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Original Research Article

In vitro sensitivity of malignant melanoma cells lines to photon and heavy ion radiation

K.P. Aninditha [a,c], K.J. Weber [a,c], S. Brons [b,c], J. Debus [a,b,c,d,e], H. Hauswald [a,b,c,d,*]

[a] Heidelberg University Hospital, Department of Radiation Oncology, Im Neuenheimer Feld 400, 69120 Heidelberg, Germany
[b] Heidelberg Ion-Beam Therapy Center (HIT), Im Neuenheimer Feld 450, 69120 Heidelberg, Germany
[c] National Center for Radiation Research in Oncology (NCRO), Heidelberg Institute for Radiation Oncology (HIRO), 69120 Heidelberg, Germany
[d] Clinical Cooperation Unit E050, German Cancer Research Center (DKFZ), 69120 Heidelberg, Germany
[e] DKTK Site Heidelberg, German Cancer Research Center (DKFZ), 69120 Heidelberg, Germany

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ABSTRACT

Background: The role of radiotherapy in malignant melanoma is still in discussion due to its relative resistance to radiation. In various literature, heavy ions show a higher relative biological effectiveness than photons. The aim of this work is to evaluate the radiotherapeutical effect from photons as well as heavy ions on malignant melanoma cells and to indicate the possible radiosensitivity based on its proliferation-inhibitory effect.

Methods: Two different cell lines of malignant melanoma, WM115 (primary tumor) and WM266-4 (metastatic site, skin) were used in this in vitro study. The cells were treated with photons or heavy ions (C_{12} and O_{16} ions). Cell-proliferation assay using hemocytometer was used for the quantitative and qualitative evaluation of cell growth. Furthermore, flow cytometry was also used to analyze the cell cycle distribution.

Results: Heavy ions compared to photons and between the two heavy ion modalities, O_{16} ions showed an improved suppression of cell growth in both cell lines. Furthermore, a G2/M arrest was detected in both cell lines after all radiotherapy modalities – with the arrest increasing with the dose applied.

Conclusion: Heavy ions showed a greater inhibitory effect on cell proliferation compared to photons and an increased G2/M arrest. Therefore, C_{12} and O_{16} heavy ions might overcome the relative radioresistance of malignant melanoma to photons. Further research is warranted.

1. Introduction

Malignant melanoma (MM) is known as the third most frequent skin cancer, following basal cell carcinoma and squamous cell carcinoma. Despite the lower incidence, it is responsible for 90% of deaths caused by cancer of the skin due to its very high metastatic potential [1,2]. The development of therapeutic options has shown promising progress during the last years. Depending on tumor stage, different adjuvant therapies can be applied for MM following surgery and have shown promising results, such as interferon alpha-2b in the early stages or vemurafenib in B-Raf V600E-mutations [1]. Furthermore, in recent years immunotherapy has been able to improve the outcome in advanced melanoma [3]. The combination of immuno- or targeted therapies with radiotherapy might further prolong survival in melanoma with brain metastases [4]. In addition, immunotherapy combined with ion beam therapy might improve immunogenic reactions [5]. However, MM is relatively resistant to photon radiation. The role of both adjuvant and definite radiotherapy in the primary setting is still being discussed. Photon radiotherapy is commonly used especially for the treatment of bone and brain metastases from MM. Different in vitro studies that were published have shown radiotherapy to be able to suppress cell growth in MM. However, the range of results reported is heterogeneous [6].

Abbreviations: DNA, deoxyribonucleic acid; DMEM, Dulbecco's modified Eagle's Medium; EDTA, ethylendiamin-tetraacetate; FCS, fetal calf serum; HIT, Heidelberg Ion-Beam Therapy Centre; KeV, kilo electron volt; LET, linear energy transfer; MM, malignant melanoma; MeV, mega electron volt; PBS, phosphate-buffered saline; RT, radiotherapy; RBE, relative biological effectiveness; RNA, ribonucleic acid.

* Corresponding author at: Department of Radiation Oncology, Heidelberg University Hospital, INF 400, 69120 Heidelberg, Germany.

E-mail addresses: Klaus-Josef.Weber@med.uni-heidelberg.de (K.J. Weber), Stephan.Brons@med.uni-heidelberg.de (S. Brons), Juergen.Debus@med.uni-heidelberg.de (J. Debus), henrik.hauswald@med.uni-heidelberg.de (H. Hauswald).

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The progressing implementation of high linear energy transfer (LET) heavy ion radiotherapy centers leads to the development of new therapeutic strategies and eventually offers advanced melanoma patients the option of being treated with heavy ions. High-LET radiotherapy might overcome the relative radioresistance of MM through a higher relative biological effectiveness (RBE). Furthermore, the unique dose profile with Bragg peaks, very steep dose gradients and less low dose exposure due to fewer beams leads to a lower dose to the healthy surrounding tissue. Therefore, the side effects of these treatments might be reduced with the same or even better therapeutic effect than photon radiotherapy [7].

In the current in vitro study, we analyzed the effects of photon and heavy ion radiotherapy on a primary and metastatic MM cell line to help implement new therapeutic options for this disease in the future.

2. Materials and methods

2.1. Cell culture

The two different MM cell lines (WM115 cells from primary site, and WM266-4 cells from a metastatic site on the skin) were obtained from ATCC, Manassas, VA, U.S.A. Cells were cultured in DMEM (Dulbecco's modified Eagle's Medium), mixed with 10% FCS (fetal calf serum) and 1% Penicillin/Streptomycin at 37 °C with 6% CO_2 and mostly passaged at 80% confluence after 7 ± 2 days. For the experiment's preparation, the cells were seeded in 25 cm^2 flasks in particular cell counts, depending on the treatment.

2.2. Photon irradiation

The seeded cells were incubated in 25 cm^2 flasks for 24 h to allow cells to adhere. The cells were then irradiated using 320 keV photons by X-RAD 320 [Precision X-Ray] with physical doses of 2.0, 4.0 and 6.0 Gy and then incubated for another 72 h, 96 h and 120 h.

2.3. Heavy ion irradiation

The high LET heavy ion radiotherapy was performed at Heidelberg Ion-Beam Therapy Centre (HIT) using a horizontal beamline in raster scanning technique. Depending on the therapy scheme the cells were irradiated with either carbon ions (C_{12}) or oxygen ions (O_{16}). The single layers of cells were irradiated in the middle of the 8 mm-extended Bragg peak for C_{12} and 10-mm-extended Bragg peak for O_{16}. The ionization energy varied between 1.47 and 1.67 GeV for carbon ions and 2.3 and 2.64 GeV for oxygen ions with LET values of 101 keV/μm and 141 keV/μm, respectively. Single physical C_{12} ion or O_{16} ion doses of 0.5, 1.0 and 2.0 Gy were used.

2.4. Cell proliferation assay

The cells were harvested from 25 cm^2 flasks with 1 ml of EDTA (ethylendiamin-tetraacetate)-trypsin at 37 °C and afterwards neutralized with 1 ml of medium, resulting in a 1:2 dilution. The absolute cell numbers were then counted using a hemocytometer and compared to a control flask, that contained identical cells but without irradiation.

2.5. Cell cycle analysis

The cell cycle distributions were analyzed using flow cytometry. At 96 h post irradiation the cells were dispersed using EDTA-trypsin, washed with phosphate-buffered saline (PBS), then fixed using 70% ethanol. Fixed cells were centrifuged, washed and incubated in RNAse and propidium iodide prior to measurement of DNA-content using a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany). The minimum of 10^4 ungated cells were analyzed using BD CellQuest Pro 4.0.2 (Becton Dickinson and Company, U.S.A.) and the cell cycle phases were evaluated using ModFit LT 3.0 (Verity Software House, Inc., U.S.A.).

2.6. Statistical analysis

Statistical analysis of the results was performed using an unpaired two-tailed *t*-test. Curves and figures were then calculated and generated using SigmaPlot 12.0 (Systat Software Inc., U.S.A.). Results are presented as mean values +/− standard deviation. $P < 0.05$ was considered as a significant difference when comparing results. RBE was defined as the ratio of an absorbed dose type X (for example C_{12} or O_{16} ions) to absorbed reference dose type Y (photons for example) resulting in the same biological effect, for example a specific survival fraction of 10% (RBE10). Raw data was determined in at least three independent experiments.

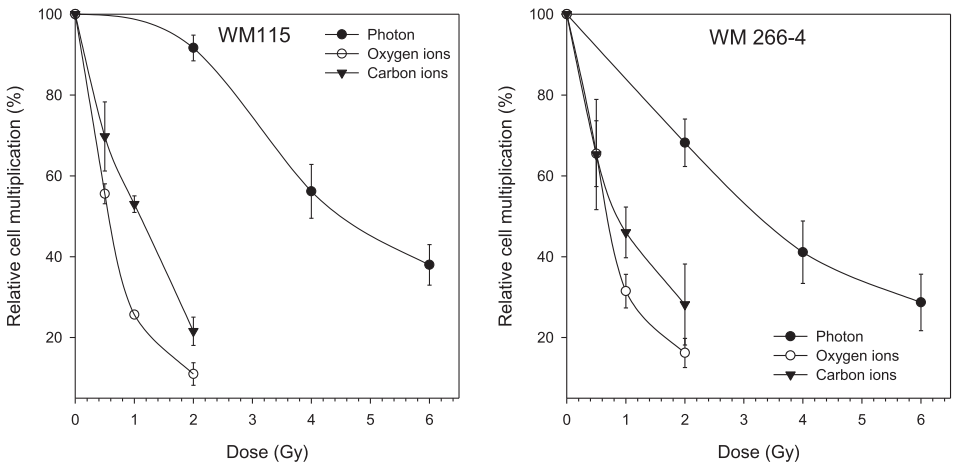


Fig. 1. Proliferation assays of two melanoma cell lines (WM115 and WM266-4), 96 h post-radiation with three different modalities (photons, carbon ions (C_{12}), or oxygen ions (O_{16})). Both heavy ion modalities (C_{12}, O_{16}) showed a relatively shifted curve to the left side, indicating greater inhibitory effects than photon irradiation. Raw data was determined in three independent experiments.

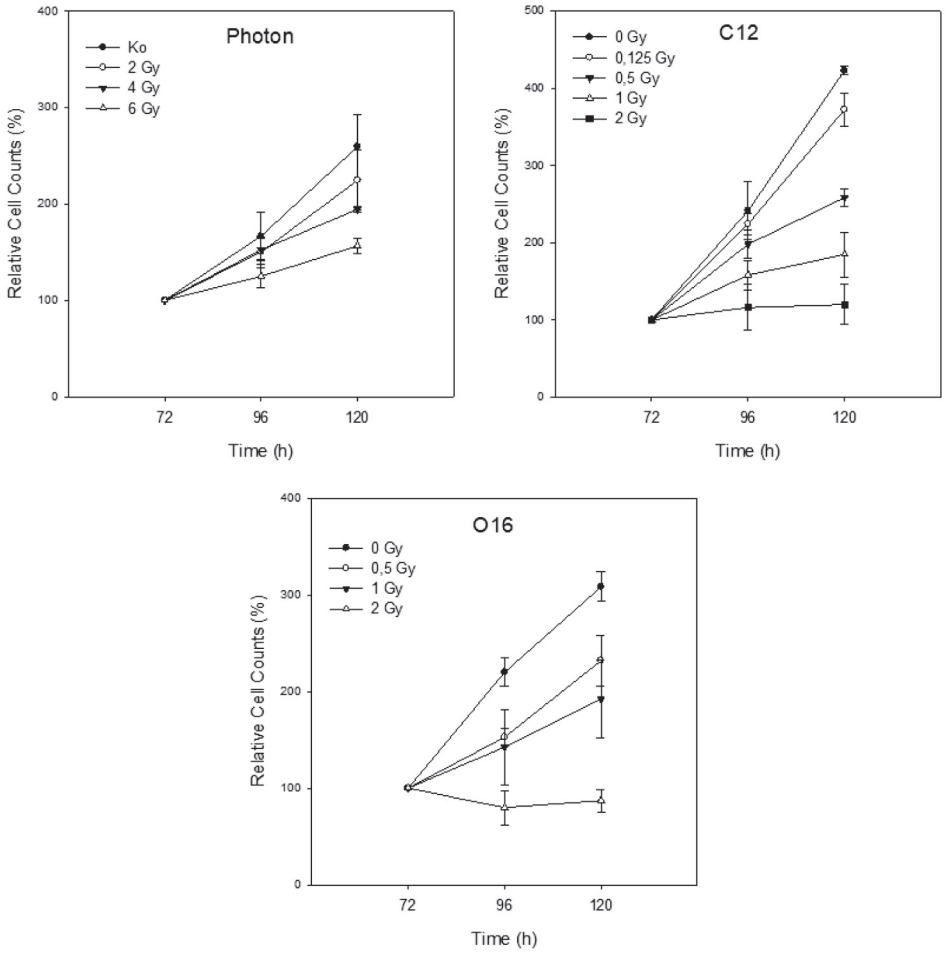
3. Results

3.1. The proliferation-inhibiting effect of heavy ions compared to photon radiotherapy

The following results were based on the cell proliferation assay at 96 h after radiotherapy (Fig. 1). The 2.0–6.0 Gy photon irradiations showed a wide range of proliferation-inhibition to both MM cell lines with 8.3–68.2%. In comparison to photon radiotherapy, both heavy ion types showed a curve significantly shifted to the left side indicating greater inhibitory effects; after 2.0 Gy C_{12} and O_{16} irradiation of both cell lines the proliferation rate was able to be suppressed to 10.9%–28.8%. The inhibitory effect on proliferation of 4.0 Gy photon radiotherapy on WM115 was relatively comparable to 1.0 Gy C_{12} and 0.5 Gy O_{16} radiotherapy (43.8% vs. 47.0% vs. 44.4%, respectively). On WM266-4 there was a similar rate of proliferation-inhibition with 68.2%, 61.8% and 68.5% (6.0 Gy photons, 2.0 Gy C_{12} and 1.0 Gy O_{16}) with this cell type.

Cell growth was evaluated by relative cell counts, and shown to be variably affected depending on radiotherapy modality and dose. Fig. 2 shows the evaluation of cell growth at 72 h, 96 h and 120 h after radiotherapy with curve flattening depending on the radiotherapy dose level and beam modality. Heavy ions compared to photons resulted in a decreased gradient in the proliferation rates after 120 h. After 120 h the growth in WM115 after 6.0 Gy photon radiotherapy was inhibited by only 37.5%. The maximum inhibition of growth after 120 h was shown to be 70.7% for 2.0 Gy C_{12} and 68.5% for 2.0 Gy O_{16}. In WM266-4 cells the maximum

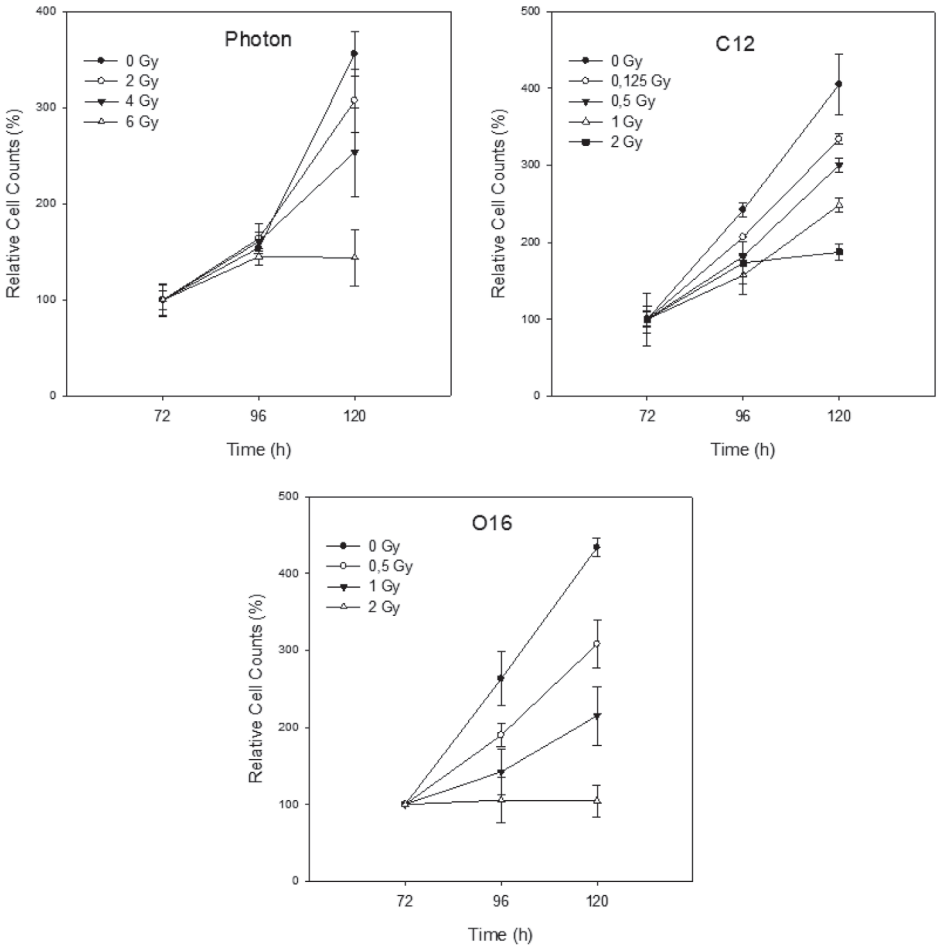
a) WM115



Photon	Growth rate in %	C_{12}	Growth rate in %	O_{16}	Growth rate in %
0 Gy	259.47± 30.0	0 Gy	422.80±4.8	0 Gy	308.31±15.36
2 Gy	270.95±30.0^{x}	0.125 Gy	356.43±30.9	0.5 Gy	231.94±26.3
4 Gy	206.83±24.2	0.5 Gy	261.94±32.3	1 Gy	192.26±40.3
6 Gy	162.14±14.2**	1 Gy	185.00±18.9**	2 Gy	86.95±11.5
		2 Gy	123.81±17.7**		

Fig. 2. Growth ratio of two melanoma cell lines a) WM115 and b) WM266-4 was evaluated at 72 h, 96 h, and 120 h post treatment with photons, carbon ions (C_{12}) or oxygen ions (O_{16}), showing curve flattening with increasing dose and linear energy transfer (LET), indicating stronger growth inhibitory effects. Raw data was determined in three independent experiments. $p < 0.05$; x: $p > 0.05$; **: $p < 0.001$.

b) WM266-4



Photon	Growth rate in %	C_{12}	Growth rate in %	O_{16}	Growth rate in %
0 Gy	355.87± 18.5	0 Gy	405.25±32.5	0 Gy	433.9±10.7
2 Gy	306.59±45.5^{x}	0.125 Gy	378.13±48.6^{x}	0.5 Gy	316.96±52.5
4 Gy	253.00±40.5	0.5 Gy	318.42±19.7	1 Gy	247.32±24.8**
6 Gy	148.81±5.5**	1 Gy	323.41±52.5	2 Gy	104.30±20.4**
		2 Gy	192.04±24.0		

Fig. 2 (*continued*)

inhibition of growth observed after 120 h was 73.4% with 2.0 Gy O_{16}, 52.6% with 2.0 Gy C_{12} and 58.2% with 6.0 Gy photons, respectively. In absolute number we can also see that the 2.0 Gy O_{16} radiotherapy resulted in a reduced number of cell counts compared to the control after 120 h.

3.2. Increased G2/M arrest on heavy ion compared to photon radiotherapy

Fig. 3 shows the cell cycle analysis of both melanoma cell lines 96 h after radiotherapy. In the control sample of WM115 and WM266-4, 12.5% and 13.8% of the cell were distributed in G2/M phase, respectively. Photon radiotherapy with 2.0–6.0 Gy resulted in an increased cell distribution in the G2/M phase, ranging from 15% to 22% in WM115 and 14% to 30% in WM266-4. Accordingly, radiotherapy with 0.5–2.0 Gy C_{12} resulted in relatively comparable 18% to 28% distribution in WM115 and 23% to 31% in WM266-4. In addition, radiotherapy with 0.5–2.0 Gy O_{16} resulted in a distribution of 12% to 41% in WM115 and 18% to 37% in WM266-4. This observation indicated a G2/M arrest in both cell lines after all radiotherapy modalities with an arrest that increased, the higher the applied dose was. The results also showed that 0.5–2.0 Gy of both heavy ion modalities resulted in a comparable distribution as 2.0–6.0 Gy of photon radiotherapy.

4. Discussion

The aim of this study was to evaluate the effectivity of heavy ion radiotherapy compared to conventional photon radiotherapy in MM. In our experiments, heavy ions and especially O_{16} ions showed an improved suppression of cell growth in both cell lines compared to photons. Furthermore, a G2/M arrest was indicated in both cell lines after all radiotherapy modalities and the arrest increased with higher dose. These in vitro analyses might help further research in radiation oncology to improve the effectiveness of radiotherapy by adding evidence for heavy ion radiotherapy in

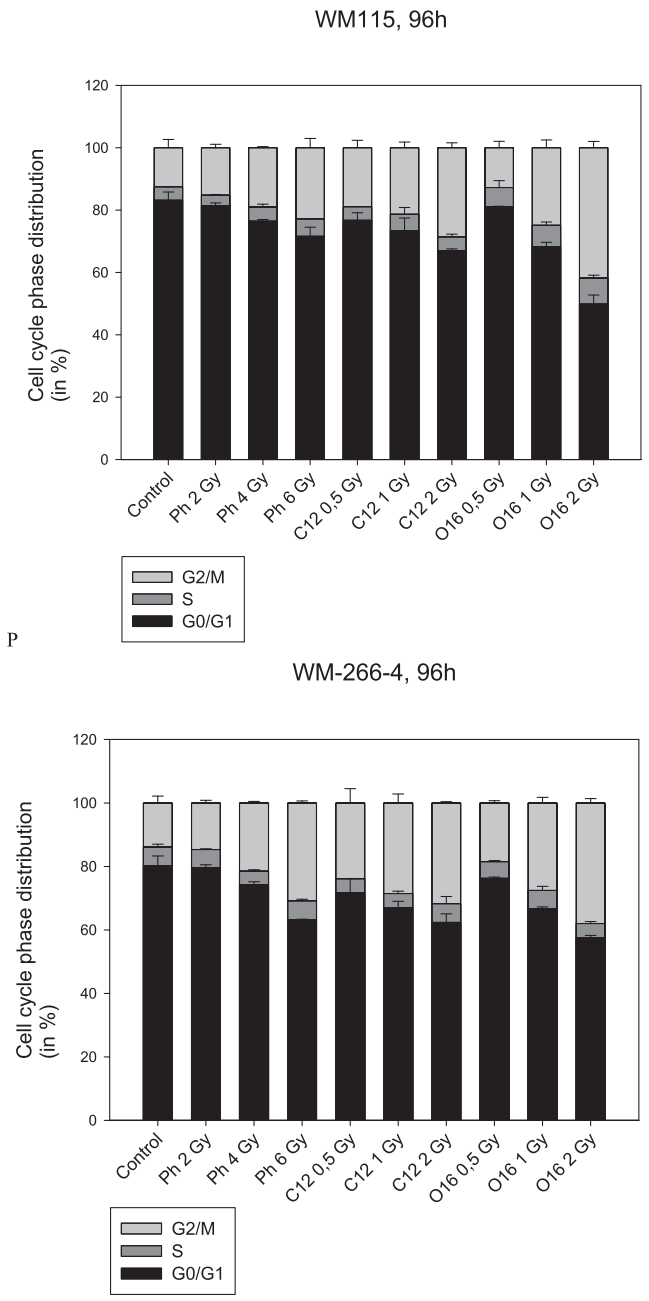


Fig. 3. Cell cycle analyses using FACS-scan at 96 h after irradiation of both melanoma cell lines (WM115 and WM266-4). Irradiation caused G2/M arrest that increased, depending on the dose and linear energy transfer (LET). Raw data was determined in three independent experiments.

MM. MM cells are considered to be relatively radioresistant compared to other cancer cells [7–9]. The broad shoulder on a melanoma survival curve indicated, that a higher radiotherapy dose is needed to achieve a significant effect. Some underlying mechanisms causing the relative radioresistance of MM are the effectiveness of its DNA repair, high proliferation capacities and hypoxic cell pools including radioresistant cancer stem cells [7].

The unique Bragg-peak dose profile combined with the possibility of using lower dose per fraction (in virtue of the high photon-relative biological effectiveness of heavy ions) might potentially contribute to better normal tissue sparing without neglecting the tumor killing effects [10]. Furthermore, the effects of high-LET ion beam radiotherapy were observed in several relative radioresistant tumors, especially MM. Prior reports of in vitro, in vivo and clinical studies have shown high-LET radiotherapy to be more effective than photon radiotherapy, for example in terms of RBE [11–14]. High-LET radiotherapy causes more direct than indirect DNA double-strand breaks, which may be the cause for it being more effective than photon radiotherapy in certain histologies [15].

Up to date, in vitro data on heavy ion radiotherapy in MM is limited. Qin et al. [11] reported on the influence of high-LET heavy ions (C_{12}) and low-LET photons on apoptosis and related proteins of MM on tumor-bearing mice under the same physical dose. The authors showed especially C_{12} to be able to promote apoptosis in MM cells and inhibit their proliferation. Moreover, C_{12} ions had significantly increased apoptosis and proliferation inhibition. One of the results shown is that high-LET radiotherapy was able to inhibit the tumor growth of B16F10 melanoma cells on mice to 95% one week after the therapy, compared to 37.5% on the photon arm. Not only was growth inhibited, the tumor volume shrunk significantly compared to the control.

In our study, we also identified the superiority of heavy ions compared to photon radiotherapy in terms of proliferation-inhibiting effects. The dose used to achieve the same effect to photon radiotherapy was notably less using heavy ion therapy (0.5 Gy for heavy ions comparable to the results of 2.0 Gy photons in our study), which means that heavy ions might be ~4 times more effective than photons. Nonetheless, with these results we were able to show the potential of heavy ions in MM. The estimation of carbon ions RBE was ~2, meaning 1.0 Gy carbon ions should cause the same biological effect as 2.0 Gy photons [7].

Moreover, between the two heavy ion modalities, O_{16} ions showed an improved suppression of cell growth in both cell lines in comparison to C_{12} ions. Up to now, the published data on O_{16} ion beams is still limited. Habermehl et al. [16] reported on hepatocellular carcinoma cell lines irradiated with photons or heavy ion beams (C_{12} and O_{16} ions). The authors had shown similar RBE_{10}-values for C_{12} and O_{16}, namely 1.9–3.3. Our comparable results of 1.0 Gy O_{16} ion beams to 2.0 Gy C_{12} ion beam and 6.0 Gy photon irradiation in WM266-4 proliferation assay indicated that O_{16} ion beams might be ~6 times more effective than photons. The significant curve shifting to the left, which means that fewer cells survive at a specific dose, might also indicate the potentials of C_{12} and O_{16} ion beams to overcome the radioresistance in MM.

The evaluation of the cell cycle distribution 96 h post radiotherapy has shown an increasing cell population in G2/M-phase, depending on dose and radiotherapy modality. One explanation for the G2/M phase increase might be a prolonged G2/M arrest indicating a high amount of DNA damage, which caused the cells unable to pass the checkpoint to proliferate and therefore stay in the G2/M phase, then undergo apoptosis [17]. The comparable results between 0.5 and 2.0 Gy heavy ions to 2.0–6.0 Gy photons also demonstrated the improved effectiveness of heavy ions. A previous study by Suetens et al. [18] compared the cell cycle patterns after radiotherapy with photons and carbon ions of both prostate and colon cancer cell line. The results also showed the permanent G2/M arrest in PC3 cells using relatively a lower dose of carbon ions (0.5–2.0 Gy).

5. Conclusion

In conclusion, heavy ions showed a greater inhibitory effect on primary and metastatic malignant melanoma cell proliferation compared to photons, and an increased G2/M arrest. Further in vitro experiments as well as clinical trials are needed for further evaluation of heavy ion beam therapy in malignant melanoma.

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Declaration of Competing Interest

None.

Acknowledgment

Not applicable.

Authors contributions

HH, KJW and JD developed and planned the study. KA, SB and KJW performed the irradiation and were responsible for management of the cells. KA, SB, KJW and HH participated in writing the manuscript and revising it. KA, KJW, JD and HH performed data analysis. KJW and HH reviewed all data and statistical analyses. All authors read and approved the final manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ctro.2019.06.002.

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