In vivo magnetic resonance imaging and spectroscopy identifies oncolytic adenovirus responders

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At present, it is not possible to reliably identify patients who will benefit from oncolytic virus treatments. Conventional modalities such as computed tomography (CT), which measure tumor size, are unreliable owing to inflammation-induced tumor swelling. We hypothesized that magnetic resonance imaging (MRI) and spectroscopy (MRS) might be useful in this regard. However, little previous data exist and neither oncolytic adenovirus nor immunocompetent models have been assessed by MRS. Here, we provide evidence that in T2-weighted MRI a hypointense core area, consistent with coagulative necrosis, develops in immunocompetent Syrian hamster carcinomas that respond to oncolytic adenovirus treatment. The same phenomenon was observed in a neuroblastoma patient while he responded to the treatment. With relapse at a later stage, however, the tumor of this patient became moderately hyperintense. We found that MRS of taurine, choline and unsaturated fatty acids can be useful early indicators of response and provide detailed information about tumor growth and degeneration. In hamsters, calprotectin-positive inflammatory cells (heterophils and macrophages) were found in abundance; particularly surrounding necrotic areas in carcinomas and T cells were significantly increased in sarcomas, when these had been treated with a granulocyte–macrophage colony-stimulating factor-producing virus, suggesting a possible link between oncolysis, necrosis (seen as a hypointense core in MRI) and/or immune response. Our study indicates that both MRI and MRS could be useful in the estimation of oncolytic adenovirus efficacy at early time points after treatment.

Key words: oncolytic, adenovirus, MRI, MRS, cancer

Additional Supporting Information may be found in the online version of this article.

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‡O.H. and R.I. contributed equally to this work

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Many cancer patients treated with oncolytic viruses show signs of antitumor effects,1–5 but there appears to be remarkable variation between individuals. Understanding which patients are likely to benefit would be helpful for clinical decision making, but reliable surrogate markers are currently not available. Thus, some patients may needlessly be subjected to inefficient therapies and side effects, whereas other patients may be prematurely withdrawn owing to temporary increase in tumor volume, which in reality might only reflect “pseudoprogression”, i.e., inflammatory swelling due to an immune reaction at or against the tumor.1 Therefore, conventional computed tomography (CT) may be suboptimal in assessing tumor response.1,2 Similarly, FDG-PET may not be optimal, because infiltrating activated lymphocytes, which preferentially utilize glucose, can create the impression of increased metabolism in the tumor. Also, virus infection can increase the synthesis and release of tumor marker peptides by lysed cells, which renders their evaluation in the serum unreliable.6,7 Although increasingly understood for
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appears hyperintense (bright) in T2-weighted MRI.\textsuperscript{12} Coagulative necrosis is replaced by viscous, often lipid-rich material, which dries. In liquefactive necrosis, tissue architecture is lost and hypointense (dark) in T2-weighted MRI as it is dense and dry. In tissue architecture is preserved. The necrotic area is distinguished after suppression of the water signal (thus also providing good contrast between different soft tissues without exposure to ionizing radiation. T2-weighted MRI can visualize swelling, because water that has accumulated in extracellular spaces (vasogenic edema) increases the T2 relaxation time, which appears bright on the resulting images. In some tumors, T2 signal intensity is influenced by coagulative or liquefactive necrosis.\textsuperscript{11} In coagulative necrosis, cell injury leads to the denaturation of proteins and lysosomal enzymes while tissue architecture is preserved. The necrotic area is hypointense (dark) in T2-weighted MRI as it is dense and dry. In liquefactive necrosis, tissue architecture is lost and cells are replaced by viscous, often lipid-rich material, which appears hyperintense (bright) in T2-weighted MRI.\textsuperscript{12} Coagulative necrosis has been suggested to correspond with responses to some antitumor treatment modalities\textsuperscript{13} but has so far not been associated with oncolytic viruses.

Magnetic resonance spectroscopy (MRS) is a noninvasive tool for gathering information on the metabolic and functional state of tissues. It measures the concentration of certain metabolites and thus can be used to indirectly evaluate parameters such as the amount of viable cells and the aggressiveness of tumors. It can thereby assist in the identification of malignant tumors that require intervention, and in the monitoring of treatment effects.\textsuperscript{14} Proton MRS produces a frequency spectrum of resonances that correspond to different molecular arrangements of $^1$H atoms. Tumors typically provide spectra with large peaks generated by water and lipids, but signals from other biochemical compounds, such as unsaturated fatty acids, choline, inositol and taurine, can be distinguished after suppression of the water signal (thus also the water content affects the results).

Choline-containing compounds are precursors and degradation products of cell membranes and produce a peak at 3.2 parts per million (ppm, unit of frequency in MRS). An increase in the resonances arising from choline-containing compounds is seen with pronounced cell membrane synthesis and has been shown in multiple settings to predict the aggressiveness of tumors.\textsuperscript{15} Taurine is a free amino acid. Several studies have indicated that, similar to choline, it can serve as a biomarker for tumor presence. High taurine in tumor measured by MRS seems to suggest malignant growth (bladder,\textsuperscript{16} breast,\textsuperscript{17} brain,\textsuperscript{18} prostate\textsuperscript{19} and colon\textsuperscript{20}), whereas some publications seem to suggest that low levels in blood serum indicate the presence of tumor (endometrium\textsuperscript{21} and breast\textsuperscript{22}).

Finally, some data also suggest that unsaturated fatty acids released from tumor cell membranes (thereby becoming better detectable by $^1$H MRS) might correlate with induction of tumor cell apoptosis\textsuperscript{23} or autophagy.\textsuperscript{24}

To evaluate T2-weighted MRI and proton MRS in assessing treatment efficacy during oncolytic virotherapy, we have used Syrian hamster ($Mesocricetus auratus$) tumor models. In contrast to other commonly used laboratory animal species, hamsters have been reported semipermissive for human adenovirus and responsive to human granulocyte–macrophage colony-stimulating factor (GM-CSF).\textsuperscript{25–29} In summary, MRI and MRS have the potential of finding tumors, evaluating their aggressiveness and monitoring treatment benefit. This, however, has not been studied before in the context of oncolytic adenoviruses.

What’s new?

Using oncolytic viruses and other immunological therapies against tumors could be a lot more successful if clinicians had a way to identify which patients would benefit from such treatments. Could magnetic resonance imaging (MRI) and spectroscopy (MRS) provide assistance? In this paper, the authors tested the ability of MRI and MRS to show how tumors responded to oncolytic viruses. Both in a hamster model and in a human patient, the imaging and spectroscopy data provided good indications of how the treatment was proceeding, suggesting these tools might be more broadly useful for monitoring the early stages of treatment with oncolytic viruses.

Magnetic resonance imaging (MRI) has been shown to provide good contrast between different soft tissues without exposure to ionizing radiation. T2-weighted MRI can visualize swelling, because water that has accumulated in extracellular spaces (vasogenic edema) increases the T2 relaxation time, which appears bright on the resulting images. In some tumors, T2 signal intensity is influenced by coagulative or liquefactive necrosis.\textsuperscript{11} In coagulative necrosis, cell injury leads to the denaturation of proteins and lysosomal enzymes while tissue architecture is preserved. The necrotic area is hypointense (dark) in T2-weighted MRI as it is dense and dry. In liquefactive necrosis, tissue architecture is lost and cells are replaced by viscous, often lipid-rich material, which appears hyperintense (bright) in T2-weighted MRI.\textsuperscript{12} Coagulative necrosis has been suggested to correspond with responses to some antitumor treatment modalities\textsuperscript{13} but has so far not been associated with oncolytic viruses.

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Material and Methods

Animals and viruses

Syrian golden hamsters ($M. auratus$) were ordered from Harlan (Haslett, MI) and quarantined for 2 weeks. EU animal regulations (Animal licence ESAVI-2010-09782/Ym-23) were followed and hamsters were sacrificed when the diameter of the tumor was more than 2 cm or when any suffering was observed.

HaP-T1 (hamster pancreatic carcinoma) cells (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and DDT1-MF-2 (hamster leiomyosarcoma) cells (American Type Culture Collection) were grown under recommended conditions in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% serum, 1% penicillin and streptomycin.

Ad5-D24 and Ad5-D24-GM-CSF virus were used in this study. Ad5-D24 (previously published with the name of Ad5Delta24TK-GFP)\textsuperscript{30} is an oncolytic virus that has a 24-bp deletion in the Rb-binding site of the E1A region. This enables the virus to replicate only in pRb pathway-disrupted cells (pRb pathway disruption is a phenomenon seen in most cancer cells\textsuperscript{31}). Ad5-D24-GM-CSF\textsuperscript{29} has the same deletion, but also harbors a GM-CSF-coding area, enabling it to produce...
this immunostimulatory cytokine after entering cells. GM-CSF is transcribed under the E3 promoter, which results in replication-associated transgene expression from \( \sim 8 \) hr after infection. E3 is intact except for a deletion of 6.7K/gp19K.

Viruses were grown to stocks of similar particle concentration (Ad5-D24-GM-CSF: \( 9.5 \times 10^{10} \) VP/ml, \( 2 \times 10^{11} \) pfu; Ad5-D24 2 \( 10^{12} \) VP/ml, \( 5 \times 10^{10} \) pfu).

Hamsters were shaven at the flanks. A solution of 10e7 HaP-T1 or 5 \( \times \) 10e6 DDT1-MF-2 cells in 50 \( \mu \)l DMEM was injected subcutaneously with a 0.5-ml syringe at both peritoneal flanks. After 7 (HaP-T1) or 14 (DDT1-MF-2) days, tumors had developed and \( \sim 50\% \) of these were sufficiently large (more than 20 mm\(^3\)) to perform imaging that would allow the spectrum to be evaluated for choline in a reasonable time (<30 min). HaP-T1 hamsters with larger tumors were randomized to the in vivo MRS groups, whereas hamsters with smaller tumors were randomized to the ex vivo MRS groups. Only little difference in the randomized HaP-T1 average tumor volumes between in vivo groups were observed at Day 0 [Ad5-D24-GM-CSF 25 mm\(^3\), Ad5-D24 29 mm\(^3\) and phosphate-buffered saline (PBS) 32 mm\(^3\)], while some difference was seen with the randomized DDT1-MF-2 tumors (GM-CSF 137 mm\(^3\), Ad5 41 mm\(^3\) and PBS 140 mm\(^3\)).

Hamsters were treated with a single injection of PBS or 1 \( \times \) 10\(^9\) virus particles per tumor (a virus dose per kg equivalent to that generally given to patients in Advanced Therapy Access Program) in 50 mm\(^3\) PBS with a 0.5-ml syringe.

**Magnetic resonance imaging and spectroscopy, in vivo follow-up**

The in vivo tumor MRI and MRS scans were obtained immediately before the treatment (Day 0) and at Days 2, 4, 7, 14 and 21 postvirus treatment. As commonly seen with human tumors, these tumors also exhibited some necrosis at the core and border. As commonly seen with human tumors, these tumors also exhibited some necrosis at the core and border postvirus treatment. As commonly seen with human tumors, these tumors also exhibited some necrosis at the core and border postvirus treatment. As commonly seen with human tumors, these tumors also exhibited some necrosis at the core and border.

Animals were anesthetized using isoflurane (1.5\%, O\(_2\)/N\(_2\)/30/70\% as carrier gas) during the MR scanning, and the body temperature was maintained at 37°C with a water-heated holder.

MRI and MRS were performed in a 7-T PharmaScan magnet (Bruker BioSpin MRI, Germany) with ParaVision 5.1 software, using a quadrature volume transmitter coil and a small single loop surface receiver coil, 10 mm in diameter.

Anatomical images of the tumors were acquired with multislice T2-weighted fast spin-echo sequence TR 2,500 msec, TE 44 msec, two averages, 256 \( \times \) 256 data matrix, 25.6 mm \( \times \) 25.6 mm FOV (100-\( \mu \)m in-plane resolution), 15 slices (collected interleaved), thickness 0.8 mm (gap 0), and using fat suppression preparation, scan time 2 min 40 sec.

Maximal MRS voxel (2–184 \( \mu \)l) that was carefully placed inside the tumors excluding the edges and the surrounding fat was used in each case. Voxel shimming procedure yielded quite variable line widths of 17 to \( \sim 50 \) Hz owing to the heterogeneous tumor composition, which also prevented the use of automated shimming approaches. No respiratory gating was needed, as the breathing did not cause movement to the subcutaneous tumors on the lower back. A single voxel \(^1\)H spectra were obtained with PRESS sequence, TR 2,500 msec, TE 12 msec, 128–512 averages depending on the tumor size, outer volume suppression preparation was used, water suppression by VAPOR and reference water peak was acquired with identical parameters without water suppression and no averaging. Quantification itself is kept as straightforward as possible and as a consequence of the high variation only the most robust group differences reach significance. The scanning time for a small tumor (<5 mm diameter) was 22 min (512 averages) and for the larger tumors (>15 mm diameter) was 5.5 min (128 averages). Absolute metabolite concentrations (in mM) were obtained using the unsuppressed tissue water spectrum (corrected for T2 decay, see below) as a reference and assuming a water volume percentage of 80%.

To quantify the T2 relaxation of water within the spectroscopy voxel, an array of water signals was acquired with TE 14, 18, 24, 30, 40, 80 and 160 msec (PRESS, TR 4,000 msec and two averages), and a T2 decay curve was fitted to the data. The ratio of \( M(t = 14\) msec)/\( M_0 \) was used as a correction coefficient to take into account the differences in T2 decay of the reference water peak between groups [\( M_0 \) is the signal intensity before any T2 decay and it is assessed from the fitted T2 decay curve at \( t = 0 \), \( M(t = 14\) msec) was approximated to be equal to \( M(t = 12\) msec) used in metabolite measurement].

**Ex vivo tumor MRS (see Supporting Information materials and methods)**

**Patient MRI.** The single patient reported here was imaged with a 1.5 T [respiratory-triggered turbo spin echo (TSE), TR 6,900/TE 94 and 5-mm slice thickness] GE Signa-MK, Oulu University Hospital, Finland. The patient was treated in an individualized Advanced Therapy Access Program, regulated by the Finnish Medicines Agency FIMEA as determined by EC/1394/2007. His parents signed written informed consent. Virus was administered intravenously and intratumorally in ultrasound guidance. This case had been reported previously.

**Analysis and statistics.** MRI analysis: In addition to qualitative image analysis, quantitative analysis was performed as follows: Tumors were manually outlined and their volumes were assessed. Using the image with the largest cross section of the tumor, the area of the dark core region and the total tumor area were outlined. The edge of the “core” region was identified by visual evaluation, where the signal intensity threshold of voxels included within the core corresponded to approximately mean – 2*SD of the signal intensity of viable surrounding tumor tissue. In Figure 2b the relative core to total area was used for calculations; similar significant results could also be found using absolute values (data not shown).

Analysis of both in vivo and ex vivo MRS data was done using PERCH NMR software (PERCH Solutions, Finland).
and assigned peak lineshapes were fitted individually (line-widths were not fixed). Integrals were normalized to the water peak assessed from the same voxel and to the number of averages, thereby taking into account the differences in spectroscopy voxel size. The ex vivo MRS peaks were normalized to processed tissue sample weight and quantified based on the known concentration of the added compound, 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt (TSP).

All values are indicated as mean ± standard error of the mean (SEM). For statistical analyses, SPSS software was used (IBM SPSS statistics, version 19). Differences between groups were calculated using Student’s t-test for two independent samples, and differences between time points by that of two related samples.

**Histology and immunohistology.** HaP-T1 tumors (Day 4: PBS N = 2, Ad5-D24 N = 0, Ad5-D24-GM-CSF N = 2; Day 8: PBS N = 2, Ad5-D24 N = 2, Ad5-D24-GM-CSF N = 3; 3 weeks: PBS N = 4, Ad5-D24 N = 0, Ad5-D24-GM-CSF N = 0) from the ex vivo MRS groups were halved and one half fixed in 4% buffered paraformaldehyde (PFA; pH 7.4) for 24 hr, then placed into 70% ethanol and routinely paraffin wax embedded. The DDT1-MF-2 tumors (Day 4: PBS N = 1, Ad5-D24 N = 2, Ad5-GM-CSF N = 2; Day 7: PBS N = 5, Ad5-D24 N = 2, Ad5-GM-CSF N = 6; Day 14: PBS N = 0, Ad5-D24 N = 3, Ad5-GM-CSF N = 1) were excised with margins and fixed in 4% buffered paraformaldehyde (PFA; pH 7.4) for 24 hr, then placed into 70% ethanol and routinely paraffin wax embedded. Sections (3–5 μm) were prepared and stained with hematoxylin–eosin (HE) for histological examination or were used for immunohistology.

Immunohistology was performed for the identification of infiltrating T cells (shown to be the predominant lymphocytes in the tumors; data not shown) and the expression of calprotectin (endogenous activator of toll-like receptor 4, S100A8/S100A9), a marker of monocytes, neutrophils and early differentiation stage macrophages that can also be induced in, among other cells, mature macrophages, fibroblasts and microvascular endothelial cells and is suspected to play a role in cancer by exerting both an antitumor or tumor/metastasis-promoting activity. The following cross-reacting primary antibodies were used: rabbit anti-human CD3 (Dako, Glostrup, Denmark) and mouse anti-human calprotectin (clone MAC387; AbDserotec, Düsseldorf, Germany; cross reaction with heterophils of guinea pigs). The streptavidin peroxidase method with heat pretreatment (citrate buffer pH 6.0) for antigen retrieval and diaminobenzidzin as chromogen was applied, following previously published protocols.

The T-cell infiltration was assessed semiquantitatively, based on the number of disseminated T cells within intact tumor areas (i.e., absence of necrosis and/or hemorrhage). This was done in a single blinded manner so that the pathologist did not know which group the tumor belonged to. T-cell numbers were graded as follows: low, low to medium, medium, medium to high or high. These were later given values 1, 2, 3, 4 or 5 from which the statistics were performed.

**Results**
Ad5-D24-GM-CSF reduces tumor volume
Tumors were treated with a single intratumoral injection of an armed virus (Ad5-D24-GM-CSF), unarmed virus (Ad5-D24) or PBS on Day 0. As known from previous experiments, oncolytic viruses, even when lacking anarming device, are expected to have antitumor efficacy, which can be potentiated by arming the virus by active transgenes such as GM-CSF. In the carcinoma model tumor volumetry revealed a significant reduction in the size of tumors treated with Ad5-D24-GM-CSF (N = 7, Day 4 p < 0.05 and thereafter p < 0.01) in comparison to those injected with PBS (control group; N = 7). For the unarmed oncolytic Ad5-D24 virus (N = 3), the average tumor volume did not significantly differ from that of the control group at any time point, although a trend of slower tumor growth was seen in the first week (Figs. 1a and 1b). In the sarcoma model (Figs. 1b and 1c), 50% (N = 4) of the armed virus (Ad5-D24-GM-CSF)-treated tumors seemed to respond partially to the treatment, as indicated by reduced growth, while all other tumors (Ad5-D24-GM-CSF N = 4, Ad5-D24 N = 7, PBS N = 6) showed faster growth (Day 4: Ad5-D24 p = 0.002, PBS p = 0.006, nonresponding Ad5-D24-GM-CSF p = 0.0001; Day 7: Ad5-D24 p = 0.001, PBS p = 0.03, nonresponding Ad5-D24-GM-CSF p = 0.01). Ad5-D24-GM-CSF seemed to reduce tumor growth more than Ad5-D24 on Day 7 (p = 0.03) but no difference was observed on Day 4 (p = 0.8). Although Ad5-D24-treated and the nonresponding tumors seemed to slow the tumor growth compared to PBS on Day 4 (p = 0.03 and p = 0.006, respectively) the significance was lost on Day 7 (p = 0.2 and p = 0.1, respectively). A significant growth difference between Ad5-D24-GM-CSF- and PBS-treated tumors was seen both on Days 4 (p = 0.03) and 7 (p = 0.03).

**Treatment responders**
In the carcinoma model, four of seven tumors treated with Ad5-D24-GM-CSF and one of three tumors treated with Ad5-D24 started to decrease in size within 7 days post-treatment. These tumors are referred to as “treatment responders,” whereas the remainder are referred to as “nonresponders.” A comparison of the volumetric growth of both groups is shown in Figure 1b. All responding tumors became undetectable by MRI between Days 14 and 28, whereas other tumors exhibited consistent growth. In the sarcoma model, the four tumors that responded partially are referred to as “partial responders.”

In summary, these results suggest that GM-CSF arming of the virus leads to varying immunological antitumor efficacy. With most, but not all, of the carcinomas treated with the
armed virus the tumors disappeared completely and the ham-

sters were cured.

MRI follow-up of carcinomas identifies treatment responders by alterations in T2 contrast

Treatment response was associated with changes consistent with coagulative necrosis before a decrease in tumor size. In T2-weighted MRI, the majority of tumors were heterogeneous, often showing focal hypointensities (dark areas in the MRI), consistent with coagulative necrosis. In the treatment responder group all tumors (N = 5) exhibited a relatively large (>1/4 of the tumor area) dark core both at 2 and 4 days after treatment, whereas in nonresponder (N = 5) and PBS (N = 7) groups, tumors exhibited either significantly smaller (p < 0.001 for both days) or no dark cores at all (Fig. 2b).

At later time points (Days 7, 14 and 21), a core of intermediate signal intensity was detected by T2-weighted MRI in all nonresponding tumors (N = 5) and in all PBS-injected tumors (N = 7) from Day 14 onward. This was not seen with any of the responding tumors (N = 5). All responding tumors retained the dark core at all later time points. The distinct core type of intermediate signal intensity was viscous (as

Figure 1. Tumor volume over time, quantified by MRI. (a and b) Carcinoma model and (c and d) sarcoma model. (a) In the untreated (PBS) control group, the tumor growth is most rapid. The group treated with the armed virus (Ad5-D24-GMCSF) shows the slowest tumor growth. The tumor volumes of the Ad5-D24 group did not differ from that of the untreated (PBS) group. (b) In five treated carcinoma tumors (four with armed Ad5-D24-GMCSF and one with unarmed Ad5-D24), the volume peaks at Days 2, 4 or 7 and starts to decrease thereafter (responders). In five treated tumors (three with armed Ad5-D24-GMCSF and two with unarmed Ad5-D24), there is no such response and consistent growth is observed (nonresponders). The tumor volumes of nonresponders did not differ from that of the untreated (PBS) group. (c) The armed virus group (Ad5-D24-GMCSF) seemed to reduce tumor growth more than the unarmed Ad5-D24 virus (p = 0.03). (d) With four sarcoma tumors (all treated with armed Ad5-D24-GMCSF virus) a partial response was observed as indicated with a significantly slower tumor growth compared to PBS on Day 4, but significance was lost on Day 7. *p < 0.05, **p < 0.01, differences between Ad5-D24-GMCSF/treatment responders/partial responders when compared to the untreated (PBS) control group. Values presented as mean ± SEM.
confirmed when cutting the tumor), had a high lipid content, which was also confirmed by \textit{ex vivo} MRS analysis (Supporting Information). The observed features are consistent with liquefactive necrosis.\textsuperscript{11–13} Taken together, the results suggest that response to oncolytic virus is associated with coagulative necrosis, seen already at early time points, whereas liquefactive necrosis develops at a later stage, likely as a consequence of rapid tumor growth.

T2 relaxation measurements of water in spectroscopy voxels revealed shorter T2 times in treatment responders than in PBS and nonresponder groups (d2–d7, \( p < 0.01 \), Fig. 3a). In responders, T2 relaxation times decreased over time, whereas they increased in the other groups (Fig. 3a). This T2 shortening may indicate a decreased amount of free water and matches the observation of coagulative necrosis in the tumor core, as coagulative necrosis is associated with cellular dehydration and protein denaturation. In the sarcoma model, MRI follow-up detected acute hemorrhages and hemorrhagic necrosis in large regions of the tumors. This led to signal loss preventing similar T2 quantification and spectroscopy of the entire tumor as achieved for carcinomas. Nevertheless, virus treatment postponed the first signs of hemorrhagic necrosis in MRI.
Figure 3. *In vivo* MR spectroscopy. (a) T2 relaxation time (quantified from MRS water peak). T2 is decreased in treatment responders from Day 2 onward. This is in line with the development of coagulative necrosis in the tumor core. Thus, quantitative T2 decrease might be useful as a marker for effective treatment. Grouping according to treatment response: significant decrease in responders vs. untreated (PBS) tumors Days 2, 4 and 7 (**p < 0.01; d14 p = 0.07, N = 2), and responders vs. non-responders Days 4 and 7 (##p < 0.01; d14 p = 0.06). (b) A representative *in vivo* spectrum, unsaturated fatty acids, taurine and choline peaks assigned (note also large lipid and cholesterol compound peaks at 0.7–2.8 ppm). The inserted MRI image shows the location of the spectroscopy voxel within the tumor. (c) Taurine, unsaturated fatty acids and choline were significantly lower in responders than in untreated (PBS) tumors or non-responders. Values presented as mean ± SEM. *p < 0.05 responders vs. untreated (PBS) tumors; #p < 0.05 responders vs. non-responders. No differences were seen between non-responders and untreated (PBS) tumors.
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To optimize the signal-to-noise ratio in 1H MRS, maximal treatment unsaturated fatty acids in carcinomas responding to MRS revealed lower levels of taurine, choline and the most significant time periods for each metabolite are in bold.

Table 1. Orientation (arrow pointing direction) and amount (number of arrows) of change between indicated time periods

<table>
<thead>
<tr>
<th>Days</th>
<th>Water T2</th>
<th>Taurine</th>
<th>Choline</th>
<th>Unsaturated fatty acids</th>
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<td>4–14</td>
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<td>Responders</td>
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The most significant time periods for each metabolite are in bold.

MRS revealed lower levels of taurine, choline and unsaturated fatty acids in carcinomas responding to treatment

To optimize the signal-to-noise ratio in 1H MRS, maximal voxel size fitting into the tumor was used (Fig. 3b). The voxel included all areas of the tumor, including heterogeneous regions featuring focal necrosis, stroma and neoplastic cell aggregates. Thus, the results represent the average amount inside the voxel. The relatively narrow water line widths (16–50 Hz) obtained at early time points indicated the absence of marked hemorrhage or a hematoma. A representative in vivo tumor spectrum (Fig. 3b and Supporting Information Fig. S1) showed high lipid content and three distinct peaks, which were subsequently chosen for further analysis (Fig. 3c): taurine (3.4 ppm), unsaturated fatty acids (5.4 ppm) and choline (3.2 ppm, comprising glycerophosphocholine, phosphocholine, free choline and myoinositol; see Supporting Information ex vivo MRS results for details).

The MRS data were grouped and analyzed either according to the treatment response (Fig. 3) or according to the type of treatment (Supporting Information Fig. S2). The graphs are somewhat similar as the treatment responder curve mimics the curve of the Ad5-D24-GM-CSF group, whereas the nonresponder curve mimics that of the Ad5-D24 group.

At 2 and 4 days post-treatment, both PBS (Tau: \(p = 0.26\), tCho: \(p = 0.09\)) and nonresponder (Tau: \(p < 0.05\), tCho: \(p = 0.08\)) showed signs of increase in taurine and choline levels in comparison to pretreatment levels (Day 0; baseline), whereas no growth was seen in the responder group.

On Days 4 (\(p < 0.05\)) and 7 (\(p < 0.05\)), the only significant difference between responders and nonresponders (both virus treated) was seen with choline. When comparing responders with PBS-injected tumors, significant differences (\(p < 0.05\)) were seen with all metabolites (taurine, choline and unsaturated fatty acids) on Day 7 post-treatment. These MRS results indicate that low levels of any of these metabolites can suggest treatment efficacy (Fig. 3 and Table 1), but with choline the results are most prominent.

At Day 14 post-treatment, the nonresponding group showed a sixfold increase (\(p = 0.06\)) in unsaturated fatty acids from that on Day 7, whereas the levels in the responding group remained at baseline. Thus, unsaturated fatty acids might be the best metabolite to analyze at 2 weeks. The rebound seen with the nonresponders might indicate recovery from the primary oncolysis and a gain in rapid tumor growth, perhaps owing to the development of resistance to the virus\(^7\) (Fig. 1b).

At 3 weeks post-treatment, responders exhibited baseline or lower levels of all metabolites and in fact most tumors disappeared, whereas the nonresponders and the PBS group showed levels threefold higher than baseline. The further increase in antitumor efficacy beyond 2 weeks post-treatment might result from adaptive immunity\(^29\).

Ex vivo histology of treated carcinomas confirms the presence of multifocal necrosis and a substantial heterophil-dominated inflammatory response

Ex vivo analysis displayed individual necrotic neoplastic cells and often also multifocal, variably sized areas of coagulative necrosis (Fig. 4a). In addition, large areas of liquefactive necrosis were seen, mainly in PBS-treated tumors (Fig. 4b). T cells (CD3\(^+\)) and heterophils (a neutrophil equivalent in hamsters; calprotectin\(^+\)) were the dominant carcinoma-infiltrating leukocytes. Although T cells were mainly disseminated throughout the tumors, heterophils were particularly numerous within areas of necrosis (Fig. 4c). Some tumors, especially in the Ad5-D24-GM-CSF treatment group, showed pronounced heterophil infiltration in necrotic tissue close to areas of necrosis (Figs. 4c and 4d). This could be the result of increased heterophil recruitment in response to either necrosis resulting from oncolysis or due to the release of GM-CSF from infected tumor cells\(^38\). Extensive focal hemorrhage, which could in theory have explained the dark cores seen in MRI, was not observed histologically in the carcinoma model. For more detailed reporting of the histology, please see Supporting Information results.

Histology of the sarcomas revealed pronounced T-cell infiltration in partially responding tumors

Histological and immunohistological examination showed that heterophils and macrophages (calprotectin\(^+\)) were sparse. T cells (CD3\(^+\)) were found disseminated between intact neoplastic cells and, often slightly more numerous, in the tumor periphery. Interestingly, the partially responding (Ad5-D24-GM-CSF treated) tumors (\(N = 4\)) exhibited a more intense T-cell infiltration than the other tumors (Fig. 4, right
The difference was significant in comparison to tumors that did not respond to the virus treatment \((N = 12, p = 0.013)\) or those injected with PBS \((N = 6, p = 0.007)\), where the T-cell infiltration was least intense, but not in comparison to Ad5-D24-GM-CSF-treated nonresponders \((N = 5, p = 0.1)\).

Figure 4. (a) Ad5-D24-GMCSF virus-treated carcinoma, Day 4 post-treatment. Focal area of coagulative necrosis, indicated by stars, with retained outline morphology of necrotic cells. (b) PBS-treated carcinoma (control), Day 20 post-treatment. The tumor exhibits large focal areas of necrosis with complete loss of cellular morphology and the presence of amorphic eosinophilic (proteinaeous) material (liquefactive necrosis, indicated by stars). (c) Ad5-D24-GMCSF virus-treated carcinoma, Day 8 post-treatment. Extensive focal necrosis (indicated by stars) with complete loss of cell structure and predominantly cell-free calprotectin expression, originating from necrotic heterophils. Cell-bound calprotectin expression is seen in intact heterophils in areas of necrosis adjacent to viable tumor tissue (arrows) and in the stroma of viable tumor tissue (arrowheads). (d) Ad5-D24-GMCSF virus-treated carcinoma, Day 8 post-treatment. Area of transition between focal area of necrosis with marked cell-free calprotectin expression (indicated by stars) and viable tumor tissue, exhibiting increased numbers of (calprotectin-positive) heterophils close to the areas of necrosis (arrows). Right column. Demonstration of T cells (CD3+) infiltrating the intact neoplastic sarcoma tissue on Day 7. Top: an armed Ad5-D24-GMCSF virus-treated tumor, showing a high number of infiltrating T cells. Middle: an unarmed Ad5-D24-treated tumor with a low number of disseminated T cells. Bottom: PBS-treated tumor with a low number of infiltrating T cells. HE stain was used in panels a and b, while streptavidin peroxidase method, Meyer's hematoxylin counterstain was used in other panels. Bars = 50 μm.
A patient responding to oncolytic virus treatment showed signs of coagulative necrosis in T2 MRI

Pretreatment and post-treatment MRI scans were available from one patient treated with oncolytic adenovirus in an Advanced Therapy Access Program. N21 was a 6-year-old boy with advanced neuroblastoma diagnosed in April 2007. He had been treated with three different chemotherapy regimens and an autologous stem cell transplant but had shown progressing disease. Oncolytic virus therapy with Ad5/3-Cox2L-D24 elicited a partial response in injected tumors and a complete response in the noninjected bone marrow. Similar to the responding tumors in the experimental hamster study, the tumor became darker in the two following T2-weighted MRI scans (Fig. 5), which is compatible with the hamster data. Three months later, cancer cells were detected in the bone marrow and a second oncolytic virus treatment...
was given in June 2008. Four months later, tumor progression was seen in MRI, and this was associated with a distinctly lighter contrast in the tumor (Fig. 5).

Discussion

Our study was performed to test the utility of MRI and MRS in identifying the response to oncolytic adenovirus. Our results indicate that T2 contrast change and T2 water relaxation time are promising methods for the identification of a treatment response at early time points. Also, these parameters could easily be adopted clinically as they can be measured with widely available conventional MRI equipment. Although more time consuming and technically more demanding, MRS of metabolites such as taurine, choline and unsaturated fatty acids might provide more detailed information on virus replication, GM-CSF production, cell lysis, immune reaction and different types of necrosis.

The antitumor effects mediated by a GM-CSF-coding adenovirus can be roughly divided into three phases. Immediately after virus injection, innate antiviral responses and effects mediated by GM-CSF—including NK cell recruitment—can be expected, while oncylisis might be most prominent in the subsequent week. The adaptive immune system (primed by oncylisis and GM-CSF) would likely contribute only after a few weeks. Although these effects are expected to overlap, we aimed to study each phase separately by examining the tumors at 0–4 days, 4–14 days and later (Table 1).

In the early phase (0–4 days), even before volumetric differences between groups emerge, differences can be seen in the water T2 relaxation time and with choline and taurine metabolites. In fact, a distinct separation is present already 2 days after treatment. Increases in taurine and choline levels have been described as associated with tumor growth and aggressiveness.\(^{15–19,22}\) Therefore, lower taurine and choline remain significant in the subsequent week. The adaptive immune system (primed by oncylisis and GM-CSF) would likely contribute only after a few weeks. Although these effects are expected to overlap, we aimed to study each phase separately by examining the tumors at 0–4 days, 4–14 days and later (Table 1).

In the intermediate phase (4–14 days), when effects are seen also in the tumor volume, progressive differences in water T2 relaxation time, choline and taurine remain significant. Interestingly, a sixfold increase in unsaturated fatty acids was seen in nonresponding tumors from 1 week onward. The reason for this remains unknown, but it coincides with a change in the slope of the tumor size curve; at early time points the slope of nonresponding tumors is distinct from PBS-treated control tumors, whereas after 2 weeks it is identical. Thus, fatty acid increase might correlate with resistance to therapy.

In the late phase (14–21 days), all metabolite concentrations in the PBS and nonresponder groups continued to be twofold to fourfold higher than baseline, thus constituting potentially useful markers to study treatment responses in clinical trials with oncolytic viruses.

As typical for cancer patients, our results suggest that some hamsters responded to treatment, whereas others did not. Also, as no difference was seen between treated nonresponders and the PBS group, some tumors might harbor currently unknown features that limit the efficacy of oncolytic adenovirus. Powerful surrogate endpoints, such as described here, could be useful for the identification of such factors. Ultimately, predictive or prognostic biomarkers would facilitate clinical translation.

We found that a decreased T2 time might be a promising predictive marker for treatment response. “Dry” areas with coagulative necrosis induce T2 shortening. T2 measurement from a single voxel is a fast and simple procedure, and even acquiring a quantitative T2 map with high spatial resolution is easy to implement in a clinical setting.

With regard to MRI, we discovered that a hypointense dark core appears in responding, but not in unresponsive tumors. Although further studies would be of utility, the available literature and our histological results suggest that this phenomenon could relate to the presence of coagulative necrosis, as opposed to liquefactive necrosis, which was seen chiefly in nonresponding and PBS-treated control tumors especially at later time points. If this data can be confirmed, and extended to humans, a hypointense tumor area appearing in T2-weighted MRI, and persisting thereafter, could be a sign of antitumor efficacy, and could possibly serve as a predictive biomarker. Our observations on the single patient reported here lend support to this concept, but formal clinical studies are needed to address this in more detail.

To our knowledge, there are no previous reports on the appearance of a dark core in tumors responding to oncolytic adenovirus, when imaged with T2-weighted MRI. However, a similar necrotic center has been described in patient tumors treated with vaccinia virus coding for GM-CSF.\(^{4,40}\) As the authors were using contrast-enhanced dynamic MRI, a method that is most suitable for studying vascular phenomena, they concluded that signal decrease resulted from acute vascular shutdown. Similarly, our MRI results suggest that responders develop hypointense “dry” areas in the tumor where the quantity of free water is diminished, a feature first seen as darkening and then as shrinking of the tumor. Thus, our data do not contradict the vascular shutdown hypothesis. Instead, it is possible that two phenomena contribute to the findings of both groups. Oncylisis and GM-CSF expression may cause both vascular shutdown and coagulative necrosis and it is not inconceivable that the former could contribute to the latter. These phenomena are distinct from the “normal” liquefactive necrosis seen in rapidly growing advanced tumors, which is possibly a consequence of hypoxia and concurrent neovascularization. In fact, an interesting hypothesis for future studies would be that oncylisis has a prominent effect against vascular growth factors, causing acute vascular shutdown and inhibiting tumor progression-related new vessel formation.\(^{41,42}\)
In our study, the type and extent of the inflammatory response seen in tumors was assessed. The immunohistochemical demonstration of calprotectin, a well-established marker for neutrophils/heterophils, monocytes and macrophages, identified a generally substantial heterophil (neutrophil in humans) recruitment into carcinoma tissue, both disseminated and in association with necrotic areas. At this point, it cannot be determined whether this represents an immune response against the tumor or whether tumor cell necrosis due to viral oncology together with GM-CSF is the dominant factor in the recruitment of these cells. However, calprotectin is known as a pivotal amplifier of infection, is secreted by activated phagocytes and is recognized by TLR-4 on monocytes. Its release leads to increased recruitment of leukocytes into inflamed tissues and direct activation of infiltrating phagocytes by calprotectin-induced cytokines. Accordingly, its release from degenerate heterophils within necrotic tumor areas—as observed in our study—might potentiate the inflammatory response.

Although the heterophil recruitment appears to be the main immune response in the carcinoma model, this was almost entirely lacking in the sarcoma model, where a pronounced T-cell infiltration was observed in partially responding tumors. This might be due to adaptive immune responses generated by the Ad5-D24-GMCSF virus. As a significant difference compared to the nonresponding virus-treated tumors was observed, we believe that this immune response is not just against virus but could also be against the tumor. The reasons for the lack of heterophil recruitment into the sarcomas are not known. As this phenomenon was observed in all treatment groups we believe this to be a model-specific finding, highlighting differences in the immunology of different tumors and tumor types. It might, however, contribute to the quantitative difference in the response between the models.

In summary, our data suggest that noninvasive in vivo MRI and MRS can be used at early time points to identify response to treatment with oncolytic adenovirus coding for GM-CSF. By integrating functional imaging with conventional size-based analysis in a clinical trial setting, these imaging modalities could be studied further with regard to their utility as biomarkers.

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