ACTA ONCOLOGICA
2023, VOL. 62, NO. 11, 1581–1586
https://doi.org/10.1080/0284186X.2023.2238550

Taylor & Francis
Taylor & Francis Group

ORIGINAL ARTICLE

Using immunotherapy to enhance the response of a C3H mammary carcinoma to proton radiation

Steffen Nielsen[a], Mateusz K. Sitarz[b], Priyanshu M. Sinha[a], Charlemagne A. Folefac[a], Morten Høyer[b], Brita S. Sørensen[a,b] and Michael R. Horsman[a]

[a]Experimental Clinical Oncology-Dept. Oncology, Aarhus University Hospital, Aarhus, Denmark; [b]Danish Centre for Particle Therapy, Aarhus University Hospital, Aarhus, Denmark

ABSTRACT

Background: The benefit of combining immunotherapy with photon irradiation has been shown pre-clinically and clinically. This current pre-clinical study was designed to investigate the anti-tumour action of combining immunotherapy with protons.

Materials and Methods: Male CDF1 mice, with a C3H mammary carcinoma inoculated on the right rear foot, were locally irradiated with single radiation doses when tumours reached 200mm^3. Radiation was delivered with an 83–107MeV pencil scanning proton beam in the centre of a 3 cm spread out Bragg peak. Following irradiation (day 0), mice were injected intraperitoneal with anti-CTLA-4, anti-PD-1, or anti-PD-L1 (10 mg/kg) twice weekly for two weeks. Endpoints were tumour growth time (TGT3; time to reach 3 times treatment volume) or local tumour control (percent of mice showing tumour control at 90 days). A Student's T-test (tumour growth) or Chi-squared test (tumour control) were used for statistical analysis; significance levels of $p < 0.05$.

Results: Untreated tumours had a mean (± 1 S.E.) TGT3 of 4.6 days (± 0.4). None of the checkpoint inhibitors changed this TGT3. A linear increase in TGT3 was seen with increasing radiation doses (5–20 Gy), reaching 17.2 days (± 0.7) with 20 Gy. Anti-CTLA-4 had no effect on radiation doses up to 15 Gy, but significantly enhanced 20 Gy; the TGT3 being 23.0 days (± 1.3). Higher radiation doses (35–60 Gy) were investigated using a tumour control assay. Logit analysis of the dose response curve, resulted in a TCD50 value (radiation dose causing 50% tumour control; with 95% confidence intervals) of 48 Gy (44–53) for radiation only. This significantly decreased to 43 Gy (38–49) when mice were treated with anti-CTLA-4. Neither anti-PD-1 nor anti-PD-L1 significantly affected tumour control.

Conclusion: Checkpoint inhibitors enhanced the response of this C3H mammary carcinoma to proton irradiation. However, this enhancement depended on the checkpoint inhibitor and radiation dose.

ARTICLE HISTORY
Received 22 May 2023
Accepted 28 June 2023

KEYWORDS
C3H mammary carcinoma; proton radiation; checkpoint inhibitors; tumour growth delay; local tumour control

Background

Various immune checkpoint proteins prevent over-activation of the normal immune system [1]. Tumours also utilise such proteins to avoid the immune system [2], especially the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and programmed cell death protein 1 (PD-1) and its ligand (PD-L1). However, antibody inhibitors to these checkpoints have been developed and undergone testing in a variety of cancer sites, especially melanoma and lung [3,4], the results of which have resulted in unprecedented improvements in outcomes in patients with a spectrum of solid tumours. This has generally established immunotherapy as the fourth modality in cancer treatment after surgery, radiation, and chemotherapy. Despite these significant improvements, many patients actually do not respond to immunotherapy [5,6]. Consequently, considerable effort is underway to find alternative treatments that combined with checkpoint inhibitors could improve patient response. The ideal candidates for improving immune therapy are those that preferentially induce significant tumour damage leading to the release of tumour antigens. These are then identified by antigen-presenting cells, which subsequently prime and activate T-cells that attack the tumour [7]. Our own studies using the vascular disrupting agent OXi4503, which induces significant indirect tumour cell killing *via* the induction of damage to the tumour vascular supply, as well as through a direct cytotoxic mechanism, reported that it could convert a C3H mammary carcinoma model, unresponsive to checkpoint inhibitors, into a responder to these agents [8].

Radiation treatment is another excellent candidate for combining with checkpoint inhibitors [9]. Not only does it induce significant tumour damage, but there is evidence that the immune system plays a significant role in the response to radiation. Several pre-clinical studies have shown that tumours grown in animals that are immune suppressed are

CONTACT Michael R. Horsman mike@oncology.au.dk Experimental Clinical Oncology-Dept. Oncology, Aarhus University Hospital, Palle Juul-Jensens Blvd. 99, C108, DK-8200 Aarhus N, Denmark

substantially less radiation sensitive than the same tumours irradiated in mice with a fully functional immune system [10,11]. Consequently, numerous pre-clinical studies have investigated the potential of combining radiation with checkpoint inhibitors and demonstrated the benefit of this approach [12–14]. Many of these checkpoint inhibitors, in combination with radiation, are under clinical evaluation [15–17].

Protons have a superior physical dose distribution than photons/electrons [18]. This would, in turn, result in a more precise delivery of radiation dose to the tumours, with significantly lower normal tissue damage. The probability of killing immune cells in the surrounding area is also reduced [19], such that anti-tumour immunity is preserved. These facts make the use of protons an even more likely candidate for combination with checkpoint inhibitors. However, before applying such a combination in patients, pre-clinical studies are required to determine the optimal combination for the greatest therapeutic benefit.

Material and methods

Animal and tumour model

C3H mammary carcinomas, subcutaneously implanted in the right rear foot of male CDF1 mice (Purchased from Janvier Labs., Le Genest-Saint-Isle, France) that were 10 to 14 weeks-old, were used for all experiments. Details of the derivation and maintenance of this model has been described previously [20]. Basically, every three months tumour material, stored in liquid nitrogen, was thawed and implanted on the flanks of mice. When large, these tumours were excised and minced with a pair of scissors under sterile conditions. Tumour material could then either be implanted on the flanks of additional mice for continued passage or 5–10 μl of this material injected into the right rear foot of mice used for experimental studies. Experiments were initiated when foot tumours had reached approximately 200mm^3 in size. This volume was achieved about three weeks after inoculation and was calculated from the formula D1 x D2 x D3 x π/6, where the D values represent the three orthogonal diameters. Attempts were made to randomise the tumour bearing mice into the different radiation-dose groups. However, since the tumours grew at different rates, they did not achieve the 200mm^3 starting volume at the same time. Consequently, some selection was necessary to ensure that tumours starting treatment on the same day were distributed among the different treatment groups. All animal studies were conducted according to the animal welfare policy of Aarhus University (http://dyrefaciliteter.au.dk), and with the Danish Animal Experiments Inspectorate's approval (License number: 2021-15-0201-01008).

Checkpoint inhibitors

The checkpoint inhibitors were anti-CTLA-4 antibody (invivoMab anti-mouse CTLA-4[CD152], clone 9H10), anti-PD-1 antibody (invivoMab anti-mouse PD-1[CD279], clone RMP1-14), and anti- PD-L1 antibody (invivoMab anti-mouse PD-L1[B7-H1], clone 10 F.9G2), and all three were purchased from NordicBioSite/BioXCells (Kristiansand, Norway). They were freshly prepared before each experiment by dissolving in sterile phosphate-buffered saline (PBS) and intraperitoneally (i.p.) injected into mice at a constant injection volume of 0.02 ml/g mouse body weight. Drug treatments were started when tumours reached 200 mm^3 (day 0) and involved injecting a standard dose of 10 mg/kg on days 1, 4, 7, and 10 regardless of the antibody. This schedule was designed to mimic experimental settings used by others [12,21].

Tumour irradiation

Single dose tumour irradiations were given as described previously [22]. All irradiations to the tumour-bearing feet were given locally to the tumours of non-anaesthetised mice, which were restrained in specially constructed Lucite jigs; the tumour bearing legs being exposed and loosely attached to the jig with tape, without impairing the blood supply to the foot [23]. Proton beam irradiations were performed with a Varian AC250 (ProBeam, Varian/Siemans Healthineers, Palo Alto, CA, USA) system at the Danish Centre for Particle Therapy (Aarhus, Denmark) in a dedicated Experimental Room with a horizontal beam line. The set-up consisted of a temperature controlled (25 °C) water phantom on top of which up to three mice were positioned with their right hind leg submerged in the water at a distance of 7 cm from the phantom wall. Single fraction fields of pencil scanning proton beam were then delivered perpendicularly to the wall, with the field size (2.5 × 10 cm^2) and energies (83-107 MeV), covering all three legs within the Spread-Out Bragg Peak (SOBP; from 5.5 to 8.5 cm depth). The mice bodies were located outside the treatment volume and were additionally shielded from scattered radiation by a brass block (2 cm thick) positioned between the mice and the phantom wall. Treatment plans were prepared in ECLIPSE Treatment Planning System (Varian, Palo Alto, CA, USA) and tested prior to the experiment with gafchromic EBT-3 films (for the rescaled plan of 5 Gy in SOBP). Film measurements confirmed good field uniformity within the planned target volume (deviation $<$1% for films positioned at phantom wall and submerged at the depth of 7 cm) and acceptable dose scattered to mice body (less than 1% of SOBP dose at films positioned 1 cm above water surface, behind the brass block).

Response endpoint

The endpoints were either tumour growth delay or local tumour control, as described previously [23]. For the tumour growth delay estimates, tumour volume was determined daily from the day of irradiation and the tumour growth time (TGT3; time taken for tumours to reach 3 times the initial treatment volume) calculated. Results were shown as mean values ± 1 standard error (± 1 S.E.). With the tumour control assay, treated mice were observed on a weekly basis and the percentage of animals in each treatment group showing local tumour control at 90 days, determined. For radiation alone, and each radiation and checkpoint inhibitor combination, full

radiation dose-response curves were produced. From these curves, logit analysis allowed us to calculate the TCD50 dose (the radiation dose necessary to induce a response in 50% of animals), with 95% confidence intervals. The ratio of these values obtained for radiation alone and radiation with checkpoint inhibitor was used to calculate enhancement ratios (± 95% confidence intervals). A Student's T-test (tumour growth) or a Chi-squared test (tumour control) was used for statistical analysis, with the significance levels being $p < 0.05$ for both methods.

Results

The effect of radiation with/without anti-CTLA-4 on tumour growth inhibition are shown in Figure 1. Control, untreated tumours, had a mean (± 1 S.E.) TGT3 of 4.8 days (± 0.1). This TGT3 increased when tumours were irradiated with single doses of between 5 to 20 Gy, the effect becoming larger as the radiation dose increased. Using all individual TGT3 values at each radiation dose resulted in a linear increase in TGT3 with dose; the slope value being 0.68, with a correlation coefficient of 0.93. Injecting mice with anti-CTLA-4 (4×10 mg/kg), resulted in a TGT3 value of 4.4 days (± 0.3), which was not significantly (T-test; $p > 0.05$) different from the control value. Combining radiation and anti-CTLA-4 also resulted in a linear increase in TGT3 with radiation dose, but the slope value (0.93) was higher than that obtained for radiation alone; the correlation coefficient being 0.95. However, despite this excellent correlation, the use of a slope value is somewhat misleading, since there was no significant (T-test; $p > 0.05$) difference between the TGT3 values for radiation alone and radiation + anti-CTLA-4 for all doses between 0 and 15 Gy. Only at the 20 Gy radiation dose was there a significant (T-test; $p < 0.05$) difference between the radiation and the radiation with anti-CTLA-4 treatments; the respective TGT3 values being 17.2 days (± 0.7) and 23.0 days (± 1.3).

We investigated the potential effects of higher radiation doses using the endpoint of local tumour control and the results are shown in Figure 1 and Table 1. For radiation only, tumour control began to occur at doses above 40 Gy, reaching a maximum 100% value at 60 Gy. Logit analysis of this dose response curve resulted in a TCD50 value (radiation dose causing 50% tumour control; with 95% confidence intervals) of 48 Gy (44–53). Irradiating tumours and then treating the mice with anti-CTLA-4 shifted the dose-response curve to the left, with the calculated TCD50 value significantly (χ^2-test; $p < 0.05$) decreased to 43 Gy (38–49). Taking the ratio of the TCD50 values for radiation alone and radiation + anti-CTLA-4 allowed us to determine an enhancement ratio (with 95% confidence intervals), which was found to be 1.12 (1.01–1.25) and this enhancement was statistically significant (χ^2-test; $p < 0.05$; Table 1).

Table 1. Summary of the effect of combining radiation and checkpoint inhibitors on local tumour control.

Treatment	TCD50[a] (95% CI)	E.R.[b] (95% CI)	*p*-value
Radiation	48 Gy (44-53)	—	—
Radiation + anti-CTLA-4	43 Gy (38–49)	1.12 (1.01–1.25)	0.025–0.05
Radiation + anti-PD-1	48 Gy (44–53)	1.00 (0.91–1.09)	0.95–0.975
Radiation + anti-PD-L1	49 Gy (41–59)	0.98 (0.86–1.12)	0.70–0.80

All results were calculated from the data shown in Figures 1 and 2.
[a]Radiation dose to give 50% tumour control with 95% confidence intervals (95% CI).
[b]Enhancement ratio (TCD50 for radiation/TCD50 for radiation + checkpoint inhibitor) with 95% CI.

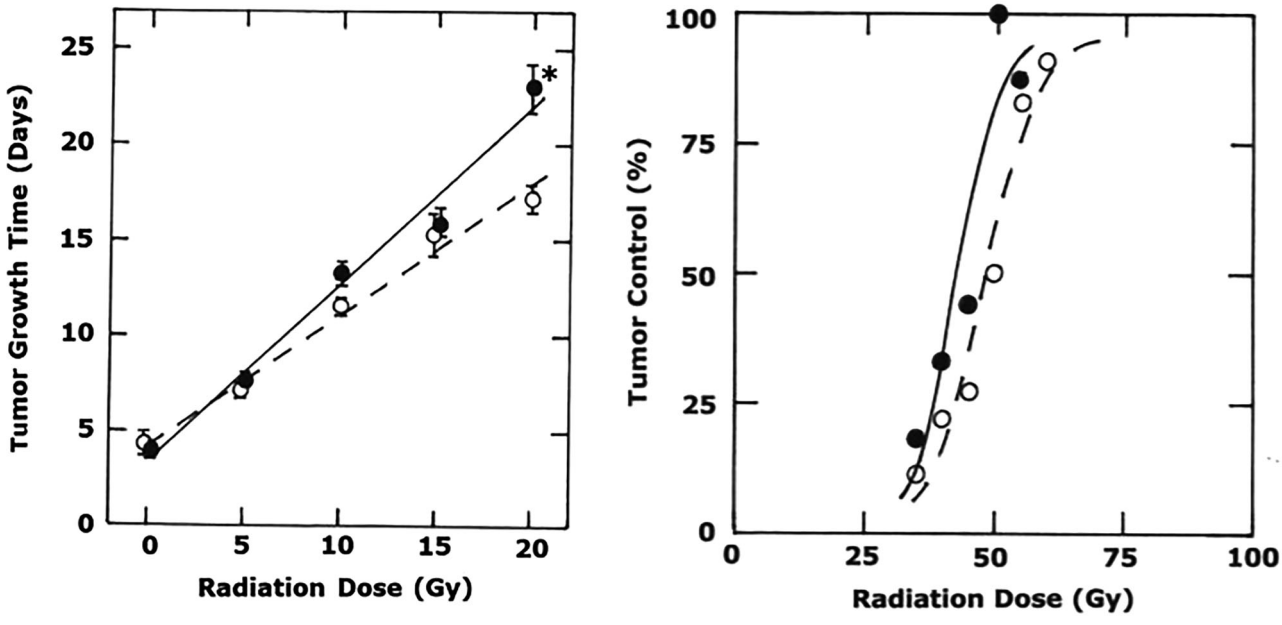

Figure 1. The effect of anti-CTLA-4 on the radiation response of a C3H mammary carcinoma. Tumour bearing mice were locally irradiated with graded single doses when foot implanted tumours had reached 200 mm^3 (day 0) and then given intraperitoneal injections with PBS or anti-CTLA-4 (10 mg/kg) on days 1, 4, 8, and 11. The left panel shows the endpoint of tumour growth time (time to reach 3 times the treatment volume), and show mean (± 1 S.E.) values for 7–8 mice per group with the lines fitted following linear regression analysis using individual data points (*indicates the radiation + anti-CTLA-4 values that were significantly different from radiation alone). The right panel shows percent local tumour control 90 days after treatment with the points based on 7–12 mice and the lines fitted following logit analysis. For both figures the symbols are for radiation alone (○) or radiation + anti-CTLA-4 (●).

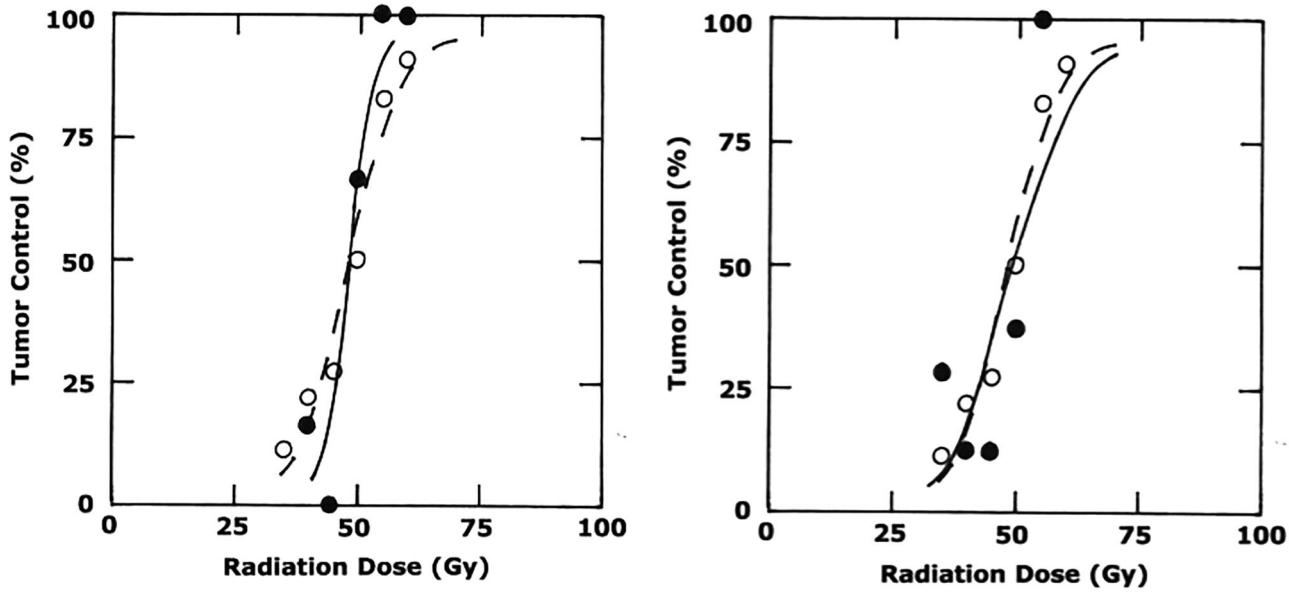

Figure 2. The effect of anti-PD-1 or anti-PD-L1 on the radiation response of a C3H mammary carcinoma. Tumour bearing mice were locally irradiated with graded single doses when foot implanted tumours had reached 200 mm^3 (day 0) and then given intraperitoneal injections with PBS or checkpoint inhibitors (10 mg/kg) on days 1, 4, 8, and 11. The results show percent local tumour control 90 days after treatment with the points based on 6–12 mice and the lines fitted following logit analysis. The left panel is for anti-PD-1 and the right panel for anti-PD-L1. For both figures the symbols are for radiation alone (○) or radiation + antibody (●).

Similar experiments were undertaken using anti-PD-1 and anti-PD-L1. When administered alone, neither agent had any significant (T-test; $p > 0.05$) effect on tumour growth compared to controls (data not shown). Neither anti-PD-1 nor anti-PD-L1 influenced local tumour control as shown in Figure 2. Here the radiation and the radiation with checkpoint inhibitor curves were almost identical. The respective TCD50 values, calculated from these curves, and the subsequent non-significant (χ^2-test; $p > 0.05$) enhancement ratios are summarised in Table 1.

Discussion

This C3H mammary carcinoma is generally considered to be non-immunogenic when implanted in CDF1 mice, since tumour take following transplantation is typically 100% and tumours reach the treatment size of 200 mm^3 typically within only two weeks. As in our previous study [8], we found this tumour model to be unresponsive to treatment with anti-CTLA-4, anti-PD-1, or anti-PD-L1, supporting the non-immunogenic suggestion.

This tumour model does respond to local tumour irradiation, with a nice dose-dependent response observed (Figure 1). When mice, with irradiated tumours, were subsequently injected with anti-CTLA-4, there was an increase in the slope of the radiation dose-response curve. However, this is somewhat misleading, since for all radiation treatments at 15 Gy and below, there was actually no statistically significant difference between the radiation-only values and the corresponding radiation + anti-CTLA-4 response. Only at 20 Gy did we observe a significant enhancement in radiation response when adding anti-CTLA-4. This suggests that for anti-CTLA-4 to be effective, the other combination treatment needs to induce a certain level of tumour damage.

To investigate the effects of radiation doses above 20 Gy we opted to use local tumour control as the endpoint (Figure 1); tumour control also being the more clinically relevant endpoint [24]. Here we clearly saw a change in the dose-response curve for radiation alone when we include anti-CTLA-4 in the treatment schedule and this change in response was statistically significant (Table 1). A previous study, in which radiation and anti-CTLA-4 were combined, showed limited effects with a 1×20 Gy treatment, but a superior effect when a lower radiation dose was administered in a fractionated schedule [13]. Anti-CTLA-4 clearly has an effect with single radiation-dose treatments in our C3H mammary model, but maybe even a greater effect would be possible if we applied radiation in a fractionated schedule. We certainly saw no effect of combining anti-CTLA-4 with 15 Gy irradiation or below, so maybe low doses per fraction are simply not able to induce sufficient damage to stimulate an immune response, suggesting that maybe one needs to apply high radiation doses in a limited fractionation schedule, such as seen with stereotactic treatments. Of course, one major difference between our study and those of others is that they used photon irradiation while we used protons and this may play a significant role. There are suggestions that there may be a difference in radiation damage produced by protons when compared to photons [25]. Protons result in clustering of energy deposition compared to the same dose of photons and this could lead to more complex DNA damage which could also be more difficult to repair. The increase in ionisation density with protons can also increase the level of reactive oxygen species when compared to the levels found with photon radiation, again affecting the level of DNA damage [25]. More significantly for our study, the differences in energy deposition between photons and protons, for the same radiation dose, have been suggested to result in differences in the inflammatory response [25] and this

could influence immunological effects. Indeed, recent *in vivo* studies reported proton modification of the immune tumour microenvironment [26,27]. One of those studies actually found an increase in the level of PD-L1 and that combining proton irradiation with anti-PD-L1 treatment enhanced tumour growth delay compared to that seen with protons alone [27]. Another major advantage of protons over photons is their superior physical dose distribution [18], which clinically should result in a reduction of the irradiated volume. This would also decrease the likelihood of irradiating and killing the host cells involved in the immune response found in areas surrounding the tumour [19].

What was surprising was that while we found an effect of anti-CTLA-4 on radiation response, the same was not seen with anti-PD-1/anti-PD-L1. Although CTLA-4 and PD-1 suppress T cell function they do so by different mechanisms; CTLA-4 preventing T cell activation and PD-1 suppressing effector T cell function further down the immune cascade [7,28]. For immune therapy to work one must overcome both blockages. One would expect that the higher the radiation dose the greater the degree of tumour cell killing and the subsequent higher release of antigens. However, so long as the CTLA-4 block exists, there will be no increase in T cell activation. Removing this initial block should increase the number of activated T cells, perhaps to a level sufficient to bypass the later PD-1/PD-L1 block. Eliminating the second block using anti-PD-1/PD-L1 alone would be insufficient because the CTLA-4 suppression of T cell activation remains in place.

The type of damage induced may also play a role. In the current study, there was only a benefit when combining proton radiation with anti-CTLA-4. Yet, in our previous study using the same C3H mammary carcinoma model where we combined vascular disrupting agents (VDAs) with checkpoint inhibitors, we found different results. Using combretastatin A-4 phosphate, which induces anti-tumour effects solely *via* the induction of vascular damage, a significant increase in tumour response to combretastatin A-4 phosphate was observed only when it was combined with anti-PD-L1, not anti-CTLA-4 nor anti-PD-1 [8]. However, with the combretastatin analogue OXi4503, which has dual functional activity, causing both a vascular mediated anti-tumour effect and direct tumour cell killing [29,30], the tumour response to OXi4503 was enhanced by both anti-CTLA-4 and anti-PD-L1, but not anti-PD-1. High dose radiation also kills tumour cells directly, but can also have an indirect cell killing effect *via* the induction of vascular damage [31]. Thus, it was a surprise that only anti-CTLA-4 worked with radiation. For OXi4503 the primary effect is vascular moderated, with the direct cell killing a secondary response that occurs due to oxidative activation to a cytotoxic quinone intermediate [29,30]. While for radiation, the primary effect is cell killing *via* the induction of DNA damage and the vascular effects secondary [31]. Histological and immunological assessment of T cell infiltration might have helped shed some light on why we observed such differences. However, this was not undertaken, because our previous study with OXi4503 showed a decrease in CD8+/CD4+ expression in C3H mammary carcinomas following drug treatment, an effect we attributed to a drug-induced reduction in tumour blood flow [8]. Interestingly, hyperthermia can also decrease tumour blood flow [32] and our unpublished results on CD8+/CD4+ expression following heat treatment showed similar decreases. Consequently, we have no reason to expect a different effect following tumour irradiation. Clearly, how the degree of cell killing, and the method of induced cell kill, influence the combination of protons with immune therapy, needs further investigation.

Pre-clinical and clinical studies have reported that larger benefits were possible if radiation was combined with checkpoint inhibitors that target both the CTLA-4 and PD-1/PD-L1 blockade [33–35]. Whether any additional enhancement would be possible with such a combination in our C3H mammary carcinoma tumour when we were only able to enhance the proton response when using anti-CTLA-4, is not clear. It might require a multimodality approach combining different checkpoint inhibitors and radiation with another treatment, like VDAs, that have an alternative mechanism of action, but are also known to enhance radiation response, such as we previously reported in this C3H mammary carcinoma model [36].

Acknowledgements

The authors thank Ms. Dorthe Grand, Ms. Maria Lynnerup Bech, and Ms. Marianne Kristiansen for excellent technical help with the experiments and animal care.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by the Danish National Board of Health's research pool to strengthen research in immunotherapy of cancer under grant number J.nr. 4-1612-236/10; project 0300 and Kræftens Bekæmpelse (grant number: R302-A17231).

Data availability statement

Data presented in this study are available on request from the corresponding author. The data are not publicly available because the results from these experiments and all other animal experiments at our institute are stored in a single data depository, therefore, access is limited to relevant qualified personnel only.

References

[1] Salama AKS, Moschos SJ. Next steps in immuno-oncology: enhancing antitumor effects through appropriate patient selection and rationally designed combination strategies. Ann Oncol. 2017;28(1):57–74. doi: 10.1093/annonc/mdw534.

[2] Dunn GP, Bruce AT, Ikeda H, et al. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol. 2002;3(11):991–998. doi: 10.1038/ni1102-991.

[3] Callahan MK, Postow MA, Wolchok JP. CTLA-4 and PD-1 pathway blockade: combinations in the clinic. Front Oncol. 2014;4:385. doi: 10.3389/fonc.2014.00385.

[4] Antonia SJ, Villegas A, Daniel D, et al. Durvalumab after chemoradiotherapy in stage III non-small-cell lung cancer. N Engl J Med. 2017;377(20):1919–1929. doi: 10.1056/NEJMoa1709937.

[5] Syn NL, Teng MWL, Mok TSK, et al. De-novo and acquired resistance to immune checkpoint targeting. Lancet Oncol. 2017;18(12): e731–741–e741. doi: 10.1016/S1470-2045(17)30607-1.
[6] Postow MA, Callahan MK, Wolchok JD. Immune checkpoint blockade in cancer therapy. J Clin Oncol. 2015;33(17):1974–1982. doi: 10.1200/JCO.2014.59.4358.
[7] Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. Immunity. 2013;39(1):1–10. doi: 10.1016/j.immuni.2013.07.012.
[8] Horsman MR, Wittenborn TR, Nielsen PS, et al. Tumors resistant to checkpoint inhibitors can become sensitive after treatment with vascular disrupting agents. Int. J. Mol. Sci. 2020;21:4778. doi: 10.3390/ijms21134778.
[9] Lauber K, Brix N, Ernst A, et al. Targeting the heat shock response in combination with radiotherapy: sensitizing cancer cells to irradiation-induced cell death and heating up their immunogenicity. Cancer Lett. 2015;368(2):209–229. doi: 10.1016/j.canlet.2015.02.047.
[10] Stone HB, Peters LJ, Milas L. Effect of host immune capability on radiocurability and subsequent transplantability of a murine fibrosarcoma. J Natl Cancer Inst. 1979;63(5):1229–1235.
[11] Lee Y, Auh SL, Wang Y, et al. Therapeutic effects of ablative radiation on local tumor require $CD8^+$ T cells: changing strategies for cancer treatment. Blood. 2009;114(3):589–595. doi: 10.1182/blood-2009-02-206870.
[12] Demaria S, Kawashima N, Yang AM, et al. Immune-mediated inhibition of metastases after treatment with local radiation and CTLA-4 blockade in a mouse model of breast cancer. Clin. Cancer Res. 2005;11(2):728–734. doi: 10.1158/1078-0432.728.11.2.
[13] Dewan MZ, Galloway AE, Kawashima N, et al. Fractionated but not single-dose radiotherapy induces an immune-mediated abscopal effect when combined with anti–CTLA-4 antibody. Clin Cancer Res. 2009;15(17):5379–5388. doi: 10.1158/1078-0432.CCR-09-0265.
[14] Dovedi SJ, Adlard AL, Lipowska-Bhalla G, et al. Acquired resistance to fractionated radiotherapy can be overcome by concurrent PD-L1 blockade. Cancer Res. 2014;74(19):5458–5468. doi: 10.1158/0008-5472.CAN-14-1258.
[15] Durante M, Reppingen N, Held KD. Immunologically augmented cancer treatment using modern radiotherapy. Trends Mol Med. 2013;19(9):565–582. doi: 10.1016/j.molmed.2013.05.007.
[16] Bernier J. Immuno-oncology: allying forces of radio- and immunotherapy to enhance cancer cell killing. Crit Rev Oncol Hematol. 2016;108:97–108. doi: 10.1016/j.critrevonc.2016.11.001.
[17] Van Limbergen EJ, De Ruysscher DK, Olivo Pimentel V, et al. Combining radiotherapy with immunotherapy: the past, the present and the future. Br J Radiol. 2017;90(1076):20170157. doi: 10.1259/bjr.20170157.
[18] Mohan R, Grosshans D. Proton therapy – present and future. Adv Drug Deliv Rev. 2017;109:26–44. doi: 10.1016/j.addr.2016.11.006.
[19] Tubin S, Yan W, Mourad WF, et al. The future of radiation-induced abscopal response: beyond conventional radiotherapy approaches. Future Oncol. 2020;16(16):1137–1151. doi: 10.2217/fon-2020-0063.
[20] Overgaard J. Simultaneous and sequential hyperthermia and radiation treatment of an experimental tumor and its surrounding normal tissue in vivo. Int J Radiat Oncol Biol Phys. 1980;6(11): 1507–1517. doi: 10.1016/0360-3016(80)90008-5.
[21] Verbrugge I, Hagekyriakou J, Sharp LL, et al. Radiotherapy increases the permissiveness of established mammary tumors to rejection by immunomodulatory antibodies. Cancer Res. 2012; 72(13):3163–3174. doi: 10.1158/0008-5472.CAN-12-0210.
[22] Sørensen BS, Bassler N, Nielsen S, et al. Relative biological effectiveness (RBE) and distal edge effects of proton radiation on early damage in vivo. Acta Oncol. 2017;56(11):1387–1391. doi: 10.1080/0284186X.2017.1351621.
[23] Horsman MR, Chaplin DJ, Overgaard J. Combination of nicotinamide and hyperthermia to eliminate radioresistant chronically and acutely hypoxic tumour cells. Cancer Res. 1990;50:7430–7436.
[24] Baumann M, Krause M, Hill R. Exploring the role of cancer stem cells in radioresistance. Nat Rev Cancer. 2008;8(7):545–554. doi: 10.1038/nrc2419.
[25] Paganetti H. Mechanisms and review of clinical evidence of variations in relative biological effectiveness in proton therapy. Int J Radiat Oncol Biol Phys. 2022;112(1):222–236. doi: 10.1016/j.ijrobp.2021.08.015.
[26] Mirjolet C, Nicol A, Limagne E, et al. Impact of proton therapy on antitumor immune response. Sci Rep. 2021;11(1):13444. doi: 10.1038/s41598-021-92942-1.
[27] Chen M-F, Chen P-T, Hsieh C-C, et al. Effect of proton therapy on tumor cell killing and immune microenvironment for hepatocellular carcinoma. Cells. 2023;12:332. doi: 10.3390/cells12020332.
[28] Parry RV, Chemnitz JM, Frauwirth KA, et al. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. Mol Cell Biol. 2005;25(21):9543–9553. doi: 10.1128/MCB.25.21.9543-9553.2005.
[29] Salmon HW, Siemann DW. Effect of the second-generation vascular disrupting agent OXi4503 on tumor vascularity. Clin Cancer Res. 2006;12(13):4090–4094. doi: 10.1158/1078-0432.CCR-06-0163.
[30] Folkes LK, Christlieb M, Madej E, et al. Oxidative metabolism of combretastatin A-1 produces quinone intermediates with the potential to bind to nucleophiles and to enhance oxidative stress via free radicals. Chem Res Toxicol. 2007;20(12):1885–1894. doi: 10.1021/tx7002195.
[31] Song CW, Glatstein E, Marks LB, et al. Biological principals of stereotactic body radiation therapy (SBRT) and stereotactic radiation surgery (SRS): indirect cell death. Int J Radiat Oncol Biol Phys. 2021;110(1):21–34. doi: 10.1016/j.ijrobp.2019.02.047.
[32] Elming PB, Sørensen BS, Oei AL, et al. Hyperthermia: the optimal treatment to overcome radiation resistant hypoxia. Cancers. 2019; 11:60. doi: 10.3390/cancers11010060.
[33] Thangamathesvaran L, Shah R, Verma R, et al. Immune checkpoint inhibitors and radiotherapy – concept and review of current literature. Ann Transl Med. 2018;6(8):155. doi: 10.21037/atm.2018.03.09.
[34] Oei AL, Korangath P, Mulka K, et al. Enhancing the abscopal effect of radiation and immune checkpoint inhibitor therapies with magnetic nanoparticle hyperthermia in a model of metastatic breast cancer. Int J Hyperthermia. 2019;36(sup1):47–63. doi: 10.1080/02656736.2019.1685686.
[35] Zhao X, Shao C. Radiotherapy-mediated immunomodulation and anti-tumor abscopal effect combining immune checkpoint blockade. Cancers. 2020;12:2762. doi: 10.3390/cancers12102762.
[36] Iversen AB, Busk M, Horsman MR. Induction of hypoxia by vascular disrupting agents and the significance for their combination with radiation therapy. Acta Oncol. 2013;52(7):1320–1326. doi: 10.3109/0284186X.2013.825050.