Carbon ion radiotherapy decreases the impact of tumor heterogeneity on radiation response in experimental prostate tumors

Christin Glowa a,b,c,*, Christian P. Karger b,c, Stephan Brons c,d, Dawen Zhao e, Ralph P. Mason e, Peter E. Huber a,c,f, Jürgen Debus a,c, Peter Peschke c,f

a Department of Radiation Oncology, University Hospital Heidelberg, Heidelberg, Germany
b Department of Molecular Radiation Oncology, German Cancer Research Center (DKFZ), Heidelberg, Germany
c National Center for Radiation Oncology (NCRO), Heidelberg Institute for Radiation Oncology (HIRO), Heidelberg, Germany
d Department of Radiation Oncology, University of Texas Southwestern Medical Center, Dallas, Texas, USA
e Department of Medical Physics in Radiation Oncology, German Cancer Research Center (DKFZ), Heidelberg, Germany
f Heidelberg Ion Beam Therapy Center (HIT), Heidelberg, Germany

ABSTRACT

Objective: To quantitatively study the impact of intrinsic tumor characteristics and microenvironmental factors on local tumor control after irradiation with carbon (12C-) ions and photons in an experimental prostate tumor model.

Material and Methods: Three sublines of a syngeneic rat prostate tumor (R3327) differing in grading (highly (-H) moderately (-HI) or anaplastic (-AT1)) were irradiated with increasing single doses of either 12C-ions or 6 MV photons in Copenhagen rats. Primary endpoint was local tumor control within 300 days. The relative biological effectiveness (RBE) of 12C-ions was calculated from the dose at 50% tumor control probability (TCD50) of photons and 12C-ions and was correlated with histological, physiological and genetic tumor parameters.

Results: Experimental findings demonstrated that (i) TCD50-values between the three tumor sublines differed less for 12C-ions (23.6–32.9 Gy) than for photons (38.2–75.7 Gy), (ii) the slope of the dose-response curve for each tumor line was steeper for 12C-ions than for photons, and (iii) the RBE increased with tumor grading from 1.62 ± 0.11 (H) to 2.08 ± 0.13 (H) to 2.30 ± 0.08 (AT1). The relative biological effectiveness (RBE) of 12C-ions was correlated with histological, physiological and genetic tumor parameters.

Conclusion: The response to 12C-ions is less dependent on resistance factors as well as on heterogeneity between and within tumor sublines as compared to photons. A clear correlation between decreasing differentiation status and increasing RBE was found. 12C-ions may therefore be a therapeutic option especially in patients with undifferentiated prostate tumors, expressing high resistance against photons.

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Introduction

Although radiotherapy with high-energy photons is one of the major treatment strategies for cancer, tumor response is sometimes transient, and therapy may fail due to recurrence of resistant tumor cells. Indeed, tumors are highly dynamic systems expressing morphological and physiological heterogeneity [1]. Clinically, such heterogeneity influences therapeutic responses, especially in radiotherapy leading to rather shallow dose-response curves for tumors. Currently, clinical decisions on the prescribed radiation dose are still population-based rather than individually, mainly because the influence of tumor-associated factors on tumor response is not sufficiently understood.

After initial experience with different ion types in at the Lawrence Berkeley Laboratory (USA) [2], carbon (12C-) ion radiotherapy was introduced during 1994 in Japan [3,4] followed by Europe [5] in 1996. The favorable depth-dose profile of 12C-ions (Bragg-peak), together with the highly advanced beam scanning technique allows the dose to be tailored to the tumor, while sparing the surrounding normal tissue [5]. Besides this gain in geometrical accuracy, the higher linear energy transfer (LET) of 12C-ions with its more clustered local energy deposition is believed to be the main reason for the increased efficacy, which is described by the relative biological effectiveness (RBE), using the response to photons as reference.

Resistance against photon therapy is associated with both intrinsic cellular factors conditioned by the evolutionary capacity of cancer phenotypes and epigenetic parameters, such as the temporal and spatial heterogeneity of the tumor microenvironment caused by dysfunctional blood flow, low pH and hypoxia [6,7]. Resulting
consequences are impairment of the DNA repair machinery, cell-cycle dysregulation, inhibition of cell death pathways and the upregulation of the endogenous cell stress system. In addition, ionizing radiation generates ROS, promotes tumor cell repopulation and exerts proangiogenic effects via the activation of prosurvival signaling cascades [8]. Intrinsic factors (e.g., genetic instability, mutation rate, and epigenetic status) and extrinsic factors (e.g., microenvironmental factors and therapy) that shape intratumor heterogeneity also influence therapeutic responses by creating tumors with a higher diversity of phenotypes for selection. Many therapies fail to eliminate all tumor cells, and recurrent cells often display greater genetic instability or emerge biological properties that lead to resistance.

As promising clinical results [3,5] suggest that high-LET particles might overcome classical photon therapy resistance factors, dose-response studies were performed in three experimental prostate tumor sublines differing in grading. The most important finding was that doses required for local tumor control differed significantly and the dose-response curves were steeper for 12C-ions, as compared to photons. As both indicate a minor impact of tumor heterogeneity on therapy outcome after 12C-ion therapy, dose-response parameters were correlated with known resistance factors like undifferentiation, hypoxia, vessel maturity and stem cell content.

Material and methods

Tumor model

Fresh fragments of tumor tissue of the syngeneic Dunning prostate adenocarcinoma sublines R3327-H, -H8 and -AT1 [9] were implanted subcutaneously in the distal right thigh of young adult male Copenhagen rats (weight 180–200 g, Charles River Laboratories Inc., Wilmington, Massachusetts, USA); All experiments were approved by the governmental review committee on animal care, and animals were kept under standard laboratory conditions. During irradiation of H- and HI-tumors, rats were kept under inhalation anesthesia with a mixture of 2.5% sevoflurane (Abbott, Wiesbaden, Germany) and oxygen at 2 l/min using an inhalation mask. In AT1-irradiation studies animals were anesthetized with an intraperitoneal injection of Ketamine hydrochloride (125 mg/kg, Pfizer Deutschland GmbH, Berlin, Germany) mixed with Xylazine hydrochloride (20 mg/kg, Bayer HealthCare AG, Leverkusen, Germany) and breathed air [10].

Irradiation setup

The experimental setup is essentially the same as previously published [10,11]. Rats were placed in a special device for accurate positioning of the tumor (see details in Reference [10]). The mean tumor diameter at treatment was 10.5 mm (range 9.0 mm to 12.0 mm). Photon irradiations were performed using a single 6MV beam of a linear accelerator (Siemens, Erlangen, Germany) with a 15 mm diameter at the isocenter (90% isodose). For 12C-ions, the tumor was positioned in the center of a single 20 mm spread-out Bragg-peak (SOBP) having a field diameter of 16.5 mm (90% isodose). The mean dose-averaged LET in the tumor was 75 keV/μm (range 64–96 keV/μm).

In the present study, 178 animals with H- or HI-tumors were irradiated with increasing single doses of photons or 12C-ions, respectively (Table 1). 36 sham-treated animals served as controls. Primary endpoint was local tumor control within 300 days, defined as no detectable tumor regrowth. Tumor volume was measured routinely using a caliper. As some tumors showed recurrences near to 300 days, the actual observation time exceeded 300 days and in case of regrowth, the interpolated starting point of regrowth was used to decide whether the tumor was controlled.

For the HI-tumor three recurrences occurred after 300, but within 320 days. Therefore, local control within 320 days was used as secondary endpoint for this tumor. While locally controlled AT1- and HI-tumors regressed completely, residual fibrotic nodules remained in case of locally controlled H-tumors. These nodules were extracted and investigated histologically for fibrotic pattern (Hematoxylin/Eosin) and proliferation (BrDU). A fibrotic pattern without proliferation was considered as secondary endpoint for locally controlled H-tumors (Supplementary Fig. S1).

Dose response analysis

For the endpoint “local tumor control”, actuarial control rates were calculated and the logistic dose-response model was fitted using the maximum likelihood fitting procedure of the software STATISTICA (version 10.0, Statsoft Inc., www.statsoft.com) (see References [10,11] for details). Incomplete follow-up of animals was considered in the fitting procedure using the method of effective sample sizes that corrects in the correlating the fit parameters was considered. In addition, 90% confidence intervals (CIs) were calculated using Fieller’s theorem [14]. The differences of the fit parameters between tumor sublines were tested using the two-sided Mann–Whitney U test for independent samples as the Shapiro–Wilk test revealed significant deviations from normal distribution for some of the underlying data. Values of p < 0.05 and p < 0.0005 were considered as significant and highly significant, respectively.

Results

Dose-response

Fig. 1 shows the adjusted dose-response curves for tumors of the three tumor sublines and all endpoints. The corresponding
TCD\textsubscript{50} and RBE-values are given in Table 2. Mean tumor regression (complete tumor volume reduction) times for the H-, HI- and AT1-tumor were ≥300 d, 42 ± 1.7 d and 110 ± 4.7 d for photons, and ≥300 d, 44 ± 1.7 d and 80 ± 2.0 d for \textsuperscript{12}C-ions, respectively. While locally controlled AT1- and HI-tumors regressed completely, tiny nodules remained in case of locally controlled H-tumors. As the H-tumors exhibit very long volume doubling times, treatment response was protracted as well and recurrences beyond the 300-day follow-up could not be ruled-out, even if no indication of regrowth was seen within this period. Using lack of proliferative activity together with a fibrotic pattern as secondary histological endpoint, the dose-response curves (Fig. 1 dotted line) were shifted markedly by 10.1 Gy for photons and only 3.2 Gy for \textsuperscript{12}C-ions (TCD\textsubscript{50}).

Three important findings can be derived from the dose-response study: (i) as quantified by the TCD\textsubscript{50}-values (Fig. 2A), the dose-response curves for all three tumor sublines (Fig. 1) were located much closer to each other for \textsuperscript{12}C-ions (23.6–32.9 Gy) than for photons (38.2–75.7 Gy), (ii) the slope of the dose-response curve (Fig. 2B) for each subline was steeper for \textsuperscript{12}C-ions than for photons, and (iii) the RBE increased with tumor grading (i.e. H vs. HI vs. AT1), independent of the considered endpoint (Fig. 2C). This RBE-increase is a direct consequence of the fact that the increase of TCD\textsubscript{50} with tumor grading is much stronger for photons than for \textsuperscript{12}C-ions.

Characterization of sublines

HE-stained histological sections of H-, HI-, and AT1-tumors exhibited marked phenotypical differences (Fig. 3A–C). H-tumors showed high stromal density and tubular structures similar to those found in normal prostate tissues. In contrast, HI-tumors tended to form irregular ring-like structures filled with large amounts of mucin, and AT1-tumors were found to be anaplastic without tubular structures. Thus, glandular differentiation was associated with slow tumor growth. Structural-functional characteristics of the three untreated tumor sublines are summarized in Fig. 3 and Table 3. Proliferation, measured as volume doubling time, decreased from 20 days for the well differentiated H- to 5 days for the anaplastic AT1-tumor (Fig. 3D–F, Table 3). For the HI-tumor both, the level of differentiation as well as the volume doubling time was intermediate. Typical for the H-tumors was a rather mature vasculature with prominent pericytes (Fig. 3G), associated with a high blood perfusion and little to no hypoxia (Fig. 3J), and a rather broad distribution of pO\textsubscript{2}-values. HI-tumors, although showing highest amount of CD31+ vascular structures with an appropriate diameter, were characterized by a lack of pericytes (Fig. 3H). Nevertheless, these tumors were well perfused with only restricted hypoxic regions (Fig. 3K), also depicted by a narrower pO\textsubscript{2}-distribution in the histogram (Fig. 3N). In contrast, few peripheral mature vessels and many small immature capillaries in central regions are the cause for extensive chronic hypoxia in AT1-tumors (Table 3, Fig. 3L). Ploidy patterns, analyzed by flow-cytometry, appeared characteristic for each subline. While the two better differentiated HI- and H-tumors contained a prominent subpopulation of peridiploid cells with a DNA-labeling index of 1.07 and an aneuploidic cell fraction with a concordant DNA-index of 1.8, the AT1-tumor was characterized by a high amount of aneuploidic cells in the near tetraploid range (Table 3). In a semi-quantitative double staining approach, expression patterns of putative stem cell surface markers (CD24\textsuperscript{+}/CD44\textsuperscript{+}/CD133\textsuperscript{+}/CD326\textsuperscript{+}) correlate with tumor grading inasmuch as the number of positive cells increase with a higher amount of genomic alterations.

Discussion

In the present study, a decreased dependence on tumor grading and steeper dose-response curves for \textsuperscript{12}C-ions were found. Consequently, the RBE increased significantly with tumor grading. While
non-controlled anaplastic AT1-tumors [11] recurred within 180 days, some HI-tumors showed regrowth around day 300. Extending the follow-up time, however, had no significant influence on TCD\textsubscript{50} and RBE (Table 2) and hence, the RBE at 300 days is considered as reliable. The extremely slow turnover of the differentiated H-tumor, with its occasionally remaining tissue nodules caused a less distinct tumor status at 300 days. We therefore used lack of proliferative activity within these nodules as secondary but still growth-related endpoint. Although the respective TCD\textsubscript{50}-values increased for both photons and \textsuperscript{12}C-ions, the RBE differed only non-significantly from that of the primary endpoint local control. The local control-based RBE of the H-Tumor can therefore be considered as reliable.

The increase of RBE with tumor grading (Fig. 2C) is primarily caused by a significantly higher radiation resistance against photons of the less differentiated tumor sublines, while the tolerance against \textsuperscript{12}C-ions remains much less affected (Fig. 2A). It has to be noted that although the TCD\textsubscript{50}-values still differ significantly (marginally for AT1 vs. HI and highly for AT1 and HI vs. H) for \textsuperscript{12}C-ions, the differences are quantitatively much smaller than for photons. In addition, the dose-response curves for the same tumor were generally steeper for \textsuperscript{12}C-ions than for photons (Fig. 2B), indicating that the effectiveness of \textsuperscript{12}C-ions is less dependent on biological heterogeneity not only between sublines, but also within tumors of the same subline.

Transferring this result to patients suggests that tumor-specific radiosensitivity factors, which prevent therapeutic success in photon radiotherapy, can be at least partially overridden by \textsuperscript{12}C-ions. To elucidate the relative impact of the different resistance factors in \textsuperscript{12}C-ion therapy as well as to identify possible underlying molecular mechanisms, detailed characterization of tumors prior and after irradiation is required. Although this was beyond the scope of the present study, the compilation of tumor characteristics in Table 3 presents candidates for these radiosensitivity factors.

Key biological features governing tumor responses to photon radiation include tumor hypoxia, DNA damage repair, angiogenesis/vasculogenesis, cancer stem cells, tumor stroma, and the immune response pathways. In contrast to in vitro studies with cell populations, possessing single defined inherent or acquired photon therapy resistance patterns, it is presently not feasible to fully assess the significance or the contribution of each biological factor to the complex dynamics of therapy resistance in vivo, where all factors interact simultaneously.

The rise of therapy resistance to photons from highly differentiated H-tumors to anaplastic AT1-tumors is associated with a

Table 2

TCD\textsubscript{50} and RBE-values for three tumor sublines of the R3327 prostate carcinoma measured in this and a previous study [11].

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>TCD\textsubscript{50} ± SE (90% CI) [Gy]</th>
<th>RBE ± SE (90% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-Tumor (this study)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LC (300 d)</td>
<td>38.2 ± 1.8 (34.0–41.1)\textsuperscript{a}</td>
<td>23.6 ± 1.1 (21.5–25.8)</td>
</tr>
<tr>
<td>Histo\textsuperscript{a}</td>
<td>48.3 ± 2.8 (41.6–54.6)</td>
<td>26.8 ± 1.0 (24.9–28.8)</td>
</tr>
<tr>
<td>HI-Tumor (this study)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LC (300 d)</td>
<td>62.4 ± 3.2 (56.6–68.9)\textsuperscript{b}</td>
<td>30.0 ± 1.1 (27.5–32.6)</td>
</tr>
<tr>
<td>LC (320 d)\textsuperscript{c}</td>
<td>63.9 ± 3.9 (57.5–74.7)</td>
<td>31.6 ± 1.6 (28.6–35.5)</td>
</tr>
<tr>
<td>AT1-Tumor [11]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LC (300 d)</td>
<td>75.7 ± 1.6 (69.9–78.6)\textsuperscript{d}</td>
<td>32.9 ± 0.9 (30.8–34.9)</td>
</tr>
</tbody>
</table>

Uncertainties are given as single standard errors and 90% confidence intervals. Endpoints for this dose-response study were “local tumor control (LC) within 300 d or 320 d” or “histological tumor control” (Histo).

\textsuperscript{a} All differences in TCD\textsubscript{50} between photons and \textsuperscript{12}C-ions for the same tumor and endpoint are highly significant (p < 0.0005).
\textsuperscript{b} All differences in TCD\textsubscript{50} at LC (300 d) between tumor sublines are highly significant (p < 0.0005).
\textsuperscript{c} Differences in TCD\textsubscript{50} at LC (300 d) relative to the H-tumor are highly significant (p < 0.0005), but only significant (p < 0.05) for HI vs AT1.
\textsuperscript{d} Differences in RBE at LC (300 d) relative to the H-tumor are highly significant (p < 0.0005) for the AT1 - and significant (p < 0.05) for the HI-tumor.

\textsuperscript{e} All differences in TCD\textsubscript{50}, RBE and HRE relative to LC (300 d).
\textsuperscript{f} No significant difference for RBE and HRE relative to LC (300 d).

Table 3

Resistance factors in the three sublines of the R3327 prostate tumor model.

<table>
<thead>
<tr>
<th>Heterogeneity factors</th>
<th>H-tumor</th>
<th>HI-tumor</th>
<th>AT1-tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume doubling time</td>
<td>&lt;20 days</td>
<td>=10 days</td>
<td>=5 days</td>
</tr>
<tr>
<td>Vascularization</td>
<td>Many big mature vessels</td>
<td>Big immature vessels</td>
<td>Less vessels, mostly immature</td>
</tr>
<tr>
<td>Number of CD31\textsuperscript{+}-vessels</td>
<td>18.35 ± 0.85</td>
<td>22.93 ± 1.91\textsuperscript{a}</td>
<td>17.31 ± 1.43</td>
</tr>
<tr>
<td>Vessel diameter [μm]</td>
<td>14.13 ± 0.26</td>
<td>14.14 ± 0.46</td>
<td>9.07 ± 0.28\textsuperscript{a}</td>
</tr>
<tr>
<td>CD31\textsuperscript{a}-area [%]</td>
<td>2.17 ± 0.23</td>
<td>4.06 ± 0.02\textsuperscript{e}</td>
<td>2.45 ± 0.17</td>
</tr>
<tr>
<td>SMA\textsuperscript{a}-area [%]</td>
<td>0.71 ± 0.11</td>
<td>0.01 ± 0.01\textsuperscript{e}</td>
<td>0.24 ± 0.07</td>
</tr>
<tr>
<td>Amplitude weighted pO\textsubscript{2} [torr]</td>
<td>136.0\textsuperscript{f}</td>
<td>52.0\textsuperscript{f}</td>
<td>14.0\textsuperscript{f}</td>
</tr>
<tr>
<td>Hypoxic fraction [pO\textsubscript{2} &lt; 10 mmHg, HFr\textsubscript{p}]</td>
<td>1%</td>
<td>1.3%</td>
<td>18%</td>
</tr>
<tr>
<td>Subpopulations\textsuperscript{f}: aneuploidic cells</td>
<td>Diploid (DI 1.0: 33.5%), peripidiploid (DI 1.07: 59.1%), hypotetraploid (DI 1.43: 7.4%)</td>
<td>Diploid (DI 1.0: 15.3%), peripidiploid (DI 1.07: 82.2%), hypotetraploid (DI 1.18: 72.3%)</td>
<td>Diploid (DI 1.0: 66.5%), hypotetraploid (DI 1.18: 33.5%)</td>
</tr>
<tr>
<td>Subpopulations\textsuperscript{f}: potential stem cell marker (CD24, CD44, CD133, CD326)</td>
<td>2.9–6.0% of cells</td>
<td>6.6–20.3% of cells</td>
<td>15.3–24.6% of cells</td>
</tr>
</tbody>
</table>

CD31\textsuperscript{+} and SMA-staining are expressed in values ± SE. The amount of vessels was calculated as absolute number of CD31\textsuperscript{+}-vessels per microscopic image (200× magnification). Aneuploidic tumor cells and potential stem cell markers were characterized by flow-cytometry.

\textsuperscript{a} Significant (p < 0.05) relative to H- and AT1-tumor.
\textsuperscript{b} Highly significant (p < 0.0005) relative to H- and HI-tumor.
\textsuperscript{c} Highly significant (p < 0.0005) relative to H- and AT1-tumor.
\textsuperscript{d} Differences in RBE at LC (300 d) relative to the H-tumor are highly significant (p < 0.0005) for HI vs AT1.
\textsuperscript{e} Differences in RBE at LC (300 d) relative to the H-tumor are highly significant (p < 0.0005) for HI vs AT1.
\textsuperscript{f} All differences in TCD\textsubscript{50}, RBE and HRE relative to LC (300 d).

Animals breathing oxygen.

Animals breathing air.
Fig. 2. Dependence of the dose-response parameters for photons and $^{12}$C-ions on the tumor subtype. The increase of radiation tolerance with increasing tumor grading is stronger for photons than for $^{12}$C-ions (A). For a given tumor grading, the slope of the dose-response curve is steeper for $^{12}$C-ions than for photons (B). The effectiveness of $^{12}$C-ions relative to photons increases with increasing tumor grade (C). These results are essentially independent of uncertainties resulting from the endpoint definition.

significant decrease in p53 [16] together with higher bcl-2-levels in anaplastic AT1-tumors [17]. Prostatic progression is often associated with changes in interactions between the androgen-dependent stromal microenvironment and tumor cells, with loss of smooth muscle cells and appearance of carcinoma-associated fibroblasts [18,19]. HI- and AT1-tumors exhibit minor requirements for stromal interaction and their malignant stroma may contribute more to maintaining the three-dimensional tumor architecture [20] than to keeping a differentiated tumor phenotype.

Tumor growth and differentiation strongly depend on blood supply, vascularization, and angiogenesis [21]. Accordingly, in the Dunning model, a negative correlation of tumor malignancy with vessel maturity, accompanied by a reduced perfusion and higher levels of interstitial pressure in anaplastic tumors, all powerful triggers of therapy resistance was observed [22]. Both differentiated tumors are normoxic, typically with $pO_2 \geq 52$ Torr and small hypoxic fractions $HF_{10} \approx 1\%$ (H) and $1.3\%$ (HI), especially when animals were breathing oxygen, as in the present study. When rats were breathing air, as in case of experiments with AT1-tumors, extensive signs of hypoxia with $pO_2$-values around 14 Torr and a hypoxic fraction $HF_{10} = 18\%$ are typical. These intratumoral heterogeneities clearly influence the steepness of dose-response curves (Fig. 3A–B). $^{12}$C-ions induce a higher level of clustered DNA-damage [23], due to a more dense energy deposition within small areas of DNA-sites. The average number of lesions per cluster tends to increase with increasing LET [24] and is considered the most important contributor to the higher effectiveness of $^{12}$C-ions [25]. Cellular repair proficiency may also be relevant. Mutant cells, possessing hampered DSB-repair capacity, do not exhibit large RBEs [26], but alterations of DNA-damage processing exhibit minor resistance to $^{12}$C-ions [27]. Finally, deregulated cell death pathways, such as survivin and bcl-2 overexpression that protect cells from apoptosis may account for therapeutic resistance [28]. Although not an absolute requirement, a higher bcl-2 protein expression is clearly associated with the progression to an androgen-independent metastatic phenotype in human tumors [29], as well as in the Dunning system [17]. The more serious damage produced by high-LET radiation might counteract such cell protective features [30,31].

Compared to photons, the response to $^{12}$C-ion treatment generally occurred faster, even in differentiated, slow growing tumors. Hence, radiation resistance mediated via fibroblasts, vascular elements and immunological factors [7], which limit treatment efficacy by providing a protective environment seems to be of minor importance. The role of tumor vasculature is difficult to judge because blood vessels are highly affected by radiotherapy, especially by high single doses [32]. Moreover, evidence emerged that high-LET irradiation enhances apoptosis by activation of Caspase-3 through Caspase-9, even in the presence of mutated p53, raising the idea of two distinct types of cell death [33,34].

A differential response of resting or slowly cycling cells is another option for the increased effectiveness of $^{12}$C-ions. As irradiation preferentially inactivates proliferating cells, it is postulated that tumor recurrence results partly from the regrowth of quiescent tumor cells that could not be sufficiently killed [35]. In contrast, for $^{12}$C-ions a more efficient inhibition of recovery in quiescent cells was detected in preclinical experiments [36,37] and in a clinical study [38].

Finally, subsets of cancer stem cells (CSCs) are assumed to be responsible for radioresistance [39–42]. While in human prostate cancer, the existence of CSCs is a matter of debate, several candidate cell populations were identified in the Dunning system [15]. For selected human tumors a higher radioresistance of CSCs due to either increased activation of DNA-repair or upregulated free radical scavenging systems is described [43,44]. Proficient DNA-damage and higher intracellular ROS-levels in CSCs make particles a promising strategy to overcome radioresistance of CSCs [45,46].
Conclusion
Intrinsic tumor characteristics contributing to radioresistance against photons have minor impact for high-LET irradiations. Candidates for these resistance factors are DNA-repair capacity, p53-status, cell death regulation, as well as microenvironmental factors. As shown in an experimental prostate model, tumor control by $^{12}$C-ions is less dependent on tumor heterogeneity. In case of unclear resistance factors to photons, $^{12}$C-ions may therefore provide a therapeutic advantage.

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Fig. 3. Comparison of H- (A,D,G,J,M), HI- (B,E,H,K,N) and AT1-tumors (C,F,I,L,O). Structural changes and the differentiation status are detected by Hematoxylin / Eosin (HE) staining (A-C). Proliferating cells were BrdU-labeled (red, D-F) and cell nuclei counterstained with DAPI (blue, D-I). Vessel diameter, structure and maturity were evaluated by staining endothelial cells with CD31 (red) and smooth muscle actin (SMA, green, G-I). For visualization of hypoxic areas, pimonidazole was used (brown, J-L). Magnification: 200×; Scale bars: 50 μm. Quantitative MRI-measurements of the oxygen concentration (FREDOM) were used to determine $pO_2$-maps (M-O, inlets). For a representative tumor of each subline, $pO_2$-values were converted to histograms.

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Conflict of interest
The authors disclose no potential conflicts of interest.

Appendix: Supplementary material
Supplementary data to this article can be found online at doi:10.1016/j.canlet.2016.05.013.