Translation and optimization of a gamma H2AX foci assay
for the prediction of intrinsic radiation sensitivity

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List of Abbreviations

Abbreviation	Definition
53BP1	p53-binding protein 1
APE1	Apurinic endonuclease 1
ATM	Ataxia-telangiectasia mutated kinase
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and rad3-related protein
BER	Base excision repair
BRCA1	Breast cancer tumor suppressor protein-1
BRCA2	Breast cancer tumor suppressor protein-2
BrdU	Bromodeoxyuridine
Cal33	A tongue squamous cell carcinoma
CDK	Cyclin dependent kinase
CDTX	Cell-line derived tumor xenograft
cfoci	Corrected foci
CHK2	Checkpoint kinase 2
CT	Computed tomography
CTCAE	Common terminology criteria for adverse events
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DNA	Deoxyribonucleic acid
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DSB	Double strand break
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
FaDu	A hypopharyngeal squamous cell carcinoma
Fas	Fas cell surface death receptor
FasL	Fas ligand
¹⁸ F-FDG	Fluorodeoxyglucose
FFPE	Formalin-fixed paraffin-embedded
GADD45	Growth arrest and DNA damage inducible alpha
hHNSCC	Human head and neck squamous cell carcinoma
HNC	Head and neck cancer
HNSCC	head and neck squamous cell carcinoma
HPV	Human papilloma virus
HR	Homologous recombination
IF	Immunofluorescence
IHC	Immunohistochemistry

Abbreviation	Definition
LIG1	Ligase I
LIG3	Ligase III
LIG4	Ligase IV
LMEM	Linear mixed-effects model
MDC1	Mediator of DNA damage checkpoint protein 1
ML	Maximum likelihood estimation
MRE11	Double-strand break repair protein MRE11
MRN	MRE11-RAD50-NBS1 complex
NBS1	Nijmegen Breakage Syndrome 1
nfoci	Normalized foci
NHEJ	Nonhomologous end joining
ODRC	Offset of dose response curve
p16INK4a	Cyclin dependent kinase inhibitor 2A
p21WIF1/CIP1	A cyclin-dependent kinase inhibitor
p53	Tumor protein p53
PARP1	Poly (ADP-ribose) polymerase 1
PCNA	Proliferating cell nuclear antigen
PET	Positron emission tomography
PNK	Polynucleotide kinase 3'-phosphatase
PP2A	Phosphatase 2A
pRb	Retinoblastoma protein
RAD50	DNA repair protein RAD50
RAD51	DNA repair protein RAD51
REML	Restricted maximum likelihood estimation
ROI	Region of interest
RPA	Replication protein A
SD	Single dose
SDRC	Slope of dose response curve
SKX	A squamous cell carcinoma derived from the floor of the mouth
SSB	Single strand break
SSBR	Single strand break repair
ssDNA	Single strand DNA
TCD ₅₀	Tumor control dose 50%
TME	Tumor microenvironment
TNF	Tumor necrosis factor

Abbreviation	Definition
TNFR1	Tumor necrosis factor receptor 1
UT-SCC-45	A squamous cell carcinoma derived from the floor of the mouth
UT-SCC-5	A tongue squamous cell carcinoma
XLF	XRCC4-like factor
XRCC1	X-ray repair cross complementing 1
XRCC4	X-ray repair cross complementing 4
γ H2AX	Phosphorylated histone variant H2AX on Serine 139 residue

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1. Introduction

Head and neck squamous cell carcinoma (HNSCC) encompasses a heterogeneous group of malignant tumors that arise in the oral cavity, nasopharynx, oropharynx, larynx, or hypopharynx. HNSCC accounts for approximately 90% of head and neck cancers (HNC). More than 300,000 new cases of HNSCC and about 150,000 deaths per year were estimated (Ferlay et al., 2015). In Germany, roughly 20,000 new cases of HNC with 10,000 deaths were registered in 2012, which accounted for about 3.4% of all cancer incidences (Kaatsch et al., 2017). Although HNSCC represents only a small proportion of the overall cancer incidence, the prognosis of patients with HNSCC remains poor, with 5-year overall survival rate of 40 - 50%. Treatment choice for an early stage HNSCC is either surgery or radiotherapy as a single-modality, whereas multimodality treatment is important for treating patients with locally advanced HNSCC in stage T3 or T4 (Colevas et al., 2018). Radiotherapy remains an indispensable and crucial element for the curative care of HNSCC. Recent developments of curative treatment of HNSCC are: a) optimization of fractionation schedules, b) the use of high precision radiotherapy modules i.e., proton therapy and intensity-modulated radiation therapy, and c) implementation of chemotherapeutics and/or immunotherapeutics in treatment plans (Haddad and Shin, 2008; Santuray et al., 2018). One of the major biological factors that influences the radiation response of tumors and normal tissues, namely intrinsic radiation sensitivity, varies among patients (West et al., 1993). Patients, hence, respond to radiation exposure differently. However, patient stratification for treatment planning relies primarily on the stage of disease and primary location, whereas intrinsic radiation sensitivity remains, in general, unconsidered (Caudell et al., 2017).

To individually tailor radiotherapy for a patient, a robust predictive assay, by which the radiation response of tumors can be determined prior to the onset of treatment, is currently in a great need (Baumann et al., 2016). Molecular signatures e.g., tumor hypoxia, cancer stem cell expression, glucose uptake rate, gene signatures, and DNA damage response related biomarkers demonstrated promising potentials as predictive biomarkers for radiotherapy (Yaromina et al., 2012). Gamma H2AX (γ H2AX) – a phosphorylated variant of histone H2AX, represent DNA double strand breaks (DSB) inflicted by genotoxic agents such as radiation (Rogakou et al., 1998). Upon DNA damage induction, accumulation of γ H2AX can be visualized as foci by antibody-based approaches. The central role of γ H2AX in DNA DSB repair mechanisms allows γ H2AX to be used as a DNA DSB biomarker (Kinner et al., 2008). Residual γ H2AX foci (24 h post irradiation) indicate unsuccessful repair of DNA DSB, leading to cell death (Banáth et al., 2010). Therefore, radiation response can be determined by the quantification of residual γ H2AX foci. Determination of residual γ H2AX foci correlated with intrinsic radiation sensitivity of cervical cancer cell lines (Banáth et al., 2004). In *in vivo* studies,

numbers of residual γ H2AX foci in irradiated tumor xenograft models demonstrated a distinct difference between a radiosensitive and a radioresistant tumor model (Menegakis et al., 2011), and correlated negatively with local tumor control values of different hHNSCC xenograft models (Koch et al., 2013). Moreover, residual γ H2AX foci numbers in *ex vivo* irradiated patient-derived materials were able to classify radiosensitivity based on clinical radiation response of the cancers (Menegakis, von Neubeck et al., 2015). Even though the *ex vivo* γ H2AX foci assay could potentially be introduced as a predictive assay for clinical trials, several technical challenges need to be addressed. A tumor biopsy serves as a representative of the corresponding bulk tumor, providing molecular and cellular information of the tumor at a certain time point. However, a question concerning the representativeness of a single biopsy in relation to the corresponding bulk tumor has arisen due to a high heterogeneous trait of tumors. In addition, it is necessary to identify whether radiation response between *ex vivo* irradiated biopsies and the corresponding *in vivo* irradiated tumors are comparable.

This study aims to further optimize and develop the *ex vivo* γ H2AX foci assay by:

1. Investigating the heterogeneity of residual γ H2AX foci among equally treated biopsies,
2. Determining the degree of heterogeneity in the formation and removal of γ H2AX foci between the *in vivo* and *ex vivo* set-up of the assay, and
3. Investigating the comparability of radiation response determined by γ H2AX foci between *in vivo* irradiated tumors and the corresponding *ex vivo* irradiated biopsies.

Human head and neck squamous cell carcinoma (hHNSCC) tumor models (SKX, UT-SCC-45, FaDu, Cal33, and UT-SCC-5) established in the pathogen-free animal facilities were used in this entire study (Experimental center and OncoRay, Faculty of Medicine, Technische Universität Dresden). Xenotransplantation was performed on NMRI nude mice that carry an autosomal recessive mutation in the *FOXn1* gene resulting in T-cell deficiency. The experiments were approved by the regulatory authorities, and followed the institutional guidelines and the German animal welfare regulations.

1.1 Effect of ionizing radiation on cellular level

Ionizing radiation e.g. photons or protons, a type of radiation that is generally applied in the clinics, is identified by its capability to penetrate biological materials resulting in ionization of intracellular water and macromolecules. Ionizing radiation generates damages on biological molecules by either a) direct energy deposition on the target molecules, or b) indirect ionization of surrounding water molecules (radiolysis of water) generating free radicals that further ionize the target molecules. The radiolysis of water was comprehensively summarized (Breen and Murphy, 1995). In brief, the initial step of radiolysis, ionization of water molecules, generates aqueous electron (e_{aq}^-) and oxygen(1+) dihydride (H_2O^+) (Eq. 1). Oxygen(1+)dihydride subsequently reacts to a water molecule (Eq. 2) forming a hydronium ion (H_3O^+) and a hydroxyl radical ($\cdot OH$). Radiation directly excites water molecules followed by hemolysis into a hydrogen radical ($\cdot H$) and a hydroxyl radical (Eq. 3). The presence of hydroxyl radicals can result in the production of hydrogen peroxide (H_2O_2) (Eq.4). In the presence of oxygen, aqueous electron reacts to oxygen molecules resulting in superoxide (Eq. 5.), thereby enhancing the effect of radiation on biomolecules (Herrmann et al., 2006).

Multiple reactive oxygen species produced during the ionization process react with all biological macromolecules such as proteins (Kumta and Tappel, 1961), membrane lipids (Konings, 1987), and DNA molecules (Schulte-Frohlinde and Bothe, 1991), generating damages on these molecules and disrupting their physiological effects. The schematic illustration of direct- and indirect damage on DNA induced by ionizing radiation is depicted (Figure 1.1). The introduction of ionizing radiation to cells affects multiple cellular and physiological functions. To date, the major target of ionizing radiation-induced damages is DNA, promoting cell death, mutagenesis, and carcinogenesis. In general, a single dose of 1 Gy X-ray irradiation can induce approximately 1000 single strand breaks (SSB) and 20-40 double strand break (DSB). Additionally, SSB on both strands in proximity can lead to a formation of a DSB. Whereas, adjacent multiple DSB can cause a clustered DNA DSB lesion, for which a tedious repair process is required (Oike et al., 2016). Shortly after DNA damage induction, cells initiate a defensive mechanism so-called DNA damage repair to rectify the damages.

Cells regain their original functions and the ability to reproduce following the complete DNA repair. Cells with unrepaired damage(s), however, will undergo one of the cell death mechanisms (Kinner et al., 2008).

1.1.1 Radiation induces cell death

Radiation-induced cell death can be processed by a number of mechanisms e.g., mitotic catastrophe, apoptosis, and necrosis. Mitotic catastrophe is the mode of cell death when damage is induced by irradiation or chemotherapeutics. The majority of damaged cells treated with therapeutic irradiation dose do not die immediately. Instead, the damaged cells remain intact and morphologically active (Herrmann et al., 2006). Subsequently, the damaged cells may proceed to mitosis causing an abnormal cell division which generally leads to the formation of multi-nucleate giant cells with uncondensed chromosome (Castedo et al., 2004). This process occurs probably due to cells entering mitosis with unrepaired or misrepaired damages. Following the abnormal mitosis, an insufficiency of critical genetic materials results in a deficiency of essential proteins leading to the loss of replicative capacity and cell death, thereafter (Vakifahmetoglu et al., 2008). Mitotic catastrophe can, also, serve as an initiator for other cell death mechanisms e.g., apoptosis or senescence (Joiner and van der Kogel, 2009).

Figure 1.1 Ionizing radiation induces damages on macromolecules

Direct damages on DNA and other macromolecules are induced by ionization of the molecules and disruption of their chemical bonds. Damages can be indirectly inflicted by ionization of surrounding water molecules, which produces free radicals, which in turn cause damages on the biomolecules.

Apoptosis is a highly regulated process and considered as one of the main cell death mechanisms following ionizing radiation exposure. Cell shrinkage, chromatin condensation, collapsed cytoplasm, nuclear envelope disassembly, and DNA fragmentation are common morphological alterations of cells undergoing apoptosis (Alberts et al., 2002). A family of protease, namely caspase, is involved in the intracellular machinery of apoptosis. To initiate apoptosis, initiator caspases (Caspase 2, 8, 9, 10) are activated followed by the activation of effector caspases (Caspase 3, 6, 7). Apoptosis is initiated via two main pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (Elmore, 2007). The extrinsic pathway begins with the docking of death ligands e.g., TNF (Tumor necrosis factor), FasL (Fas ligand) on their cellular specific receptors e.g., TNFR1 (TNF receptor 1), Fas (Fas cell surface death receptor), resulting in the activation of initiator caspase 8 and 10, which subsequently triggers the effector caspase 3. In contrast, the intrinsic pathway requires the release of mitochondrial proteins e.g., cytochrome-C in order to initiate the process. The release of mitochondrial proteins leads to the activation of initiator caspase 9 and subsequently the effector caspase 3. Finally, adjacent parenchymal cells, neoplastic cells or macrophages execute the end state of apoptosis - phagocytosis of apoptotic cells. (Elmore, 2007). The mitochondrial pathway is more relevant to radiation-induced apoptosis since the induction of the process is dependent on DNA damages and an increase of p53 expression (Herrmann et al., 2006).

In contrast to apoptotic cell death where the dying process is well regulated and does not trigger cytotoxicity, cells that die as a result of acute injury typically swell and burst, resulting in the release of cytoplasmic contents to the surrounding area (Alberts et al., 2002). The release of cytoplasmic contents generally causes a local inflammatory response. Cell death driven by necrosis occurs in cells that are heavily damaged by, for example, irradiation, severe changes of physiological conditions e.g., hypoxia, or a lack of ATP synthesis. Necrosis is considered as a passive mechanism since the execution of necrosis requires no additional protein synthesis and minimal energy. Morphological alteration of necrotic cells is characterized by extensive cell swelling, cell membrane disruption, release of cytoplasm, endocytosis of plasma membrane, and random DNA degradation (Syntichaki and Tavernarakis, 2002).

Senescence refers to a state of permanent cell cycle arrest where cells are irreversibly interrupted in cell propagation (Campisi, 2013). A gradual loss of replicative potential, so-called replicative senescence, is associated with aging process and correlates with telomere shortening at the end of chromosomes during proliferation. Alternatively, the introduction of cellular stresses such as activation of oncogene or DNA damages induced by irradiation can drive cells to enter premature senescence (Joiner and van der Kogel, 2009). Senescent cells

are limited in cell propagation, cells are, however, intact and metabolically survive with an increased expression of senescence-associated β -galactosidase. Although, senescent cells are metabolically active, they do not contribute to tumor and tissue survival due to the permanently suspended cell progression. The master regulators of senescence are p53 and p16INK4a-pRb tumor suppressor pathways (Pawlikowski et al., 2013). Loss of p53 in the vast majority of cancers promotes cells to bypass senescence by which increasing cancer cell propagation, although a subset of tumors undergoes senescence post irradiation (Schmitt, 2007). Radiation-induced senescence frequently occurs in fibroblast cells resulting in fibrogenesis (Hall and Giaccia, 2012).

The main purpose of radiotherapy is to diminish cell proliferation and eradicate tumor cells. Tumor cells that possess the abilities to form clones (clonogenicity) and maintain the infinite cell division capacity are defined as clonogenic cells (Joiner and van der Kogel, 2009). Clonogenic cell death is determined by the loss of clonogenicity and infinite reproduction, by which tumor cells become non-malignant. The term “clonogenic cell death” caused by irradiation covers multiple possible mechanisms (Herrmann et al., 2006). After irradiation, most cells attempt to proliferate, thereby, the major cell death mode in clonogenic cell death is mitotic catastrophe (mitotic cell death). Alternatively, cells can undergo other cell death mechanisms i.e., apoptosis, necrosis, autophagy, or senescence before an onset of mitosis is possible (pre-mitotic cell death). Besides the cell death mechanisms, cells can be deactivated (loss of original function) by the acceleration of cell differentiation due to radiation exposure, so called terminal differentiation. Cell terminal differentiation induced by radiation is frequently observed in normal tissues in form of the development of fibrosis, which drastically reduces patients' quality of life (Rodemann and Bamberg, 1995; Delanian and Lefaix, 2007).

1.1.2 Cell-cycle arrest mediated by radiation

The second countermeasure against radiation-induced DNA damages is the activation of cell-cycle checkpoints (Joiner and van der Kogel, 2009). Cells exposed to ionizing radiation activate cell cycle progression delays through G1, S, and G2 phases (Bernhard et al., 1995). To preserve genetic integrity, damaged cells block the progression through the next phase, hence providing additional time for repairing DNA damages. Cell cycle progression is regulated by cyclin-dependent kinases (CDK), which become active after associating with their cyclin partners. The activation of cell-cycle checkpoints by radiation is a result of cyclin-CDK inhibition via either direct inhibition of a cyclin-CDK complex or indirect modulation by dephosphorylation of CDK (Joiner and van der Kogel, 2009). The major mediator of G1/S phase transition post radiation exposure is p53, which is activated in ATM (ataxia-telangiectasia mutated kinase) dependent manner. p53 mediates G1 phase arrest by a transactivation of p21WIF1/CIP1, an inhibitor of essential cyclins and CDKs for G1/S phase transition, and GADD45 (growth arrest

and DNA damage inducible alpha), a gene mediating growth arrest and DNA damage repair (Bernhard et al., 1995). G1 phase arrest induced by irradiation occurs infrequently due to mutation of p53 in tumors. Consequently, a loss of functional p53 in a wide range of cancers contributes to tumor radioresistance and aggressiveness (McIlwrath et al., 1994). In contrast to G1 phase arrest, cells can initiate G2 phase arrest independent of p53 and p21 status in response to DNA damage induction (Kastan et al., 1991). Nevertheless, p53 and p21 are necessary for sustaining G2 phase arrest after DNA damages (Bunz et al., 1998). In radiosensitive cell lines, a longer G2 phase arrest was observed in relation to matched normal or radioresistant cell lines (Pawlik and Keyomarsi, 2004). Considering G2 phase arrest in tumor cells, the delay might offer a time-extension for repair process by which cell survival after sublethal damages is ensured. As such, disruption of G2 phase arrest by inhibitors e.g., caffeine or pentoxifylline, induces mitotic catastrophe in irradiated tumor cells resulting in radio- and chemo-sensitization effect (Herrmann et al., 2006; On et al., 2011; Filippi-Chiela et al., 2013).

1.2 DNA damage repair

Within a cell, more than 70,000 endogenous lesions, including 20 DSB, occur daily during natural metabolism, serving as a source of spontaneous mutations (Tubbs and Nussenzweig, 2017). Besides, the exposure of cells to exogenous agents e.g., radiation and chemical agents, generates DNA damage. Eukaryotic cells possess a unique DNA repair machinery with a high fidelity to counteract endogenous and exogenous DNA damage. DNA DSB is the most lethal damage produced by irradiation. Cells utilize predominantly two major mechanisms i.e. non-homologous end joining (NHEJ) and homologous recombination (HR) to rectify DNA DSB (Willers et al., 2004). A defect in one of the pathways is strongly associated with an extensive genomic instability and a high radiosensitivity, particularly in patients suffering from defective DNA repair functions (Pollard and Gatti, 2009). Under normoxic condition, cells detect DNA DSB through the MRE11-RAD50-NBS1 (MRN) complex, leading to the activation of DNA-PKcs (DNA-dependent protein kinase catalytic subunit), ATR (Ataxia telangiectasia and Rad3 related), and ATM (Bristow and Hill, 2008). Thereafter, ATM or DNA-PKcs phosphorylates histone H2AX resulting in the formation of γ H2AX in the DSB vicinity. The activation of ATM mediated by the MRN complex and the γ H2AX formation is regulated in a feedback loop (Uziel et al., 2003). DNA damage sensing factors e.g., MDC1 (mediator of DNA damage checkpoint 1), and 53BP1 (p53-binding protein 1), and DNA damage repair proteins involved in HR and NHEJ are, subsequently, recruited within 1-6 h post radiation exposure (Bristow and Hill, 2008). The competition of DNA DSB pathways between NHEJ and HR is partly regulated by 53BP1 (p53-binding protein 1) and BRCA1 (breast cancer tumor suppressor protein-1). 53BP1, an ATM kinase substrate, demotes the end resection mediated by the MRN complex

to favor NHEJ. Conversely, BRCA1, a tumor suppressor gene, promotes the end resection and removes 53BP1 from DSB to initiate HR in S/G2 phase (Daley and Sung, 2014).

1.2.1 Non-homologous end joining (NHEJ)

NHEJ is initiated by the binding of Ku heterodimers (Ku70-Ku80) to both ends of the break preventing extensive DNA end resection (Figure 2). Subsequently, Ku heterodimers recruit DNA-PKcs to the DSB sites forming the Ku/DNA-PKcs complex. The complex formation stimulates the kinase activity of DNA-PKcs and coordinates the repair pathway NHEJ (Mladenov et al., 2016). Artemis, an endonuclease, is phosphorylated and recruited to the DSB sites by DNA-PKcs. Artemis and Pol β (polymerase β) modify DNA ends, providing compatible DNA ends for ligation (Löbrich and Jeggo, 2005). Ligation, the final step of NHEJ that is mediated by DNA-PKcs, is carried out by LIG4 (Ligase IV) accompanied with XRCC4 (X-ray repair cross complementing 4), XLF (XRCC4-like factor), and PNK (Polynucleotide kinase 3'-phosphatase). NHEJ is considered as an error-prone mechanism, in which two ends of a DSB break are rejoined without the use of homology. This process is a fast process but less accurate, thereby causing small deletions or insertions. Despite of the enhanced risk of error, gene coding and regulatory regions account only a minimal fraction of the total genome (ENCODE Project Consortium, 2012), minimizing the probability of damage occurring in protein coding regions. Thereby, the consequence of minor deletions or insertions caused by NHEJ are minimal or non-essential (Joiner and van der Kogel, 2009). Moreover, NHEJ is active throughout the entire cell cycle providing a flexible repair mechanism that increases the chance of survival of damaged cells (Mladenov et al., 2016).

1.2.2 Homologous recombination (HR)

In contrast to NHEJ, HR requires sequence homology of sister chromatid serving as a template to rejoin broken ends. HR is a slow process (require ca. 6 that occurs in G2/S phase due to the requirement of a sister chromatid. However, it provides cells with a highly accurate DSB repair mechanism. ATM phosphorylates BRCA1, which is subsequently recruited to DNA DSB ends (Figure 2). The MRN complex generates 3'single stranded DNA (ssDNA) overhangs, which are immediately coated by RPA (replication protein A). BRCA2 promotes the replacement of RPA by RAD51 resulting in nucleoprotein filaments. RAD52 is attracted to RAD51-coated nucleoprotein filaments to prevent exonucleolytic degradation. RAD51-nucleoprotein filaments invade onto the neighboring sister chromatid leading to the formation of a Holliday junction. Finally, Holliday junction is resolved by helicases. Germline or somatic mutations of HR repair genes are strongly associated with hereditary and sporadic tumorigenesis. For instance, BRCA1/2 mutated genes likely dictate the risk of breast cancer, prostate cancer, and ovarian cancer development (Levy-Lahad and Friedman, 2007).

Moreover, patients with a hereditary disease such as Fanconi anemia, Bloom disease, and Werner disease, which are caused by defects in HR genes, are reported with an extremely high radiation sensitivity (Pollard and Gatti, 2009; Krejci et al., 2012).

Figure 1.2 Overview of DNA double strand break (DSB) repair by NHEJ and HR

Nonhomologous end joining (NHEJ) begins with sensing of DNA ends by Ku heterodimers (Ku70 and Ku 80), which subsequently recruit DNA-PKcs. Artemis, an endonuclease recruited by DNA-PKcs, and Polβ prepare DNA ends for ligation. The final step of NHEJ is ligation, which is conducted by LIG4 with the support of XRCC4, XLF, and PNK. Homologous recombination (HR) starts with the resection on DNA breaks to generate single strand DNA (ssDNA) by MRN complex. The formation of ssDNA prevents DNA DSB repair via NHEJ, and ssDNA is coated by RPA. RPA is replaced by RAD51 with the support of BRCA2. The RAD51 nucleoprotein filaments mediate strand invasion on the neighboring sister chromatid leading to the formation of Holliday junction. The final step of the process is the resolution of the Holliday junction into two DNA duplexes.

1.2.3 Base damage repair and single strand break repair

Base damage repair (BER) and single strand break repair (SSBR) appear to be important for ionizing radiation as mutations or defects on the pathways resulting in an increased radiosensitivity (Murcia et al., 2003). Depending on the cause of damages, base damage and single strand break (SSB), generally, can be repaired by several mechanisms. BER and SSBR pathways are relevant for rectifying damages induced by ionizing radiation (Joiner and van der Kogel, 2009). About 50 times more SSBs than DSBs are generated by 1 Gy of X-ray irradiation, and a great number of endogenous SSBs or base damages occur daily. This implies a strong impact of BER and SSBR in maintaining genomic integrity. BER and SSBR pathways are illustrated in Figure 3. In brief, BER begins with base damage recognition and removal, which are generated by a glycosylase/ DNA lyase. The removal process cut out damaged base without removing DNA backbone resulting in an abasic site (sugar residue). The residual DNA backbone is subsequently removed by APE1 (apurinic endonuclease 1). In case of multiple damaged nucleotides, up to ten nucleotides are removed by APE1. Subsequently, a complex of PCNA (proliferating cell nuclear antigen), and DNA polymerases δ and ϵ synthesizes DNA to replace the missing bases. The DNA strand is rejoined by ligase 1 (long patch). A single damaged nucleotide, in contrast, is replaced by DNA polymerase β , and completed by DNA ligase 3–XRCC1–mediated ligation (short patch) (Joiner and van der Kogel, 2009). Detection of SSB is carried out by PARP1 (Poly(ADP-Ribose)-Polymerase 1) with the support of XRCC1. SSBR subsequently initiates an additional end-processing step mediated by PNK to shape damage ends, resulting in a repair (nicked) intermediate. Thereafter, nucleotide replacement via short or long patch process is carried out to complete the repair process.

Even though SSBs and base damages are unrelated to cell death, unrepaired damages occurred during S phase can lead to DSB and genomic instability (Saleh-Gohari et al., 2005). In addition, radiation is able to inflict a simple SSB accompanied by a base damages (Nikjoo et al., 2001) resulting in a non-DSB clustered damage. In case that radiation-induced SSB are located on the opposite side of base damages, processing of damaged bases by BER can generate DSB (Lomax et al., 2013). Silencing of PARP1 and XRCC1 in HeLa cells considerably increased radiation susceptibility in S phase cells as well as asynchronous cells (Godon et al., 2008). Mutation, deletion, and inhibition of PNK, LIG3 or LIG1 can result in hyper-radiosensitivity (Webster et al., 1992; Lu et al., 2016). Especially, inhibition of PARP1 enhances the effectiveness of treatment for BRCA1/BRCA2 deficient tumors, whose HR pathway is impaired. This implies a synthetic lethality between HR and BER/SSBR repair mechanisms (Joiner and van der Kogel, 2009)

Figure 1.3 Schematic illustration of base excision repair (BER) and single strand break repair (SSBR) pathways

In BER, glycosylase recognizes and removes different base damages as the first step, whereas, PARP and XRCC1 mediate the SSB detection in SSBR. APE1 and PNK subsequently generate a repair intermediate for BER and SSBR, respectively. The repair intermediate is, thereafter, processed by one of two sub-pathways (short patch or long patch).

1.2.4 Role of γ H2AX in DNA damage repair

Histone, a family of positively charged proteins, consists of H1, H2A, H2B, H3, and H4. Two of each histone form a histone octamer, which wraps DNA double helix forming a nucleosome. Histone H2AX, a subtype of the histone H2A family, is a substrate of phosphoinositide 3-kinase-related protein kinases (PIKKs) such as ATM, ATR, and DNA-PKcs (Podhorecka et al., 2010). Cellular content of H2AX varies in a wide spectrum, ranging from 2 to 25% depending on cell types. Upon DNA DSB induction, histone H2AX becomes phosphorylated (γ H2AX) over 1 - 2 megabases surrounding a DSB end (Rogakou et al., 1998). The phosphorylation of H2AX at serine 139 in the SQEY carboxy-terminal tail readily occurs within 1 - 3 min and reaches the highest peak at 1 - 2 h post irradiation depending on cell types (Paull et al., 2000). Phosphatase 2A (PP2A) or PP2A related enzymes dephosphorylate γ H2AX after the complete DNA damage repair (Chowdhury et al., 2005; Chowdhury et al., 2008). Residual or persistent γ H2AX is considered as unrepaired DNA DSB, which potentially induce damaged cells to initiate one of the cell death mechanisms (Bonner et al., 2008; Banáth et al., 2010). Gamma H2AX plays a central role in DNA DSB repair by recruiting multiple DNA damage repair

proteins and retaining those proteins at DNA DSB sites until the damage is successfully repaired (Paull et al., 2000; Podhorecka et al., 2010). As such, deficiency in γ H2AX and its regulators are related to: a) an impaired recruitment of NBS1, BRCA1, and 53BP1 to DNA damage sites, b) an increased risk of chromosome aberration and genomic instability, and c) an alteration in DNA damage repair capability (Bassing et al., 2002; Celeste et al., 2002; Celeste et al., 2003). These lead to an increased radiosensitivity. For instance, SKX, a hHNSCC cell line isolated from the floor of the mouth and alveolar bone of a 83-year old patient, exhibits an extreme radiosensitivity due to a defect in NHEJ pathway and ATM downregulation via miR-421 (Kasten-Pisula et al., 2009; Mansour et al., 2013). Conversely, a high expression of endogenous γ H2AX in colorectal cancer and non-small cell lung carcinoma patients detected by an immunohistological-based method was associated with a poor prognosis compared to its counterpart (Matthaios et al., 2012; Lee et al., 2015). It is, therefore, suggested that tumors endogenously induce DNA damages to customize their molecular mechanisms by which favoring cell proliferation and survival (Tubbs and Nussenzweig, 2017).

1.3 Prediction of tumor radioresponsiveness

To achieve a personalized radiotherapy, assessment of radioresponsiveness of the tumor prior to the start of radiotherapy is crucial, but it is currently unavailable in the clinics (Baumann et al., 2016). It has become evident that radioresponsiveness is strongly correlated with intrinsic radiation sensitivity (Deacon et al., 1984; Fertil and Malaise, 1985; Torres-Roca et al., 2005). Consequently, a large number of attempts to establish an assay using molecular biomarkers and/or omic profile of tumors to predict radiation response for clinical use has been reported (West et al., 1993; Vasireddy et al., 2010; La Thangue and Kerr, 2011; Forker et al., 2015; Menegakis, von Neubeck et al., 2015; Willers et al., 2015; Eze et al., 2017; Reddig et al., 2018). Assessment of DNA damage capacity showed a promising potential in patient classification according to intrinsic radiation sensitivity (Willers et al., 2015; Reddig et al., 2018). Tumor-omics profiling, as well, provides a substantial information concerning treatment response of tumors. Several reports demonstrated that genomic signature of patients were associated with treatment responses and overall survival (Torres-Roca et al., 2015; El Naqa et al., 2017; Scott et al., 2017). Besides, radiomics exhibit a promising potential for personalization of radiotherapy. A predictive model based on over thousands of radiomic features extracted from ^{18}F -FDG-PET (Fluorodeoxyglucose positron emission tomography) and CT (computed tomography) images showed a high potential in the risk assessment of tumor outcomes in HNSCC patients (Vallires et al., 2017). Another study reported a high accuracy of 80% in the treatment response prediction by using radiomic features of treatment planning CT scans and a machine learning approach in colorectal cancer patients receiving chemo-radiotherapy (Bibault et al., 2018).

1.3.1 Prediction of tumor radiation sensitivity by γ H2AX

A potential application of γ H2AX as a radiosensitivity predictor was firstly reported in a cohort of six cervical cancer cell lines (Banáth et al., 2004). The study reported a correlation between residual γ H2AX level and survival fraction after radiation exposure of 2 Gy, and a difference of repair kinetics in a cell line- and p53 status-dependent manner. Clinical applicability of γ H2AX foci as a marker for treatment response prediction was tested in tumor biopsies from cohorts of advanced uterine cervix carcinomas (Bañuelos et al., 2009; Olive et al., 2010). Out of 47 untreated and eight patients treated with chemo- or radiotherapy, residual γ H2AX foci were detected; on average, only 25% of tumor nuclei exhibited one or more γ H2AX foci pre-treatment and 74% after the initiation of treatment (Bañuelos et al., 2009). Out of 26 patients, $24 \pm 19\%$ and $38 \pm 19\%$ of cells contained γ H2AX foci in biopsies taken before treatment and 24 h after exposure to the first fraction of radiotherapy (1.8–2.5 Gy), respectively. No correlation between the local tumor control and the fraction of cells that retained γ H2AX foci was, however, identified (Olive et al., 2010). The studies elicited a complexity in the quantification of γ H2AX foci in patient materials, for which tumor heterogeneity could, probably, be responsible (Cyll et al., 2017).

Preclinical studies of the γ H2AX foci assay as a predictive method for the assessment of tumor radiation response in hHNSCC models were reported (Menegakis et al., 2009; Menegakis et al., 2011; Koch et al., 2013; Menegakis, von Neubeck et al., 2015). Numbers of residual γ H2AX foci determined in two hHNSCC cell lines i.e. FaDu and SKX, showed a strong correlation with clonogenic cell survival fraction *in vitro* ($r^2 > 0.9$). In addition, a good correlation between observed and predicted survival fraction based on residual γ H2AX foci was concluded (Menegakis et al., 2009). In hHNSCC subcutaneous xenograft models of FaDu and SKX, residual γ H2AX foci in a well-perfused area i.e., cells within a distance of 45 μ m from the nearest vessel, correlated with radiation doses ($r^2 > 0.75$). The slopes of dose response curve of residual γ H2AX foci could differentiate FaDu and SKX according to their radiation sensitivity (Menegakis et al., 2011). In nine hHNSCC tumor xenograft models, a robust negative correlation between residual γ H2AX foci and the necessary dose to locally control 50% of the tumors was reported (Koch et al., 2013). The tumor microenvironment (TME) dependent evaluation of γ H2AX foci was successfully translated to *ex vivo* irradiated tumor biopsies and patient-derived materials. Residual γ H2AX foci quantified in *ex vivo* irradiated tumor pieces were capable of classifying three tumor entities i.e., seminoma, prostate, and glioblastoma according to their clinical radiosensitivity (Menegakis, von Neubeck et al., 2015).

2 Tumor heterogeneity determined with a γ H2AX foci assay: A study in human head and neck squamous cell carcinoma (hHNSCC)

Table 2.1 Literature information of publication I

Title	Tumor heterogeneity determined with a γ H2AX foci assay: A study in human head and neck squamous cell carcinoma (hHNSCC)
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2.1 Summary of the publication

Radiation therapy requires a method by which radiosensitivity can be predicted to support physicians in planning individualized therapy and improving therapeutic outcomes. Among predictive biomarkers, the DNA double strand break marker, γ H2AX, is a promising predictor of individual radiosensitivity. The clinical relevance of the γ H2AX foci assay was demonstrated in preclinical studies. Subsequently, a clinically relevant *ex vivo* set-up of γ H2AX foci assay based on tumor specimens and biopsies was established. However, the representativeness of tumor biopsies for the bulk tumor in the *ex vivo* γ H2AX foci assay need to be validated. The aim of this study was to investigate the intra- and intertumoral heterogeneity of residual γ H2AX foci. In addition, the sample preparation step prior to *ex vivo* radiation exposure and the foci evaluation approach were optimized. Excised tumors of hHNSCC xenograft models with different radiosensitivity i.e., SKX, FaDu, and UT-SCC-5, were cut into eight pieces and reoxygenated in culture medium for a) 10 h or b) 24 h. Tumor samples were irradiated *ex vivo*; fixed and paraffin embedded 24 h post irradiation. Two consecutive 3 μ m sections of the central part of the tumor sample were immunostained for a) pimonidazole and BrdU or b) γ H2AX and DAPI. The foci counting was performed manually in oxic tissue regions. The normalized residual γ H2AX foci were fitted to a linear mixed-effects model (LMEM). The intra- and intertumoral heterogeneity was evaluated on each level of the analysis cascades i.e., a) tumor samples of all mice (intramodel), b) tumor samples of a particular mouse (intertumoral interspecimen), and c) the microscopic field of views within one tumor sample relative to the treatment conditions time and radiation dose (intertumoral intraspecimen). The result showed no significant effect of the reoxygenation time (10 h or 24 h) on normalized residual γ H2AX foci. No intramodel heterogeneity was found exempt in FaDu. In contrast, statistically significant intertumoral inter- and intraspecimen heterogeneities were detected in all models. This finding indicated a pronounced intertumoral heterogeneity of residual γ H2AX foci. By applying the newly developed statistic approach, which analyzes the entire factors of interest i.e. the γ H2AX foci heterogeneity among microscopic fields of view, tumor samples, mice, tumor lines, and the treatment conditions simultaneously. The impact of inter- and intraspecimen heterogeneity could be differentiated. Thereby, a more reliable statistical analysis could be achieved. In a clinical situation, patient-derived tumor biopsies with a greater heterogeneity relative to experimental models are expected. Therefore, a comparable or higher numbers of randomly selected nuclei from tumor specimens should be evaluated to define patient-specific heterogeneity for radiosensitivity prediction. Ideally, analysis on multiple tumor specimens that are equally treated under the same *ex vivo* condition is suggested.

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Molecular radiobiology

Tumor heterogeneity determined with a γ H2AX foci assay: A study in human head and neck squamous cell carcinoma (hHNSCC) models

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ABSTRACT

Purpose: This study aimed to analyze the *intra-tumoral* heterogeneity of γ H2AX foci in tumor specimens following *ex vivo* radiation to evaluate the potential of γ H2AX foci as predictors for radiosensitivity.

Material and methods: γ H2AX foci were quantified in tumor specimens of 3 hHNSCC tumor models with known differences in radiosensitivity after reoxygenation in culture medium (10 h, 24 h), single dose exposure (0 Gy, 4 Gy), and fixation 24 h post-irradiation. Multiple, equally treated samples of the same tumor were analyzed for foci, normalized and fitted in a linear mixed-effects model.

Results: The *ex vivo* reoxygenation time had no significant effect on γ H2AX foci counts. A significant *intra model* heterogeneity could be shown for FaDu ($p = 0.033$) but not for SKX ($p = 0.167$) and UT-SCC-5 ($p = 0.082$) tumors, respectively. All tumor models showed a significant *intra-tumoral* heterogeneity between specimens of the same tumor ($p < 0.01$) or among microscopic fields of a particular tumor specimen ($p < 0.0001$).

Conclusion: Similar results for *ex vivo* γ H2AX foci between 10 h and 24 h reoxygenation time support the applicability of the assay in a clinical setting. The high *intra-tumoral* heterogeneity underlines the necessity of multiple analyzable samples per patient and therewith the need for an automated foci analysis.

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Introduction

The biological heterogeneity of tumors leads to patient-dependent treatment response [1–6]. Entering the era of personalized radiation oncology, prediction of radiation response is essential for patient stratification to different treatments which urgently need suitable assays [3]. Among the radiobiology-based biomarker candidates, the phosphorylated histone variant H2AX (γ H2AX) has particularly drawn attention as a DNA double strand break (DSB) marker; a potentially lethal DNA damage. Upon radiation-induced DSB, rapid γ H2AX accumulation along both sites of the damage plays a central role in DNA damage repair machin-

ery [7]. Previous publications showed an evident correlation between persistent γ H2AX, i.e. unrepaired DNA damage, and radiation response in cell-based and tissue-based studies [8–19]. The analysis of γ H2AX foci could be extended as a predictor for normal tissue radiation response in tumor diseases [16,20–22]. To predict the radiosensitivity of the tumor, the evaluation of γ H2AX foci in tumor biopsies or surgical specimens is possible. We previously validated the clinical relevance of the γ H2AX foci assay and developed an *ex vivo* set-up for the γ H2AX foci assay based on tumor specimens [11,13,14,19].

The aim of this study was to validate the γ H2AX foci assay in tumor specimens, e.g. biopsies, for their representativeness of the whole tumor. Thus, *inter model*, *intra model* as well as *intra-tumoral* heterogeneities of γ H2AX foci were examined by applying a linear mixed-effects model. Additionally, the clinical applicability of the assay was optimized by studying foci normalization and *ex vivo* reoxygenation time prior to radiation exposure.

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Material and methods

Experimental tumor models

Tumor xenograft models FaDu, SKX and UT-SCC-5 were generated as described previously [11,13,23,24]. In brief, 4 Gy whole body irradiated 7–14 weeks old male and female NMRI (nu/nu) nude mice from the pathogen-free animal breeding facility (Experimental Center and OncoRay, Faculty of Medicine, Technische Universität Dresden, Germany) were transplanted subcutaneously on the hind leg with tumor pieces taken from cryoconserved tumor material after passing on source animals. The animal facility and the experiments were approved by the regulatory authorities and followed the institutional guidelines as well as the German animal welfare regulations.

Tumor sample preparation

Sixteen FaDu tumors with a maximum diameter of $\sim 10 \times 10$ mm (~ 1 cm³) were excised and cut into 8 pieces. From 9 SKX and 10 UT-SCC-5 tumors with a diameter of ~ 12 – 14 mm (~ 1 – 1.5 cm³), 8 fine needle biopsies per tumor were taken (Bio-Pince 18 gauge, 1.3 mm, Angiotech-Medical Device Technologies Inc., Gainesville, FL, USA) from anesthetized animals (Ketamine ([Ketamin 500 Curamed® Cu-ramed] 100 mg/kg) and Xylazine ([Rompun®] 10 mg/kg)). Subsequently, the tumors were excised from sacrificed animals and cut in 8 pieces similar to FaDu tumors. The tumors of each model were randomly allocated into 2 arms with a tissue reoxygenation time of 10 h and 24 h, respectively.

For tissue reoxygenation, the biopsies and tumor specimens were immediately transferred into 1.5% agarose (A9539,

Fig. 1. (A) Overview of the experimental workflow, (B) scanned IHC sections in a ROI, (C) superimposed images of IHC scans with corresponding IF images, and (D) IF images with marked nuclei for foci analysis of each tumor model. The shown tumor specimens were exposed to 4 Gy and fixed 24 h post-irradiation. IHC: BrdU (proliferation marker, nuclear brown stain), pimonidazole (hypoxic marker, cytoplasmic red stain), and tissue counterstain with hematoxylin (blue). IF: γ H2AX foci were visualized in green (Alexa 488) and DNA in blue (DAPI). IHC and IF images were superimposed to determine oxic and hypoxic areas. The red (B, C) or turquoise (D) line distinguishes between oxic and hypoxic regions. Nuclei were numbered and randomly selected for foci counting. Original magnification is 100 $\times$ and 400 $\times$ for IHC and IF, respectively. IHC: immunohistochemistry staining, IF: immunofluorescence staining.

Sigma–Aldrich, Germany) coated petri dishes containing 2.5 ml of Dulbecco's MEM culture medium supplemented with 2% HEPES, 1% Na–pyruvate, 1% non-essential amino acids, 1% penicillin–streptomycin (all Biochrom AG, Berlin, Germany) and 10% FCS (Sigma–Aldrich, Taufkirchen, Germany) for a) 6 h or b) 20 h. Prior to irradiation, tumor specimens were incubated for 4 h in medium supplemented with 8.5 μ mol pimonidazole (hypoxia marker, Natural Pharmacia International, Belmont, MA, USA) and 5 μ mol Bromodeoxyuridine (BrdU, proliferation marker, SERVA electrophoresis, Heidelberg, Germany) to determine the oxygenation status of the tissue at the time of irradiation. From 8 tumor specimen per tumor, 4 samples received no radiation while the remaining 4 were *ex vivo* exposed to a single dose of 4 Gy (X-rays, Maxishot 200 Y.TU/320-D03, Yxlon Int. GmbH, Hamburg, Germany; 200 kV, 20 mA; 0.5 mm Cu filter; dose rate 1 Gy/min). Immediately after irradiation, culture medium was exchanged to remove surplus pimonidazole and BrdU. Samples were fixed in 4% formalin and embedded in paraffin 24 h post-irradiation (Fig. 1A).

Immunohistochemistry/immunofluorescence staining

The staining procedures have been described elsewhere [11,13,14]. In brief, 2 consecutive 3 μ m sections of the central part of the tumor specimen were either stained for a) pimonidazole, BrdU and hematoxylin (immunohistochemistry, IHC) or b) 4',6-diamidino-2-phenylindole (DAPI) and γ H2AX at Ser139 (immunofluorescence, IF; Merck Millipore, Upstate, clone JBW301, Darmstadt, Germany). ARK™ (Animal Research Kit; Agilent Technologies Deutschland GmbH, Hamburg, Germany) and TSA™ Kit #2 (Alexa488, Life Technologies GmbH, Darmstadt, Germany) were used for IHC and IF staining, respectively. A Zeiss Axio Imager MI fluorescence microscope controlled by AxioVision 4.9 software (Carl Zeiss, Jena, Germany) was used to acquire IHC and IF images. The complete IHC section was scanned with a digital color camera (Axiocam MRc, Carl Zeiss, Jena, Germany, 100X) and 5–7 regions of interest (ROI) were marked for IF image acquisition. In the corresponding IF stained section, 17 individual images per ROI were acquired with a step size of 0.25 μ m (z-stack) using a monochrome digital camera (AxioCam MRm, Carl Zeiss, Jena, Germany; motorized scanning stage, Maerzhäuser, Wetzlar, Germany, 400X). The individual images were merged into a single image for γ H2AX foci analysis.

The evaluation of γ H2AX foci was performed solely in oxic regions (pimonidazole negative tissue) as the number of γ H2AX foci is highly dependent on tumor oxygenation [11]. Viable oxic tumor areas were manually defined by BrdU positive cells indicating proliferation. From 5–7 ROI per tumor specimen, approximately 50–70 randomly selected nuclei were evaluated for γ H2AX foci and nuclear area analysis.

Statistical analysis

Counted foci numbers were corrected for the individual nucleus area as well as for a mean nucleus area of the tumor model (see below) and normalized to the time and dose matching control [12–14]. In brief, the corrected foci number was calculated from:

$$cfoci = \left(\frac{foci_i}{area_i} \right) \times \overline{area}_i$$

where $area_i$ is the area of the individual nucleus and its foci number ($foci_i$). To elucidate the influence of the mean area ($\overline{area}$) on the statistically relevant intra model and intra-tumoral heterogeneity, $\overline{area}$ was either calculated as the mean area of all randomly selected nuclei in a) the corresponding tumor of the currently analyzed nucleus ($\overline{area}_i$) or b) of all tumors of the tumor model treated under the same experimental conditions ($\overline{area}_T$) which was also used in

[11–14]. For a potential clinical application of the γ H2AX foci assay, only $\overline{area}_i$ could be calculated since for a particular patient only one sampling i.e. surgery, is performed.

A mean foci background value of the unexposed tumor specimens $\overline{cfoci}_0$ was calculated for each individual tumor. The corrected foci $cfoci$ of an exposed sample were then normalized by:

$$nfoci = cfoci - \overline{cfoci}_0$$

In the following, $nfoci$ calculated with $\overline{area}_i$ or $\overline{area}_T$ are referred to as $Nfoci_1$ and $Nfoci_2$, respectively. Negative $nfoci$ values were set to zero under the assumption of no negative irradiation induced foci.

Impact of the correction approach on the γ H2AX foci assay

To optimize foci normalization for the *ex vivo* γ H2AX foci assay, two foci correction approaches were conducted using a tumor specific mean nucleus area ($Nfoci_1$) or a global tumor-model specific mean nucleus area ($Nfoci_2$) according to treatment conditions, i.e., reoxygenation time and dose [12–14]. As the normalized foci population showed a positively skewed distribution, a paired sample sign test with a significance level of 0.05 was carried out to test the statistical difference of the correction approaches. A significant difference in normalized foci was found when comparing the correction methods with $Nfoci_1$ leading to increased foci values (Suppl. Fig. 1). To progress toward personalized radiosensitivity estimation and enhance the clinical relevance of the assay, a tumor specific mean nucleus area ($\overline{area}_i$), i.e. a patient specific nucleus area, was applied for further foci analysis ($Nfoci_1$).

Statistical analysis was performed with SPSS 23 (IBM Deutschland GmbH, Ehningen, Germany). Graphs were plotted with OriginPro 8G (OriginLab Corp. Northampton, MA, USA). Paired-sample sign tests were used to determine the influence of the correction methods. To investigate the intra- and intertumoral heterogeneity in γ H2AX foci assay, a linear mixed-effects model, which accounts for the complete range of variations in a single model, was applied in this study. Reoxygenation time and cell line were defined as fixed effects, whereas tumor, specimen and ROI were defined as random effects. The statistically significant threshold was defined as $p \leq 0.05$. In the following *inter model* refers to differences between the 3 hHNSCC models (SKX vs. FaDu vs. UT-SCC-5), *intra model* to differences among tumors of the same model (e.g. FaDu tumor 1 vs FaDu tumor 2) and *intra-tumoral* to differences within one particular tumor (e.g. Specimen 1 vs specimen 2 (*inter-specimen*); ROI 1 vs. ROI 2 (*intra-specimen*)).

Results

Characteristics of tumor specimens

Fig. 1 shows representative tissue sections of the 3 analyzed models with proliferating BrdU positive cells in the outer, oxic tissue rim and a pimonidazole positive central hypoxic tissue core which is in line with our previous study [14]. From 9 SKX, 16 FaDu and 10 UT-SCC-5 xenograft tumors, 86%, 94% and 99% of all processed tumor specimens could be evaluated for γ H2AX foci, respectively (Table 1). In SKX and UT-SCC-5, 33.9% and 63.3% of the biopsies, which were taken prior to tumor excision, could be analyzed. If the biopsies were not assessable due to necrotic or differentiated tissue, the corresponding tumor specimens, which were excised and processed in parallel to the biopsies, were evaluated in addition. Overall, 273 (95% confidence interval (CI): 199–226, SKX), 343 (95% CI: 321–363, FaDu) and 213 nuclei (95% CI 199–226, UT-SCC-5) per tumor specimen were suitable for analysis and 21%, 19% and 31.9% of all analyzable nuclei were evaluated for γ H2AX foci, respectively (Table 1).

Table 1
Characteristics of tumors and the analyzed tumor specimens. *n* = number.

Tumor model	Grade	TCD ₅₀ [95% C.I.] (Gy)	<i>n</i> of tumors	Total <i>n</i> of tumor specimens	Analyzable tumor specimens (%)	Total <i>n</i> of analyzable nuclei	Analyzed nuclei (%)	Mean <i>n</i> of analyzable nuclei/specimen [95% C.I.]
SKX	G2	14.9 [10.9; 18.9][11]	9	72	62 (86%)	$\sim 1.69 \times 10^4$	620 (21%)	273 [199–226]
FaDu	G4	31.0 [27; 39][25]	16	128	120 (94%)	$\sim 4.11 \times 10^4$	1200 (19%)	343 [321–363]
UT-SCC-5	G3	35.7 [16; 41][25]	10	80	79 (99%)	$\sim 1.68 \times 10^4$	790 (32%)	213 [199–226]

Heterogeneity of γ H2AX foci

The *inter model* comparison of Nfoci₁ showed significant differences ($p < 0.0001$; data not shown). For all tumor models, a significant difference in γ H2AX cfoci between exposed and unexposed samples were found with 4 Gy irradiated samples showing higher foci values 24 h post exposure (SKX $p < 0.0001$; FaDu $p = 0.003$; UT-SCC-5 $p < 0.0001$; data not shown). The most sensitive tumor model SKX showed the highest Nfoci₁ values after exposure to 4 Gy in comparison to the other 2 hHNSCC models which are characterized by similar 50% tumor control doses (TCD₅₀, Table 1).

To investigate the *intra model* and *intra-tumoral* heterogeneity of γ H2AX foci in SKX, FaDu and UT-SCC-5, a linear mixed-effects model was applied. The linear mixed-effects model evaluates Nfoci₁ on each level of the analysis chain which were a) all tumors per model (*intra model*), b) all tumor specimens of a particular tumor (*intra-tumoral inter-specimen*) and c) the microscopic ROI within one tumor specimen (*intra-tumoral intra-specimen*) relative to the reoxygenation times (Fig. 2A). The reoxygenation time had no significant effect on Nfoci₁ (Table 2, Suppl. Fig. 2). A significant *intra model* heterogeneity was exclusively seen for FaDu (Fig. 2B, Table 2). For all three models, statistically significant *intra-tumoral inter-specimen* as well as *intra-tumoral intra-specimen* differences in Nfoci₁ were found (Table 2). This result suggests a higher *intra-tumoral* than *intra model* heterogeneity as well as a pronounced *inter model* heterogeneity of Nfoci₁ in hHNSCC models.

Discussion

The current study is aiming to further increase the clinical applicability of the γ H2AX foci assay as a predictive tool for tumor radiosensitivity. Before onset of primary radio(chemo)therapy, only tumor biopsies, i.e. a limited amount of tumor tissue is available for γ H2AX foci analysis. The same holds sometimes true when surgery is preceding radiotherapy and most of the tissue is used for routine histological diagnosis or depending on the tumor entities, e.g. glioblastomas, large areas of necrotic tissues are surgically removed which are not suitable for analysis of viable tissue reactions such as DNA repair. To test the hypothesis that γ H2AX foci from individual tumor specimens are representative for the bulk tumor, the impact of *intra model* and *intra-tumoral* heterogeneity in the clinically relevant *ex vivo* set-up of the γ H2AX foci assay was investigated in 3 hHNSCC xenograft models.

The *ex vivo* set-up of the assay is based on the random selection of analyzable ROI from an individual tumor specimen [14]. For analysis with different sources of variability, standard linear regression models are not appropriate [26–28]. Linear mixed-effects models are extensions of linear regression models and allow for investigation of multiple variabilities. In addition to fixed effects, they include random effects and repeated measurements associated to the experimental design and can therefore determine data heterogeneity [27–30].

To the best of our knowledge, this is the first study analyzing heterogeneity of γ H2AX foci with a linear mixed-effects model providing a statistical framework that accounts for multiple sources of variation [27]. Here, significant *intra model* differences in Nfoci₁ were found for FaDu only while *intra-tumoral* differences

were evident for all 3 models among tumor specimens of an individual tumor (*inter-specimen*) as well as among microscopic ROI (*intra specimen*, Fig. 2, Table 2). The highest contribution to the overall detected significant Nfoci₁ variations were provided by the differences among tumor specimen and ROI. This suggests heterogeneity in micromilieu parameters affecting repair efficacy and clonal composition as well as random distribution of DNA damage induced by radiation [14,15,31,32].

In our previous study, experimental tumor specimens were reoxygenated for 0.5–12 h prior to exposure to 4 Gy leading to a stable plateau of residual γ H2AX foci values after 6 h of reoxygenation [14]. However, tumor sample processing after a reoxygenation time of 6–12 h is challenging in a routine diagnostics context as this would not fit in one-shift pathology lab organization. Here, we could show that a reoxygenation time of 10 h or 24 h had no statistical significant effect on residual γ H2AX foci. This dramatically enhances the clinical application of the assay by providing a high time flexibility in sample processing. We and others could show that evaluation of γ H2AX foci in clinical samples is feasible following a 24 h reoxygenation period [12,14].

The dose-dependent induction of γ H2AX foci is highly dependent on tumor oxygenation [11]. Although endogenous hypoxia markers such as Hypoxia-inducible factor 1- α , Glucose transporter 1, Carbonic anhydrase 9 might not always correlate with pimonidazole, it has been extensively used for assessment of (chronic-) hypoxia due to its robustness and specificity [33–38]. Differentiation of oxygenation status based on pimonidazole labeling has been successfully demonstrated for specimens of experimental tumors [11,13] and patient biopsies [12,14,39].

In the *in vivo* set-up of the γ H2AX foci assay, a global tumor-model specific mean nucleus area was used since an *intra-tumoral* control was experimentally not available following *in vivo* irradiation [11,13]. However, in this study, the area of randomly selected nuclei differed significantly across tumors from the same experimental condition (Suppl. Fig. 1), underlining the necessity for a tumor-specific mean nucleus area. Differences in nuclear area within one tumor model might be attributed to adaptations of the tumor micromilieu to the *ex vivo* culture conditions. The stimulation of *ex vivo* growth of transiently suppressed tumor cells *in vivo*, e.g. due to low oxygen or nutrition status, might lead to changes in the cell cycle distribution. Due to pan-nuclear γ H2AX staining of S-phase cells, analyzable tumor cells are in G1- and G2-phase of the cell cycle. Increased numbers of G2-phase cells would lead to a higher mean nucleus area. With regard to clinics, an inter-patient heterogeneity in tumor cell size, i.e. area, should be expected. Using a tumor- and treatment specific mean nucleus area in the foci correction seems therefore reasonable for individualized prediction of radiosensitivity.

On occasion, mean cfoci values of unirradiated control samples were higher than time matching individual foci numbers of irradiated samples. The resulting negative nfoci values were set to zero anticipating no negative irradiation induced DNA damage. However, the predictive value of cfoci in unirradiated patient derived tumors specimens should be analyzed in a clinical context. Previously, we could show that the basal γ H2AX foci numbers from unirradiated tumor samples differed among 9 hHNSCC models.

Fig. 2. (A) Analysis chain of the linear mixed-effects model where the heterogeneity of Nfoci₁ among (B) all tumors of the same model (*intra model*), (C) tumor specimens of a particular tumor (*intra-tumoral inter-specimen*), and (D) region of interest within a tumor specimen (*intra-tumoral intra-specimen*) were sequentially investigated. Bold letters indicate the tumors and specimens of the example box plots, respectively. T: tumor, S: specimen, ROI: region of interest.

Table 2

Statistical analysis of Nfoci₁ fitted to a linear mixed-effects model for the hHNSCC tumor models SKX, FaDu and UT-SCC-5. The reoxygenation time was defined as fixed effect whereas tumor, tumor specimen and microscopic ROI were defined as random effect.

Tumor model		p Value		
		SKX	FaDu	UT-SCC-5
Fixed effect	Reoxygenation time	0.479	0.142	0.120
Random effect	Tumor (<i>intra model</i>)	0.167	0.033	0.082
	Tumor × Specimen (<i>inter-tumoral inter-specimen</i>)	0.007	<0.0001	0.006
	Tumor × Specimen × ROI (<i>inter-tumoral intra-specimen</i>)	<0.0001	<0.0001	<0.0001

Particularly the human papilloma virus (HPV) positive UT-SCC-45 model showed the highest basal foci values in conjunction with lowest fractionated tumor control dose of the investigated models with no known mutation in DNA repair pathways [13]. Recently, it was shown that the HPV status is a prognostic biomarker for local-regional control in HNSCC patients treated with postoperative or primary radiochemotherapy [40,41]. Thus, foci numbers of unirradiated samples could have clinically relevant predictive values.

Quantification of γ H2AX foci is time consuming and subjected to inter-observer variations. Those short-comings have been overcome for *in vitro* samples and liquid biopsies by open source image

processing or commercially available software [9,10,42,43]. There is still no automated software solution available for the analysis of γ H2AX foci in highly variable solid tumor samples (e.g. due to overlapping cells, different cell types, complex microenvironment) which currently hinders a clinical application of the assay.

In conclusion, significant *inter model*, *intra model* (FaDu only) and *intra-tumoral* heterogeneity in γ H2AX foci were found in 3 hHNSCC tumor models with the applied linear mixed-effects model, which could be extended to be a standard statistical approach to analyze γ H2AX foci data. The cellular composition of established tumor models is considered to be relatively homogeneous due to clonal

selection during tumor passaging [44]. In patient derived biopsies, a higher degree of heterogeneity should be expected caused by the complex tumor niche [44–46]. Thus, a comparable or higher number of randomly selected nuclei per tumor specimen as in this study should be analyzed to define patient specific heterogeneity in γ H2AX foci for prediction of intrinsic radiosensitivity; ideally from multiple tumor specimens treated under the same *ex vivo* conditions. Nonetheless, a potential application of the assay in the clinic needs a sophisticated automated software solution for foci analysis.

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Declarations

Ethical approval and consent to participate: The local committee on ethics of animal experimentation approved all experiments.

Conflict of interest

The authors have no conflict of interest to declare.

Availability of data and material

The dataset generated during the current study is available from the corresponding author on reasonable request.

Competing interest

The authors declare that they have no competing interests.

Author's contribution

TR and CvN performed all the experiments and wrote the manuscript. TR analyzed the data. CvN designed the animal study. SL and UR were major contributors to the statistical analysis. MK and MB had the project idea, wrote the concept for grant application, supervised the study and contributed to data interpretation. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.radonc.2017.06.027>.

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3 Heterogeneity of γ H2AX foci increases in *ex vivo* biopsies relative to *in vivo* tumors.

Table 3.1 Literature information of publication II

Title	Heterogeneity of γ H2AX Foci Increases in Ex vivo Biopsies Relative to In vivo Tumors
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3.1 Summary of the publication

Evaluation of radiation induced γ H2AX foci demonstrates a great potential to be developed as a predictive assay, by which treatment individualization and improvement of therapeutic outcome could be achieved. Tumor heterogeneity, however, poses a significant complexity in the determination of tumor radiation response. This study aims to investigate the representativeness of a single biopsy and the translatability of *ex vivo* obtained foci results to *in vivo* tumors. Here, γ H2AX foci from two previously published datasets with comparable experimental designs exempt sampling and radiation exposure i.e. *in vivo* and *ex vivo*, were compared. Endogenous (control), initial (30 min post irradiation) and residual foci (24 h post irradiation) were corrected, normalized and subjected to LMEM, which fixed- and random effects were incorporated in the analyses. In addition, correlation between cellular geometry distribution and detected foci was investigated by linear regression. The statistical reassessment revealed that radiation and the experimental settings induced alteration in cell geometry distribution. In both experimental settings, a slight enlargement of nucleus size after radiation exposure was found in most of the analyzed tumor lines. Difference of cell geometry distribution was present between *in vivo* and *ex vivo* set-up depending on tumor lines. A positive linear correlation between detected foci number and nucleus size distribution was found in irradiated samples. Significant intratumoural heterogeneity i.e., heterogeneity among microscopic field of views within one tumor specimen, was observed in initial γ H2AX foci for the *in vivo* set-up in most of evaluated tumor lines. Residual γ H2AX foci of the *ex vivo* set-up exhibited a greater degree of intratumoural heterogeneity relative to the *in vivo* set-up. Solely in FaDu, comparable residual γ H2AX foci between the experimental settings were found, where the sample size of the *ex vivo* set-up is twice as high as the *in vivo* set-up. In conclusion, the altered cellular geometry distribution of tumor cells post irradiation implies cell-cycle phase alterations. The linear relationship between detected foci numbers and nucleus size distribution supports the relevance of integrating cellular geometry in data processing by which relative foci numbers could be estimated. The greater degree of heterogeneity in γ H2AX foci for the *ex vivo* set-up and the difference in nucleus size between the experimental settings indicated a strong influence of the culture condition in cellular adaptation and DNA damage induction. For an accurate prediction of tumor radiation response based on the *ex vivo* γ H2AX foci assay, multiple biopsies per tumor and multiple microscopic field of views per biopsy should be accounted in the evaluation.

Article

Heterogeneity of γ H2AX Foci Increases in Ex Vivo Biopsies Relative to In Vivo Tumors

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Abstract: The biomarker for DNA double strand breaks, gammaH2AX (γ H2AX), holds a high potential as an intrinsic radiosensitivity predictor of tumors in clinical practice. Here, two published γ H2AX foci datasets from in and ex vivo exposed human head and neck squamous cell carcinoma (hHNSCC) xenografts were statistically re-evaluated for the effect of the assay setting (in or ex vivo) on cellular geometry and the degree of heterogeneity in γ H2AX foci. Significant differences between the nucleus areas of in- and ex vivo exposed samples were found. However, the number of foci increased linearly with nucleus area in irradiated samples of both settings. Moreover, irradiated tumor cells showed changes of nucleus area distributions towards larger areas compared to unexposed samples, implying cell cycle alteration after radiation exposure. The number of residual γ H2AX foci showed a higher degree of intra-tumoral heterogeneity in the ex vivo exposed samples relative to the in vivo exposed samples. In the in vivo setting, the highest intra-tumoral heterogeneity was observed in initial γ H2AX foci numbers (foci detected 30 min following irradiation). These results suggest that the tumor microenvironment and the culture condition considerably influence cellular adaptation and DNA damage repair.

Keywords: radiation; predictive biomarker; DNA damage response; mixed model statistics

1. Introduction

Cytogenetic biomarkers or molecular (protein-) biomarkers have been extensively investigated in experimental and translational research [1–5] to determine the effect of radiation at the cellular

level. However, previous attempts to utilize biomarker data for radiation dose calculation in each individual patient have been unsuccessful. For individualized radiotherapy to be successful, an assay which reliably predicts radiation response is urgently needed to allow for patient stratification and treatment personalization [4,6–10]. GammaH2AX, a phosphorylated variant of the histone H2A family, plays a central role in DNA damage repair pathways. The generation of the phosphorylated histones in the proximity of DNA damage (mainly DNA double strand breaks (DSB)), can be visualized as distinct foci by immunological-based approaches [11]. In addition to the role of γ H2AX foci as a DNA DSB biomarker, several biological functions can have an impact on γ H2AX foci, e.g., changes in chromosome structure [12] and alteration in the p53-p-21-WIP1 pathway [13]. However, monitoring of γ H2AX foci using epifluorescent microscopy is a fast, inexpensive and reliable method for DNA DSB detection [6,8]. Besides the high sensitivity and straightforwardness of the γ H2AX foci assay, residual γ H2AX foci were demonstrated to correlate with intrinsic radiosensitivity in experimental tumor models [5,14,15], as well as patient-derived materials [16–18]. The recently developed ex vivo analysis of γ H2AX foci based on tumor biopsies showed promising potential in being used as a clinical predictive assay [5,6,16–18]. Despite the high capability of the assay, tumor heterogeneity—one of the major complexities in cancer [19–22]—challenges the reliability of the assay in radiosensitivity prediction. This leads to controversial concerns addressing the representativeness of ex vivo biopsies relative to in vivo (xenograft) models and patient materials. In addition, the in vivo and ex vivo set-up of the assay relies on γ H2AX foci detection in sectioned specimens, leading to only partially analyzed cell nuclei. Thereby, the geometrical changes of cell nuclei due to the in vivo or ex vivo set-up need optimization of γ H2AX foci data processing for clinical application of the assay.

This study aims to further optimize the assay by determining the effects of radiation, experimental settings and cellular geometry on γ H2AX foci by statistical means. Two published datasets with comparable experimental design but performed with in vivo tumors [14] and ex vivo tumor biopsies [5] were analyzed with a linear mixed-effects model (LMEM) as well as linear regression. The LMEM extends linear regression by including random effect terms in the model. This statistical approach permits the incorporation of multiple sources of variation in the analysis so that complex experimental designs can be comprehensively evaluated and heterogeneity of data can be determined [23–25]. Thus, the degree of heterogeneity in γ H2AX foci and the influence of the culture condition on the cellular geometry were assessed in two γ H2AX foci datasets.

2. Results

2.1. Impact of Extrinsic Influences on Cellular Geometry

In both datasets, cell nucleus areas and foci numbers were determined. The logarithmically transformed nucleus areas of tumors from different hHNSCC models were fitted by the LMEM, where on average ($\pm$ SD) 415 ± 59 (in vivo) and 3050 ± 1073 (ex vivo) nuclei per tumor model and treatment cohort were analyzed (Table S1). For the in vivo cohort, no statistically significant increase in the mean nucleus area was found in 30 min post exposure samples (4 Gy) relative to unexposed controls. However, the means of nucleus area increased significantly for in vivo (~14%) and ex vivo (~6%) at 24 h post irradiation (4 Gy) in four out of nine (in vivo) and two out of three (ex vivo) evaluated tumor models (Figure 1A,B). The nucleus areas of UT-SCC-5 tumors were not affected in both settings. Independent of treatment, the nucleus areas of FaDu and SKX tumors were statistically significantly larger in the in vivo cohort compared to the ex vivo setting, but smaller in UT-SCC-5 tumors (Figure 1C).

The nucleus areas were classified in six area categories and foci numbers were plotted accordingly. Linear regression analysis of the nucleus categories and foci numbers showed a significantly linear increase of the foci number with the nucleus area in exposed samples but not in controls for all tumor lines in both cohorts (Figure 2 and Figure S1). To summarize, the experimental setting and the radiation exposure have a significant impact on cell geometry measured as cell nucleus area.

Figure 1. Impact of radiation exposure (4 Gy) and experimental setting (in vivo versus ex vivo) on nucleus area distribution. Scatter plots of nucleus areas from different hHNSCC xenograft models in vivo (A) and ex vivo (B). Comparison of nucleus areas between the experimental settings (open-circle: in vivo, red: ex vivo) in three hHNSCC tumor lines (FaDu, SKX, UT-SCC-5) (C). Nucleus areas were logarithmically transformed and fitted by a linear mixed-effects model (LMEM). Treatment conditions (control, 4 Gy exposure, time point post irradiation) or experimental settings (in vivo versus ex vivo) were defined as fixed effects; tumor, specimen, as well as region of interest (ROI) as random effects. Multiple pairwise comparisons (in vivo) with Bonferroni correction following LMEM analysis were performed. Solid bars represent means. (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$). p values can be found in Supplementary Tables S2 and S3.

Figure 2. Number of foci linearly increases with the nucleus area (x_i) categories (C). Exemplary box plots of foci number as a function of the nucleus area categories in FaDu (**upper row**), SKX (**middle row**), and UT-SCC-5 (**lower row**) of control (0 Gy) and exposed (4 Gy) tumors. Nucleus areas were classified into six categories with an interval size of 40 μm². Foci number of controls and exposed tumors, which were fixed 30 min and 24 h post exposure for the in vivo set-up (**left panels**) as well as controls and exposed tumor fixed at 24 h post exposure for the ex vivo set-up (**right panels**) are shown. P value of the linear regression analysis is shown. The linear regression analysis outputs as well as data for the remaining six hHNSCC models are presented in supplementary Figure S1 and Table S4. Please note the different Y-axis for 30 min post-exposure in the in vivo set-up.

2.2. Heterogeneity of γ H2AX Foci in Two Experimental Settings

2.2.1. In Vivo Cohort

The heterogeneity of γ H2AX foci was analyzed with LMEM in the in vivo cohort (Figure 3). Foci numbers were corrected and scaled based on the individual nucleus area as well as the mean of the nucleus area of the tumor (cfoci). Normalized foci (nfoci_g) were calculated by subtracting cfoci of an individual cell with the mean cfoci of the unexposed tumors (for details, see Section 4). The analysis revealed inter-tumoral (among tumors) homogeneity of cfoci in most of the tumor models. This was indicated by insignificant variations in endogenous (control), initial (30 min post exposure) and residual (24 h post exposure) cfoci with the exception of initial cfoci of UT-SCC-14 (Figure 3A, Tumor). In contrast, the variations in cfoci among microscopic regions of interest (ROI) i.e., intra-tumoral heterogeneity were considerably pronounced in initial cfoci (Figure 3A, ROI). However, endogenous and residual cfoci were mostly homogenous among microscopic ROI except endogenous cfoci in XF354 and residual cfoci of SKX. Inter-tumoral homogeneity based on nfoci_g was similar to cfoci with initial nfoci_g of UT-SCC-14, showing sole significant heterogeneity (Figure 3B, Tumor). Intra-tumoral heterogeneity based on microscopic ROI was found in most of the tumor hHNSCC lines for initial nfoci_g and in SKX and SAS tumors for residual nfoci_g (Figure 3B, ROI).

Figure 3. Heterogeneity of corrected foci (cfoci, upper row) and normalized foci (nfoci_g, lower row) for the in vivo set-up determined by a linear mixed-effects model (LMEM). Scatter plots of $-\log_{10}P$ values according to the analysis chain of LMEM (black-square: control, red-circle: initial foci, blue-triangle: residual foci) for cfoci (A) and nfoci_g (B). No fixed effect was defined; tumor and ROI were set as random effects. cfoci represents the ratio of foci number and nucleus area of an individual cell multiplied by the mean nucleus area of the corresponding tumor, whereas nfoci is cfoci of an individual nucleus normalized by the mean cfoci of the unexposed tumors (for details see Section 4). Solid lines indicate the significant threshold ($p = 0.05$, above the line: $p < 0.05$). Exact p values are given in Supplementary Tables S5 and S6.

2.2.2. Ex Vivo Cohort

Inter-tumoral homogeneity of cfoci (among tumors of one hHNSCC model) could be observed in SKX and UT-SCC-5 tumors, but not in FaDu tumors (Figure 4A, Tumor). Intra-tumoral inter-specimen heterogeneity of cfoci (variation among specimens of one tumor) was present in all samples (Figure 4A, Specimen), whereas intra-tumoral intra-specimen heterogeneity of cfoci (variation among ROI of one tumor) was detected in all samples, except endogenous cfoci of SKX and UT-SCC-5 (Figure 4A, ROI). The outcome of the statistical analysis for nfoci_g in the ex vivo cohort is similar to the previous report although a different normalization approach was applied [5]. Considering intra-tumoral heterogeneity of cfoci and nfoci_g between the in- and ex vivo set-up of the assay, a higher degree of variation can be seen in the ex vivo cohort.

Figure 4. Heterogeneity of corrected foci (cfoci, **A**) and normalized foci (nfoci_g, **B**) for the ex vivo set-up determined by a linear mixed-effects model (LMEM). Scatter plot of $-\log_{10} P$ value according to the analysis chain of LMEM for cfoci (**A**) and nfoci_g (**B**) in three tumor models (black-square: control, blue-triangle: residual foci). No fixed effect was defined; tumor, specimen, and ROI were set as random effects. cfoci represents the ratio of foci number and nucleus area of an individual cell multiplied by mean nucleus area of the corresponding tumor, whereas nfoci is cfoci of individual nuclei normalized by the mean cfoci of unexposed tumors (for details, see Section 4). Solid lines indicate the significant threshold ($p = 0.05$, above the line: $p < 0.05$). Exact p values are given in Supplementary Tables S5 and S6.

The comparison of cfoci and nfoci_g in the in vivo and the ex vivo cohort shows that the results of the γ H2AX foci assay are dependent on the experimental setting. An insignificant effect of the culturing condition in residual cfoci and nfoci_g was only detected in FaDu tumors (Figure S3). Altogether, these results suggest a higher degree of variation in cfoci and nfoci_g of the ex vivo set-up compared to the in vivo set-up of the γ H2AX foci assay.

3. Discussion

The γ H2AX foci assay has been considered as one of the most promising approaches in radiation dosimetry [26–28] and intrinsic radiosensitivity prediction [2,3,5,6,14–18,29]. Previously, we showed a significant negative correlation between residual γ H2AX foci following in vivo exposure and the TCD₅₀ (dose to control 50% of tumors locally) [14]. Furthermore, we demonstrated that the slopes of the dose-response curves of γ H2AX foci in ex vivo cultured patient-derived and experimental tumor-derived biopsies differentiated the tumors according to their intrinsic radiosensitivity [16]. The described ex vivo assay relies on ex vivo irradiated tumor specimens of patient or experimental tumors obtained by surgery or punch biopsies. Potential drawbacks for a clinical application of the assay could, however, be the representativeness of a single biopsy and the translatability of ex vivo

obtained foci results to an in vivo tumor [30,31]. Here, the representativeness of the γ H2AX foci assay was investigated by comparing two published datasets (in vivo [11] and ex vivo [5]), in which the experimental conditions are comparable. In addition, the influence of cellular geometry e.g., the nucleus area, on foci normalization was analyzed. Both questions were statistically approached with a LMEM.

The current study is limited to two experimental datasets generated in a single institution causing an inevitable bias due to e.g., staining procedures, imaging, and γ H2AX foci quantification. To the best of our knowledge, there is no published comparable dataset from a different laboratory that is applying the same independent factors i.e., radiation dose, time points, and tumor models. The constant experimental factors minimize their influence on the statistical analysis and enable a unique comparison investigating the effect of the ex vivo cultivation/exposure relative to in vivo exposed tumors. Thus, first conclusions on the influence of the assay setting on the assay readout (γ H2AX foci) can be drawn. The results will have major implications: if the ex vivo γ H2AX foci assay is not able to reflect the in vivo reaction of the tumor, the assay no longer has the potential to be translated in a clinical routine method.

In a previous publication, γ H2AX foci were corrected by the nucleus area and the mean of the nucleus area as weighting and scaling factors, respectively [5,14,16]. Radiation can influence the size of a cell nucleus by e.g., initiating a cell cycle arrest [32–34] or alterations in chromatin condensation [35]. Due to the pan-nuclear γ H2AX signal in the S-phase of the cell cycle [36,37] and in apoptotic cells [38–40], intact- and analyzable nuclei are restricted to G₁- or G₂-phase of the cell cycle. In histological sections, a change in nucleus size due to an alteration in the cell cycle would be reflected by an increase or decrease of the nucleus area on average. In clinics, a high variation in tumor cell size, i.e., area, due to inter- and intra-patient heterogeneity should be anticipated. Moreover, the size of the nucleus might change in a dose and time-dependent manner, i.e., intensification and/or protracted release of a G₂-phase cell cycle arrest after high dose exposure. The incorporation of the nucleus area into a predictive assay or a mathematical model is therefore crucial. Normalizing the foci data on a tumor- and treatment specific mean nucleus area appears to be a reasonable approach for personalized radiosensitivity prediction [5].

Here, we could show alterations of the nucleus area distribution depending on the experimental setting and time after irradiation. In the in vivo and ex vivo cohorts, four out of nine and two out three models showed a significant increase in nucleus area 24 h post exposure (Figure 1), respectively, which was also represented by a switch in cell nucleus categories, i.e., towards higher categories (Figure S2). Both findings most likely indicate a G₂-phase cell cycle arrest. Interestingly, the change of tumor microenvironment (from in vivo to ex vivo) induced a significant reduction in cell nucleus area in FaDu and SKX, while UT-SCC-5 tumors increased their cell nucleus areas (Figure 1C) independent of the radiation treatment. Especially, the area changes seen in control samples suggest that cells adapted to the new culture condition by modulating proliferation and progression through the cell cycle.

Determination of intrinsic radiosensitivity by γ H2AX cfoci and nfoci on tumor sections relies on immune-histopathological approaches [5,14,16,17]. Relative to the in vitro situation where foci formation of an entire nucleus can be observed, solid tissue sections have a common limitation: the representation of a three-dimensional tumor structure in a two-dimensional top-view projection. Cells and cell nuclei are sectioned in different anatomical planes depending on the positioning of tissue during the embedding process. This circumstance is more relevant in tumors, as nucleus size variation is a common feature [41–43]. This study demonstrated a linear correlation between foci number and nucleus area after DNA damage induction, indicating a dependency of foci detection on measured nucleus area. This emphasizes the relevance of incorporation of a cellular geometry in the data processing chain [44] by applying e.g., a LMEM. Analysis of foci cluster, size, or volume could potentially provide additional information on (complex-) DNA damage and intrinsic radiosensitivity, since foci are significantly dependent on radiation quality [44,45] and cell lines [46]. This indicates a functionality of foci geometry as an indicator for DNA damage complexities [47]. Estimating foci

volume in conjunction with foci number per nucleus area in tumor specimens could, therefore, be a promising approach in the assessment of tumor radiation response.

Following ionizing radiation, the γ H2AX foci signal reaches the intensity peak at approximately 30 min post DNA damage induction in a dose-dependent manner [48,49]. Pronounced intra-tumoral heterogeneity of initial cfoci and nfoci could be seen in the in vivo cohort for most of the evaluated tumor models (Figure 3A,B). The indistinctive morphology of foci at early time points post exposure i.e., overlapping foci and giant foci, contributes to the data variation [14,33,48], which might call for a higher sample size for initial foci detection to minimize the variability.

Tumor heterogeneity is one of the common complexities in cancer research and therapy [19,20]. Generally, a higher degree of heterogeneity would be expected in vivo, where a plethora of uncontrollable factors e.g., oxygen supply, nutrition supply, and tumor microenvironment can potentially affect the biological read-out [30,31,50,51]. However, in this study, both cfoci and nfoci_g showed a more pronounced heterogeneity in the ex vivo set-up, compared to the in vivo set-up. Based on these results, dissecting and transferring a tumor sample into a new microenvironment with supposable optimal oxygen and nutrition supply led to the intensification of heterogeneity in γ H2AX foci. An asynchronous adaptation of cells to the ex vivo culture condition could possibly contribute to the higher degree of heterogeneity in γ H2AX foci. A LMEM approach includes random and fixed effects of a data acquisition chain, which are frequently disregarded in classical parametric or non-parametric models, although they might have an influence on the statistical outcome [5,23–25]. In this study, main contributors for data variation were intra-tumoral (in vivo), and intra-tumoral/inter- and intra specimen (ex vivo). As a consequence, certain amounts of tumor specimen, ROI, and nuclei should be analyzed to obtain a precise prediction of the radiation response measured with γ H2AX foci [5,17]. Indications for an adjustment of the foci analysis from the in vivo to the ex vivo situation can be drawn from the FaDu, SKX and UT-SCC-5 cohorts. For the latter two models, a significant difference in residual nfoci_g was found, while for FaDu no significant difference in residual nfoci_g between the in vivo and ex vivo set-up was detected (Figure S3). Considering the sample size of FaDu (Table S1), for the ex vivo cohort ~ten times more nuclei were analyzed for foci relative to the in vivo cohort (4390 versus 450 nuclei); for SKX and UT-SCC-5 ~five to seven times more nuclei were scored for foci (2000 versus 450 nuclei (SKX), 2830 versus 400 nuclei (UT-SCC-5)). This implies that a sample size of ≥ 10 for the ex vivo situation is needed to obtain comparable results to the in vivo situation.

In summary, this statistical study applied a LMEM to show that the nucleus area and the heterogeneity of γ H2AX foci in hHNSCC models depends on the experimental set-up (in vivo, ex vivo) and possibly on the cellular adaptation of the tumor specimen to the new microenvironment [50,52]. From the translational perspective, patient-derived materials are expected to have a higher degree of heterogeneity compared to preclinical in vivo models, where clonal selection during passaging potentially diminishes the native tumor heterogeneity [21,31,50–53]. Thereby, multiple samples, ROI per specimen, and nuclei per ROI should be accounted for in γ H2AX foci analysis [5]. For a precise and non-biased radiosensitivity prediction, a sophisticated semi-automated foci counting software is necessary for bridging the gap between laboratory and clinic.

4. Materials and Methods

4.1. Tumor Handling and Data Acquisition

The heterogeneity of γ H2AX foci from in vivo irradiated tumors [14] and ex vivo irradiated tumor biopsies [5] were assessed in two published datasets. In both studies, hHNSCC tumor xenografts were used. GammaH2AX foci analysis was solely performed in oxic and viable tumor regions based on immunohistochemical detection of pimonidazole and bromodeoxyuridine (BrdU) incorporation. Nucleus area was extracted from the DNA staining with DAPI (Figure 5, graphical abstract).

The in vivo cohort comprising nine hHNSCC tumor xenograft models (Cal33, FaDu, SAS, SKX, XF354, UT-SCC-5, UT-SCC-8, UT-SCC-14, UT-SCC-45) were exposed to 4 Gy, excised and fixed 30 min

or 24 h post exposure. GammaH2AX foci were analyzed in 4–10 tumors/model and in 5 randomly selected nuclei from 10 oxic ROI/tumor (50 nuclei/tumor) [14]. For the ex vivo cohort, up to sixteen tumor specimens from subcutaneous hHNSCC tumors (SKX, FaDu, UT-SCC-5) were taken and cultured ex vivo. Half of the specimens/tumor were exposed to 4 Gy. All samples were fixed and paraffin embedded 24 h later. GammaH2AX foci were scored in 10 randomly selected nuclei in 5–7 oxic ROI/specimen (50–70 nuclei/specimen) [5].

Figure 5. Overview of experimental designs applied in the published datasets of the in vivo [14] and ex vivo [5] cohort. For the in vivo set-up, tumor-bearing mice were exposed to X-ray irradiation (IR). Tumors were excised, fixed in formalin, and embedded in paraffin (FFPE) 30 min or 24 h post IR (**upper panel**). For the ex vivo set-up, tumor specimen were excised, cultured in petri-dished containing medium, and irradiated. Specimens were fixed and embedded 24 h post IR (**lower panel**). Immunohistochemistry and immunofluorescence staining were performed with an identical procedure. Foci evaluation was performed exclusively in oxic and viable parts of the tumor.

4.2. Data Processing

Foci number from both datasets were corrected and normalized as described previously [5]. In brief, corrected foci were calculated as:

$$cfoci_i = \frac{foci_i}{area_i} \times \overline{area_t} \quad (1)$$

where $area_i$ is the area of an individual cell nucleus and the corresponding foci number $foci_i$. $\overline{area_t}$ is the tumor- and dose specific mean nucleus area of the corresponding tumor. Due to the experimental setting, a tumor-specific unexposed control is unavailable for the in vivo set-up. Therefore, the overall mean of the corrected foci of all control tumors ($cfoci_0$) was calculated for each tumor model and used for normalizing the corrected foci of a radiation exposed sample as follows:

$$nfoci_g = cfoci - \overline{cfoci_0} \quad (2)$$

In the following, negative $nfoci_g$ were set to zero under the assumption that radiation does not induce negative DNA damage. Statistical analysis was performed with SPSS 24 (IBM Deutschland GmbH, Ehningen, Germany). Graphs were plotted with OriginPro 8G (OriginLab Corp. Northampton, MA, USA).

4.3. Statistical Analysis

4.3.1. Analysis on Cellular Geometry and Foci Number

Alterations in the nucleus area are crucial in the correction and normalization procedures of γ H2AX foci data. Therefore, extrinsic influences (i.e., radiation dose, experimental cohort) on the nucleus area were analyzed by fitting the log-transformed nucleus area by the LMEM. The nucleus area distribution was determined by classifying the data into six categories with a bin size of $40 \mu\text{m}^2$. The foci number of individual nuclei was grouped according to the categories. Linear regression analysis was performed to describe the correlation between foci number and nucleus area categories.

4.3.2. Heterogeneity of γ H2AX Foci

The LMEM was applied to determine inter-tumoral (between tumors of a model) and intra-tumoral (within one tumor) heterogeneity. Fixation time points (control, 30 min (only in vivo), 24 h) or experimental settings (in vivo, ex vivo) were defined as fixed effects, whereas tumor, specimen (only ex vivo), and ROI were defined as random effects. Since the in vivo cohort only comprises of tumor and ROI but not a specimen in the analysis chain, ROI and tumor were repeatedly included to generate the missing factor in the LMEM for direct comparison between the experimental settings. In case of data fitting without a fixed effect, restricted maximum likelihood estimation (REML) was applied. Otherwise, maximum likelihood estimation (ML) was employed. For multiple pairwise comparisons, Bonferroni adjustment was conducted. The varying reoxygenation times in the ex vivo cohort were excluded from the analysis, as an insignificant influence was previously found [5]. The statistically significant threshold was defined as $p \leq 0.05$. The p values of the statistical analyses and input datasets are presented in supplementary information (Tables S1, S5–S7).

Supplementary Materials: Supplementary materials can be found at <http://www.mdpi.com/1422-0067/19/9/2616/s1>.

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Abbreviations

γ H2AX	Gamma H2AX
hHNSCC	Human head and neck squamous cell carcinoma
LMEM	Linear mixed effects model
ROI	Region of interest
REML	Restricted maximum likelihood estimation
ML	Maximum likelihood estimation
BrdU	Bromodeoxyuridine
cfoci	Corrected foci
nfoci	Normalized foci

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4 Comparable radiation response of *ex vivo* and *in vivo* irradiated tumor samples determined by residual γ H2AX foci

Table 4.1 Literature information of manuscript

Title	Comparable radiation response of <i>ex vivo</i> and <i>in vivo</i> irradiated tumor samples determined by residual γ H2AX foci
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4.1 Summary of the manuscript

The clinically relevant *ex vivo* γ H2AX foci assay demonstrates a promising potential as a predictive tool for tumor radiation response. Nevertheless, evidence whether biopsies can reflect and predict the radiation response of the corresponding bulk tumor is still missing. In this study, fine needle biopsies of hHNSCC tumor xenograft models (SKX, FaDu, Cal33, UT-SCC-5, UT-SCC-45) were taken prior to a single dose irradiation to the tumor. The biopsies were maintained under cell culture condition for 24 h, and subsequently exposed to radiation with equivalent doses (0, 2, 4 or 8 Gy). Residual γ H2AX foci were evaluated in well-perfused regions of *in vivo* irradiated tumors and *ex vivo* irradiated biopsies. This experimental design provides a possibility to uniquely compare radiation response between both cohorts. Linear regression analysis of residual γ H2AX foci showed comparable slopes of dose response curves (SDRC) of residual γ H2AX foci between *in vivo* irradiated tumors and *ex vivo* irradiated biopsies in four out of five models. In addition, a good degree of comparability in foci numbers between *ex vivo* irradiated biopsies and the corresponding *in vivo* irradiated tumors was observed. To assess the predictive capability of the *ex vivo* γ H2AX foci assay, the slopes were compared with the previously reported intrinsic radiation sensitivity (TCD_{50}) of the tumor models. The result showed that the SDRC values of residual γ H2AX foci were able to distinguish between radiosensitive and radioresistant tumor models. In conclusion, *ex vivo* irradiated biopsies can reflect radiation response of *in vivo* irradiated tumors determined by the γ H2AX foci assay, with the exception of FaDu tumors. SDRC value might be used as a predictor for radiation response in the clinics. The outcome of this study, however, remains to be validated in (pre-) clinical studies with a larger cohort. To further enhance the prediction accuracy, refinement of the assay by incorporating additional biomarkers would be necessary.

Comparable radiation response of *ex vivo* and *in vivo* irradiated tumor samples determined by residual γ H2AX

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Running title: Radiation response *in vivo* vs. *ex vivo*

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Figure: 3

Table: 1

Supplementary figure: 4

Supplementary table: 2

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Declarations:

Ethics approval and consent to participate:

The local committee on ethics of animal experimentation approved all experiments.

Availability of data and material:

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Author's contribution:

TR and CvN performed the experiments and wrote the manuscript. TR analyzed the data and performed the statistical analysis. CvN designed the animal study. SL validated the statistical analysis. CvN, MK and MB had the project idea, wrote the concept for grant application, supervised the study and contributed to data interpretation. All authors read and approved the final manuscript.

Abstract

Purpose: a) To investigate if an *ex vivo* cultured and irradiated tumor biopsy reflects and predicts the radiation response of the corresponding *in vivo* irradiated tumor measured with the DNA double strand break marker γ H2AX foci.

Materials and methods: Five human head and neck squamous cell carcinoma (hHNSCC) xenograft models were used. Fine needle biopsies were taken from anesthetized tumor-bearing NMRI nude mice prior to *in vivo* single dose irradiation (0, 2, 4, or 8 Gy) under ambient blood flow. Biopsies were *ex vivo* reoxygenated and irradiated with equivalent doses. Tumors and biopsies were fixed 24 h post irradiation, and γ H2AX foci were assessed in oxygenated tumor regions.

Results: Linear regression analysis showed comparable slopes of the residual γ H2AX foci dose response curves in four out of five hHNSCC models when *in vivo* and *ex vivo* cohorts were compared. The slopes from *ex vivo* biopsies and *in vivo* tumors could classify the respective tumor model as sensitive or resistant according to the intrinsic radiation sensitivity (TCD₅₀).

Conclusion: The ability of *ex vivo* irradiated tumor biopsies to reflect and predict the intrinsic radiation response of *in vivo* tumors increases the translational potential of the *ex vivo* γ H2AX foci assay as a diagnostic tool in clinical practice.

Introduction

The standard of care for advanced HNSCC comprises surgery followed by radio(chemo)therapy or, alternatively, primary radio(chemo)therapy with a total radiation dose of ~70 Gy for combined fractionation. Although a routine pathological investigation classifies tumor grade and stage prior to initiation of radio(chemo)therapy, the classification generally disregards the patient-dependent intrinsic radiosensitivity, which importantly influences treatment outcome [1–4]. Thus, a predictive assay that supports physicians to prescribe an individualized radiation dose could improve treatment outcome and reduce excess toxicity [1–7].

One of the available molecular biomarkers for the determination of intrinsic radiosensitivity is the phosphorylated histone H2AX (γ H2AX). Phosphorylation occurs within seconds after irradiation at serine 139 in vicinity of a DNA double strand break (DSB) [8]. The formed γ H2AX foci are microscopically detectable and serve as a specific, sensitive, robust, and straightforward DNA DSB biomarker [9–14]. GammaH2AX foci demonstrated a promising capability in stratifying tumors according to their intrinsic radiosensitivity. Previously, residual γ H2AX foci (24 h post irradiation) predicted the radiation response of *in vivo* irradiated

hHNSCC xenograft tumors [15,16]. Based on this, a clinical relevant *ex vivo* γ H2AX foci assay was established, showing its predictive potential in specimens of established tumor models and surgical patient-derived biopsies by grouping them according to the known clinical radiosensitivity [17–20]. However, evidence that an *ex vivo* cultured and irradiated tumor biopsy can reflect and predict the radiation response of the corresponding *in vivo* irradiated tumor is still missing. In this study, fine needle biopsies were taken from xenotransplanted hHNSCC prior to a single dose irradiation to the tumor. The biopsies were exposed to radiation with equivalent doses and γ H2AX foci, as a marker of DNA double strand breaks, were assessed in both *in vivo* tumors and *ex vivo* biopsies. This experimental design allowed for a matching comparison of radiation response between the *in vivo* and *ex vivo* scenario. The experimental tumor models were categorized according to the slopes of the dose-response curves (SDRC) of residual γ H2AX foci, a potential radiation sensitivity predictor [15,18,19], and their radiation sensitivity based on previously determined data [15,16,21] to assess the prediction capability of the *ex vivo* assay.

Materials and Methods

Animal and experimental tumor models

Five established hHNSCC lines i.e., SKX, FaDu, Cal33, UT-SCC-5, and UT-SCC-45 were investigated. Characteristics of these tumor models [15,22,23] as well as their xenotransplantation were previously described [15,21,24]. In brief, 7 - 14 weeks old male and female NMRI (nu/nu) mice from the pathogen-free animal breeding facilities (Experimental Center and OncoRay, Faculty of Medicine, Technische Universität Dresden, Germany) were whole body irradiated with 4 Gy 2 - 5 days prior to transplantation (X-rays, Maxishot 200 Y.TU/320-D03, Yxlon Int., Hamburg, Germany; 200 kV, 20 mA; 0.5 mm Cu filter; dose rate 1 Gy/min). Small pieces of a source tumor were subcutaneously transplanted on the hind-leg of anesthetized mice (Ketamine ([Ketamin 500 Curamed®, Curamed Pharma, Karlsruhe, Germany] 100 mg/kg) and Xylazine ([Rompun®, Bayer Healthcare, Leverkusen, Germany] 10 mg/kg)). Microsatellite, histology, and volume doubling times were assessed for validation of tumor lines. The animal facilities and the experiments were approved by the regulatory authorities and followed the institutional guidelines as well as the German animal welfare regulations.

In vivo and *ex vivo* experimental settings

Figure 1 summarizes the workflow of the *in vivo* and *ex vivo* procedures. Animals bearing tumors with a maximum diameter of $\sim 10 \times 10$ mm (~ 1 cm²) were anesthetized and randomly allocated to the treatment arms. Fine needle biopsies (BioPince 18G, 1.3 mm, Angiotech-Medical Device Technologies Inc., Gainesville, USA) were taken. Tumor biopsies were

immediately transferred into petri dishes coated with 1.5% agarose (A9539, Sigma–Aldrich, Taufkirchen, Germany)). Biopsies were maintained in 2.5 ml Dulbecco's MEM culture medium supplemented with 2% HEPES, 1% Na pyruvate, 1% non-essential amino acids, 1% penicillin streptomycin (all Biochrom, Berlin, Germany); and 10% FCS (Sigma-Aldrich, Taufkirchen, Germany) at 37°C and 5% CO².

In vivo irradiation: After recovery from anesthesia, animals were intraperitoneally injected with 0.1 mg/g body weight pimonidazole (hypoxia marker; Natural Pharmacia International, Belmont, MA, USA) and 3.75 mg bromodeoxyuridine (BrdU; proliferation marker; SERVA electrophoresis, Heidelberg, Germany) 1 h prior to irradiation. Tumor-bearing legs were locally irradiated with single doses of 0, 2, 4, or 8 Gy of X-rays under ambient blood flow. Animals were sacrificed 24 h post irradiation. Tumors and the overlying skin were excised; sebaceous glands served as positive controls for pimonidazole incorporation. Tumors were fixed in 4% formalin and embedded in paraffin.

Ex vivo irradiation: tumor biopsies were incubated for 20 h prior to media supplementation and 4 h incubation with 8.5 µmol pimonidazole and 5 µmol BrdU. Four biopsies from SKX, FaDu, and UT-SCC-5 tumors were *ex vivo* exposed to single doses of 0, 2, 4, or 8 Gy (one dose/biopsy). Due to experimental complications e.g., high necrotic fraction and low numbers of viable cells within a biopsy, using two biopsies per dose per tumor was more applicable for γH2AX foci evaluation. Therefore, two biopsies from Cal33 and UT-SCC-45 tumors received the corresponding dose of the *in vivo* tumor. Immediately after radiation exposure, culture medium was exchanged to remove excess pimonidazole and BrdU. Tumor biopsies were fixed in 4% formalin and embedded in paraffin 24 h post radiation exposure.

Staining, imaging acquisition, and γH2AX evaluation

The immunohistochemical and immunofluorescence staining procedures were previously described [15–17,20]. In brief, two consecutive 3 µm sections of the central part of tumors or biopsies were stained for a) immunohistochemistry (IHC): pimonidazole (Hypoxyprobe, Burlington, MA, USA), BrdU (Agilent Technologies, clone Bu20a, Hamburg, Germany), and counterstained with hematoxylin; or b) immunofluorescence (IF): γH2AX at serine 139 (Merck Millipore, Upstate, clone JBW301, Darmstadt, Germany) and counterstained with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, Karlsruhe, Germany). ARKTM Kit (Animal Research Kit; Agilent Technologies Deutschland, Hamburg, Germany) and Tyramide Signal Amplification (TSA) Kits #2 (Alexa 488, Life Technologies, Darmstadt, Germany) were applied for IHC and IF, respectively. Image acquisition for IHC and IF was carried out by a fluorescence microscope (Axio Imager M1) equipped with dual cameras (digital color camera: AxioCam MRc, monochrome camera: AxioCam MRm; Carl Zeiss Jena, Jena, Germany) and a

motorized scanning stage (Maerzhaeuser Wetzlar, Wetzlar, Germany). The microscope was controlled by the AxioVision 4.9 software (Carl Zeiss Jena, Jena, Germany). IHC stained sections were scanned with a magnification of 100X using the digital color camera. Subsequently, ten (*in vivo*) regions of interest (ROI) were selected for IF image acquisition. Due to the characteristic of xenograft tumors in which the core of the tumor is highly necrotic, and viable tumor cells are located at the growing outer cell layers, biopsy taken from xenograft tumors contained a low number of analyzable cells located at each end of the biopsy. As a result, five to seven ROIs/ biopsy (*ex vivo*) could be evaluated. The criteria for the ROI selection were as follows; (a) *in vivo*: a single mouse blood vessel surrounded by multiple BrdU positive but pimonidazole negative (oxic) cells; (b) *ex vivo*: tissue at the outer rim of the biopsy with multiple BrdU positive but pimonidazole negative (oxic) cells. For each ROI, a focus stack image (17 individual images with a focus interval of 0.25 μm) at 400X magnification was acquired. All focal planes of the stack image were calculated to a single extended depth of focus image for γH2AX foci evaluation. Within each ROI, oxic cells (*in vivo*: $\leq 45 \mu\text{m}$ from vessel) with intact nuclei were numbered for randomization and five to ten (*in vivo*) or ten (*ex vivo*) of these nuclei were randomly selected for foci determination. Manual and blinded evaluation of residual γH2AX foci and nuclear area was carried out. Necrotic, apoptotic, S-phase, and differentiated cells were excluded from analysis. Supplement figure 1 shows exemplary images for IHC and IF staining with annotations for foci analysis. Supplement table 1 provides information on total and analyzed numbers of tumors, biopsies and nuclei.

Data processing and statistical analysis

As the nucleus area of the randomly selected nuclei is not homogenously distributed, data were corrected for the nucleus area [15–17,20,25]. Residual corrected foci of an individual nucleus ($cfoci_i$) was calculated by multiplying residual γH2AX foci numbers of an individual nucleus ($foci_i$) with the quotient of the mean nucleus area of the corresponding tumor or biopsy ($\overline{area}$) and the area of the individual nucleus ($area_i$) (Eq. 1).

$$cfoci_i = foci_i \times \left(\frac{\overline{area}}{area_i} \right) \quad (1)$$

Residual normalized foci of an individual nucleus ($nfoci_i$) from an exposed sample were calculated by subtracting $cfoci_i$ from the exposed sample with a mean cfoci of the unexposed tumors or biopsies ($\overline{cfoci_0}$) (Eq. 2). Negative $nfoci_i$ were set to zero under the assumption of no negative irradiation induced foci.

$$nfoci_i = cfoci_i - \overline{cfoci_0} \quad (2)$$

Statistical analysis was performed with SPSS 23 (IBM Deutschland, Ehningen, Germany). Graphs were plotted with GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA).

A mean residual cfoci or nfoci per tumor and biopsy was calculated. The correlation of the mean residual cfoci or nfoci and the radiation doses was determined by linear regression analysis. Analysis of covariance (ANCOVA) was applied to compare the regression slopes and constants (estimated endogenous foci - offsets). The regression slopes and offsets were plotted against previously reported local tumor control data [15,16,21] after single (TCD₅₀(SDambient)) or fractionated (30 fractions, 6 weeks; TCD₅₀(30fx/ 6weeks)) radiation exposure under ambient blood flow. The comparability between the procedures was assessed by Bland-Altman analysis. The threshold for statistical significance was defined as $P < 0.05$.

Results

The characteristics of analyzed samples of tumors (*in vivo*) and biopsies (*ex vivo*) were summarized in supplement table 1. Due to predominant necrotic tissue or tumor cell differentiation, analyzable tumors and biopsies ranged between 42.5% (SKX) - 94.4% (Cal33) for the *in vivo* cohort as well as 50.7% (SKX) - 88.9% (Cal33, UT-SCC-45) for the *ex vivo* cohort, respectively. Beside the variations in usable samples, tumor cell density in ROI influenced the total analyzable nuclei ranging *in vivo* from 5,571 (SKX) - 18,790 (Cal33) nuclei and *ex vivo* from 9,851 (SKX) - 37,876 (UT-SCC-5) nuclei. However, equal or lower amounts of analyzable cell nuclei could be found in *ex vivo* biopsies relative to *in vivo* tumors. Supplement figure 1 shows representative images of *in vivo* tumors and *ex vivo* biopsies. The latter showing BrdU positive cells in the outer, oxic tissue rim and a central hypoxic tissue core while *in vivo*, BrdU positive and oxic cells surround the vessels similar to previous reports [15,17,20].

We investigated whether residual γH2AX foci determined in *ex vivo* irradiated biopsies can reflect radiation response of *in vivo* irradiated tumors. Comparison of residual cfoci between tumors and the corresponding biopsies exposed to equivalent doses were analyzed. In all tumor models, a good degree of comparability was observed as indicated by Bland-Altman analysis (Suppl. Fig. 2). A dose dependent linear increase of residual γH2AX cfoci and nfoci was observed in most of tumor models following *in vivo* and *ex vivo* radiation exposure, exempt residual cfoci of *ex vivo* irradiated UT-SCC-5. (cfoci: Suppl Fig 3; Suppl. Table 2; nfoci Fig. 2, Table 1).

The slopes of dose response curves (SDRC) of residual nfoci ranged from 0.38 (FaDu) - 1.50 (UT-SCC-45) for *in vivo* irradiated tumors, and from 0.19 (FaDu) - 1.48 (UT-SCC-45) for *ex vivo* irradiated biopsies. With the exemption of FaDu, no statistical difference between the slopes of the *in vivo* and *ex vivo* cohort could be found (Table 1). *Ex vivo* irradiated FaDu biopsies exhibited a significant, ~2-fold lower dose response compared to *in vivo* irradiated tumors (slope of *in vivo*/*ex vivo*; $P < 0.0001$). A similar result was observed in the regression slopes of

residual cfoci except for a significant difference in the offsets of dose response curves (ODRC) between *in vivo* and *ex vivo* cohorts of UT-SCC-45 (Suppl. Table 2).

As previously reported, SDRC of residual nfoci could potentially be used as a radiation response predictor [17]. The SDRC were plotted against the dose needed to control 50% of *in vivo* irradiated tumors locally i.e., TCD₅₀, following fractionated (TCD_{50(30fx/6weeks)}, Fig. 3A) or single dose irradiation (TCD_{50(SDambient)}, Fig. 3B). A range of possible cut-off values for the SDRC (0.55 – 0.7) was determined as it discriminates the tumor models for both TCD₅₀ values in a resistant and a sensitive population. Moreover, tumor models with a TCD_{50(30fx/6weeks)} above the clinical standard dose for primary radiotherapy of ~70 Gy showed lower SDRC (≤ 0.55 ; UT-SCC-5, Cal33, FaDu) while tumor models with lower TCD_{50(30fx/6weeks)} exhibit higher SDRC (≥ 0.7). In contrast, SDRC and ODRC of residual cfoci demonstrated an insufficient potential in the differentiation of radiosensitivity (Suppl. Fig 4).

Discussion

This study is aiming to further enhance the relevance and to translate the γ H2AX foci assay as a clinically predictive tool for determining intrinsic radiation sensitivity in tumors. Depending on their intrinsic radiosensitivity, the response to radiation among patients varies considerably [1–7]. Applying a patient-individualized dose based on molecular determinants, omics data, and mathematical modelling could offer an improvement of therapeutic outcomes and an alleviation of normal tissue toxicity [1,4–6,11,14,26,27]. Among molecular-biomarker based assays for the determination of radiation response, the γ H2AX foci assay holds a promising potential to become a predictive biomarker in clinical practice [14,16–20,25]. However, it is currently unclear whether γ H2AX foci determined in biopsies can reflect and predict the radiation response of the corresponding bulk tumor [14,20,25]. Here, of five hHNSCC models with known radiosensitivity, residual γ H2AX foci in irradiated tumors *in vivo* and the corresponding biopsies taken from untreated tumors and irradiated *ex vivo* were evaluated. Radiation response was determined by SDRC of residual γ H2AX foci. To assess the predictive value of the assay, the slopes were compared with the previously reported tumor radiosensitivity (TCD₅₀) data [15,16,21].

In previous studies, SDRC of residual γ H2AX foci demonstrated predictive potential for stratifying experimental tumors and patient-derived tumors based on clinically known intrinsic radiosensitivity tumors [17–19]. However, those studies exclusively assessed radiation induced residual γ H2AX foci following *ex vivo* exposure. To our knowledge, this is the first study showing a matching comparison of radiation-induced residual γ H2AX foci between locally irradiated tumor xenografts (*in vivo*) and tumor biopsies (*ex vivo*) taken from xenograft tumors prior to radiation exposure. The result demonstrates comparable SDRC of residual

nfoci and cfoci of the *in vivo* irradiated tumors and the *ex vivo* irradiated biopsies in four out of five tumor models. The SDRC values of residual γ H2AX nfoci were able to distinguish between radioresistant and radiosensitive models. Generally, a total dose up to 70 Gy is applied in primary radio(chemo)therapy for head and neck cancer (HNC) [28]. Of note, all the evaluated radioresistant tumor models (UT-SCC-5, Cal33, and FaDu) have a fractionated TCD₅₀ value higher than the conventional therapeutic dose of 70 Gy. Moreover, the radioresistant tumor models showed that the SDRCs of residual γ H2AX nfoci were lower than the proposed cut-off range of 0.55 – 0.7, while the SDRCs of the radiosensitive tumor models (UT-SCC-45 and SKX) were higher than the proposed range. Therefore, a SDRC value might be a suitable indicator for discriminating radiosensitive and radioresistant tumors in the clinics. This is in line with a recent study on HNC patient-derived biopsies reported a similar cut-off of 0.7 for SDRC of residual nfoci [29]. An investigation on additional hHNSCC tumor models and patient-derived materials is ongoing to verify the proposed cut-off range.

The human papillomavirus (HPV) infection status has been shown to be a significant prognostic marker for radiation response of HNSCC. HPV positive HNSCC patients demonstrated a favorable prognosis [30–34] and HPV positive HNC cells are more highly susceptible to radiation compared to HPV negative HNC cells [35,36]. In this study, offsets of dose response curves (ODRC) of residual γ H2AX foci did not correlate with the radiation response of the tumor models (Suppl. Fig 4). The HPV negative, and due to a defective DNA damage machinery [23,37], highly radiosensitive model SKX showed a low endogenous γ H2AX foci number [16] and low ODRC in the both cohorts. In contrast, the HPV positive UT-SCC-45 model demonstrated an increased radiosensitivity and a high number of endogenous foci [16], which is possibly related to the replication process of HPV. DNA damage proteins including γ H2AX, 53BP1 and others are activated and recruited to HPV replication sites to promote viral genome amplification and stability [38,39].

Tumor heterogeneity is among the cellular complexities that greatly encumbers the translation of molecular biomarkers to the clinics [40,41]. In our previous report, a high intratumoral heterogeneity of residual γ H2AX foci was detected in hHNSCC models (UT-SCC-5, FaDu, SKX) [20]. Likewise, intratumoral heterogeneity in residual γ H2AX foci was more pronounced as compared to intertumoral heterogeneity (data not shown). In line with our previous report, the intra- and intertumoral heterogeneity in the γ H2AX foci assay was more pronounced in the *ex vivo* irradiated biopsies than in the *in vivo* irradiated tumors, which probably contributes to the significant difference in the SDRC of residual nfoci and cfoci between the FaDu cohorts [20,25].

Despite the high sensitivity of the γ H2AX foci assay, its quantification is labor-intensive and observer-dependent [14]. (Semi-) automated foci counting algorithms [13,42–44] or commercialized software [12,45,46] were implemented in basic- and translational researches. Those algorithms and software are, however, currently unsuitable for tissue specimens due to complex morphological organization of tumor tissues e.g., cells overlap, varying cell types, and complex micromilieu. The evolution of artificial intelligence (AI) has permitted researchers to apply machine learning techniques in microscopy [47], radiology [48], detection of melanomas [49] as well as outcome prediction in colorectal cancer based on recognition of tissue patterns [50]. The advance of AI technology might enable us to overcome the technical challenges in cell and foci recognition process. This could pave the way for the development of a non-biased, reliable automatic foci-counter for solid tissue specimens therewith the assay could potentially be translated, standardized, and implemented as a clinical application in precision radiotherapy [11,14].

In conclusion, with the exception of FaDu tumors, *ex vivo* irradiated biopsies reflected radiation response of *in vivo* irradiated tumors determined by the γ H2AX foci assay. Comparable outcomes in γ H2AX foci as well as the slopes of dose response curves of residual γ H2AX nfoci and cfoci between *in vivo* and *ex vivo* cohorts were observed. Moreover, the SDRC of residual γ H2AX nfoci were capable of differentiating radiosensitive and radioresistant tumors based on their intrinsic radioresponsiveness. The outcome of this study needs to be validated in (pre-) clinical studies with a larger cohort. For further classification into more radiation response groups, refinement of the assay by modelling with further biomarkers would be necessary.

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Competing interest:

In the past 5 years, Dr. Baumann attended an advisory board meeting of MERCK KGaA (Darmstadt), for which the University of Dresden received a travel grant. He further received funding for his research projects and for educational grants to the University of Dresden by Teutopharma GmbH (2011-2015), IBA (2016), Bayer AG (2016-2018), Merck KGaA (2016-2030), Medipan GmbH (2014-2018).

Dr. Baumann, as former chair of OncoRay (Dresden) and present CEO and Scientific Chair of the German Cancer Research Center (DKFZ, Heidelberg), signed/s contracts for his

institute(s) and for the staff for research funding and collaborations with a multitude of companies worldwide.

For the German Cancer Research Center (DKFZ, Heidelberg) Dr. Baumann is on the supervisory boards of HI-STEM gGmbH (Heidelberg).

For the present study, Dr. Baumann confirms that none of the above mentioned funding sources were involved in the study design or materials used, nor in the collection, analysis and interpretation of data nor in the writing of the paper.

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Fig. 1. Overview of the experimental workflow and γ H2AX foci analysis procedures

In vivo: after recovery from anesthesia, mice were injected with pimonidazole and BrdU 1 h prior to a single dose radiation exposure with 0, 2, 4, or 8 Gy. *Ex vivo*: Four biopsies from anesthetized mice bearing SKX, FaDu, and UT-SCC-5 tumors were taken and each biopsy was *ex vivo* exposed to a single dose irradiation of 0, 2, 4, or 8 Gy (one biopsy/ dose). Due to experimental complications e.g., high necrotic fraction and low numbers of viable cells within a biopsy, the experimental protocol was adapted for Cal33 and UT-SCC-45. Here two biopsies per tumors were taken and exposed to a single dose irradiation corresponding to the dose applied for the *in vivo* tumor. Tumors and biopsies were fixed in formalin and embedded in paraffin. Gamma H2AX foci were enumerated and analyzed. (IHC: Immunohistochemistry, IF: Immunofluorescence, FFPE: Formalin-Fixed Paraffin-Embedded)

Fig. 2. Dose response curves of residual γ H2AX nfoci of *in vivo* irradiated tumors and *ex vivo* irradiated biopsies.

Linear regression analysis of residual γ H2AX nfoci (24 h post irradiation) as a function of radiation dose was performed across five hHNSCC tumor models. Symbols (open circle: *in vivo*; open triangle: *ex vivo*) and error bars represent mean nfoci and standard error of mean

(SEM), respectively. The regression constant was adjusted to zero. ANCOVA was applied to compare between the slopes of the dose response curves of irradiated tumors and irradiated biopsies for each tumor model (Table 1).

Fig. 3. Classification of radiation sensitivity based on tumor control dose 50% (TCD_{50}) and slope of dose response curve (SDRC) of residual γ H2AX nfoci

$TCD_{50}(30fx/6weeks)$ (A) or $TCD_{50}(SD_{ambient})$ (B) and slopes of the residual γ H2AX nfoci dose response curves of the *in vivo* (open circle) and *ex vivo* (open triangle) cohort were plotted. The TCD_{50} values were previously published [15,16,21]. Error bars represent 95% confidence intervals (95% CI) and standard errors (SE) for TCD_{50} values and the SDRC, respectively. The vertical dotted lines at SDRC = 0.55 and 0.7 represent a possible range of the SDRC for the classification between resistant and sensitive tumors. The horizontal dotted line represents the conventional clinical radiation dose (CRD = 70 Gy) for head and neck cancer treatment. Note: Different scaling on y-axis, $TCD_{50}(12fx/6weeks)$ was used for SKX due to its high radiosensitivity.

Table 1. Linear regression analysis of residual γ H2AX nfoci dose response of *in vivo* irradiated tumors and *ex vivo* irradiated biopsies from five hHNSCC models. The differences of the slopes of the dose response curves (SDRC) between the *in vivo* irradiated tumors and the *ex vivo* irradiated biopsies for each tumor model were determined by ANCOVA. TCD_{50} after irradiation with single dose under ambient condition ($SD_{ambient}$) and 30 fractions within 6 weeks (30fx/ 6 weeks) are shown.

		Resistant			Sensitive	
		UT-SCC-5	Cal33	FaDu	UT-SCC-45	SKX
<i>in vivo</i>	Slope [$\pm$ SE]	0.45 ± 0.07	0.49 ± 0.04	0.38 ± 0.03	1.5 ± 0.10	0.79 ± 0.10
	95% CI	0.31; 0.59	0.42; 0.56	0.31; 0.45	1.31; 1.70	0.58; 1.00
	P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
<i>ex vivo</i>	Slope [$\pm$ SE]	0.40 ± 0.05	0.51 ± 0.03	0.19 ± 0.03	1.48 ± 0.12	0.81 ± 0.06
	95% CI	0.31; 0.50	0.44; 0.58	0.13; 0.24	1.23; 1.72	0.68; 0.94
	P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
P value (Significance between slopes)		0.649	0.666	<0.001	0.847	0.855
TCD_{50} (30fx/ 6 weeks) ^[16,21]		117.2	105.2	85.2	45.4	11.76
[95%CI]		[103; 140]	[90; 141]	[77; 96]	[38; 52]	[11.3; 12.2] ^{a,b}
TCD_{50} ($SD_{ambient}$) ^[15,21]		42.7	38.1	38.9	23.3	14.9
[95%CI]		[38; 48]	[32; 45]	[35; 44]	[15; 29]	[10.9; 18.9]

^a Standard deviation

^b TCD_{50} fx values after irradiation with 12 fractions within 6 weeks

5 Discussion

Prediction of tumor radiation response has been considered as an essential approach for treatment individualization. The *ex vivo* γ H2AX foci assay possesses a high potential as a predictive tool for tumor radiosensitivity (Menegakis, von Neubeck et al., 2015). To improve the clinical relevance of the assay, five hHNSCC xenograft models i.e., SKX, UT-SCC-45, FaDu, Cal33, and UT-SCC-5, were used in this study. The study focused on 2 aspects: a) tumor heterogeneity of γ H2AX foci, and b) prediction capability of *ex vivo* irradiated tumor biopsies. To investigate tumor heterogeneity of residual γ H2AX foci, multiple equally *ex vivo* treated tumor derived specimens were evaluated for γ H2AX foci. Data were subjected to a LMEM. The result demonstrated a pronounced heterogeneity in residual γ H2AX foci in *ex vivo* irradiated tumor specimens (Rassamegevanon et al., 2017) implying the need of substantial amounts of nuclei for γ H2AX foci evaluation. The degree of heterogeneity in the *ex vivo* irradiated tumor specimens and the *in vivo* irradiated tumors were determined by statistically re-analysis of two published datasets. In addition, the alteration of cellular geometry distribution was investigated. The statistical re-assessment suggested the necessity of multiple biopsies for an accurate tumor radiosensitivity prediction since a greater heterogeneity of residual foci was observed in the *ex vivo* cohort relative to the *in vivo* cohort (Rassamegevanon et al., 2018). The capability of *ex vivo* irradiated tumor biopsies to reflect radiation response of the corresponding tumor was assessed. Radiation response of tumors estimated in *ex vivo* irradiated biopsies was comparable to the corresponding *in vivo* irradiated tumor. This finding supports the clinical applicability of the *ex vivo* γ H2AX foci assay as a predictive approach for individualized radiotherapy.

5.1 Tumor heterogeneity in γ H2AX foci assay

Phenotypic and genetic heterogeneity in cancer have been considered as one of the hallmarks of cancer (Hanahan and Weinberg, 2011). Thereby, evaluation of cellular expression of certain biomarkers in a limited number of biopsies frequently fails to represent tumor heterogeneity within and among patients (Bedard et al., 2013; Jamal-Hanjani et al., 2015; Cyll et al., 2017; Tanaka et al., 2017). Like other available biomarkers for the determination of radiation response, the evaluation of γ H2AX foci demonstrated a considerable magnitude of heterogeneity. Here, residual γ H2AX foci of multiple equally treated tumor specimens were fitted in LMEM (Rassamegevanon et al., 2017). LMEM accounts multiple fixed- and random effects that are generally neglected in classical parametric or non-parametric models, although they could statistically affect the assessment (Oberg and Mahoney, 2007; Duricki et al., 2016). As a result, radiation effect might be over/ underestimated leading to inaccurate patient stratification. Intratumoral heterogeneity either interspecimen (among specimens of a tumor)

or intraspecimen (among ROIs of a specimen) significantly contributed to the variations in the *ex vivo* γ H2AX foci assay. Intratumoral inter- and intra-specimen were evident in all evaluated tumor lines (SKX, FaDu, UT-SCC-5). Two out of three models, exempting FaDu, exhibited an insignificant heterogeneous γ H2AX foci number in intramodel level (among tumors from a model). The outcomes reflected a pronounced intensity of tumor heterogeneity in γ H2AX foci, implying an insufficient representativeness of a single biopsy. As such, multiple biopsies from different lesion sites are needed to capture an accurate molecular picture of a tumor (Wentzensen et al., 2015; Tanaka et al., 2017). In a preclinical study on biopsies of prostate cancer patients, the *ex vivo* γ H2AX foci assay was afflicted with inter- and intra- patient heterogeneity (De-Colle et al., 2017), which is consistent with this study. The complex tumor niche in patient-derived materials is likely associated with the high variations in the quantification of γ H2AX foci (Cassidy et al., 2015; Forker et al., 2015).

Cell-line derived tumor xenograft (CDTX) models fail to capture clonal heterogeneity because of the clonal selection process during serial passaging (Adeegbe and Liu, 2017). Nevertheless, CDTX possesses a higher degree of complexity (e.g. tumor microenvironment (TME) and vascularization). In contrast, 2-dimensional, 3-dimensional cell culture or *ex vivo* explant models preclude the preservation of TME causing lacks of tumor complexity (Majumder et al., 2015; Meijer et al., 2017). Statistical reassessment of γ H2AX foci by LMEM in *ex vivo* irradiated tumors and *in vivo* irradiated tumors revealed an intriguing result (Rassamegevanon et al., 2018). Intratumoral heterogeneity in endogenous and residual γ H2AX cfoci or nfoci was not significantly presented in the *in vivo* cohort, exempt a pronounced variation in initial cfoci and nfoci (30 min following irradiation). Conversely, cfoci and nfoci of the *ex vivo* cohort demonstrated a greater degree of intratumoral heterogeneity. The results indicate the monoclonal character of *in vivo* irradiated CDTX reflected by homogenous DNA damage repair capacity. For the *ex vivo* cohort, the sample processing steps i.e. tumor dissection and reoxygenation in presumably optimal conditions for cell growth, potentially led to an asynchronous cellular adaptation. Consequently, the degree of variation in DNA damage repair capacity in the *ex vivo* irradiated tumor biopsies was intensified. Despite multiple ROIs as well as a substantial number of randomly selected nuclei, numerous analyzable nuclei per ROI were excluded due to practical reasons. Additional specimens and nuclei for foci analysis would be required to potentially minimize the variation of γ H2AX foci assay in the *ex vivo* cohort or assay set-up.

5.2 Alteration of nuclear area post irradiation

Radiation exposure perturbs cell cycle progression in normal cells (Bernhard et al., 1995) and cancer cells (Strasser-Wozak et al., 1998). In tumors with p53 deficiency, G2 phase arrest predominantly manifests in radiation exposed cells due to the necessity of p53 in mediating G1 phase arrest (Kastan and Bartek, 2004). The p53 status of cell line used in this study is showed (Table A.1). Four models possess mutation in p53 while the status of UT-SCC-45 is unknown. A statistical reassessment by fitting the nuclear area of (un-) irradiated tumors cells to LMEM was carried out (Rassamegevanon et al., 2018). Nuclear area determined by DAPI staining in irradiated tumors displayed a greater nuclear area, on average, compared to unirradiated tumors regardless of the experimental set-ups i.e., *in vivo* and *ex vivo* exposure. In cell-cycle dynamics, nuclei in G2-phase possesses the largest nuclear volume due to the completion of DNA replication (Umen, 2005; Huber and Gerace, 2007; Maeshima et al., 2011). An increase in nuclear area in irradiated tumors supports the assumption that tumor cells underwent G2-phase arrest. In addition, differences in means of nuclear area between the experimental set-ups were detected. Clearly, alteration of TME to the *ex vivo* condition affects nuclear area distribution independent of radiation treatment. The changes in nuclear area suggests that cells adapted to the *ex vivo* conditions by regulating cell proliferation and progression through the cell cycle.

The γ H2AX foci assay relies on visualization foci formation on tumor specimens by immuno-histopathological approaches. A common limitation of the histo-pathological approach is, however, that a three-dimensional tumor is represented in a two-dimensional structure. Cells and cell nuclei are sectioned in different anatomical planes depending on tumor positioning during embedding, thereby exhibiting different nuclear areas. The deviation of nuclear area is predominant in cancers in which an abnormal nuclear size and morphology is a common trait (Taira et al., 2012; Edens et al., 2013). In this study, foci numbers increased linearly with increasing detected nuclear area in all evaluated tumor lines independent of the experimental set-ups (Rassamegevanon et al., 2018). The outcome indicates the dependency of foci numbers on measured nuclear area, and highlights the relevance of incorporating measured nuclear area in the data analysis (Costes et al., 2006).

The individual nuclear area and treatment conditions influence the biological read-out of γ H2AX foci numbers. Therefore, the incorporation of the nuclear area in the data processing chain of the assay or a mathematical modeling is crucial. In the γ H2AX foci assay, foci numbers were corrected by applying an individual nuclear area and a mean of nuclear areas of a tumor model or a tumor as a weighting factor and a scaling factor, respectively (Menegakis et al., 2011; Koch et al., 2013; Menegakis, von Neubeck et al., 2015; Rassamegevanon et al., 2017;

Rassamegevanon et al., 2018). Since treatment conditions contribute to the change in nuclear area as well as inter- and inpatient heterogeneity of nuclear area are highly possible, normalization of foci numbers on a tumor- and treatment specific mean nucleus area is, therefore, a feasible approach for personalized prediction of radiosensitivity (Rassamegevanon et al., 2017; Rassamegevanon et al., 2018).

5.3 Clinical relevance of the γ H2AX foci assay

Monitoring of γ H2AX foci is considered as a fast and straightforward approach to determine DNA DSB (Willers et al., 2015; Reddig et al., 2018). Moreover, the visualization and the quantification of γ H2AX foci on formalin-fixed paraffin-embedded tissues (FFPE) require solely a conventional IF staining and an epifluorescent microscope. Hence, the simplicity and accessibility of the assay underline its clinical applicability. Clinical relevance of the γ H2AX foci assay was demonstrated. A previous study showed that γ H2AX foci formation and removal were highly dependent on tumor micromilieu (Menegakis et al., 2011). Initial- and residual γ H2AX foci in two hHNSCC tumor xenograft models, FaDu and SKX, decreased linearly with increasing distance from the nearest perfused vessel. Additionally, a linear dose-dependent response to irradiation was solely observed in residual γ H2AX foci quantified within the perfused area. Thereafter, investigation of γ H2AX foci in nine hHNSCC tumor xenograft model with known radiation sensitivity was performed (Koch et al., 2013). Residual γ H2AX foci of *in vivo* irradiated tumors negatively correlated with the intrinsic radiation sensitivity (TCD_{50}). Those studies suggested a high potential of γ H2AX foci as a predictor of radioresponsiveness. Consequently, an *ex vivo* set-up of the γ H2AX foci assay was, henceforth, established (Menegakis, von Neubeck et al., 2015). In the study, experimental tumor specimens and patient-derived materials were *ex vivo* exposed to irradiation. Influence of reoxygenation in a cell culture medium (0.5 - 12 h) on DNA damage induction of tumor biopsies and predictive capacity of the *ex vivo* γ H2AX foci assay were studied. The result demonstrated that residual nfoci of *ex vivo* irradiated tumor biopsies reached a plateau after 6 h of reoxygenation. The slope of the dose response curve (SDRC) of residual γ H2AX nfoci was able to differentiate between radiosensitive and radioresistant tumor entities, thereby supporting clinical feasibility of the *ex vivo* γ H2AX foci assay.

Following the establishment, the sample processing step of the *ex vivo* γ H2AX foci assay was optimized. Previously studied reoxygenation times of tumor specimens prior to irradiation were, however, impracticable for clinical routine (Menegakis, von Neubeck et al., 2015). Here, the reoxygenation time was extended to 24 h without significant influence on residual γ H2AX nfoci relative to 10 h in all three evaluated tumor lines i.e. SKX, FaDu and UT-SCC-5 (Rassamegevanon et al., 2017). Clinical practicability of the *ex vivo* γ H2AX foci assay was

greatly enhanced due to the high flexibility of the assay. Furthermore, the comparability of radioresponsiveness determined by γ H2AX foci assay between *in vivo* irradiated tumors and the corresponding *ex vivo* irradiated biopsies was investigated. Four out of five tumor models showed a comparable SDRC of residual γ H2AX nfoci and cfoci. Additionally, SDRC of nfoci was able to classify tumors according to their radiation sensitivity. A range of predictive cut-off scores of 0.55 - 0.7 for SDRC of residual γ H2AX nfoci is proposed since the score was capable of distinguishing tumors according to intrinsic radiation sensitivity (Yaromina et al., 2010; Menegakis et al., 2011). This range of cut-off values is in good agreement with a recent study on HNC patient-derived biopsies reporting a similar cut-off of 0.7 for SDRC of residual nfoci (Meneceur et al., 2019). In contrast, the offset of the dose response curve (ODRC) of residual cfoci was unable to differentiate tumors based on their radiation sensitivity. HPV burden of patients is considered as a promising prognostic biomarker for patients with locally advanced HNSCC, in which HPV positive is associated with a better prognosis (Lohaus et al., 2014; Lohaus et al., 2015; Linge et al., 2016). UT-SCC-45, a radiosensitive HPV positive tumor model, exhibited a greater ODRC relative to SKX, a radiosensitive HPV negative tumor model. The replication process of HPV requires the activation and recruitment of DNA damage proteins including γ H2AX, 53BP1 and others to facilitate viral genome amplification and stability (Gautam and Moody, 2016; Spence et al., 2016), implying a potential association with the high ODRC of γ H2AX foci in HPV positive tumors.

An investigation on *ex vivo* irradiated patient-derived materials of ten different tumor entities was performed (Menegakis, De Colle et al., 2015). Ranking of SDRC of residual γ H2AX foci was in a good agreement with the anticipated radioresponsiveness of the different tumor entities. The result emphasizes clinical feasibility of the assay, and supports the concept that intrinsic radiation sensitivity is associated with radiation response of tumors. In addition, the result of the investigation is consistent with this study, in which SDRC demonstrated a promising predictive capability. Altogether, the γ H2AX foci assay holds a great potential to become a clinical decision support method.

5.4 Technical challenges and limitations of the assay

One of the main biological functions of γ H2AX is recruitment and retention of DNA damage repair proteins such as the MRN complex, MDC1, 53BP1, and BRCA1 to DNA DSB sites (Podhorecka et al., 2010). Phosphorylated H2AX shares multiple biological functions in addition to the canonical function in the DNA damage repair pathway (Turinetti and Giachino, 2015). In cells undergoing senescence, persistent “DNA damage foci” including γ H2AX, 53BP1, ATM and ATM/ATR substrates were observed without detectable DNA damage (Rodier et al., 2009). The authors suggested that “DNA damage foci” in senescent cells do not

directly represent DNA damages, but the activation of DNA damage signaling mediated by senescence. Without exogenous DNA damage induction, a substantial number of phosphorylated H2AX mediated by DNA-PKcs, CHK2 (Tu et al., 2013), and ATM (McManus and Hendzel, 2005) was detected in cells undergoing mitosis. An additional function of γ H2AX in the maintenance of genome integrity was reported (Yu et al., 2006; Mirzayans et al., 2015). According to the versatile functions of γ H2AX, a γ H2AX focus does not necessarily represent a single DNA DSB (Lukas et al., 2011; Turinetto and Giachino, 2015). In this study, the determination of DNA DSB was restricted to the detection of γ H2AX foci dependent on tumor microenvironment in FFPE tumor specimens. Gamma H2AX signals that were unrelated to radiation induced DNA damages were excluded from analysis by the definition of analyzable tumor cells. Apoptotic cells generally show γ H2AX pan-nuclear and apoptotic ring staining (Solier and Pommier, 2014). Similarly, S-phase showed pan-nuclear staining post DNA damage induction (de Feraudy et al., 2010). DAPI staining and the corresponding IHC staining provide additional information pertaining to cell cycle. Mitotic, detectable apoptotic and necrotic cells were excluded. As a result, the analysis of γ H2AX was primarily based on G1/G2-phase cells in this experimental setting. (Menegakis et al., 2011; Koch et al., 2013; Rassamegevanon et al., 2017). The cell segregation approach is, however, unable to rule out DSB unrelated γ H2AX foci in multiple other scenarios e.g. senescent cells or early apoptotic cells in which γ H2AX formation is irrelevant in the DNA DSB repair context.

Since the discovery of γ H2AX (Rogakou et al., 1998), the γ H2AX foci assay has found its place in laboratories, where DNA damage repair is evaluated. The assay possesses a high sensitivity and straightforwardness, thereby, enhancing the functionality of the assay in various research fields e.g., drug discovery and development (Hopp et al., 2017), biodosimetry (Barnard et al., 2015), molecular radiobiology (Rieckmann et al., 2013), and translational cancer research (Ivashkevich et al., 2012; Koch et al., 2013). Common laboratory devices without special requirement can conveniently be used for the γ H2AX foci assay. Nevertheless, the high variability of γ H2AX foci quantification i.e., inter-observer variation needs to be considered. Two studies reported distinct differences between numbers of γ H2AX foci enumerated by different investigators (Menegakis et al., 2011; Koch et al., 2013). FaDu and SKX tumors were processed, visualized and detected by identical procedures. However, the percentage differences of means of residual γ H2AX foci between the studies accounted for approximately 50% and 80% for FaDu and SKX, respectively. In a multi-laboratories investigation of γ H2AX foci in irradiated human lymphocytes, an extensive variation among laboratories was found regardless of foci scoring methods i.e. manual or automated (Barnard et al., 2015). An investigation of γ H2AX foci in lymphocytes in four different laboratories based on individual protocols showed dramatically varied results; approximately 50% variation among the

laboratories (K. Rothkamm et al., 2013). Clearly, the performance of the assay is limited due to the lack of standardized procedure and reproducibility. Hence, a standardized protocol, a standardized foci scoring method and analysis procedure, as well as a multi-institutional study are required to validate and to improve clinical applicability of the assay (Williams et al., 2012).

Several semi-automated foci counting algorithms are currently available as commercial software (Runge et al., 2012; Kai Rothkamm et al., 2013) or open-access platforms (Lapytsko et al., 2015; Oeck et al., 2015; Feng et al., 2017). Most of the algorithms are based on image transformation, segmentation, and intensity-threshold configuration (Ivashkevich et al., 2011). As a result, multiple foci with excessively high or low intensity are excluded from the evaluation. However, those foci would be counted in manual evaluation. Moreover, overlapping foci or greatly adjacent foci are enumerated as a single focus (Lapytsko et al., 2015). As a result, automated foci counters might underestimate cellular response upon treatment. Furthermore, the available algorithms applied in either basic- or translational research are restricted to *in vitro* models or liquid biopsies. In such models, the homogenous distribution and uniform structure of cells facilitate cell and DNA damage foci recognition. In addition, high-resolution images acquired by e.g. confocal microscopy are preferable to obtain a well-correlated result between manual and automated counting (Ivashkevich et al., 2011; Lapytsko et al., 2015; Oeck et al., 2015; Feng et al., 2017). Despite the technical restrictions, (semi-) automated foci counters allow the foci determination in a non-biased and high-throughput fashion, therewith, increasing clinical relevance of the γ H2AX foci assay. In this study, tumor specimens, which present a highly complex morphology, were analyzed. In solid tumors, heterogeneous cell nuclear size distribution, overlapping cell nuclei and foci, and complex tumor microenvironment hindered the development of a reliably automated foci counter and the applicability of the available foci counter algorithms so far. Manual foci quantification, despite the practical limitations, currently remains the most feasible approach for the determination of DNA damage foci in solid tumors (Willers et al., 2015; De-Colle et al., 2017; Rassamegevanon et al., 2017; Rassamegevanon et al., 2018; Reddig et al., 2018).

The determination of cellular response to treatment by the *ex vivo* γ H2AX foci assay relies on solid tumor biopsies (Menegakis, von Neubeck et al., 2015; De-Colle et al., 2017; Rassamegevanon et al., 2017; Rassamegevanon et al., 2018). A tissue biopsy is considered as the gold standard procedure for cancer diagnosis. However, invasive methods are needed to obtain tumor materials, which can be challenging if the tumor is deep-seated e.g. pancreatic cancer. Consequently, liquid biopsies are being extensively studied for treatment response prediction and cancer early detection (Chaudhuri et al., 2015; Rostami and Bratman, 2017; Wyatt et al., 2017) since e.g. blood samples are minimally invasive accessible. Studies using patient liquid biopsy for the determination of radiation sensitivity with the γ H2AX foci assay

were reported. Quantification of γ H2AX foci in lymphocytes of patients with colorectal cancer elicited a significant potential in identifying patients with high risk of late adverse effect following radiotherapy (Kroeber et al., 2015). A retrospective study on lymphocytes from patients with prostate cancer demonstrated that γ H2AX foci disappearance rate correlated with radiation toxicity based on CTCAE 4.0 system (van Oorschot et al., 2017). Similar to a study in patients with head and neck cancer, γ H2AX foci analysis on lymphocytes suggested a promising potential in predicting acute normal tissue complications prior to radiation exposure (Goutham et al., 2012). However, the quantification of γ H2AX foci in lymphocytes reflects the radioresponsiveness of normal tissue following radiation exposure (Vasireddy et al., 2010; Palla et al., 2017). The translation of the results to the radioresponsiveness of the tumor is questionable. Moreover, a study reported a poorly comparable result on the analysis of genetic alterations determined in liquid biopsies by two clinically approved laboratories using a similar test. (Torga and Pienta, 2018). As a result, liquid biopsies should not be considered as an alternative material for tumor tissue but should rather be used as complementary clinical testing (Mino-Kenudson, 2016; Jung and Kirchner, 2018; Ratner, 2018). In summary, the necessity of tumor biopsies for radiation response determination is emphasized. An individualized predictive model, in which tumor radiosensitivity and risk of normal tissue complication are incorporated, might be developed by a simultaneous assessment of radiation response in liquid and tissue biopsies.

5.5 Conclusion and Outlook

The purpose of the current study was to further develop and optimize the *ex vivo* γ H2AX foci assay for a clinical application. The results suggest that the *ex vivo* γ H2AX foci assay possesses a promising capability in prediction of radioresponsiveness. The high flexibility in the sample pre-treatment step (10 h and 24 h reoxygenation in medium) tremendously enhanced clinical relevance of the assay. Importantly, *ex vivo* irradiated tumor biopsies were capable of reflecting radiation response of *in vivo* irradiated matching tumors in four out of five hHNSCC tumor models (exception FaDu). The good concordance between both cohorts suggests that quantification of residual γ H2AX foci in biopsies can predict radiation response of the corresponding tumor. In addition, SDRC of residual γ H2AX foci could be a potential predictor for the radiation sensitivity-based classification of tumors. Based on previously published TCD₅₀ values, a range of cut-off scores for the SDRC of 0.55 – 0.7 is proposed as it was able to distinguish between radioresistant models and radiosensitive models. Nonetheless, the proposed cut-off remains to be validated in larger cohorts of experimental models of different tumor entities and patient-derived materials.

The determination of radiation response by the *ex vivo* γ H2AX foci assay was primarily influenced by intratumoral heterogeneity, causing difficulties in an accurate prediction of tumor radiation response. The assessment of intertumoral- and intratumoral heterogeneity in γ H2AX foci from different experimental set-ups (*ex vivo*, *in vivo*) revealed that the degree of heterogeneity in the *ex vivo* cohort was greater relative to the *in vivo* cohort. These results emphasize the need of multi-focal and multi-position analysis for a reliable prediction of tumor radiation response via the γ H2AX foci assay. Before the onset of radio(chemo-)therapy, tumor materials available for the γ H2AX foci assay are frequently limited. Consequently, to obtain a robust SDRC for the prediction of tumor radiation response, applying a set of, at least, four radiation doses with an increment of 4 Gy up to 12 Gy is suggested. This could avoid the administration of a dose range that might fails to induce sufficient residual foci number for linear regression analysis.

The emerging era of personalized radiation therapy leads to an urgent need in clinical predictive assays to support an individualized dose prescription (Lambin et al., 2013; Baumann et al., 2016). Currently, preclinical predictive approaches e.g. mathematical modeling (McMahon et al., 2016), omics profiling (Herskind et al., 2016), and molecular biomarker expression (Forker et al., 2015) are under extensive development and optimization. The *ex vivo* based γ H2AX foci assay, as well, has been extensively studied and optimized to meet clinical requirements. To validate the proposed cut-off and to increase clinical applicability of the assay, a further investigation of residual γ H2AX foci in additional tumor models, patients-derived tumor tissues, and patient-derived xenografts are requisite. The outcome of residual γ H2AX foci together with the tumor and the patient's characteristics e.g., treatment modalities and response, tumor size, invasiveness, and prognostic biomarkers, can be incorporated. In addition, analysis of DNA and RNA expression of DNA damage repair-related genes in tumors and normal tissues present a promising capability in treatment response prediction (Herskind et al., 2016; El Naqa et al., 2017; Scott et al., 2017). As a result, a multivariate-based prediction model could be developed to maximize precision in the prediction (Kirsch et al., 2018). The inconsistency in foci enumeration among observers and laboratories poses complications in the translation of the assay (K. Rothkamm et al., 2013). Standardization of the γ H2AX foci assay i.e., sample preparation, staining procedure, foci visualization, image acquisition, and foci enumerating procedure, is required to minimize inter-observer and inter-laboratory variations. Considering the limitations of the γ H2AX foci assay, a non-biased, automated foci counting algorithm that facilitates the foci counting process is of great need. The availability of the platform would be of major benefit for clinical translation of the assay (Willers et al., 2015), thereby bridging the gap between laboratories and clinics.

6 Abstract

Radiotherapy remains one of the most important treatment modalities for cancer therapy. Malignant tumors show an extended spectrum of intrinsic radiation sensitivity even among tumors of the same entity or with similar histological features. Predicting intrinsic radiation sensitivity might improve treatment outcome and allow individualized treatment. Hence, an assay that provides a predictive information of the tumor's intrinsic radiation sensitivity is of great need. Histone H2AX, a histone variant of histone H2A family, is rapidly phosphorylated upon DNA double strand break (DSB) induction resulting in gamma H2AX (γ H2AX). Gamma H2AX accumulates at DNA DSB sites and subsequently recruits DNA damage repair factors. Formation of γ H2AX is visualized by an immunohistology-based approach and detected as foci under an epifluorescent microscope. Gamma H2AX foci represent DNA DSBs, while residual γ H2AX foci (foci detected 24 h post irradiation) are considered as unrepaired damages. In previous studies, the γ H2AX foci assay showed a high potential as a predictive method for radiosensitivity. This thesis aims to further translate and optimize the *ex vivo* γ H2AX foci assay for a clinical applicability.

In this study, all experiments were performed using human head and neck squamous cell carcinoma (hHNSCC) xenograft models. For *ex vivo* investigations, tumors on the hind legs of nude mice were excised and cut into multiple pieces, or fine-needle biopsies of the tumors were taken. Tumor biopsies were reoxygenated in culture medium for 10 h or 24 h followed by radiation exposure of 0 - 8 Gy. Tumor biopsies were fixed and embedded in paraffin 24 h post irradiation. For the γ H2AX foci assay under *in vivo* conditions, tumors-bearing mice were irradiated with single doses of 0 - 8 Gy. Tumors were excised, fixed, and paraffin embedded 24 h post irradiation. Manual quantification of γ H2AX foci was performed exclusively in perfused areas, which were identified by pimonidazole (hypoxic marker) and BrdU (proliferation marker) staining. Foci number was corrected, normalized, and statistically analyzed by a linear-mixed effects model (LMEM), linear regression model or analysis of covariance.

To investigate tumor heterogeneity in the *ex vivo* γ H2AX foci assay, γ H2AX foci were enumerated in four equally treated tumor specimens per group i.e. unirradiated and *ex vivo* irradiated with 4 Gy. Strong intratumoral heterogeneity in γ H2AX foci was determined with a minor intertumoral heterogeneity. No significant effect of reoxygenation between 10 h or 24 h was observed, enhancing clinical practicability of the assay. The effect of experimental settings was studied by analyzing data from this study (*ex vivo*) and from comparable published data (*in vivo*) with LMEM. Radiation-induced nuclear area alteration was detected in some of the evaluated tumor models in under both experimental conditions. A greater intra- and

intertumoral heterogeneity were observed in the *ex vivo* set-up compared to the *in vivo* set-up. Radiation response determined by the γ H2AX foci assay in *ex vivo* irradiated biopsies and in the corresponding *in vivo* irradiated tumors was evaluated. Between *in vivo* and *ex vivo*, four out of five tumor models showed comparable slopes of dose response curves (SDRC) of normalized and corrected γ H2AX foci. SDRC of normalized γ H2AX foci was able to classify tumors according to their intrinsic radiation sensitivity (TCD_{50}).

In conclusion, the *ex vivo* γ H2AX foci assay holds a promising potential for predicting radiation sensitivity in solid tumors. The comparable radiation response assessed by γ H2AX foci of *in vivo* irradiated tumors and the matching *ex vivo* irradiated tumor biopsies supports clinical applicability of the assay. Using SDRC of γ H2AX foci as a predictor of radiosensitivity, radioresistant and radiosensitive tumors could be classified. The significant intratumoral heterogeneity in the *ex vivo* γ H2AX foci assay suggests a limited representativeness of a single biopsy for radiosensitivity prediction. Additionally, the change of tumor microenvironment modulated cellular adaptation and DNA damage repair capability. The outcomes suggested that a sufficient number of cells, regions of interest, and biopsies are required to obtain a solid prediction.

7 Zusammenfassung

Die Strahlentherapie ist eine der wichtigsten Behandlungsmethoden für die Krebstherapie. Maligne Tumore zeigen ein großes Spektrum in der intrinsischen Strahlenempfindlichkeit auch bei Tumoren ähnlicher Herkunft oder mit ähnlichen histologischen Eigenschaften. Die Vorhersage der Strahlenempfindlichkeit von Tumoren könnte die Tumorheilungsraten verbessern und eine individuelle Behandlung ermöglichen. Die Bestimmung der Strahlenantwort mittels DNA-Doppelstrangbruch (DSB) Biomarkern zeigt ein vielversprechendes Potential für die Vorhersage der intrinsischen Strahlenempfindlichkeit. Histon H2AX, eine Histon-Variante der Histon H2A Familie, wird bei DNA-DSB-Induktion rasch phosphoryliert, was zu gamma H2AX (γ H2AX) führt. Gamma H2AX akkumuliert an DNA-DSB-Stellen und rekrutiert anschließend DNA-Schadensreparaturmoleküle. Die Bildung von γ H2AX wird durch einen immunhistologisch basierten Ansatz sichtbar gemacht und als Foci mikroskopisch nachgewiesen. Gamma H2AX Foci repräsentieren DNA-Doppelstrangbrüche, während residuale γ H2AX Foci (detektierbare Foci 24 h nach Bestrahlung) als nicht reparierte Schäden betrachtet werden können. In früheren Studien zeigte der γ H2AX Foci Assay ein hohes Potenzial als prädiktive Methode für die Strahlenempfindlichkeit. Diese Arbeit zielt darauf ab, den *ex vivo* γ H2AX Foci Assay weiter zu entwickeln und zu optimieren, wodurch die klinische Anwendbarkeit des Assays verbessert wird.

Im Rahmen dieser Arbeit wurden Experimente mit humanen Kopf-Hals-Plattenepithelkarzinom (hHNSCC) Xenograft Modellen durchgeführt. Für *ex vivo* Untersuchungen wurden hHNSCC Tumoren auf dem Hinterlauf von Nacktmäusen transplantiert, bei ausreichender Größe entnommen und in mehrere Stücke geschnitten oder direkt biopsiert. Die Tumorbiopsien wurden in Zellkulturmedium für 10 h oder 24 h reoxygeniert, gefolgt von einer Bestrahlung mit 0 - 8 Gy. Anschließend wurden die Tumorbiopsien 24 h nach Bestrahlung fixiert und in Paraffin eingebettet. Für Untersuchungen des γ H2AX Foci Assays unter *in vivo*-Bedingungen wurden tumortragenden Mäuse mit 0 - 8 Gy lokal bestrahlt. Die Tumore wurden 24 h nach der Bestrahlung exzidiert, fixiert und in Paraffin eingebettet. Die manuelle Quantifizierung von γ H2AX Foci wurde ausschließlich im perfundierten Bereich durchgeführt. Die Diskriminierung zwischen oxischen und hypoxischen Regionen wurde durch Färbung mit Pimonidazol (Hypoxie-Marker) und BrdU (Proliferationsmarker) bestimmt. Die Foci-Anzahl wurde korrigiert, normalisiert und statistisch durch ein lineares gemischtes Modell (LMEM), ein lineares Regressionsmodell oder die Analyse der Kovarianz analysiert.

Um die Tumorerheterogenität im *ex vivo* γ H2AX Foci Assay zu untersuchen, wurden vier gleichbehandelte Tumorproben pro Gruppe, d. h. unbestrahlt und *ex vivo* mit 4 Gy bestrahlt, auf γ H2AX Foci analysiert. Es wurde eine ausgeprägte intratumorale Heterogenität der γ H2AX

Foci bei einer geringen intertumoralen Heterogenität gefunden. Zwischen den Reoxygenierungszeiten von 10 h oder 24 h wurde kein signifikanter Unterschied in der Foci-Anzahl beobachtet, wodurch die klinische Durchführbarkeit des Assays verbessert wurde. Der Effekt der experimentellen Ansätze wurde untersucht, indem Daten dieser Studie (*ex vivo*) und vergleichbare veröffentlichte Daten (*in vivo*) mit dem LMEM analysiert wurden. Eine strahleninduzierte Veränderung der Zellkernfläche wurde in einigen ausgewerteten Tumorkliniken in experimentellen Ansätzen gefunden. Eine größere intra- und intertumorale Heterogenität wurde im *ex vivo* Ansatz relativ zum *in vivo* Ansatz gezeigt. Zusätzlich wurde die Strahlenantwort mit dem γ H2AX Foci Assay in *ex vivo* bestrahlten Biopsien und in entsprechenden *in vivo* bestrahlten Tumoren evaluiert. Das Ergebnis zeigte bei vier von fünf Tumormodellen einen vergleichbaren Anstieg der Dosis-Wirkungs-Kurven (SDRC) von normalisierten und korrigierten γ H2AX Foci Werten. Die SDRC normalisierter Foci konnte Tumoren basierend auf der intrinsischen Strahlenempfindlichkeit (TCD_{50}) in radiosensible und radioresistente Gruppen klassifizieren.

Zusammenfassend konnte gezeigt werden, dass der *ex vivo* γ H2AX Foci Assay ein vielversprechendes Potenzial zur Vorhersage der Strahlenempfindlichkeit in soliden Tumoren besitzt. Die vergleichbaren Strahlenantworten, die durch γ H2AX Foci von *in vivo* bestrahlten Tumoren und den entsprechenden *ex vivo* bestrahlten Tumorbiosien evaluiert wurde, unterstützen die klinische Anwendbarkeit des Assays. Unter Verwendung der SDRC der γ H2AX Foci als Prädiktor der Radiosensitivität konnten resistente und empfindliche Tumore klassifiziert werden. Die signifikante intratumorale Heterogenität im *ex vivo* γ H2AX Foci Assay legte eine begrenzte Repräsentativität einer einzelnen Biopsie für die Vorhersage der Radiosensitivität nahe. Darüber hinaus modulierte die Veränderung des Tumormikromilieus die zelluläre Adaptation und die Fähigkeit zur Reparatur von DNA-Schäden. Die Ergebnisse deuten darauf hin, dass eine ausreichende Anzahl von Zellen, Auswertungsregionen und Biopsien erforderlich sind, um eine solide Vorhersage zu erhalten.

8 Bibliography

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Appendices

Part A: Materials

The materials listed below were used for the entire study including the publications and the manuscript

A.1 Tumor lines

Table A.1 Characteristics of tumor lines

Tumor lines	SKX	UT-SCC-45	FaDu	Cal33	UT-SCC-5
Origin	University Hospital Hamburg-Eppendorf	University of Turku, Finland	ATCC, Rockville, USA	DMSZ Braunschweig	University of Turku, Finland
Anatomic site of origin	Floor of mouth	Floor of mouth	Hypopharyngeal	Tongue	Tongue
Grading	G2	n.a.	G4	G2	G3
TNM Stage	T4N2M0	T3N1M0	n.a.	n.a.	T1N1M0
P53 status	mutated	n.a.	mutated	mutated	mutated
HPV status	negative	positive	negative	negative	negative
TCD₅₀ (30fx/ 6wk)	11.76	45.4	85.2	105.2	117.2
[95%CI]	[11.3; 12.2] ^{a,b}	[38; 52]	[77; 96]	[90; 141]	[103; 140]
TCD₅₀ (SDambient)	14.9	23.3	38.9	38.1	42.7
[95%CI]	[10.9; 18.9]	[15; 29]	[35; 44]	[32; 45]	[38; 48]

a: Standard deviation, b:TCD₅₀ fx values after irradiation with 12 fractions within 6 weeks,

n.a.: not available

TCD₅₀ values were taken from: Yaromina et al., 2010; Menegakis et al., 2011; Koch et al., 2013

A.2 Chemicals and Materials

Table A.2 Chemicals

Chemicals	Manufacturer
Faramount, Mounting Medium, Aqueous	Agilent Technologies Deutschland
Fluoromount, Mounting Medium, Aqueous	GmbH, Hamburg, Germany
Xylazine Rompun®	Bayer Healthcare GmbH, Leverkusen, Germany
Ethanol 96.3%, denatured	Berkel AHK, Berlin, Germany
Dulbecco's Modified Eagle Medium (DMEM)	Biochrom AG, Berlin, Germany

Chemicals	Manufacturer
Non-essential amino acids	Biochrom AG, Berlin, Germany
Penicillin/Streptomycin	
HEPES Buffer	
Sodium Pyruvate	
Cutasept F	Bode Chemie GmbH, Hamburg, Germany
Sodium chloride, >99.5%, p.a.	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Ketamine 500 Curamed®	Curamed Pharma GmbH, Karlsruhe, Germany
Potassium chloride	Fluka Feinchemikalien GmbH, Neu-Ulm, Germany
Saline solution	Fresenius Kabi AG, Bad Homburg, Germany
DAPI(4',6-diamidino-2-phenylindole), 1mmol/l	Invitrogen, Karlsruhe, Germany
Disodium hydrogen phosphate	Merck KGaA, Darmstadt, Germany
Potassium dihydrogen phosphate	
Citric acid monohydrate	
Hematoxylin	
Pimonidazole (HP3-1000, Omni-Kit)	Natural Pharmacia International Inc., Burlington, MA, USA
Tarivid® (Ofloxacin)	Sanofi-Aventis Deutschland GmbH, Frankfurt am Main, Germany
Bromodeoxyuridine (BrdU)	SERVA electrophoresis GmbH, Heidelberg, Germany
Agarose	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Fetal Calf Serum (FCS)	
Xylene	VWR International GmbH, Darmstadt, Germany
Formaldehyde (4 %)	

Table A.3 Kits

Kits	Manufacturer
Animal Research Kit (ARK™)	Agilent Technologies Deutschland GmbH, Hamburg, Germany
Tyramide Signal Amplification (TSA) Kits #2 (Alexa 488)	Life Technologies GmbH, Darmstadt, Germany

Table A.4 Materials

Materials	Manufacturer
Fine needle biopsies, BioPince 18G, 1.3 mm	Argon Medical Devices Inc., Frisco, TX, USA
Embedding cassettes, for biopsies	Engelbrecht Medizin- und Labortechnik GmbH, Edermünde, Germany
Coverslips No. 1, 24×60 mm, 24×32 mm	
Microscope slides, StarFrost®, Advanced Adhesive	
Falcon™ conical centrifuge tubes 50 ml, 15ml	Greiner Bio-One International GmbH, Frickenhausen, Germany
Nunc™ Petri dishes 35 mm	Thermo Fisher Scientific GmbH, Dreieich, Germany

Table A.5 Antibodies

Antibodies	Manufacturer	Concentration
Anti-Bromodeoxyuridine (Clone Bu20a), mouse, monoclonal	Agilent Technologies Deutschland GmbH, Hamburg, Germany	1:50
Anti-phospho-Histone H2A.X (Ser139) (JBW301), mouse, monoclonal	Merck Chemicals GmbH, Schwalbach am Taunus, Germany	1:1000
Anti-Pimonidazole, mouse, monoclonal	Natural Pharmacia International Inc., Burlington, MA, USA	1:50

A.3 Devices and Software

Table A.6 Devices

Devices	Manufacturer
Microscope Axiolmager M1	Carl Zeiss Jena GmbH, Jena, Germany
Axiocam MRc (Digital color camera)	
Axiocam MRm (Monochrome camera)	

Devices	Manufacturer
Vortex Mixer VF2	IKA-Werke GmbH & Co. KG, Staufen, Germany
Dosimeter UNIDOS Universal Dosemeter 11767	PTW Freiburg GmbH, Freiburg, Germany
Biological safety workbench Herasafe™	Thermo Fisher Scientific GmbH, Dreieich, Germany
Spin Tissue Processor Microm STP120	
CO ₂ Incubator Heracell™	
Rotary Microtome Microm 766	
Yxlon Y.TU320-D03 X-Ray tube	Yxlon International GmbH Hamburg, Germany
Laboratory balance, BP221S	Sartorius AG, Göttingen, Germany
Laboratory balance, CPA225D	

Table A.7 Software

Software	Developer
Zen 2.6	Carl Zeiss Jena GmbH, Jena, Germany
AxioVision SE64 Rel. 4.9.1	
GraphPad Prism 6	GraphPad Software Inc., San Diego, CA, USA
SPSS 23	IBM Deutschland GmbH, Ehningen, Germany
OriginPro8G	OriginLab Corp., Northampton, MA, USA
Rstudio	RStudio, Inc, Boston, MA, USA

Part B: Supplementary materials

B.1 Supplementary materials of publication I

Suppl. Fig 1. Exemplary scattered- and box plots of γ H2AX foci in four specimens of an individual (A) SKX, (B) FaDu and (C) UT-SCC-5 tumor. Foci were corrected with a tumor specific mean nucleus area (Nfoci1) or a global tumor model specific mean nucleus area (Nfoci2). Foci normalization was carried out with tumor specific cfoci0. Comparison of the correction approaches was performed by paired-sample sign test with a significance level of 0.05. (***) $p < 0.0001$

Suppl. Fig. 2 The reoxygenation time had no statistical significance on the distribution of Nfoci1 in SKX, FaDu and UT-SCC-5 determined by the linear mixed-effects model.

B.2 Supplementary materials of publication II

Figure S1 Number of foci linearly increase with the nucleus area (x_i) categories (C). Box plots of foci number of six hHNSCC models were classified into six nucleus area categories with an interval size of $40 \mu\text{m}^2$. Foci number of controls and exposed (4 Gy) tumors, which were fixed 30 min and 24 h post exposure for the *in vivo* set-up are shown. P value of linear regression analysis is shown. Linear regression analysis outputs is presented in supplementary Table S4. Please note the different Y-axis for 30 min post-exposure.

Figure S2 Culmulative frequency percentage plots of nucleus area (x_i) classified into six categories with a bin size of $40 \mu\text{m}^2$. Nucleus area distribution for the *in vivo* set-up of which the statistical anlaysis by LMEM showed significant (A) and insignificant (B) differences among treatment groups, and for the *ex vivo* set-up (C). Comparison of the culmulative percentage nucleus area distribution between the experimental settings in three tumor lines (D).

Figure S3. Comparison of mean cfoci $\pm$ standard error of mean (SEM) (A) and nfoci $\pm$ SEM (B) in three tumor models subjected to both experimental settings of the γ H2AX assay. Data were fitted into LMEM where the experimental setting was defined as fixed effect and tumor, specimen, and ROI were defined as random effects. (**: $P < 0.01$, ***: $P < 0.001$). ROI was repeatedly included to generate the missing factor (specimen) for the *in vivo* set-up. Exact P values are presented in Supplementary Table S7.

Table S1. Summary of the input data for the statistical analysis

	Tumor line	Condition	No. of tumors	No. of specimen	No. of ROI	Total data input
<i>in vivo</i>	FaDu	Control	9	-	90	450
		30 min post IR	9	-	90	450
		24 h post IR	8	-	80	400
	SKX	Control	9	-	90	450
		30 min post IR	10	-	100	500
		24 h post IR	9	-	90	450
	UT-SCC-5	Control	8	-	80	400
		30 min post IR	9	-	90	450
		24 h post IR	8	-	80	400
	Cal33	Control	9	-	90	450
		30 min post IR	8	-	80	400
		24 h post IR	9	-	90	450
	SAS	Control	8	-	80	400
		30 min post IR	8	-	80	400
		24 h post IR	8	-	80	400
	UT-SCC-8	Control	8	-	80	400
		30 min post IR	7	-	70	350
		24 h post IR	9	-	90	450
	UT-SCC-14	Control	9	-	90	450
		30 min post IR	10	-	100	500
		24 h post IR	9	-	90	450
	UT-SCC-45	Control	8	-	80	400
		30 min post IR	8	-	80	400
		24 h post IR	9	-	90	450
	XF354	Control	7	-	70	350
		30 min post IR	4	-	40	200
		24 h post IR	7	-	70	350
<i>ex vivo</i>	FaDu	Control	16	60	438	4380
		24 h post IR	16	60	439	4390
	SKX	Control	9	31	217	2170
		24 h post IR	9	31	200	2000
	UT-SCC-5	Control	10	39	253	2530
		24 h post IR	10	40	283	2830

IR: Irradiation

Table S2. Summary of the statistical output of nucleus area analyzed by a LMEM. Bonferroni correction was applied for multiple comparisons.

	Tumor line	Condition	Mean	Standard deviation	P value ^a		
					Control	30 min	24 h
<i>in vivo</i>	FaDu	Control	116.4466	33.34720	-	0.804	1.000
		30 min post IR	122.2892	32.38627	-	-	0.297
		24 h post IR	112.6412	30.59298	-	-	-
	SKX	Control	100.2680	25.86748	-	0.281	0.001
		30 min post IR	107.1704	26.17204	-	-	0.084
		24 h post IR	119.1873	33.93800	-	-	-
	UT-SCC-5	Control	65.2466	15.69384	-	0.701	1.000
		30 min post IR	69.4444	18.16860	-	-	0.739
		24 h post IR	66.1853	19.15192	-	-	-
	Cal33	Control	80.2138	27.38123	-	1.000	0.120
		30 min post IR	81.1026	28.31106	-	-	0.156
		24 h post IR	94.9764	35.76368	-	-	-
	SAS	Control	93.8444	24.26456	-	1.000	0.000
		30 min post IR	93.5702	24.84296	-	-	0.000
		24 h post IR	108.7612	28.34736	-	-	-
	UT-SCC-8	Control	89.5806	28.27604	-	1.000	0.004
		30 min post IR	90.7311	26.55216	-	-	0.017
		24 h post IR	103.3802	33.47841	-	-	-
	UT-SCC-14	Control	85.7874	31.78737	-	1.000	0.787
		30 min post IR	90.5895	29.16455	-	-	1.000
		24 h post IR	94.9591	35.63261	-	-	-
	UT-SCC-45	Control	97.3420	24.88855	-	1.000	0.109
		30 min post IR	93.9249	23.99820	-	-	0.011
		24 h post IR	106.2284	28.73631	-	-	-
	XF354	Control	73.1351	19.25533	-	1.000	0.001
		30 min post IR	73.2460	17.68541	-	-	0.004
		24 h post IR	89.1600	23.93514	-	-	-
<i>ex vivo</i>	FaDu	Control	85.596336	25.218263	-	-	0.001
		24 h post IR	91.355702	26.967857	-	-	-
	SKX	Control	75.658134	30.057774	-	-	0.040
		24 h post IR	79.744765	31.672199	-	-	-
	UT-SCC-5	Control	75.021162	25.737746	-	-	0.522
		24 h post IR	75.603470	26.228616	-	-	-

^a Statistical significance was determined by fitting logarithmic nucleus area into a LMEM with condition as fixed effect and tumor and ROI as random effects. IR: Irradiation

Table S3. Statistical output of the comparison between the experimental settings of logarithmic nucleus area in three tumor lines. Tumor or ROI was assigned as random effects in the LMEM repeatedly to complete the missing factor for *in vivo* set-up.

	Tumor line	Condition	Experimental set-up (I)	Experimental set-up (J)	Mean Difference (I-J)	Standard error of mean	Degree of Freedom	P Value
Repeat tumor	FaDu	Control	<i>in vivo</i>	<i>ex vivo</i>	0.132	0.008	2536.809	0.000
		24 h	<i>in vivo</i>	<i>ex vivo</i>	0.095	0.018	108.437	0.000
	SKX	Control	<i>in vivo</i>	<i>ex vivo</i>	0.137	0.011	738.752	0.000
		24 h	<i>in vivo</i>	<i>ex vivo</i>	0.186	0.019	44.917	0.000
	UT-SCC-5	Control	<i>in vivo</i>	<i>ex vivo</i>	-0.006	0.009	1134.012	0.515
		24 h	<i>in vivo</i>	<i>ex vivo</i>	-0.076	0.028	35.043	0.011
Repeat ROI	FaDu	Control	<i>in vivo</i>	<i>ex vivo</i>	0.123	0.007	2241.822	0.000
		24 h	<i>in vivo</i>	<i>ex vivo</i>	0.092	0.012	41.231	0.000
	SKX	Control	<i>in vivo</i>	<i>ex vivo</i>	0.141	0.010	1121.132	0.000
		24 h	<i>in vivo</i>	<i>ex vivo</i>	0.187	0.017	20.967	0.000
	UT-SCC-5	Control	<i>in vivo</i>	<i>ex vivo</i>	-0.043	0.008	1502.768	0.000
		24 h	<i>in vivo</i>	<i>ex vivo</i>	-0.054	0.024	19.827	0.033

Table S4. Linear regression coefficient and R² of γ H2AX foci number as a function of nucleus area categories from different tumor lines and treatment conditions

	Tumor line	Condition	Slope	Sig. (Slope)	Intercept	Sig. (Intercept)	R Square
<i>in vivo</i>	FaDu	Control	0.836	0.000	-0.231	0.716	0.046
		30 min post IR	8.624	0.000	2.229	0.240	0.383
		24 h post IR	2.582	0.000	-2.038	0.062	0.141
	SKX	Control	1.256	0.000	-1.710	0.007	0.077
		30 min post IR	9.090	0.000	10.581	0.000	0.193
		24 h post IR	4.161	0.000	0.610	0.641	0.227
	UT-SCC-5	Control	0.447	0.063	-0.323	0.533	0.009
		30 min post IR	8.560	0.000	0.546	0.769	0.202
		24 h post IR	2.423	0.000	-3.169	0.000	0.097
	Cal33	Control	1.750	0.000	-1.782	0.026	0.068
		30 min post IR	11.749	0.000	3.815	0.038	0.424
		24 h post IR	3.616	0.000	-3.436	0.010	0.131
	SAS	Control	2.924	0.000	-4.669	0.000	0.127
		30 min post IR	11.078	0.000	4.342	0.027	0.402
		24 h post IR	2.548	0.000	-1.263	0.395	0.076
	UT-SCC-8	Control	0.954	0.000	-0.739	0.241	0.044
		30 min post IR	13.023	0.000	3.265	0.117	0.481
		24 h post IR	5.601	0.000	-5.535	0.000	0.264
	UT-SCC-14	Control	2.046	0.000	-2.374	0.006	0.090
		30 min post IR	11.170	0.000	0.774	0.638	0.432
		24 h post IR	5.242	0.000	-5.701	0.000	0.214
	UT-SCC-45	Control	5.855	0.000	-3.560	0.045	0.200
		30 min post IR	10.828	0.000	4.836	0.007	0.442
		24 h post IR	6.041	0.000	2.127	0.214	0.222
	XF354	Control	0.498	0.010	-0.421	0.360	0.019
		30 min post IR	16.135	0.000	-7.835	0.051	0.320
		24 h post IR	1.376	0.001	0.487	0.665	0.032
<i>ex vivo</i>	FaDu	Control	1.792	0.000	-1.289	0.000	0.051
		24 h post IR	2.652	0.000	-2.570	0.000	0.100
	SKX	Control	0.562	0.000	-0.415	0.005	0.041
		24 h post IR	2.239	0.000	-1.448	0.000	0.136
	UT-SCC-5	Control	0.445	0.000	-0.373	0.028	0.016
		24 h post IR	1.706	0.000	-0.743	0.003	0.091

IR: Irradiation

Table S5. Summary of the statistical analysis output analyzed by a random effect model for cfoci from the *in vivo* and *ex vivo* datasets

	Tumor line	Condition	Mean	Standard error of mean	P value			
					Tumor	Tumor x ROI	Tumor x Specimen	Tumor x Specimen x ROI
<i>in vivo</i>	FaDu	Control	2.569	1.335	0.128	0.136	-	-
		30 min post IR	33.216	1.335	0.057	0.000	-	-
		24 h post IR	6.335	1.416	0.144	0.300	-	-
	SKX	Control	1.939	1.303	0.134	^a	-	-
		30 min post IR	39.707	1.236	0.070	0.000	-	-
		24 h post IR	15.186	1.303	0.086	0.009	-	-
	UT-SCC-5	Control	0.599	1.013	0.127	^a	-	-
		30 min post IR	19.984	0.955	0.064	0.001	-	-
		24 h post IR	1.908	1.013	0.155	0.234	-	-
	Cal33	Control	2.473	0.663	0.142	^a	-	-
		30 min post IR	34.516	0.703	0.245	0.003	-	-
		24 h post IR	6.596	0.663	0.382	0.160	-	-
	SAS	Control	3.404	0.848	0.101	0.695	-	-
		30 min post IR	35.748	0.848	0.178	0.004	-	-
		24 h post IR	6.417	0.848	0.108	0.070	-	-
	UT-SCC-8	Control	1.880	1.103	^a	0.213	-	-
		30 min post IR	39.868	1.180	0.107	0.064	-	-
		24 h post IR	11.156	1.040	0.276	0.050	-	-
	UT-SCC-14	Control	2.847	1.568	0.128	^a	-	-
		30 min post IR	31.766	1.488	0.043	0.000	-	-
		24 h post IR	8.956	1.568	0.074	0.320	-	-
	UT-SCC-45	Control	13.367	1.231	0.137	0.237	-	-
		30 min post IR	36.032	1.231	0.074	0.051	-	-
		24 h post IR	21.072	1.160	0.108	0.290	-	-
	XF354	Control	0.723	0.576	0.166	0.037	-	-
		30 min post IR	29.431	0.762	0.384	0.131	-	-
		24 h post IR	4.117	0.576	0.559	0.792	-	-
<i>ex vivo</i>	FaDu	Control	3.343	0.672	0.028	-	0.000	0.000
		24 h post IR	4.715	0.817	0.015	-	0.000	0.000
	SKX	Control	0.931	0.216	0.146	-	0.007	0.050
		24 h post IR	4.208	0.680	0.123	-	0.007	0.000
	UT-SCC-5	Control	0.666	0.084	^a	-	0.003	0.070
		24 h post IR	3.348	0.353	0.079	-	0.005	0.000

^a: This covariance parameter is redundant. The test statistic and confidence interval cannot be computed.
IR: Irradiation

Table S6. Summary of the statistical analysis output analyzed by a random effect model for nfoci_g from the *in vivo* and *ex vivo* datasets

	Tumor line	Condition	Mean	Stand ard error of mean	P value			
					Tum or	Tumor x ROI	Tumor x Specime n	Tumor x Specime n x ROI
<i>in vivo</i>	FaDu	30 min post IR	30.648	2.330	0.057	0.000	-	-
		24 h post IR	4.191	0.521	0.144	0.284	-	-
	SKX	30 min post IR	37.768	1.944	0.070	0.000	-	-
		24 h post IR	13.286	1.060	0.087	0.009	-	-
	UT-SCC-5	30 min post IR	19.391	1.642	0.064	0.001	-	-
		24 h post IR	1.623	0.361	0.156	0.308	-	-
	Cal33	30 min post IR	32.043	1.093	0.245	0.003	-	-
		24 h post IR	4.839	0.527	0.384	0.181	-	-
	SAS	30 min post IR	32.344	1.092	0.178	0.004	-	-
		24 h post IR	3.800	0.778	0.118	0.045	-	-
	UT-SCC-8	30 min post IR	37.988	2.199	0.107	0.064	-	-
		24 h post IR	9.411	0.607	0.292	0.056	-	-
	UT-SCC-14	30 min post IR	28.750	2.332	0.043	0.001	-	-
		24 h post IR	6.547	1.124	0.078	0.284	-	-
	UT-SCC-45	30 min post IR	22.665	1.791	0.074	0.051	-	-
		24 h post IR	8.619	0.773	0.130	0.410	-	-
	XF354	30 min post IR	28.711	1.669	0.384	0.132	-	-
		24 h post IR	3.611	0.341	0.581	0.872	-	-
<i>ex vivo</i>	FaDu	24 h post IR	2.823	0.642	0.014	-	0.000	0.000
	SKX	24 h post IR	3.598	0.625	0.126	-	0.007	0.000
	UT-SCC-5	24 h post IR	2.862	0.329	0.079	-	0.006	0.000

IR: Irradiation

Table S7A. Summary of the statistical analysis output analyzed by LMEM for cfoci for direct comparison between *in vivo* and *ex vivo* datasets. Tumor or ROI was assigned as random effects in the LMEM repeatedly to complete the missing factor for *in vivo* set-up

	Tumor line	Condition	Experimental set-up (I)	Experimental set-up (J)	Mean Difference (I-J)	Standard error of mean	Degree of Freedom	P Value
Repeat tumor	FaDu	Control	<i>in vivo</i>	<i>ex vivo</i>	-3.235	0.297	2670.483	0.000
		24 h	<i>in vivo</i>	<i>ex vivo</i>	1.988	1.287	50.761	0.129
	SKX	Control	<i>in vivo</i>	<i>ex vivo</i>	1.687	0.278	826.754	0.000
		24 h	<i>in vivo</i>	<i>ex vivo</i>	11.170	0.897	13.885	0.000
	UT-SCC-5	Control	<i>in vivo</i>	<i>ex vivo</i>	-0.325	0.202	1461.697	0.107
		24 h	<i>in vivo</i>	<i>ex vivo</i>	-1.457	0.397	60.007	0.001
Repeat ROI	FaDu	Control	<i>in vivo</i>	<i>ex vivo</i>	-1.465	0.285	2530.096	0.000
		24 h	<i>in vivo</i>	<i>ex vivo</i>	1.444	0.922	28.381	0.128
	SKX	Control	<i>in vivo</i>	<i>ex vivo</i>	1.004	0.265	1795.228	0.000
		24 h	<i>in vivo</i>	<i>ex vivo</i>	11.291	1.037	18.506	0.000
	UT-SCC-5	Control	<i>in vivo</i>	<i>ex vivo</i>	0.069	0.182	1451.606	0.705
		24 h	<i>in vivo</i>	<i>ex vivo</i>	-1.405	0.356	19.670	0.001

Table S7B. Summary of the statistical analysis output analyzed by LMEM for nfoci_g for direct comparison between *in vivo* and *ex vivo* datasets. Tumor or ROI was assigned as random effects in the LMEM repeatedly to complete the missing factor for the *in vivo* set-up.

	Tumor line		P value		
			SKX	FaDu	UT-SCC-5
Repeat tumor	Fixed effect	Experimental setting	< 0.0001	0.206	0.022
	Random effect	Inter-tumoral	0.051	0.004	0.038
		Intra-tumoral inter-specimen	0.033	< 0.0001	0.0056
		Intra-tumoral intra-specimen	< 0.0001	< 0.0001	< 0.0001
Repeat ROI	Fixed effect	Experimental setting	< 0.0001	0.159	0.016
	Random effect	Inter-tumoral	0.019	0.003	0.021
		Intra-tumoral inter-specimen	0.000	< 0.0001	0.003
		Intra-tumoral intra-specimen	0.009	< 0.0001	< 0.0001

B.3 Supplementary materials of manuscript

Suppl. Fig. 1. Representative images of irradiated tumors (*in vivo*) and the corresponding irradiated biopsies (*ex vivo*) of radiosensitive (UT-SCC-45) and radioresistant (Cal33) model. Scan of immunohistochemistry staining (IHC, 100X magnification; upper panel) sections with corresponding immunofluorescence staining (IF, 400X magnification; lower panel). Tumors and biopsies were exposed to X-Ray radiation (4 Gy) and fixed 24 h post radiation exposure. IHC: BrdU (proliferation marker, nuclear brown stain), pimonidazole (hypoxia marker, cytoplasmic red stain), and tissue counterstain with hematoxylin (blue); IF: γH2AX foci (Alexa 488, green) and DNA (DAPI, blue). The red solid lines define oxic biopsies: the differentiation was defined based on superimposed images of IHC images and IF images. All analyzed nuclei were numbered and randomly selected for foci evaluation.

Suppl. Fig. 2. Bland-Altman plots showing mean and difference of residual cfoci of tumors and corresponding biopsies that have undergone *in vivo* and *ex vivo* radiation exposure with equivalent doses. Solid lines represent mean of difference (bias) and the top and bottom dotted lines represent 95% limits of agreement (bias $\pm$ 1.96 standard deviation (SD)).

Suppl. Fig. 3. Residual γ H2AX cfoci dose response of *in vivo* irradiated tumors (*in vivo*: open circle) and *ex vivo* irradiated biopsies (*ex vivo*: open triangle). Linear regression of the residual γ H2AX cfoci dose response was performed across the tumor models. Slopes and offsets of the linear regression between *in vivo* irradiated tumors and *ex vivo* irradiated biopsies were compared by applying ANCOVA (Suppl. Table 2).

Suppl. Fig. 4. Diagram of TCD₅₀(30 fx/6weeks) or TCD₅₀(SDambiant) compared with the slopes (SDRC) or the offsets (ODRC) of dose response curves of the residual yH2AX cfoci for *in vivo* irradiated tumors (*in vivo*: open circle) and *ex vivo* irradiated biopsies (*ex vivo*: open triangle). Error bars represent 95% confidence interval (95% CI) for TCD₅₀ and standard error (SE) for slopes and offsets. Note: different scaling on x axis (slope and offset) and y-axis (TCD₅₀ values).

Suppl. Table 1. Summary of the analyzed tumors (*in vivo*) and biopsies (*ex vivo*). *n* = number.

Tumor line	Tumor (<i>in vivo</i>)				Biopsy (<i>ex vivo</i>)					
	<i>n</i> of tumors	Analyzabl e tumors (%)	Total <i>n</i> of analyzable nuclei	Analyzed nuclei (%)	Mean <i>n</i> of nuclei/ tumors (95%CI)	<i>n</i> of biopsies	Analyzabl e biopsies (%)	Total <i>n</i> of analyzable nuclei	Analyzed nuclei (%)	Mean <i>n</i> of nuclei/ analyzed biopsy (95%CI)
UT-SCC-5 ^a	40	25 (62.5%)	9,901	1,290 (13.03%)	379 (346;411)	160	104 (65.00%)	37,876	7,390 (19.51%)	364 (340; 389)
Cal33 ^b	54	51 (94.44%)	18,790	2,660 (14.56%)	347 (327; 367)	108	96 (88.89%)	11,104	3,040 (27.38%)	231 (207; 256)
FaDu ^a	45	28 (62.22%)	7,161	2,910 (40.64%)	226 (227; 285)	180	80 (44.44%)	18,455	4,699 (25.46%)	231 (202; 260)
UT-SCC-45 ^b	54	46 (85.19%)	17,912	2,440 (13.62%)	389 (371; 408)	108	96 (88.89%)	10,743	3,180 (29.60%)	224 (199; 249)
SKX ^a	36	17 (42.5%)	5,571	895 (16.07%)	328 (303; 353)	144	73 (50.69%)	9,851	3,290 (33.40%)	135 (118; 152)

ⁱ: 4 biopsies/ tumor. ⁱᵇ: 2 biopsies/ tumor

^a: 4 biopsies/tumor, ^b: 2 biopsies/tumor

Suppl. Table 2. Summary of the statistical output of the linear regression analysis on residual γ H2AX cfoci dose response of *in vivo* irradiated tumors and *ex vivo* irradiated biopsies from five hHNSCC models. Comparisons of the slopes and the offsets of the linear regressions on residual γ H2AX cfoci dose response between the *in vivo* irradiated tumors and the *ex vivo* irradiated biopsies were determined by ANCOVA.

	Resistant			Sensitive	
	UT-SCC-5	Cal33	FaDu	UT-SCC-45	SKX
Tumor (<i>in vivo</i>)	Slope [$\pm$ SE]	0.34 $\pm$ 0.12	0.45 $\pm$ 0.059	0.36 $\pm$ 0.07	1.36 $\pm$ 0.21
	95% CI of slope	0.08; 0.59	0.33; 0.57	0.21; 0.51	0.94; 1.77
	Offset [$\pm$ SE]	2.23 $\pm$ 0.55	1.10 $\pm$ 0.27	1.25 $\pm$ 0.34	10.77 $\pm$ 0.93
	95% CI of offset	1.10; 3.36	0.56; 1.63	0.55; 1.95	8.89; 12.66
	R ²	0.2438	0.5496	0.4885	0.4910
	P value	0.0121	<0.0001	<0.0001	0.0022
Biopsy (<i>ex vivo</i>)	Slope [$\pm$ SE]	0.15 $\pm$ 0.08	0.44 $\pm$ 0.06	0.12 $\pm$ 0.05	1.08 $\pm$ 0.23
	95% CI of slope	-0.01; 0.31	0.33; 0.55	0.02; 0.21	0.63; 1.54
	Offset [$\pm$ SE]	3.32 $\pm$ 0.36	1.11 $\pm$ 0.25	0.62 $\pm$ 0.24	9.54 $\pm$ 1.01
	95% CI of offset	2.61; 4.03	0.60; 1.61	0.15; 1.09	7.51; 11.56
	R ²	0.03318	0.5871	0.07019	0.3343
	P value	0.0642	<0.0001	0.0175	<0.0001
P value (Significance between slopes)					
0.3225					
P value (Significance between offsets)					
0.3867					
0.9421					
0.0174					
0.8794					

n.a.: not available due to the pronounced significant difference in the slopes.

Technische Universität Dresden
Medizinische Fakultät Carl Gustav Carus
Promotionsordnung vom 24.10.2014

Erklärungen zur Eröffnung des Promotionsverfahrens

1. Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht.
2. Bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts habe ich Unterstützungsleistungen von folgenden Personen erhalten:
Prof. Dr. med. Mechthild Krause, Prof. Dr. med. Michael Baumann, PD Dr. Steffen Löck, Dr. rer. nat. Cläre von Neubeck, Ursula Range
3. Weitere Personen waren an der geistigen Herstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich nicht die Hilfe eines kommerziellen Promotionsberaters in Anspruch genommen. Dritte haben von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.
4. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.
5. Die Inhalte dieser Dissertation wurden in folgender Form veröffentlicht:

Vortrag:

- i) Rassamegevanon T. 2015. Investigation of residual γ H2AX foci as a predictor of radiation responses in *ex vivo* irradiated tumour biopsies and *in vivo* irradiated tumour. Annual HZDR PhD Seminar, Altenberg, Germany
- ii) Rassamegevanon T., von Neubeck C, Löck S, Range U, Krause M, Baumann M. 2016. Intratumoral and intertumoral heterogeneity of γ H2AX foci determined in *ex vivo* irradiated tumor biopsies. 25. Symposium „Experimentelle Strahlentherapie und Klinische Strahlenbiologie“, Dresden, Germany

Poster:

- i) Rassamegevanon T., von Neubeck C, Löck S, Range U, Krause M, Baumann M. 2016. Intratumoral and intertumoral heterogeneity of γ H2AX foci determined in *ex vivo*

irradiated tumor biopsies. Workshop in Biomarkers for Radiation Oncology, Dresden, Germany

- ii) von Neubeck C, Rassamegevanon T, Meneceur S, Löck S, Range U, Richter D, Krause M, Baumann M. 2016 The γ H2AX Foci Assay – Prediction Radiation Response based on DNA Repair Capacity. Symposium on Personalized Cancer Care, Oslo, Norway
- iii) von Neubeck C, Meneceur S, Rassamegevanon T, Richter D, Löck S, Range U, Elimport A, Hiemann R, Krause M, Baumann M. 2016 The γ H2AX Foci Assay – improvements for a possible clinical application. Annual Meeting of the Society for Biological Radiation Research – GBS, Erlangen, Germany
- iv) Rassamegevanon T, von Neubeck C, Meneceur S, Richter D, Löck S, Range U, Krause M, Baumann M. 2016 Prediction of radiation responses via the γ H2AX foci assay: translation for a clinical application. DTK Annual Retreat 2016, Heidelberg, Germany
- v) Rassamegevanon T, von Neubeck C, Löck S, Range U, Krause M, Baumann M. 2016. Translation of the gamma H2AX (γ H2AX) foci assay for clinical use: a study in head and neck squamous cell carcinomas (hHNSCC). Summer School in Translational Cancer Research, Albufeira, Portugal
- vi) Rassamegevanon T, Löck S, Range U, Krause M, Baumann M, von Neubeck C. 2017. Tumor heterogeneity determined with a γ H2AX foci assay: a study in human head and neck squamous cell carcinoma (hHNSCC) models. The 15th International Wolfsberg Meeting on Molecular Radiation Biology/Oncology, Wolfsberg, Switzerland
- vii) Rassamegevanon T, Löck S, Range U, Krause M, Baumann M, von Neubeck C. 2017. Heterogeneous radiation response determined with a γ H2AX foci assay in human head and neck squamous cell carcinoma (hHNSCC) tumor models. Annual Meeting of the European Radiation Research Society (ERRS)/ the Society for Biological Radiation Research (GBS), Essen, Germany
- viii) Rassamegevanon T, Löck S, Krause M, Baumann M, von Neubeck C. 2018. Statistical reassessment of DNA damage response determined with γ H2AX foci in *ex-* and *in vivo* irradiated tumor specimen. 27. Symposium „Experimentelle Strahlentherapie und Klinische Strahlenbiologie“, Hamburg, Germany
- ix) Rassamegevanon T, Löck S, Krause M, Baumann M, von Neubeck C. 2018. Statistical reanalysis of tumour radiation response determined with γ H2AX foci assay reveals a pronounced difference in heterogeneity between *in-* and *ex vivo* experimental set-ups. Radiation Break-through: from DNA damage responses to precision cancer therapy, Oxford, United Kingdom

- x) Rassamegevanon T, Löck S, Krause M, Baumann M, von Neubeck C. 2018. Radiation response predicted via the γ H2AX foci assay: translation and optimization for a clinical application. Summer School in Bioinformatics, Hixton, United Kingdom
- xi) Rassamegevanon T, Graja A, Lorat Y, Müller J, Löck S, Baumann M, Rübe C E, Krause M, von Neubeck C. 2019. Prediction of tumor radiation sensitivity - translation and optimization of γ H2AX foci assay for clinical use. 1st German Cancer Research Congress (GCRC), Heidelberg, Germany.

Publikation:

- i) Rassamegevanon T, Löck S, Range U, Krause M, Baumann M, von Neubeck C. 2017. Tumor heterogeneity determined with a γ H2AX foci assay: A study in human head and neck squamous cell carcinoma (hHNSCC) models. *Radiother Oncol*, 124(3):379–385 DOI: 10.1016/J.RADONC.2017.06.027.
 - ii) Rassamegevanon T, Löck S, Baumann M, Krause M, von Neubeck C. 2018. Heterogeneity of γ H2AX Foci Increases in Ex Vivo Biopsies Relative to In Vivo Tumors. *Int J Mol Sci*, 19(9):2616 DOI: 10.3390/IJMS19092616.
 - iii) Rassamegevanon T, Löck S, Baumann M, Krause M, von Neubeck C. 2019. Comparable radiation response of *ex vivo* and *in vivo* irradiated tumor samples determined by residual γ H2AX foci. *Submitted to Radiotherapy and Oncology* (Ref No.: RO-D-19-00258)
6. Ich bestätige, dass es keine zurückliegenden erfolglosen Promotionsverfahren gab.
 7. Ich bestätige, dass ich die Promotionsordnung der Medizinischen Fakultät der Technischen Universität Dresden anerkenne.
 8. Ich habe die Zitierrichtlinien für Dissertationen an der Medizinischen Fakultät der Technischen Universität Dresden zur Kenntnis genommen und befolgt.
 9. Ich bin mit den "Richtlinien zur Sicherung guter wissenschaftlicher Praxis, zur Vermeidung wissenschaftlichen Fehlverhaltens und für den Umgang mit Verstößen" der Technischen Universität Dresden einverstanden.

Dresden, den

Unterschrift des Doktoranden

Anlage 2

Hiermit bestätige ich die Einhaltung der folgenden aktuellen gesetzlichen Vorgaben im Rahmen meiner Dissertation

- ☐ das zustimmende Votum der Ethikkommission bei Klinischen Studien, epidemiologischen Untersuchungen mit Personenbezug oder Sachverhalten, die das Medizinproduktegesetz betreffen
Aktenzeichen der zuständigen Ethikkommission: entfällt
- ☒ die Einhaltung der Bestimmungen des Tierschutzgesetzes
Aktenzeichen der Genehmigungsbehörde zum Vorhaben/zur Mitwirkung:
Landesdirektion Sachsen, AZ: DD24-5131/289/19
- ☐ die Einhaltung des Gentechnikgesetzes
Projektnummer: entfällt
- ☒ die Einhaltung von Datenschutzbestimmungen der Medizinischen Fakultät und des Universitätsklinikums Carl Gustav Carus.

Dresden,

Unterschrift des Doktoranden