CD44 Isoform Status Predicts Response to Treatment with Anti-CD44 Antibody in Cancer Patients

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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 (HA) and how differential isoform expression impacts antitumoral 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 were obtained from the publicly available TCGA RNASeq dataset as well as a phase I 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.

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, hematologic 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. Although 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 MET, EGFR, ERBB2, 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 and colleagues, 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 SLC7A11 which protects the cell against oxidative burst (9). Similarly, CD44 acquires a new function when the variable...
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 anticancer 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 in vitro as well as 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.

HA–CD44 binding assay

A solution of HA (Sigma #H5388; rooster comb; ~1,500 kD) of 2.5 mg/mL was obtained by equilibration of 30 mg HA in 12 mL 50 mmol/L bicarbonate buffer (pH 9.6, 50 mmol/L: 1.59 g Na2CO3 and 2.93 g NaHCO3 in 1,000 mL water) under shaking at 37°C for 12 hours and used to coat ELISA plates (100 μL/well) for 12 hours at 4°C washed 4 × with PBS. Plates were blocked for 2 hours at 37°C and then 50 μL of a CD44s-Fc solution (ranging from 0.001–100 μg/mL) was added and incubated at room temperature for 1 hour in duplicates. After washing (4 × 300 μL), 100 μL of the detection antibody (goat f(ab′)2; fragment against human IgG; Jackson #109-036-098; 1:5,000 in blocking buffer, 200 μL PBS + 3% BSA) was added and shaken at room temperature for another hour. After washing (4 × 300 μL), the tetramethylbenzidine (TMB) substrate was added and allowed to develop for 15 minutes at room temperature 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 1 hour 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). Briefly, 96-well plates were coated with HA from human umbilical cord (Calbiochem; #385902; ~3,000 kD) dissolved in sterile Dulbecco’s phosphate-buffered saline (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 preincubated 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 (1.4 × 105 cells) was added into each well. Effect of antibodies on adherence of cancer cells to HA-coated plates is expressed as the change in Cl (cell index), a dimensionless parameter derived from the relative change in the measured electrical impedance.

Fluorescence microscopy

Cells were rinsed twice with PBS, pH 7.4, at 37°C and fixed for 25 minutes at room temperature in 4% paraformaldehyde and 4% sucrose in 120 mmol/L Na-phosphate buffer. For cell staining, cell culture medium was aspirated and washed, including a 5-minute incubation with 120 mmol/L Na-phosphate buffer followed by two wash steps with low salt (LS) Na-phosphate buffer (150 mmol/L NaCl, 10 mmol/L Na-phosphate) and two additional wash steps with high salt (HS) Na-phosphate buffer (500 mmol/L NaCl, 20 mmol/L Na-phosphate). After 30 minutes in 1xGDB (16.7% goat serum, 0.3% Triton X-100, 20 mmol/L Na-phosphate, 450 mmol/L NaCl), first antibody was added (100 μL final volume per chamber) and incubated for 2 hour in a humidity chamber. Cells were washed thrice with HS Na-phosphate buffer, and then the secondary antibody (goat anti-mouse/human, ALEXA488-labeled), diluted in 1xGDB, was added for 90 minutes. Cells were washed thrice with HS Na-phosphate buffer and once with LS Na-phosphate buffer, including 5-minute incubation interval for each wash step. Finally, cells were washed with 120 mmol/L Na-phosphate buffer, covered, and stored at 4°C until used. Standard staining with directly labeled antibodies (10 μg/mL) and cell monitoring was done in a microscope-attached

Materials and Methods

Tumor cell lines

Authenticated cell lines from different providers (as indicated in Supplementary Table S1) were obtained from the Roche internal cell bank and cultivated for periods no longer than 6 months or reauthenticated by the PCR-based short tandem repeat method. Cultivation media were either RPMI-1640 supplemented with 2 mmol/L 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 DMEM supplemented with 2 mmol/L l- Glutamine and 10% heat-inactivated FBS. All media and supplements were purchased from PAN Biotech.

exon 6 is expressed allowing for the interaction of CD44v6 with HGF and the subsequent activation of the MET signaling pathway in cancer stem cells [6]. The interaction of CD44s (*standard* CD44) and its splice variants (CD44v4) with HA has been shown to promote tumor cell migration and homing. Because 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 CD44 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 I clinical trial in patients with solid tumors suggest that CD44 isoform status may be used as a response prediction biomarker for RG7356 treatment.

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incubator at 37°C and 5% CO2. Recordings were taken in 10- to 15-minute 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 with 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 mMol/L Tris, pH 8.0, 150 mMol/L NaCl, 1 mMol/L EGTA, 1% Triton-X-100, 10% glycerol, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mMol/L PMSF, and 0.4 mMol/L 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) according to the manufacturer’s instructions. All HA levels measured can be found in Supplementary Table S1.

**Generation of HEK293 clones expressing CD44s**

For cloning and expression of human CD44 (EMBL accession number M24915), the sequence was codon optimized (Geneart), and CD44s were expressed using a pcDNA3.1(−) vector (Invitrogen). PCR was performed with a denaturation step at 94°C for 2 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 68°C for 45 seconds, 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 with the High Fidelity PCR Kit; Roche. The ligation was performed according to the manufacturer’s instructions. The obtained lysates were 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) according to the manufacturer’s instructions. All HA levels measured can be found in Supplementary Table S2.

**Cell line RNASeq—data generation**

RNA isolation was performed using RNeasy Minikit adding QiAashereder and RNase-free DNase set (Qiagen). 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 RNA Integrity Number (RIN) value >7 and with at least 500 ng total RNA were used for library preparation using the TrueSeq 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 reads per kilobase per million mapped reads (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 Fig. S2).

**The Cancer Genome Atlas data and CD44 isoform prevalence**

We obtained RNASeq data (v2) for 14 indications without publishing restrictions through The Cancer Genome Atlas (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 I clinical trial) and RNASeq in patient samples
Data were taken from a recently completed multicenter, open-label dose-escalation phase I clinical study of RG7356 at 6 study sites in France, the Netherlands, and the United States (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 all applicable regulatory and ethical requirements. All sub-

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 (Fig. 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 with CD44 alone or isotype control (Fig. 1B). Blocking of the CD44–HA interaction depends on RG7356 concentration as shown in Supplementary Fig. S1. 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 (Fig. 1C).

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, because 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 Materials and Methods and illustrated in Fig. 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

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 CD44s/HA−/− HEK293 cells with expression vectors that code for CD44s. In total, 12 transfected clones were generated, and CD44s expression was assessed using Cy5-labeled RG7356 (Fig. 2A). In addition, HA levels in lysates and supernatants were measured and compared with wild-type HEK293 cells (see Supplementary Fig. S1). All CD44s expressing HEK293 clones produced >100-fold higher levels of HA compared with native HEK293 cells (Fig. 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 4,191 genes to be significantly deregulated (absolute fold change >2 and P value < 0.01) upon CD44 transfection with 1,652 genes up-regulated and 2,539 genes downregulated in the HEK293-CD44s clone (Supplementary Table S3). HA is produced by synthetases HAS1, HAS2, and HAS3, and the latter two were found to be more than 10-fold upregulated 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 (Fig. 2C) whose activation/inhibition explains many of the downstream expression changes observed. Colocalization 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 signaling 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 underlying the importance of this interaction for various cellular pathways and processes.
levels are on average 7.5-fold higher ($P$ value $= 0.0004$) in cells expressing predominantly CD44s compared with cells predominantly expressing CD44v (Fig. 3B).

We next compared the baseline CD44 isoform status of 37 tumor cell lines in vitro with the antitumor effect of RG7356 in the corresponding xenograft models. As shown in Fig. 3C, 8 of 37 tumor xenograft models respond to RG7356 treatment with significantly impaired tumor growth, whereas 29 models did not respond. Predominant CD44 isoform expression in the tested cell lines is similarly represented with 19 of 37 expressing CD44s and 18 of 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 ($8/19 = 42\%$), whereas none of the models with predominant CD44v tumor cells respond to RG7356. This overrepresentation of CD44s in the responsive and CD44v in the nonresponsive group is highly significant ($\chi^2$ 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. These data show a very good agreement of protein level data with the isoform status determined by RNAseq (Supplementary Methods and Supplementary Figs. S3–S6). 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 with CD44v isoform expression

We next investigated the distribution of CD44 isoforms in different tumor indications. RNAseq data from TCGA (23) were 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 acute myeloid leukemia (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 (Fig. 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 (24), lung squamous carcinoma (LUSC; ref. 25), and uterine corpus endometrioid carcinoma (UCEC; ref. 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.
resulted in 188 breast carcinoma patients (53 CD44s and 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 versus CD44v expressing tumors (Supplementary Table S4, full datasets can be found in Supplementary Tables S5–S7), which show a high overlap between the three cancer types. We found 274 genes to be commonly deregulated in all three indications and additional 671 genes which are deregulated in two of the three indications. Interestingly, for all three indications, the main differences between patients expressing CD44s or CD44v are genes involved in immune response regulation, and, most significantly, the upstream regulator genes activated in CD44s patients (identified by Ingenuity) are TGFB1, TNF, and IL2 (Fig. 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, pretreatment tumor biopsies of patients from a recently completed phase I dose-escalation clinical trial (clinicaltrials.gov identifier NCT01358903) were tested for their predominant CD44 isoform expression by RNASeq and compared with the respective clinical activity of RG7356 observed in those advanced cancer patients. In total, we analyzed pretreatment samples from a cohort of 13 patients with CD44+ colorectal carcinoma 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 6 weeks on study treatment. This subgroup of patients belongs to the phase I dose-escalation part of and therefore represents a heterogeneous population with regard to the different doses received. Among the patients within the described subgroup, 3 of 13 had stable disease as best response and 2 of 13 showed a reduction in tumor size measured as “best percent” tumor change on target lesions (Fig. 5A). In 12 of 13 patients, CD44 isoform assessment via RNASeq could be performed resulting in 3 patients with predominant CD44s and 9 patients with predominant CD44v 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 (Figs. 4A and 5B).

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

The patient with stable disease and the highest percent reduction on target lesions (−22%) also showed the highest CD44v expression level as well as the highest observed ratio between CD44s transcripts and CD44v isoforms (Fig. 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 1990s (28–30), but usually those analyses were limited by their assays to detect a specific isoform. Upregulation of certain isoforms in the context of pancreatic, colorectal, and breast cancer was 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 toward CD44v in epithelial–mesenchymal transition (34), hepatocellular carcinoma (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. Because 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 nonconclusive. 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 correlate with stage of disease or poor prognosis. Banky and colleagues (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 nonresponse 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 need further investigation.

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. BRCA, breast cancer; COAD, colon adenocarcinoma; GBM, glioblastoma; HNSCC, head and neck squamous cell carcinoma; KIRC, kidney renal clear cell carcinoma; LUAD, lung adenocarcinoma; SKCM, skin cutaneous melanoma; OV, ovarian cancer; READ, renal adenocarcinoma; STAD, stomach adenocarcinoma; THCA, thyroid cancer. B, mechanistic network of regulators predicted to explain changes between patients expressing predominantly CD44s or CD44v in the TCGA breast cancer cohort by IPA (activation score 4.49, P value 1.63E–39). Gray genes are predicted to be activated in CD44s patients while SMAD7 is predicted to be inhibited. Arrows, known activating relationship between regulators.
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 does not impair RG7356 binding; however, conformational changes after RG7356 binding could impact signaling or, e.g., Fc-mediated antitumor immune responses.

Disclosure of Potential Conflicts of Interest

F. Birzele, E. Voss, A. Nopora, K. Honold, S. Weigand, F. Heil, and M. Cannarile are co-inventors on a patent, owned by Roche, on CD44 isoforms as markers for responsiveness to anti-CD44 antibodies. No potential conflicts of interest were disclosed by the other authors.

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Development of methodology: F. Birzele, A. Nopora, K. Honold, S. Lohmann

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Voss, A. Nopora, K. Honold, C. Le Tourneau, J.-P. Delord, C. van Herpen, D. Mahalingam, A.L. Coveler, V. Meresse

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Birzele, E. Voss, K. Honold, F. Heil, C. Le Tourneau, D. Mahalingam, V. Meresse, S. Weigand, M. Cannarile


Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Honold

Study supervision: F. Heil, D. Mahalingam, V. Weigand

Other (design of HA interaction assay, WB confirmation of CD44s and CD44v expression): E. Voss

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Figure 5.

CD44 isoform status might be of clinical relevance during treatment with RG7356. A, best percent 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 gray 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 are not available.
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