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Ex vivo γH2AX radiation sensitivity assay in prostate cancer: Inter-patient and intra-patient heterogeneity

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ABSTRACT

Introduction: The aim of the study is to assess inter-patient and intra-patient heterogeneity in tumour cell radiosensitivity using the *ex vivo* γH2AX assay in prostate cancer specimens.
Methods: Excised specimens from untreated prostate cancer patients were cultivated 24 h in media, irradiated *ex vivo* and fixed after 24 h. Residual γH2AX foci were counted and the slope of the dose response was calculated. Intra-patient heterogeneity was studied from three to seven different biopsies.
Results: In pathology-confirmed tumour samples from 21 patients the slope of residual γH2AX foci and radiation dose showed a substantial heterogeneity ranging from 0.82 to 3.17 foci/Gy. No correlation was observed between the slope values and the Gleason score ($p = 0.37$), prostate specific antigen ($p = 0.48$) and tumour stage ($p = 0.89$). ANOVA indicated that only in 1 out of 9 patients, biopsies from different tumour locations yielded statistically significant differences. Variance component analysis indicated higher inter-patient than intra-patient variability. Bootstrap simulation study demonstrated that one biopsy is sufficient to estimate the mean value of residual γH2AX per dose level and account for intra-patient heterogeneity.
Conclusions: In prostate cancer inter-patient heterogeneity in tumour cell radiation sensitivity is pronounced and higher than intra-patient heterogeneity supporting the further development of the γH2AX *ex vivo* assay as a biomarker for individualized treatment.
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Prostate cancer is the most common tumour type among men, having a worldwide incidence rate of 130 cases per 100.000 population and represents the second cause of cancer-related death [1]. Almost 40% of all prostate cancer patients receive radiation therapy (RT) as primary curative treatment [2]. The Prostate Specific Antigen (PSA), the Gleason Score (GS) and the tumour stage (T stage) are the most important clinico-pathological prognostic factors for patient's outcome and are routinely used in clinical practice. Together, they confer in the stratification of prostate cancer patients in groups according to the risk of relapse (low-, intermediate-, and high-risk) [3] and are crucial for selecting RT doses, volumes and association with hormonal therapy (HT). Despite recent advances in RT, the 5-year biochemical failure rate still reaches up to 30%, especially in the high risk-patient group [4,5] underlying the need for further improvement in treatment. On the other hand, high dose RT bears the risk of higher intestinal and genitourinary toxicities [6,7]. Therefore, it is an important task to develop methods that allow individualized radiation dose prescriptions, i.e. lower doses to sensitive tumours and higher doses to resistant tumours [8].

Despite recent advances in genomics of prostate cancer [9,10], no predictive biomarkers specifically for radiation sensitivity have been established. In general, response of tumours to fractionated irradiation depends on several biological factors including repopulation, reoxygenation, recovery, redistribution, and intrinsic radiation sensitivity [11]. The latter factor is associated with the number of radiation-induced DNA double strand breaks (DBSs) that are still remaining upon completion of the DNA repair process [12–19]. One of the earliest events occurring in the DNA damage response (DDR) upon DSB induction is the phosphorylation of H2AX histone

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(referred as γH2AX), that marks the site of chromatin where DSB is introduced, facilitating the recruitment of repair factors [20–25] and remaining at the site of damage until the DSB repair is fully resolved before being dephosphorylated [26–29]. γH2AX quantitative evaluation directly correlates with the residual DSBs and has been shown to correlate with cellular radiation sensitivity *in vitro* and *in vivo* [13–17,18,19,30–32].

We have developed an *ex vivo* protocol of the γH2AX assay in patient-derived tumour specimens (PDTS) that allows utilization of the technique in a more clinical scenario [33,34]. In our previous proof-of-principle study, quantitative assessment of residual γH2AX foci in PDTS across 10 different tumour types indicated that the assay could assist in discriminating between radiosensitive and radioresistant tumour types of known responsiveness to radiotherapy. The next important step for integration of the assay in strategies for individualized radiotherapy would be to study the heterogeneity of intrinsic cellular radiation sensitivity within the same tumour type or histology. Here we use *ex vivo* γH2AX assay to detect inter-patient differences in intrinsic radiation sensitivity among prostate cancer patients. Another important issue regarding the robustness of the assay is the potential sampling error, as in the clinical setting often only a limited number of biopsies is available. Evidence supports the co-existence of distinct clonal subpopulations within the same tumour carrying different DNA mutations or post-transcriptional DNA modifications [35,36]. It is a matter of debate, if the genetic intra-tumoural heterogeneity leads to functionally different phenotypes and how much it influences tumour diagnosis and therapeutic outcomes [37–39]. To address the issue of intra-patient heterogeneity in intrinsic radiation sensitivity, we compared results from multiple biopsies with inter-patient variability.

Materials and methods

Study design and processing of tumour specimens

Patients undergoing radical prostatectomy as primary treatment for prostate adenocarcinoma were included in the study. Previous radio- and/or chemotherapy were exclusion criteria. The study was approved by the Ethics Committee of the Medical Faculty of the University of Tübingen (426/2013BO1). All patients signed informed consent. Specimens were collected immediately after prostatectomy. Tumour areas were manually detected by an expert urologist (JH) and biopsies or tumour parts (PDTS) were retrieved for γH2AX foci analysis. The presence of malignant cells in the PDTS was approved on haematoxylin and eosin (H&E) stained sections by a prostate-expert pathologist (MS). Collection, cultivation and staining of PDTS were performed as previously described [33,40]. Briefly, PDTS were collected, either as fine needle biopsies or cut tumour pieces from the cancer-invaded parts of the prostate gland (biopsy needles: 200 mm length, diameter: 1.2 mm, 18 G; Vitesse Biopsy System, OptiMed global care, Ettlingen, Germany) and placed in Falcon tubes containing DMEM medium supplemented with 10% Foetal calf Serum (FCS), 2% Hepes buffer, 1% antibiotics, 1% sodium pyruvate and 1% non-essential amino acids (all Biochrom AG, Berlin, Germany). Tumour specimens were cut manually and placed in 3.5 cm diameter agarose-coated petri dishes containing 3 ml culture media. After 20 h of cultivation (37 °C, 95% humidity and 5% CO_2), medium containing the proliferation marker 5-Bromo-2′-deoxyuridine (BrdU; Serva electrophoresis GmbH, Heidelberg, Germany) and the hypoxic marker pimonidazole (Hypoxyprobe Inc, hpi, Middlesex, Burlington, USA) was added to PDTS for 4 h in final concentration of 10 μM for each marker. Immediately after *ex vivo* irradiation the medium was exchanged (without markers) and PDTS were further cultivated for 24 h, then fixed in 4% formaldehyde and embedded into paraffin.

Staining and imaging of tumour specimens

Three consecutive 3 μm thickness cross-sections from the paraffin-embedded tumour material were stained for: (a) histology verification with H&E; (b) microenvironmental parameters with anti-BrdU (mouse monoclonal, Clone Bu20a, Dako Deutschland GmbH, Hamburg, Germany, dilution: 1:50) and anti-pimonidazole (mouse monoclonal, Natural Pharmacia International, Belmont, MA, USA, dilution 1:100) with ARKTM Kit (animal research kit; Dako Deutschland GmbH, Hamburg, Germany) and VECTAstain Kit, respectively (Vectastain Elite ABC kit, PK-6102, Mouse IgG, Vector Laboratories, Inc., 30 Ingold Road, Burlingame, CA 94010 USA); (c) visualization of γH2AX foci with anti- γH2AX at Ser139 (Merck Millipore, Upstate, clone JBW301, Darmstadt, Germany, dilution 1:1000) with TSATM Kit (T20912, containing goat anti-mouse IgG and tyramide labelled with Alexa 488, Life Technologies GmbH, Molecular probes, Invitrogen, Darmstadt, Germany) and counterstained with 4,6-diamidino-2-phenylindole (DAPI). Complete H&E and immunohistochemistry (IMH) sections were scanned with a digital colour camera 3-chip TDI camera on a NanoZoomer 2.0 HT (Hamamatsu Photonics K.K., Hamamatsu, Japan, Software: NDP.view (Nanozoomer Digital Pathology), Hamamatsu Photonics K.K., Hamamatsu, Japan) and fields with viable (BrdU-positive) and oxic tumour areas (pimonidazole-negative) for γH2AX foci analysis were identified and marked. Subsequently, in the adjacent immunofluorescence (IMF) section the marked fields were identified and 17 individual images per area were taken every 0.25 μm on the *Z*-axis (z-stack) using a monochrome digital camera (AxioCamMRm, Carl Zeiss, Jena, Germany; motorized scanning stage, Maerzhaeuser, Wetzlar, Germany, 400_, EC Plan Neofluar) on a Zeiss Axio Imager Z1 Apotome fluorescence microscope controlled by AxioVision 4.8 software (Carl Zeiss, Jena, Germany). For analysis, the maximal intensity projection z-stack image per field was used and only in case of required clarification, the single images were used. The number of γH2AX foci per nucleus was evaluated manually in pathology-confirmed areas containing exclusively tumour cells (Fig. 2 & Suppl. Fig. 1).

Assessing inter-patient heterogeneity

Inter-tumour heterogeneity of residual γH2AX foci was assessed as previously published [33,40]. PDTS were collected, cut into 5 pieces and distributed in 5 petri-dishes. PDTS were irradiated with 0, 2, 4, 6, 8 Gy, 24 h later fixed in 4% formaldehyde and embedded into paraffin. Five to seven IMF-stacks were taken and 50 cells (in the pimonidazole-negative and BrdU-positive outer rim of the sample) per radiation dose per PDTS were randomly selected for analysis. The foci counting was performed independently by two blinded observers (AM and CD).

Assessing intra-patient heterogeneity

Multiple biopsies from at least 2 different locations of the same prostate gland per patient were included from a different patient cohort. A total of 2 to 4 suspicious palpable tumour nodules areas were impaled by suture-containing needles (Fig. 1A). Three to four biopsies were performed in the vicinity of each needle representing thereafter a *location*, namely biopsies taken from the same part of the tumour. Biopsies were then placed in 15 ml Falcon tubes containing culture medium. The needles were then removed, maintaining in place the sutures that were marked with coloured-immunohistochemistry compatible ink (black, blue, green and yellow) using sterile gauze. Through the sutures, the