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Cytoplasmic HMGB1 and HMGB1-Beclin1 complex are increased in radioresistant oral squamous cell carcinoma

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Abstract

Cytoplasmic high mobility group box 1 (HMGB1) is an autophagy regulator, and autophagy is important in the radioresistance of various solid cancers. We evaluated the degree of autophagy and cytoplasmic HMGB1 in radioresistant oral squamous cell carcinoma (SCC) by culturing the SCC15 and quasiliquid layer 1 (QLL1) SCC cell lines that originate from cancer of the oral tongue and a metastatic lymph node, respectively, and then delivered radiation to induce radioresistance to cells. We then compared the degree of autophagy between non-irradiated control and radioresistant cancer cells using a western blot assay. We also compared the total and cytoplasmic concentrations of HMGB1 between the non-irradiated control and radioresistant cancer cells by western blot assay, and extracellular concentrations of HMGB1 with an enzyme-linked immunosorbent assay (ELISA). Formation of an HMGB1-Beclin1 complex was evaluated by immunofluorescence and co-immunoprecipitation assays. Autophagy increased in the radioresistant SCC15 cells (compared with non-irradiated control SCC15 cells) but not in the radioresistant QLL1 cells. The total amount of HMGB1 expression within cells did not differ; however, the degree of cytoplasmic HMGB1 expression was higher in radioresistant SCC15 cells than in non-irradiated control SCC15 cells. The HMGB1-Beclin1 complex, which is a main regulator of autophagy, was also increased in radioresistant SCC15 cells compared with non-irradiated control SCC15 cells. Autophagy flux and cytoplasmic HMGB1-Beclin1 increased after the acquisition of radioresistance in oral SCC.

Keywords: HMGB1; autophagy; radioresistance; oral squamous cell carcinoma

Introduction

Cancers of the oral cavity account for 2%–3% of all malignancies and are associated with both poor survival and poor quality of life.[1] Typical treatments for oral squamous cell carcinoma (SCC) are resection, radiation, chemotherapy, or a combination.[2] Although some patients respond to radiotherapy a large proportion do not, and local recurrence and distant metastases remain major limitations of the treatment.[3] However, the mechanisms that underlie radioresistance in patients with oral SCC are poorly understood.

Alterations in various signalling pathways have been reported in oral SCC, and autophagy has recently been thought to be involved in radioresistance in malignancies of the head and neck.[4] Autophagy is a lysosome-mediated, self-degradation process that is responsible for degrading organelles and long-lived proteins. It can be activated as an adaptive response to adverse environmental conditions such as malnutrition, hypoxia, and various therapeutic stresses, including chemotherapy and radiotherapy,[5] and is considered to be a therapeutic target for the treatment of cancers, particularly solid tumours that are resistant, because it sensitises them to treatment.[6] Previous reports have indicated that inhibition of autophagy could alter the intrinsic radiosensitivity of cancer cells.[7]

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High mobility group box 1 (HMGB1) is a nuclear protein that has various roles, depending on its location. In the nucleus, HMGB1 combines with DNA and functions as a nucleosome stabiliser, and it plays a chaperone-like function in the cytoplasm, by preventing the aggregation of proteins.[8] After it has been released into the extracellular space it acts as a damage-associated molecular pattern that promotes pro-inflammatory signalling pathways.[9]

HMGB1 is involved in oncogenic pathways through various processes such as cellular replication, inflammation, angiogenesis, and metastasis.[10] Cytoplasmic translocation of HMGB1 from the nucleus is a vital characteristic of a disordered tumour microenvironment.[11]

Previous work showed that increased expression of HMGB1 was strongly associated with poor prognosis in patients with cancer.[10] In a recent study, it was found to be a key molecule in predicting responsiveness to radiotherapy,[3] but its role in the radioresistance of oral SCC has not been identified.

We hypothesised that increased autophagy flux might be involved in radioresistant oral SCC, and that HMGB1 might be involved in the regulation of autophagy. We have previously established radioresistant oral SCC cell lines that resemble a real radiotherapy regimen after a cumulative dose of 60 Gy.[2]

We found that autophagy increased in radioresistant oral SCC compared with non-irradiated control SCC cells, and the cytoplasmic translocation of HMGB1 upregulated the formation of the HMGB1-Beclin1 complex in radioresistant oral SCC.

Material and methods

Cell lines and culture

SCC15 (squamous carcinoma cell lines that originated from the tongue) were bought from the American Type Culture Collection, and QLL1 (an SCC cell line that originated from metastatic lymph nodes in cancer of the oral cavity) were a generous gift from Dr. J. Shah (Memorial Sloan-Kettering Cancer Center). They were cultured according to our previous protocol.[2] Cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum, L-glutamine 2 mmol, penicillin 50 mg/ml, and streptomycin 50 mg/ml. Bafilomycin A1 (Sigma-Aldrich) was applied to the culture medium to evaluate the autophagy flux in oral SCC.

Establishment of radioresistant oral SCC lines

Radioresistance was acquired by following our previous protocol for radiation.[2] Cells were cultured in a T75 flask until they were 80% confluent, and 2 Gy radiation was delivered at room temperature with a linear accelerator (21iX, Varian). Afterwards, 2 Gy doses of radiation were delivered repetitively as cells grew to 80% confluence after the previous radiation until a cumulative dose of 60 Gy was reached.

Western blot assay

Cells were lysed with radioimmunprecipitation assay (RIPA) buffer (Millipore) for 30 minutes at 4 °C. Extracts were centrifuged at 12,000 × g for 15 minutes at 4 °C, and the supernatant collected. The bicinchoninic acid (BCA) protein assay kit (Pierce Scientific Ltd) was applied for the quantification of protein. Proteins 20 μg were loaded on 12% sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE), transferred to nitrocellulose membranes, and blocked with 10% skim milk. After the proteins had been washed three times, primary antibodies against HMGB1, β-actin, and LC3IIB (Abcam) were added and left overnight. Isotype-matched secondary antibodies were applied sequentially. Relative band intensity was measured using ImageJ (IBM).

Nuclear-cytoplasmic fractionation

Cytosolic and nuclear extracts were prepared using the Nuclear/Cytosol Fractionation Kit (BioVision Inc) according to the manufacturer's instructions. Extracted proteins were quantified by BCA assay, and 20 μg of the extracts were loaded on 12% SDS-PAGE gel and transferred on to nitrocellulose membranes to compare the amount of HMGB1 in each compartment.

Concentration of HMGB1 in culture supernatant and ELISA using supernatant

Culture supernatant from 2×10^5 cells was collected and concentrated to compare the amount of secreted HMGB1. All the cell culture supernatant was harvested and concentrated using a centrifugal filter unit (Millipore). After concentration, supernatant 50 μL was analysed by western blot assay using an anti-HMGB1 antibody. Culture supernatant that was not concentrated was also obtained and used for an ELISA to compare the amounts of HMGB1 protein secreted. The HMGB1 ELISA kit (Shino-Test Corp) was used, and all procedures followed the manufacturer's protocol.

Immunofluorescence analysis

Cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature, and 0.2% Triton X-100 was applied for 10 minutes for permeabilisation. Blocking was done with 1% BSA-PBS for one hour at room temperature. Samples were then incubated with a primary antibody against HMGB1 and Beclin1 (Abcam) in 1% BSA-PBS overnight at 4 °C.

Alexa Fluor 488- or 594-conjugated secondary antibody (Invitrogen) was added to the cells for one hour at room temperature. Vectashield mounting solution was used for mounting and DAPI staining (Vector Laboratories). Fluo-

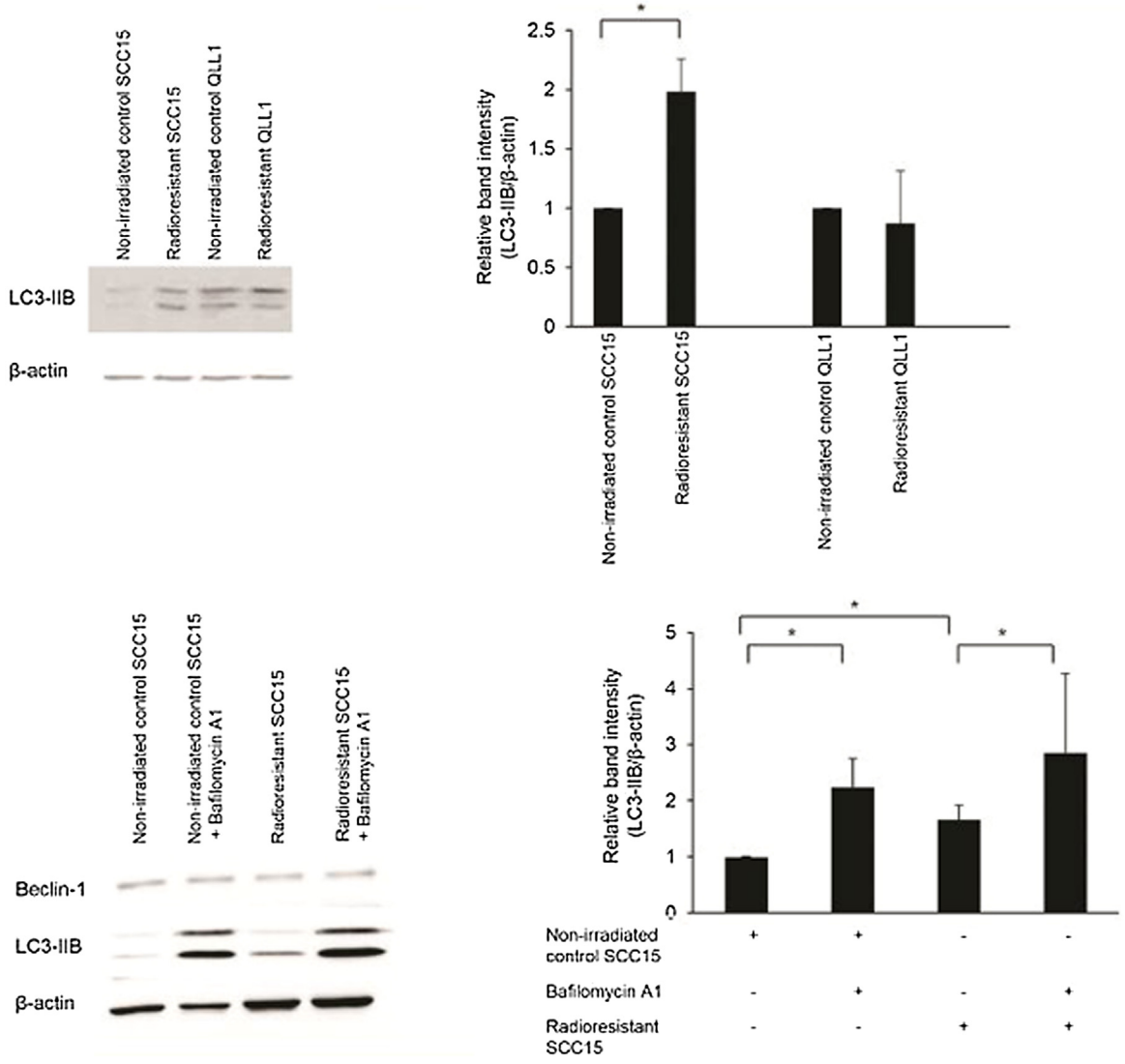

Fig. 1. Autophagy increased in radioresistant SCC15.
Two types of resistant oral SCC cell lines were established and cultured until they had received a total of 60 Gy of radiation. Whole cell lysates were analysed by western blot for LC3II and β-actin (as a loading control). Western blot analysis was repeated three times, and the relative band intensity was calculated. Non-irradiated control and radioresistant SCC15 were treated with bafilomycin A1 (20 nmol for 24 hours), and whole cell lysates were analysed by western blot assay. The western blot analysis was repeated three times, and the relative band intensity was calculated.

rescence images were acquired using an FV1000 confocal microscope (Olympus).

Co-immunoprecipitation analysis

Cells were lysed at 4 °C in RIPA buffer (Millipore). Cell lysates 200 μg were precleared by protein A Sepharose® beads (Millipore) for two hours at 4 °C and subsequently incubated overnight at 4 °C with isotype matched control antibodies or anti-Beclin1 antibodies. Immune complexes bound to the anti-Beclin1 antibody were precipitated using protein A Sepharose® beads and then washed three times with PBS. The immune complexes that formed were then eluted from the beads. Samples were prepared and then subjected to western blot analysis.

Statistics

All results are expressed as the mean (SD), and the significance of differences between two groups was analysed with Student's *t* test. The significance of comparisons of more than two groups were analysed by one-way ANOVA. Probabilities of less than 0.05 were accepted as significant. Statistical analyses were made with the aid of GraphPad Prism 6.0 (GraphPad Software Inc).

Results

Autophagy is increased in radioresistant SCC 15

To assess the level of autophagy in radioresistant oral SCC, we cultured two types of radioresistant oral SCC cell lines

(SCC15, QLL1). Fig. 1 shows that on western blot analysis the concentration of LC3IIB increased in radioresistant SCC15 compared with that in non-irradiated control SCC15, but not in radioresistant QLL1 compared with non-irradiated control QLL1.

We repeated those experiments, and our results again showed that the increase in concentration of LC3IIB was significant only in radioresistant SCC15 ($p = 0.025$). To further confirm that real autophagy flux increased in radioresistant SCC15, we treated cells with an autophagy-lysosomal inhibitor, bafilomycin A1, for 24 hours. This caused the LC3IIB concentration to increase further in radioresistant SCC15 compared with the non-irradiated control SCC15, which in turn confirmed the increase in autophagy flux. We repeated these experiments three times, and the difference was significant each time (column 1 compared with column 2, $p = 0.044$; column 1 compared with column 3, $p = 0.042$; and column 3 compared with column 4, $p = 0.041$).

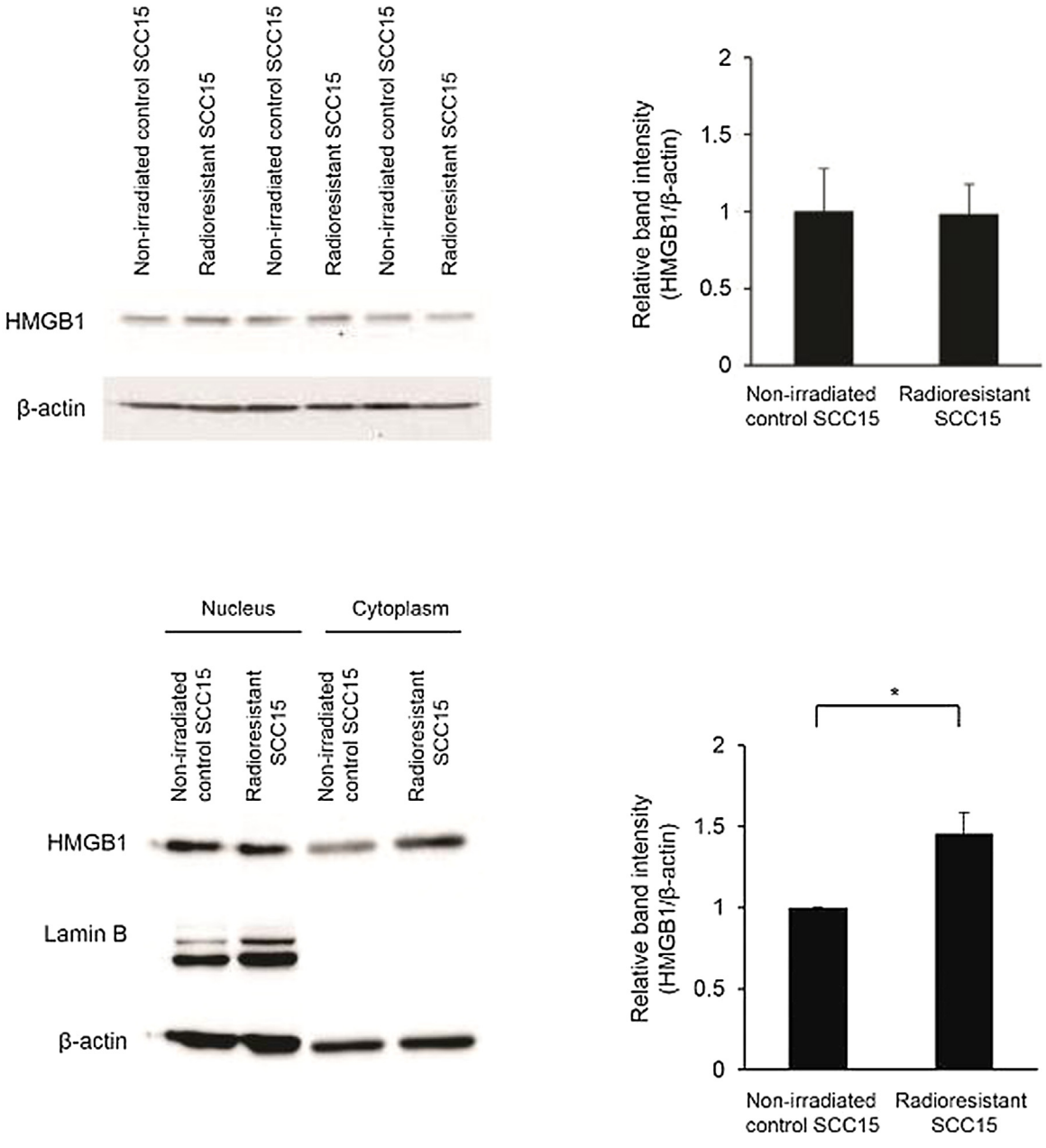

Fig. 2. HMGB1 is translocated into the cytoplasm in radioresistant SCC15.
Non-irradiated control and radioresistant SCC15 were established and cultured until the treatment group had received a total of 60 Gy of radiation. Whole cell lysates were subjected to western blot analysis for HMGB1 and β-actin (as a loading control). The western blot analysis was repeated three times, and relative band intensity calculated. Nuclear extracts and cytoplasmic fractions were separated from whole cell lysates and analysed by western blot for HMGB1. Lamin B and β-actin were used as loading controls for nuclear extracts and the cytoplasmic fraction. Western blot analysis was repeated three times, and relative band intensity was calculated.

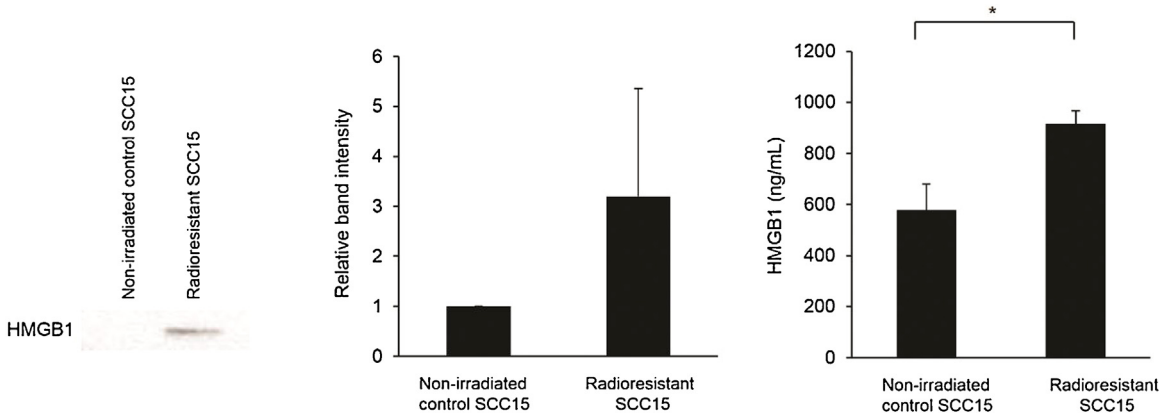

Fig. 3. HMGB1 is secreted into extracellular area in radioresistant SCC15.
Culture supernatant was harvested from non-irradiated control and radioresistant SCC15 and then concentrated and analysed for HMGB1 by western blot assay. The western blot analysis was repeated three times, and relative band intensity was calculated. Culture supernatant was harvested from radioresistant and non-irradiated control SCC15 and then concentrated and analysed for HMGB1 by ELISA.

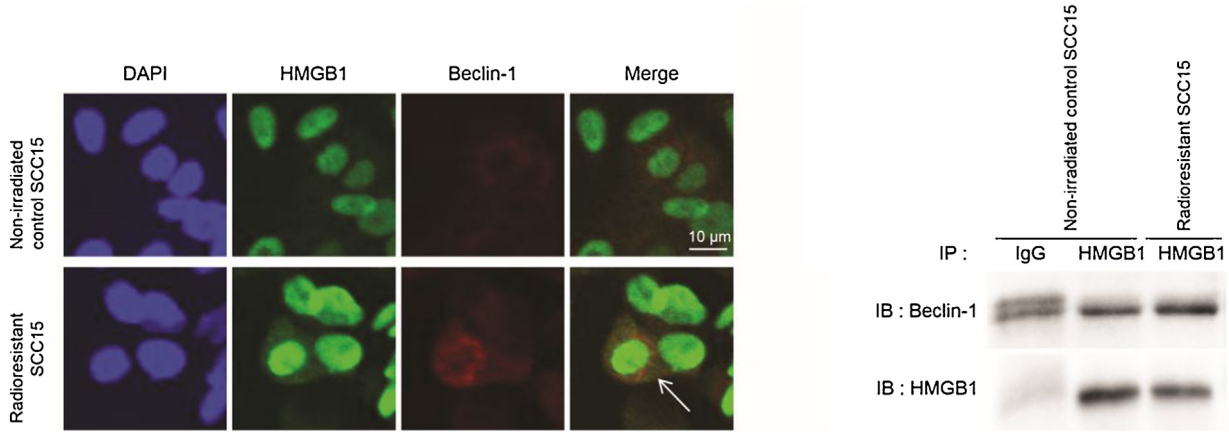

Fig. 4. Interaction between HMGB1 and Beclin1 increased in radioresistant SCC15.
Non-irradiated control and radioresistant SCC15 were cultured, and an immunofluorescence assay made to evaluate the interaction between HMGB1 (green) and Beclin1 (red). DAPI (blue) was used to stain the nucleus. Non-irradiated control and radioresistant SCC15 were cultured, and whole cell lysates were harvested. A co-immunoprecipitation analysis was made to evaluate the formation of the HMGB1 and Beclin1 immune complex.

HMGB1 expression in baseline and radioresistant SCC15

To establish the role of HMGB1 in the regulation of autophagy in radioresistant SCC15, we analysed the expression of HMGB1 protein and location in radioresistant and non-irradiated control SCC15. Although the expression differed between the two groups of cells, we did not find a significant difference in the amount of HMGB1 protein between the radioresistant SCC15 and non-irradiated control SCC15 (Fig. 2). When we repeated the experiment three times, we found no significant difference. Because the role of HMGB1 varies according to its subcellular site, we separated whole cell lysates into nuclear and cytoplasmic proteins and did a western blot assay, which showed that the concentration of HMGB1 increased in the cytoplasm of radioresistant SCC15 but not in the non-irradiated control SCC15, and that the difference in the concentration of cytoplasmic HMGB1 was significant ($p = 0.024$).

Extracellular HMGB1 increased in radioresistant SCC15

Because HMGB1 was translocated from the nucleus to the cytoplasm in radioresistant SCC15, we next collected the culture supernatant to investigate whether HMGB1 was secreted into the extracellular area. In western blot assays we found that extracellular HMGB1 increased in radioresistant SCC15 compared with non-irradiated control SCC15 (Fig. 3). We further showed, using an ELISA, that the level of HMGB1 in the extracellular area increased in radioresistant SCC15 (915.73 (51.48) ng/ml) compared with the non-irradiated control SCC15 (579.4 (100.90) ng/ml) ($p = 0.037$).

Association between HMGB1 and Beclin1 increased in radioresistant SCC15

Because the association of HMGB1 with Beclin1 has been established as an important modulator of autophagy,[12] we evaluated the interaction of HMGB1 with Beclin1. In our

immunofluorescence assay, we found that HMGB1 was translocated into the cytoplasm in radioresistant SCC15, where it was co-localised with Beclin1 (Fig. 4). In our coimmunoprecipitation analysis, we showed that binding between HMGB1 and Beclin1 increased in radioresistant SCC15 compared with that in non-irradiated control SCC15.

Discussion

Although radiotherapy is useful in the treatment of cancers of the head and neck, tumour cells develop adaptive responses such as autophagy to evade radiation-induced damage to tumour cells. Autophagy has two primary, opposing, functions in their treatment – one is a cytoprotective function that enables the tumour cells to survive, and the other is a cytotoxic function that induces them to die.[13]

Previous experiments about the role of autophagy after exposure to radiation have shown divergent results. Irradiation did not induce autophagy in colorectal, mammary, and lung carcinoma, or cervical adenocarcinoma.[7] A single dose of irradiation did not change the level of autophagy flux, and the effect of irradiation depended on the cancer cell line being tested.[7] Other studies have shown that various types of solid cancers, such as those of the colon, nasopharynx, and breast, show an upregulation of autophagy that is associated with radioresistance.[14–16] Autophagy also facilitates the effect of radiation in cancer treatment, such as for oral SCC.[17] We found that autophagy increased in radioresistant SCC15 compared with non-irradiated control SCC15, which suggests that autophagy is associated with the radioresistance of oral SCC.

The conditions of exposure to radiation and type of tumour cells have differed in each study, which might explain the contradictory results about the role of autophagy after radiation. We previously developed our own radioresistant oral SCC cell lines that mimic a real radiotherapy protocol for patients. Most patients with head and neck cancer are given radiotherapy of 2 Gy at a time, up to a total of 50–70 Gy. We therefore used a total of 60 Gy to resemble the clinical setting. Based on our results, our radioresistant SCC15 cell line showed an increased level of autophagy flux and increased cytoplasmic HMGB1-Beclin1 interaction after the acquisition of radioresistance. Interestingly, that result was not the same for QLL1, the other type of oral SCC cell line that we tested (Fig. 1). SCC15 and QLL1 originated from different cancer cells (SCC15 from the tongue and QLL1 from a metastatic lymph node). We think that the difference in the stage of the cancer could be one of the reasons why the results for primary and metastatic cancer cells differed in our experiments. Metastatic cancer cells have also been through epithelial mesenchymal transition (EMT), but primary cells have not. HMGB1 is known to be important in EMT during the progress of cancer,[18] and the presence of EMT could affect the different results between primary and metastatic cancer cells.

Signalling pathways that are activated in response to radiotherapy for head and neck cancer have been evaluated, and a significant association found between p-Akt staining and the local control rate of radiotherapy.[19] HIF1-α was significantly associated with local recurrence and survival in advanced head and neck cancer.[20] We add another possible mechanism: autophagy might contribute to radioresistance in oral SCC, and cytoplasmic translocation of HMGB1 could contribute to an increased level of autophagy.

Cytoplasmic translocation of HMGB1 has been shown to be an important inducer of autophagy in various solid cancers.[12] Although HMGB1 is usually located in the normal nucleus, it is highly expressed in cytoplasm in cancer cells.[21] It was also shown to bind with Beclin1, which then promoted the formation of a Beclin1-PI3KIII complex.[12] HMGB-mediated autophagy is a significant contributor to resistance to chemotherapy in several types of cancer cells.[11,22] For example, the HMGB1-Beclin1 complex reduced the oxidative stress caused by chemotherapy.[23] Those authors showed that HMGB1 exerted a protective effect against oxidative stress-mediated apoptosis by regulating autophagy.[23] We think that HMGB1 is translocated outside of the nucleus, where it interacts with Beclin1 in the cytoplasm of radioresistant oral SCC, thereby increasing autophagy flux.

Although cytoplasmic HMGB1 regulates autophagy, autophagy also regulates the selective release of HMGB1 in some cancer cells. For example, gastric cancer cells undergoing autophagy release HMGB1, and extracellular HMGB1 interacts with RAGE signalling through ERK 1/2 phosphorylation to contribute to the survival of cancer cells.[24] In our data the level of extracellular HMGB1 was raised in radioresistant SCC15 cells compared with non-irradiated control SCC15. It is known that HMGB1 could be released into the extracellular area actively by apoptosis and passively by necrosis. The extracellular space, which is known as the tumour microenvironment, is much more complicated, and the mechanism and immunological function of secreted HMGB1 should be further investigated.

A limitation of our study is that we did not evaluate the effect of autophagy inhibition on radioresistance. Previous studies have evaluated the effect of inhibition of autophagy on the viability of cancer cells after radiation. The growth rate of Beclin1 knockdown cells was slower than that of control cells after acute radiation,[25] and we still could not get the results after inhibition of HMGB1 expression. As cells should be cultured for a long time to acquire radioresistance, we are still struggling to set the condition of HMGB1 knock down using short interference RNA. The effect of inhibition of autophagy and HMGB1 should be studied to evaluate the role of autophagy and cytoplasmic HMGB1 in the radioresistance of oral SCC. Finally, our result was obtained from only one type of oral SCC cell line, and it was not proved in any other type. The study of the role of HMGB1-mediated autophagy should be further extended to other types of SCC cell lines in the head and neck.

Conclusion

Our data have shown that cytoplasmic HMGB1-mediated autophagy is involved in radioresistance in oral SCC, and suggest the need to evaluate the role of autophagy in the management of radioresistant cancer cells in the head and neck.

Conflict of interest

We have no conflicts of interest.

Ethics statement/confirmation of patients' permission

Neither is applicable.

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