01358903). We analyzed the link between HA production and CD44 isoform expression as well as the consequences of blocking the CD44-mediated cell adhesion to HA using RG7356. The correlation between CD44 isoform expression and anti-tumor response to RG7356 treatment was investigated in the corresponding murine xenograft in vivo models as well as in a subset of patients treated with RG7356 from a recently completed phase 1 clinical trial.

**Results:** CD44 isoform expression, in particular expression of CD44s, is associated with HA production and predicts response to treatment with RG7356 in tumor xenograft models. Furthermore, patient data suggest that CD44 isoform status is a potential predictive biomarker for clinical response to treatment with RG7356.

**Conclusions:** We provide new insights into the close interplay between CD44 and HA and a potential biomarker to enrich patient responses to RG7356 in the clinic.

**Introduction**

CD44 is a transmembrane glycoprotein with a major role in cell adhesion that has been linked to poor prognosis in several tumor indications like pancreas (1) and breast (2) cancer, hematological malignancies (3) and hepatocellular carcinoma (4). Although CD44 itself is not a signaling molecule per se, it is reported to be involved in a variety of functions that promote tumor development, progression and cancer stem cells (5, 6) by forming complexes with other signaling molecules. While its main ligand is hyaluronic acid (HA), one of the main components of the extracellular matrix that contributes to cell migration and proliferation, CD44 has also been found to form transmembrane complexes with molecules like cMET, EGFR, HER2 and VEGFR, thereby influencing signaling cascades through association with cytoskeleton components (7) or even acting as a transcription (co-
factor via its intracellular domain (8). Furthermore, CD44 is highly N- and O-glycosylated and extensive alternative splicing gives rise to a large number of isoforms that have been described to play various roles in cancer development and progression (5). According to Williams et al. the most important feature of CD44 alternative splicing is that through the introduction of up to 9 variable exons in the extracellular region. CD44 can interact in cis with a great variety of cognate receptors, thus, contributing to the activation of different signaling pathways which are key for tumor progression and dissemination (6). For example, the expression of the variable exons 8 and 10 in CD44v8-10 enables its interaction with the glutamate-cysteine transporter xCT which protects the cell against oxidative burst (9). Similarly, CD44 acquires a new function when the variable exon 6 is expressed allowing for the interaction of CD44v6 with HGF and the subsequent activation of the cMET signaling pathway in cancer stem cells [6]. The interaction of CD44s (“standard” CD44) and its splice variants (CD44v) with HA has been shown to promote tumor cell migration and homing. Since the pericellular HA matrix is physically attached to the cytoskeletal filaments, perturbations in the cell-matrix interaction (for example, by disrupting the CD44-HA binding) can cause distortions of the cellular structure leading to changes in gene expression patterns (10).

In this study we show that RG7356, a humanized monoclonal antibody directed against the constant region of CD44 (11), is able to block the interaction between CD44 and HA and subsequently prevents cell adhesion to HA coated plates. Furthermore, HA production can be induced in CD44-negative HEK293 cells by CD44 transfection leading to pronounced transcriptional changes downstream of CD44. We provide evidence in a large panel of cancer cell lines that CD44 isoform expression is directly associated with HA production levels and further predictive for response to treatment with RG7356 in tumor xenograft models. In line with these preclinical findings, preliminary data from a recently completed Phase 1 clinical trial in patients with solid tumors, suggest that CD44 isoform status may be used as a response prediction biomarker for RG7356 treatment.
Materials and Methods

Tumor cell lines

Authenticated cell lines from different providers (as indicated in Supplementary Table 1) were obtained from the Roche internal cell bank and cultivated for periods no longer than 6 months or re-authenticated by the PCR-based STR method. Cultivation media were either RPMI1640 supplemented with 2 mM L-Glutamine, 2 g/l NaHCO3, 100 IU/ml penicillin, 100 µg/ml streptomycin (Penicillin G, sodium salt, streptomycin sulfate) and 10% heat-inactivated FBS or Dublecco’s modified Eagle’s medium (DMEM) supplemented with 2 mM L-Glutamine and 10% heat-inactivated FBS. All media and supplements were purchased from PAN Biotech (Aidenbach, Germany).

HA – CD44 binding assay

A solution of HA (Sigma #H5388, rooster comb, ~1500 kD) of 2.5 mg/ml was obtained by equilibration of 30 mg HA in 12 ml 50 mM bicarbonate buffer (pH 9.6, 50mM: 1.59g Na2CO3 and 2.93g NaHCO3 in 1000 ml water) under shaking at 37°C for 12h and used to coat ELISA plates (100 µl/well) for 12h at 4°C washed 4x with PBS. Plates were blocked for 2h at 37°C and then 50 µl of a CD44s-Fc solution (ranging from 0.001-100 µg/ml) was added and incubated at RT for 1h in duplicates. After washing (4x 300 µl), 100 µl of the detection antibody (goat F(ab’)2 fragment against human IgG, Jackson #109-036-098,1:5000 in blocking buffer, 200 µl PBS + 3% BSA) was added and shaken at RT for another hour. After washing (4x 300 µl), the TMB substrate was added and allowed to develop for 15 min at RT under shaking. The colorimetric reaction was stopped by addition of 50 µl 2N sulfuric acid and absorption was measured at 450 nm versus 620 nm. In order to test the ability of RG7356 to interfere with the HA-CD44 binding, the same assay was performed with prior addition of RG7356 (2- or 5-fold excess) or an isotype control (5-fold excess, Sigma #M9269) to the CD44s-Fc-solution and pre-equilibration for 1h at 37°C before use.

Cell adhesion to HA coated plates

Surface attachment of cells was determined using an xCELLigence System / Real Time Cell Analyzer MP Instrument (Roche, Germany). Briefly, 96-well plates were coated with HA from human.
umbilical cord (Calbiochem #385902, ~3000 kD) dissolved in sterile DPBS (4 mg/ml) for 2 hours at 37°C. Background signal was measured in the presence of 100 μl cell culture medium. Cells were pre-incubated with RG7356 or control antibody in tubes for 2 hours at 37°C to allow for antibody binding. To start measurement, 100 μl of cell suspension (1.4x10^5 cells) was added into each well. Effect of antibodies on adherence of cancer cells to HA-coated plates is expressed as the change in CI (Cell Index), a dimensionless parameter derived from the relative change in the measured electrical impedance.

**Flourescence microscopy**

Cells were rinsed twice with PBS, pH 7.4, at 37°C and fixed for 25 min at RT in 4% paraformaldehyde and 4% sucrose in 120 mM Na-phosphate buffer. For cell staining cell culture medium was aspirated and washed, including a 5 min incubation with 120 mM Na-phosphate buffer followed by two wash steps with low salt (LS) Na-phosphate buffer (150 mM NaCl, 10 mM NA phosphate) and two additional wash steps with high salt (HS) Na-phosphate buffer (500 mM NaCl, 20 mM Na phosphate). After 30 min in 1xGSDB (16.7% goat serum, 0.3% Triton X-100, 20 mM Na phosphate, 450mM NaCl) first antibody was added (100 μl final volume per chamber) and incubated for 2 h in a humidity chamber. Cells were washed thrice with with HS Na-phosphate buffer and then the secondary antibody (goat anti-mouse/human, ALEXA488-labeled), diluted in 1xGSDB, was added for 90 min. Cells were washed thrice with HS Na-Phosphate buffer and once with LS Na-phosphate buffer, including 5 minutes incubation interval for each wash step. Finally, cells were washed with 120 mM Na-phosphate buffer, covered and stored at 4°C until used. Standard staining with directly labeled antibodies (10μg/ml) and cell monitoring was done in a microscope-attached incubator at 37°C and 5% CO2. Recordings were taken in 10 to 15 min intervals for up to 48 hours using Nikon’s Perfect Focus System. For transmission recordings a 488 nm laser was used.

**Determination of HA levels produced by cells in vitro (cell lysate and supernatant)**

A T75 cell culture flask was seeded with 1 million cells in RPMI 1640 and grown at 37°C, 5% CO₂ for 3 days for each cell line. Supernatants were collected, centrifuged, and stored at -80°C. Cells were washed with PBS, trypsinized, counted and lysed accordingly (100 μl lysis buffer per 1
million cells). The lysis buffer contained 50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EGTA, 1% Triton-X-100, 10% glycerol, 10 μg/ml leupeptine, 10 μg/ml aprotinin, 1mM PMSF and 0.4mM ortho-vanadate. The total protein concentration of the lysates was analyzed by BCA-assay (Thermo Scientific). The obtained lysates were split into aliquots and stored at -80°C. HA quantification was performed using the Corgenix HA-ELISA Kit (Corgenix, UK) according to the manufacturer’s instructions. All HA levels measured can be found in Supplementary Table 2.

**Generation of HEK293 clones expressing CD44s**

For cloning and expression of human CD44 (EMBL accession number M24915) the sequence was codon optimized (Geneart, Germany) and CD44s was expressed using a pcDNA3.1(-) vector (Invitrogen, Germany). PCR was performed with a denaturation step at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 68°C for 45 sec, with a final extension of 7 minutes at 68°C (Expand High Fidelity PCR Kit, Roche). The PCR product and the vector were digested with EcoRI/HindIII restriction endonucleases, and DNA fragments were resolved by 1% agarose gel electrophoresis, purified from gels (GFX PCR DNA and Gel Band Purification Kit) and ligated (Rapid DNA Ligation Kit, Roche). Vectors were transferred into XL-10 Gold super-competent cells (Stratagene, The Netherlands) by heat shock and transformed cells were isolated from agar plates containing 100 μg/ml Ampicillin. Vector DNA from transfected cells was isolated using the High Pure Plasmid Isolation Kit (Roche, Germany).

HEK293-CD44s Clone 4, expressing an average level of HA, was picked for profiling gene expression using Affymetrix Human Genome U133plus 2.0 arrays. Total RNA was prepared using the RNeasy Mini Kit (Qiagen, Germany), Microarray RNA Target Synthesis Kit T7 and RNeasy Mini-Spin Columns (Roche, Germany) and a hybridization mix was created consisting of 30 μl of fragmented cRNA solution mixed with control oligonucleotide, staggered control cRNAs, herring sperm DNA, acetylated BSA and hybridization buffer in a total volume of 300 μl. All kit procedures were performed according to the manufacturers’ instructions. The hybridization mix was loaded onto the arrays and incubated in a roller device for 16 hrs. Hybridization solution was removed, arrays were washed, stained and scanned with an Affymetrix GeneChip Scanner 3000 (7G). Partek
Genomics suite was used to process CEL files, perform normalization and QC as well as to identify differentially regulated genes between native and CD44 transfected HEK293 cells using ANOVA. Raw and processed data are accessible through GEO (GSE63862).

**Mouse Xenograft models and TGI computation**

Immunodeficient SCID/bg mice were purchased from Charles River (Sulzfeld, Germany). Animals used in experiments were between 8 and 16 weeks of age. All experiments were conducted in accordance with local governmental regulations (Regierung von Oberbayern) and Roche internal guidelines. *In vitro* passage number 4-5 were used for inoculation. All details for each xenograft model (number of cells, inoculation site and treatment regimen) can be found in **Supplementary Table 1**.

To determine tumor diameter, first its longest and smallest dimension (ideally in an off-axis angle of 90° to the longest dimension) was measured using a PHASIS system electronic slide caliper. Primary tumor volume (TV) was calculated according to the NCI protocol (TV = (length x width²) / 2), where “length” and “width” are long and short diameters of tumor mass in mm (12). Calculation was done from staging until study termination, and values were documented as medians and inter-quartile ranges (IQR) defined as differences of the third and first quartile.

For calculation of percentage tumor growth inhibition (TGI) during the treatment period with RG7356, ranging from staging (day y) until last study day (day x), the median change of tumor volume in every treated group was compared with its respective vehicle control. The following formula was applied:

\[
TGI \,[\%] = 100 - \frac{\text{median}(TV(\text{treated})_{day \, x}) - TV(\text{treated})_{day \, y}}{\text{median}(TV(\text{resp. control})_{day \, x}) - TV(\text{resp. control})_{day \, y}} \times 100,
\]

where \(TV_{day \, x}\) represents the tumor volume of a study group on study day x.
Cell line RNASeq – data generation

RNA isolation was performed using RNeasy Minikit adding QIAshredder and RNase-free DNase set (Qiagen, Germany). Kit procedures were performed according to manufacturer’s protocol. RNA samples were quantified using Nanodrop ND-8000 and RNA integrity was analyzed using the Agilent 2100 BioAnalyzer. Only samples with a RIN value > 7 and with at least 500ng total RNA were used for library preparation using the TruSeq RNA Sample Prep Kits (v2). Cluster generation and sequencing in 50 bp paired-end mode were run on a HiSeq2000 machine according to the manufacturer’s instructions generating on average 30 Mio reads per sample.

RNASeq – data analysis

Reads for each sample were processed using the following steps: First, reads were aligned against the Human protein coding transcriptome (Ensembl v60) using Bowtie2 (13) with sensitive settings. In a second step, yet unmapped reads were aligned to the Human genome (hg19) and both mappings to genome and transcriptome were combined using in-house software. Gene expression levels were computed as RPKM values (14) using in-house tools. Isoform expression levels for CD44 were estimated using MMSeq (15) and confirmed by manual inspection of read coverage plots (see Supplementary Figure 2).

TCGA data and CD44 isoform prevalence

We obtained RNASeq data (v2) for 14 indications without publishing restrictions through the TCGA data portal (16). CD44 isoform frequencies per patient were estimated based on unique junction counts linking the variable exons v2 to v10 to the flanking constant exons of CD44 (for v-variants) or by the junction count skipping all variable exons (for CD44s). Based on those estimates we selected the subgroup of patients from each indication that either solely expresses CD44s or CD44v with an estimated frequency of more than 80% in the respective tumor samples leading to two groups per indication. We then used RNASeq data for all genes (gene level read counts) and computed differential gene expression between the two groups using DESeq (17). Only genes which had an absolute fold-change > 1.4 and a p-value < 0.05 were used for further analysis in Ingenuity Pathway Analysis (IPA).
Patient data (Phase 1 clinical trial) and RNASeq in patient samples

Data were taken from a recently completed multicenter, open-label dose-escalation Phase 1 clinical study of RG7356 at 6 study sites in France, the Netherlands and the USA (clinicaltrials.gov identifier NCT01358903). The study was conducted in accordance with the Declaration of Helsinki, current International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines and all applicable regulatory and ethical requirements. All subjects provided written informed consent before study-related procedures were performed. Patients with metastatic and/or locally advanced, CD44-expressing, malignant solid tumors who were not amenable to standard treatment were enrolled and received RG7356 on a q2w or qw schedule as an intravenous infusion. Tumor response was evaluated according to RECIST (version 1.1) criteria up to 28 days prior to the first drug administration and every 6 weeks ± 7 days while a patient was on study.

A tumor biopsy was taken from each patient before enrollment to assess CD44 protein expression by immunohistochemistry (Ventana SP37 Rabbit Monoclonal Primary Antibody, Cat-No 790-4537). Collection of biopsies was guided by ultrasound or computer tomography using an 18 Gauge needle to provide cores of at least 20 mm in length. Total RNA was prepared from fresh frozen tumor tissue of at least 6-8 sections 10 µm thick. In cases of insufficient material, the entire biopsy was used. RNA isolation was performed using RNeasy Minikit adding QIAshredder and RNase-free DNase set (Qiagen, Germany). Kit procedures were performed according to manufacturer’s protocol. Each sample was centrifuged at 16,000 g for 2 min, aliquoted and analyzed via NanoDrop. RNA quality was checked using Agilent Bioanalyzer. Library preparation from cDNA was performed using the Illumina TruSeq Stranded Total RNA Kit using 0.1–1µg or total RNA. The generated libraries were applied for RNA Sequencing using an Illumina HiSeq2000 instrument generating on average 50 Mio reads of 50 bp paired-end reads per sample. RNASeq data was analyzed as described above.
Results

RG7356 binds to all CD44 isoforms and inhibits the interaction between CD44 and HA

RG7356 binds to the constant region of the extracellular domain of CD44 (11) which is present in all isoforms and is known to be responsible for the CD44-HA interaction. Using tumor cell lines we observed that RG7356 binding could be detected on cells expressing CD44s as well as a variant CD44 isoform (Figure 1A). This indicates that, as expected, binding of RG7356 to CD44⁺ cells is independent of the CD44 isoform status. In addition, RG7356 binding blocks the interaction between CD44 and HA. This could be observed by a reduced absorption signal of CD44 to HA coated plates when RG7356 was present resulting in a shift of the CD44 binding curve compared to CD44 alone or isotype control (Figure 1B). Blocking of the CD44-HA interaction depends on RG7356 concentration as shown in Suppl. Figure 1. Furthermore, in an in vitro cell adhesion assay RG7356 was shown to inhibit cell adhesion of CD44⁺ cells (MDA-MB-231) to HA coated plates in a concentration dependent manner (Figure 1C).

Transfection of CD44 into HEK293 cells leads to HA production and significant changes on the transcriptional level

To further characterize the interaction of CD44 and HA, we transfected wild-type CD44neg/HAneg HEK293 cells with expression vectors that code for CD44s. In total, 12 transfected clones were generated and CD44s expression was assessed using Cy5-labelled RG7356 (Figure 2A). In addition, HA levels in lysates and supernatants were measured and compared to wild-type HEK293 cells (see Supplementary Figure 1). All CD44s expressing HEK293 clones produced >100-fold higher levels of HA compared to native HEK293 cells (Figure 2B). In order to elucidate the molecular mechanisms leading to de novo HA production we profiled genome-wide transcriptional changes between wild type HEK293 cells and one HEK293-CD44s clone using Affymetrix arrays. We found 4191 genes to be significantly de-regulated (absolute fold-change > 2 and p-value < 0.01) upon CD44 transfection with 1652 genes up- and 2539 genes down-regulated in the HEK293-CD44s clone (see Supplementary Table 3). HA is produced by synthetases HAS1, HAS2 and HAS3, and the latter two were found to be more than 10-fold up-regulated in HEK293-CD44s cells explaining the
increased HA production on the mRNA level. In addition, forced expression of CD44 and/or augmented production of HA have significant downstream effects. In particular, using Ingenuity’s IPA upstream analysis (18), we found strong evidence for a network of upstream regulators consisting of TGFB1, TP53, TNF, SMAD, ERBB2 and other genes (Figure 2C) whose activation/inhibition explains many of the downstream expression changes observed. Co-localization of CD44, TGFB1R1 and ERBB2 has been described (19, 20) whereas interaction of CD44 and TGFB1R1 was found to facilitate modulation of SMAD-dependent and independent TGFB1-signalling by HA (19). TNF was described to modulate expression of CD44 in ovarian cancer cells (21) and TNF-induced p38 MAPK activation is involved in the regulation of functionally active HA-binding CD44 (22). Overall, these data display the strong relationship between CD44 and HA underlining the importance of this interaction for various cellular pathways and processes.

**CD44 isoform expression is associated with HA production levels and predictive for response to treatment with RG7356 xenograft models**

As shown above, expression of CD44s in HEK293 cells induces HA production followed by a large number of transcriptional changes. However, since CD44 has multiple isoforms we aimed to test whether the CD44 isoform status correlates with HA production in 36 solid tumor cell lines. HA levels were determined by ELISA in lysates and supernatants while CD44 isoforms were measured by RNA sequencing (RNASeq) as described in the Methods section and illustrated in Figure 3A. It is noteworthy that the majority of cell lines tested express a mixture of CD44 isoforms with usually one predominant “major isoform”. For simplicity, in the following we only distinguish between CD44v isoforms (containing any combination of the variable exons) and CD44s (no variable exon). Interestingly, HA levels are on average 7.5-fold higher (p-value = 0.0004) in cells expressing predominantly CD44s compared to cells predominantly expressing CD44v (Figure 3B).

We next compared the baseline CD44 isoform status of 37 tumor cell lines in vitro with the anti-tumor effect of RG7356 in the corresponding xenograft models. As shown in Figure 3C 8/37 tumor xenograft models respond to RG7356 treatment with significantly impaired tumor growth while 29 models did not respond. Predominant CD44 isoform expression in the tested cell lines is similarly
represented with 19/37 expressing CD44s and 18/37 CD44v. Surprisingly, the correlation of isoform status with xenograft response data reveals that only models using cell lines expressing predominantly CD44s are responsive to RG7356 treatment in vivo (8/19 = 42%). While none of the models with predominant CD44v tumor cells respond to RG7356. This overrepresentation of CD44s in the responsive and CD44v in the non-responsive group is highly significant (Chi²-test: p-value: 0.0004). Higher tumor growth inhibition rates are significantly associated with the expression of CD44s as major variant (Wilcoxon – Test, p-Value < 0.0001). In order to confirm the CD44 isoform status on protein level we generated Western Blots for 35 cell lines as well as tumor material from 9 xenograft models. This data shows a very good agreement of protein level data with the isoform status determined by RNAseq (see Supplementary material and Suppl. Figures 3-6). In conclusion, all eight responding models exhibit clear expression of CD44s as major isoform and no or little (PC3) CD44v variant expression on protein and mRNA level. Therefore, CD44s mRNA and protein is a necessary, although not sufficient, criterion for response to treatment with RG7356.

Differential gene expression pattern in tumor patients with predominant CD44s compared to CD44v isoform expression.

We next investigated the distribution of CD44 isoforms in different tumor indications. RNASeq data from The Cancer Genome Atlas (TCGA) (23) was used to identify CD44 isoform expression patterns in patients across various tumor indications. Interestingly, CD44 isoform expression patterns highly differ between tumor types ranging from indications like AML and Glioblastoma, expressing solely CD44s, to indications like colorectal cancer where more than 75% of the patients express CD44v8-v10 as the predominant isoform (Figure 4A).

In order to elucidate further potential differences in patients expressing predominantly CD44s or CD44v in a given tumor type, we then selected three tumor indications, namely invasive breast carcinoma (BRCA) (24), lung squamous carcinoma (LUSC) (25) and uterine corpus endometrioid carcinoma (UCEC) (26), in which we find larger subgroups of patients expressing a particular isoform. From each indication, we selected a subset of patients with an estimated major isoform frequency greater than 80%, therefore showing a relatively clear CD44s or CD44v expression pattern.
This resulted in 188 BRCA patients (53 CD44s, 135 CD44v), 46 LUSC patients (12 CD44s and 34 CD44v) and 54 UCEC patients (24 CD44s and 30 CD44v), for which we computed transcriptome wide expression differences based on RNASeq data.

For all three indications we found a surprisingly large number of differentially expressed genes in CD44s vs. CD44v expressing tumors (Supplementary Table 4, full datasets can be found in Supplementary Tables 5-7), which show a high overlap between the three cancer types. We found 274 genes to be commonly de-regulated in all three indications and additional 671 genes which are de-regulated in two out of the three indications. Interestingly, for all three indications the main difference between patients expressing CD44s or CD44v are genes involved in immune response regulation and, most significantly, the up-stream regulator genes activated in CD44s patients (identified by Ingenuity) are TGFB1, TNF and IL2 (Figure 4B) which are strikingly similar to the regulators identified in the HEK293-CD44s clones, as discussed above.

**Early signs for the clinical relevance of CD44 isoform expression pattern as response prediction marker for RG7356 treatment**

To confirm CD44s as response prediction marker in the clinical setting, pre-treatment tumor biopsies of patients from a recently completed Phase 1 dose-escalation clinical trial (clinicaltrials.gov identifier NCT01358903) were tested for their predominant CD44 isoform expression by RNASeq and compared to the respective clinical activity of RG7356 observed in those advanced cancer patients. In total, we analyzed pre-treatment samples from a cohort of 13 patients with CD44+ colorectal carcinoma (CRC) who received every two weeks (q2w) administration of dose-escalated RG7356. The selection of patients was based on their primary tumor indication and available tumor assessment according to RECIST1.1 after six weeks on study treatment. This subgroup of patients belongs to the Phase 1 dose escalation part of and therefore represents a heterogeneous population with regards to the different doses received. Among the patients within the described subgroup, 3/13 had stable disease (SD) as best response and 2/13 showed a reduction in tumor size measured as “best percent”-tumor change on target lesions (Figure 5A). In 12/13 patients CD44 isoform assessment via RNASeq
could be performed resulting in 3 patients with predominant CD44s and 9 patients with predominant CD44v8-v10 isoform expression. Despite the low number of patients analyzed, the distribution of 25% of patient tumors expressing predominantly CD44s and 75% CD44v matches the major isoform distribution obtained from the TCGA colorectal cancer cohort (Figure 4A and 5B).

Interestingly, 2/3 CRC patients with predominant CD44s expressing tumors (patients 4, 12 and 13) belonged to the patients that had SD as best response and showed minor clinical activity of RG7356 as measured by reduction of target lesions. All 9 CRC patients with predominant CD44v8-10 expression on their tumors had progressive disease as best response and 7/7 show an increase in tumor target lesions (Figure 5A and B).

Finally, the distribution of CD44 isoforms (besides the described predominant isoform) and overall expression level of CD44 transcripts was variable in the analyzed patient group (Figure 5B). One out of the three patients with a predominant CD44s isoform expression tumor did not benefit from RG7356 treatment underlining CD44s to be a necessary though not sufficient criterion for response. Nevertheless, it needs to be mentioned that this patient received the second lowest dose of RG7356 within the dose escalation cohorts among all tested CRC patients. The patient with SD and the highest % reduction on target lesions (-22%) also showed the highest CD44 expression level as well as the highest observed ratio between CD44s transcripts and CD44v isoforms (Figure 5B). However, a general correlation between the overall expression of CD44 and the distribution of isoforms within one tumor cannot be established.

**Discussion**

In this work we have shown that response to treatment with RG7356, an antibody targeted against CD44 that is able to inhibit the interaction of CD44 and HA, depends on the expression of CD44s as predominant isoform in xenograft models and colorectal cancer patients. It was recognized early on that CD44 is not a single molecule but a diverse family of molecules originating from alternative splicing and subsequent posttranslational modifications in different cell types (27). Prevalence and expression patterns of CD44 isoforms in normal tissue and disease were discussed in the early 1990’s
but usually those analyses were limited by their assays to detect a specific isoform. Up-regulation of certain isoforms in the context of pancreatic, colorectal and breast cancer were described((29), (31), (32)) but only the emergence of RNA sequencing and the collection of large sets of patient samples in TCGA made it possible to analyze splice patterns of CD44 in an unbiased fashion. Our analysis reveals that major isoform expression patterns are tissue specific and indeed do not differ significantly between primary tumors and matched normal tissues although we have not performed an in depth analysis of potential changes in minor isoform expression.

The functional relevance of CD44 isoforms for cancer cells is still unclear with several clinically relevant functions described. While CD44–HA interaction is one important factor (33), a shift towards CD44v in epithelial-mesenchymal transition (34), HCC (35), squamous cell carcinoma (36), a role of CD44v6 in preparation of the metastatic niche (37) or CD44-mediated drug resistance (38-40) in ovarian cancer (41) were reported.

Transcriptome profiling of TCGA samples expressing predominantly CD44s or CD44v provides strong evidence that CD44s or CD44v expressing patients differ significantly in their immune cell infiltration and/or activation level. Since HA production in tumor cell lines correlates with the CD44 isoform status and HA is described as a strong activator of dendritic cells and regulator of T-cell function (42), we believe that CD44 isoform expression significantly contributes to shape the inflammatory tumor microenvironment.

The prognostic relevance of CD44 and its isoforms is currently non-conclusive. While a number of reports describe unfavorable prognosis of CD44 overexpression for thyroid cancer, non-small cell lung carcinoma and others (43), there are contradictory reports indicating that CD44 expression does not or even negatively correlates with stage of disease or poor prognosis. Banky et al. (44) suggest the whole CD44 isoform pattern expression rather than specific isoform identification might be necessary to succeed in the characterization of the prognostic and predictive value of CD44 isoforms.

Here, we suggest that predominant CD44s expression is a necessary criterion for response whereas predominant CD44v expression can be considered a non-response marker for RG7356 treatment. As
the biological explanation for this finding is still unclear, alterations in the cellular signaling pathways and the resulting effects within the tumor microenvironment due to RG7356 binding to either CD44s or CD44v isoforms needs further evaluation. However, we believe that the blocking capacity of RG7356 with regard to CD44 and HA interaction plays a central role as it is more relevant for CD44s than for CD44v expressing tumor cells, the latter expressing lower levels of HA in vitro. Furthermore, since higher levels of HA are linked to inflammatory processes, macrophages and other immune cells may be critical factors in the response of CD44s expressing tumor cells to treatment with RG7356. Structural variability of different CD44 isoforms (45) does not impair RG7356 binding, however conformational changes after RG7356 binding could impact signaling or e.g. Fc-mediated anti-tumor immune responses.

References


Figure Legends

Figure 1. RG7356 binds to CD44-expressing cells and blocks the interaction with HA. A) Binding of Cy5-labeled RG7356 (10 µg/ml, 24 h) to cells expressing various CD44 isoforms: MDA-MB-231 expresses CD44s, BxPC3 expresses predominantly CD44v2-v10 while PL45 and MDA-MB-468 express predominantly CD44v8-v10. B) CD44-HA interaction can be efficiently blocked by
RG7356 in comparison to IgG isotype control or without antibody. C) Concentration dependent blockage of MDA-MB-231 cellular adhesion to HA-coated plates in ACEA assay.

Figure 2. CD44 expression enhances HA production in HEK293 cells. A) Binding of Cy5-RG7356 to native and CD44s-transfected HEK293 cells (10 µg/ml, 24 h). B) HEK293 cells –which do not normally produce HA- start producing this glycosaminoglycan upon transfection with a CD44-expressing vector. C) Mechanistic network of regulators predicted to explain changes between HEK293-CD44s and native HEK293 cells by Ingenuity Pathway analysis. Grey genes are predicted to be activated in CD44s transfected cells while white regulators are predicted to be inhibited.

Figure 3. CD44s correlates with higher HA levels and response to treatment with RG7356 in vivo. A) Exemplary representation of RNASeq data corresponding to the CD44 isoform status of two cell lines: while MDA-MB231 expresses almost solely CD44s, MDA-MB-468 expresses a mixture of CD44v8-v10 and CD44v3-v10. Percentage values correspond to isoform frequencies predicted by MMSeq. B) HA levels measured in a panel of 36 cancer cell lines are significantly higher in cells expressing CD44s compared to cells expressing predominantly CD44v variants (p-value = 0.0004). C) In vivo response to treatment with RG7356 in xenograft models (measured as %TGI, tumor growth inhibition) is highly associated with CD44s major isoform status. CD44s expressing models are shown as grey bars, CD44v expressing models as white bars. Relative CD44s levels (compared to CD44v and as measured by MMSeq) are shown as black squares. Non-responding models (%TGI=0) were set at 5% to allow for visualization of the CD44 major isoform status.

Figure 4. Differential gene expression in patients with CD44s- or CD44v-expressing tumors points out at differences in the immune status. A) CD44 isoform frequencies (predominant isoforms) in patients from indications sequenced in the course of the TCGA project. CD44 isoform distribution highly depends on cancer indication. Abbreviations: AML= acute myeloid leukemia; BRCA= breast cancer; COAD= colon adenocarcinoma; GBM= glioblastoma; HNSCC= head and neck squamous cell carcinoma; KIRC= kidney renal clear cell carcinoma; LUAD= lung adenocarcinoma; LUSC= lung squamous cell carcinoma; SKCM= skin cutaneous melanoma; OV=
ovarian cancer; READ= renal adenocarcinoma; STAD= stomach adenocarcinoma; THCA= thyroid cancer; UCEC= uterine corpus endometrioid carcinoma. B) Mechanistic network of regulators predicted to explain changes between patients expressing predominantly CD44s or CD44v in the TCGA breast cancer cohort by Ingenuity Pathway analysis (activation score 4.49, p-value 1.63E-39). Grey genes are predicted to be activated in CD44s patients while SMAD7 is predicted to be inhibited. Arrows indicate known activating relationship between regulators.

Figure 5. CD44 isoform status might be of clinical relevance during treatment with RG7356. A) Best % tumor change on target lesion (RECIST) in 13 patients with colorectal cancer treated with RG7356. Please note that for patients 1 and 2 the information on change in target lesion is not available. Patients bearing predominantly CD44s- and CD44v-expressing tumors are indicated in grey and black, respectively. Stripped columns indicate patients with stable disease. B) Normalized read counts (RNASeq) for unique junctions for different CD44 isoforms. Height of bars indicates total expression level of CD44 which varies significantly between patients. RNASeq data for patient 3 is not available.
Figure 1

A

MDA-MB-231 (CD44s)  BxPC3 (CD44^v2-v10)  MDA-MB-468 (CD44^v8-v10)  PL45 (CD44^v8-v10)

B

CD44s-Fc alone
CD44s-Fc+IgG control
CD44s-Fc+2-fold RG7356
CD44s-Fc+5-fold RG7356

C

Buffer Ctrl
Isotype Ctrl [20 μg/ml]
RG7356 [1 μg/ml]
RG7356 [20 μg/ml]

Absorption vs. CD44s-Fc [μg/ml]
Cell Index vs. Time (min)
Figure 2

A

HEK293

HEK293_CD44s

B

ng HA / mg Total protein

CD44s

HEK293 nativ

C

JUN*

STAT3*

RELA*

TP53*

beta-estradiol

estrogen receptor

NOTCH1*

ERBB2*

ESR1*

SP1*

SMAD7

EP300*

SMAD4*

NFKBIA

NFKB (complex)

TNF

SMAD3*

TGFBI*

IFNG

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Figure 3

A

MDA-MB-231

B

MDA-MB-468

C

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Figure 4

A

% of patients with CD44 predominant isoform

AML  BRCA  COAD  GBM  HNSCC  KIRC  LUAD  LUSC  SKCM  OV  READ  STAD  THCA  UCEC

CD44s  CD44 v2-v10  CD44 v3-v10  CD44 v8-v10

B

Network diagram showing interactions between genes and cytokines.}

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Figure 5

A

%Best Tumor Change on Target lesion (RECIST)

Patient ID

B

CD44v2-v10  CD44v3-v10  CD44v8-v10  CD44v10  CD44s

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CD44 isoform status predicts response to treatment with anti-CD44 antibody in cancer patients

Fabian Birzele, Edgar Voss, Adam Nopora, et al.

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