Radiotherapy and Oncology 124 (2017) 370–378

Contents lists available at ScienceDirect

Radiotherapy and Oncology

journal homepage: www.thegreenjournal.com

Molecular radiobiology

# Head and neck cancer cell radiosensitization upon dual targeting of c-Abl and beta1-integrin

Philipp Koppenhagen [a], Ellen Dickreuter [a], Nils Cordes [a,b,c,d,*]

[a] OncoRay – National Center for Radiation Research in Oncology, Faculty of Medicine and University Hospital Carl Gustav Carus, Technische Universität Dresden, Helmholtz-Zentrum Dresden – Rossendorf; [b] Department of Radiotherapy and Radiation Oncology, Faculty of Medicine and University Hospital Carl Gustav Carus, Technische Universität Dresden; [c] Helmholtz-Zentrum Dresden – Rossendorf, Institute of Radiooncology – OncoRay; and [d] German Cancer Consortium (DKTK), partner site Dresden, and German Cancer Research Center (DKFZ), Heidelberg, Germany

ARTICLE INFO

*Article history:*
Received 4 November 2016
Received in revised form 7 May 2017
Accepted 9 May 2017
Available online 31 May 2017

*Keywords:*
Beta1-integrin
c-Abl
Imatinib
Ionizing radiation
DNA-repair

ABSTRACT

Integrin-mediated cell adhesion to extracellular matrix (ECM) critically contributes to cancer cell therapy resistance and DNA double strand break (DSB) repair. c-Abl tyrosine kinase has been linked to both of these processes. Based on our previous findings indicating c-Abl hyperphosphorylation on tyrosine (Y) 412 and threonine (T) 735 upon beta1-integrin inhibition, we hypothesized c-Abl tyrosine kinase as an important mediator of beta1-integrin signaling for radioresistance. In a panel of 8 cell lines from different solid cancer types grown in 3D laminin-rich ECM cultures, we targeted beta1 integrin with AIIB2 (mAb) and c-Abl with Imatinib with and without X-ray irradiation and subsequently examined clonogenic survival, residual DSBs, protein expression and phosphorylation. Single or combined treatment with AIIB2 and Imatinib resulted in cell line-dependent cytotoxicity. Intriguingly, we identified a subgroup of this cell line panel that responded with a higher degree of radiosensitization to AIIB2/Imatinib relative to both single treatments. In this subgroup, we observed a non-statistically significant trend between the radioresponse and phospho-c-Abl Y412. Mechanistically, impairment of DNA repair seems to be associated with radiosensitization upon AIIB2/Imatinib and AIIB2/Imatinib-related radiosensitization could be reduced by exogenous overexpression of either wildtype or constitutively active c-Abl forms relative to controls. Our data generated in more physiological 3D cancer cell culture models suggest c-Abl as further determinant of radioresistance and DNA repair downstream of beta1-integrin. For solid cancers, c-Abl phosphorylation status might be an indicator for reasonable Imatinib application as adjuvant for conventional radio(chemo)therapy.



c-Abl non-receptor tyrosine kinase is an intracellular regulator of cell proliferation, differentiation, adhesion, migration and DNA-repair [1,2]. Altered c-Abl activity was first discovered in hematological malignancies like chronic myelogenous leukemia in which the fused and consequently constitutively active oncoprotein BCR-ABL drives leukemic tumorigenesis [3]. On this pathophysiological background, Imatinib, a c-Abl tyrosine kinase inhibitor, represented the first successful causal cancer treatment, today known as molecular therapy [4,5].

Owing to numerous functions promoted by dysregulated c-Abl kinase signaling such as cell polarity disruption, cell proliferation, survival and invasiveness, c-Abl is also more and more emerging as potential target in solid cancers [6,7]. Clinical trials testing for the efficacy of Imatinib or derivatives have been conducted, for example, in melanoma, breast carcinoma and colorectal carcinoma [8–10]. The overexpression and hyperactivity of c-Abl as a consequence of oxidative stress, aberrant chemokine signaling, growth factor receptor activity or missing inhibitory stimuli offer a reasonable therapeutic window for c-Abl targeting strategies [11].

In the context of loss of cell polarity through malignant transformation, it is of particular interest that c-Abl interacts with integrin cell adhesion molecules [12,13]. Cancer cell therapy resistance is a well-known consequence of integrin-mediated adhesion to extracellular matrix (ECM) [14–16]. Integrins are heterodimeric transmembrane cell surface receptors composed of alpha and beta subunits [17]. On the one hand, they mechanically connect the ECM with the intracellular actin cytoskeleton to regulate adhesion and migration, on the other hand, integrins signal to control various cell processes essential for cancer development and progression [18,19]. Based on these facts, integrins serve as potential cancer targets and numerous studies have evidently shown the

* Corresponding author at: OncoRay – National Center for Radiation Research in Oncology, Medical Faculty Carl Gustav Carus, Dresden University of Technology, Fetscherstrasse 74/PF 41, 01307 Dresden, Germany.
*E-mail address:* Nils.Cordes@OncoRay.de (N. Cordes).

http://dx.doi.org/10.1016/j.radonc.2017.05.011
0167-8140/

potential of integrin inhibition for radiochemosensitization in head and neck, brain, lung, breast and leukemia cancer cell models [20–25].

The cytotoxic effects mediated by radiotherapy and various chemotherapeutics elicit from DNA damage, especially DNA double strand breaks (DSB) [26]. DSB are mainly repaired via homologous recombination (HR) and non-homologous end-joining (NHEJ). The error-prone NHEJ repair is conducted throughout the whole cell cycle and involves binding of the Ku70/80 heterodimer to the break ends to recruit DNA-dependent protein kinase catalytic subunit (DNA-PKcs) [27]. Recently, we were able to demonstrate in head and neck cancers that cell adhesion to ECM per se and particularly beta1-integrins influence radioresistance and DNA-PK-dependent NHEJ via a specific signaling cascade involving FAK and JNK [28,29]. There is also mounting evidence about a complex signaling network acting between c-Abl and the DNA-repair machinery including DNA-PKcs, Ataxia telangiectasia mutated (ATM) and RAD51 [30–32].

In the present study, we hypothesize c-Abl tyrosine kinase as an important part of the beta1-integrin signaling cascade and a determinant of DSB repair in solid cancer. In a panel of 8 human cancer cell lines from different origin grown under more physiological 3D ECM-based conditions, we demonstrate (i) radiosensitization and (ii) compromised DSB repair after combined beta1-integrin/c-Abl inhibition in a subset of tested cancer cell lines. In this subset, we observed a non-statistically significant association between radioresponse and levels of phosphorylated c-Abl Y412.

## Material and methods

### *Cell culture and irradiation*

Human head and neck squamous carcinoma (HNSCC) cell lines were kindly provided by R. Grenman (Turku University Central Hospital, Finland). HeLa and A431 cell lines were purchased from ATCC. HaCaT cells were kindly provided by N. Fusenig (DKFZ, Heidelberg, Germany). MCF-7 and MDA-MB-231 were purchased from the Scientific Instrumentation Centre of the University of Granada. The origin and stability of the cells were routinely monitored by short tandem repeat analysis (microsatellites). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; PAA) containing glutamax-I supplemented with 10% fetal calf serum (FCS; PAA) and 1% non-essential amino acids (PAA) at 37 °C in a humidified atmosphere containing 8.5% $CO_2$. Asynchronously growing cells were used in all experiments within passages 2–10. Three dimensional (3D) cell culture was accomplished by imbedding cells in 0.5 mg/ml laminin-rich extracellular matrix (lrECM (Matrigel™); BD) as published [29,33,34]. Irradiation was performed at room temperature using single doses of 200-kV X-rays (Yxlon Y.TU 320; Yxlon; dose rate ~1.3 Gy/min at 20 mA) filtered with 0.5 mm Cu. The absorbed dose was measured using a Duplex dosimeter (PTW).

### *Antibodies and reagents*

Antibodies for c-Abl (#2862P), c-Abl Y412 (#2865S; used for Western Blot), c-Abl T735 (#2846S), MEK1/2 (#9122) and Ku80 (#2180) were obtained from Cell Signaling. β-actin was from Sigma-Aldrich (#A1978); Ku70 (#ab3114), mCherry (#ab183628) and c-Abl Y412 (for immunofluorescence staining; #ab47315) were from Abcam; 53BP1 was from Novus Biologicals (#NB100-904); beta1-integrin antibody for Western Blot analyses was from BD (#610468); beta1-integrin antibody for immunofluorescence staining was from Calbiochem (#CP26). Horseradish peroxidase-conjugated donkey anti-rabbit (#NA-934) and sheep anti-mouse (#NA-931) secondary antibodies were from GE Healthcare; Alexa Fluor 594 anti-mouse (#A-11032), Alexa Fluor 488 anti-rabbit (#A11034), Alexa Fluor 488 anti-mouse (#A-11001) was from Invitrogen, nonspecific isotype control antibody IgG anti-rat (#sc-2026) was from Santa Cruz. ECL SuperSignal® West Dura Extended Substrate was from Pierce, Complete protease inhibitor cocktail from Roche, the phosphatase inhibitors $Na_3VO_4$ and NaF from Sigma, Vectashield/DAPI mounting medium was from Alexis, oligofectamine was from Invitrogen and dimethyl sulfoxide (DMSO) was from AppliChem. For β1 integrin blocking, the inhibitory monoclonal antibody AIIB2 was used as published and at a concentration of 10 μg/ml [28].

### *Phosphoprotein microarray analysis*

The Phospho Explorer Antibody Microarray was conducted by Full Moon BioSystems Inc., as published [20].

### *siRNA transfection*

c-Abl siRNA Abl#1 (sequence: 5′-GGCCAUCAACAAACUGGAGt t3′) and Abl#2 (sequence 5′- ACGCACGGACAUCACCAUGtt-3′) as well as Silencer® Negative Control siRNA (AM4635) were obtained from Ambion. Cells were transfected with siRNA (20 nM) 24 h after plating using oligofectamine and Opti-MEM (Invitrogen) under serum-free conditions for 8 h [29,35]. 24 h post-transfection the cells were used for colony formation assay.

### *Inhibitors*

Inhibition of c-Abl kinase was achieved by Imatinib (selleckchem). Imatinib was diluted in DMSO at a concentration of 20 mM and stored at −20 °C. For foci assay and 3D colony formation assay, a concentration of 12 μM Imatinib was used and the following treatment schedule applied: Cells were treated with Imatinib and AIIB2 as single or in combination (DMSO or IgG as control) followed by irradiation after 1 h. For 53BP1 foci assay cells were fixed 24 h after irradiation. For 3D colony formation, cells were washed with media at 24 h after treatments and incubated at 37 °C for several days for colony formation.

### *Immunofluorescence staining*

For immunofluorescence staining, $4 \times 10^5$ cells were plated onto cover slips in 24 well plates (BD). After 24 h cells were fixed using 3% formaldehyde (Merck), followed by permeabilization with 0.25% Triton-X-100 (Roth). Cells were immunostained with specific antibodies and representative pictures were taken with the LSM 510 meta (Zeiss) as reported [28].

### *Total protein extraction, Western blotting*

3D grown cells were treated with antibodies (AIIB2 versus IgG control; 10 μg/ml) and/or c-Abl kinase inhibitor (Imatinib versus DMSO control; 12 μM), followed by irradiation after 1 h of incubation. Cell lysis was performed 1 h post-irradiation and whole cell lysates were used for Western blotting as previously described [28]. To objectify protein expressions, densitometric analysis with respective non-irradiated/non-treated controls set to 1.0 was utilized.

### *Protein fractionation*

An amount of $1.6 \times 10^6$ cells were seeded into 100 mm cell culture dishes (BD). After 24 h, cells were treated with AIIB2 (or IgG isotype control antibody; 10 μg/ml) for 1 h followed by irradiation (non-irradiated controls). At 1 h post irradiation, proteins were