

# The kinetics of $\gamma$ -H2AX during radiotherapy of head and neck cancer potentially allow for prediction of severe mucositis

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**Background.** The aim of the study was to evaluate the changes in  $\gamma$ -H2AX expression in peripheral blood lymphocytes (PBL) according to severity of radiation-induced mucositis.

**Patients and method.** Fifty patients with head and neck cancer treated with radiotherapy (RT) or chemoradiation were included in the study. Blood samples were collected before treatment to measure baseline  $\gamma$ -H2AX levels. Second sample was taken 45 minutes after the first RT fraction and then once a week, 45 min after irradiation. In patients treated with chemoradiation the blood sample was taken the day after chemotherapy. Mucositis was evaluated once a week and reported according to CTCAE v4 and RTOG/EORTC scales. PBL were analyzed with flow cytometry and level of H2AX phosphorylation at every time point was evaluated.

**Results.** In 35 patients mild to moderate (grade 1–2) mucositis was observed and 15 patients developed severe (grade 3) mucositis. No cases of grade 4 mucositis were observed. The difference in baseline levels of  $\gamma$ -H2AX between groups with mild and severe mucositis was statistically insignificant ( $p = 0.25$ ). The statistically significant difference in  $\gamma$ -H2AX level was observed in week 7 of treatment ( $p = 0.01$ ). No significant differences in  $\gamma$ -H2AX level were found neither between group treated with concomitant chemoradiation or RT alone neither between groups with and without common comorbidities. In the analysis of the kinetics of  $\gamma$ -H2AX during treatment, a statistically significant difference ( $p = 0.0088$ ) between groups with mild and severe mucositis was observed. After fourth week of treatment levels of  $\gamma$ -H2AX decreased significantly in the group with severe mucositis and increased in patients with mild side effects. The observed difference was not caused by the decrease in peripheral lymphocyte count, which was similar in both groups.

**Conclusions.** Presented results indicate that severity of radiation-induced mucositis does not correlate directly with  $\gamma$ -H2AX levels measured *in vivo* in PBL. Prediction of mucositis grade based on  $\gamma$ -H2AX level is not yet possible, either before treatment or early during treatment, but preliminary results, indicating significant differences in  $\gamma$ -H2AX kinetics between groups, encourage further studies.

Keywords:  $\gamma$ -H2AX; peripheral blood lymphocytes; mucositis

## Introduction

Radiotherapy and chemoradiation remain the major treatment modalities of head and neck cancer

either as a primary treatment or in postoperative adjuvant setting.

Despite the clear therapeutic benefits, acute and late side effects of radiation and chemora-

diation still limit the quality of life of patients. Furthermore, there is significant heterogeneity in response to radiation, both in tumours and healthy tissues. It would therefore be of great value for the clinicians to predict the response not only in the primary tumour volume, but also in the surrounding normal tissues.

Radiation damage in any tissue occurs primarily through single or double DNA strand break (DSB), which lead to cell death due to chromosomal aberrations and apoptosis if not repaired. Phosphorylated histone H2AX (called  $\gamma$ -H2AX) is an indirect protein marker of DSB. It has been shown that DSB in cell nucleus triggers H2AX phosphorylation in position Ser139 (*i.e.*  $\gamma$ -H2AX). Phosphorylated H2AX is a trigger for DNA repair signaling pathways (*e.g.* through ATM, MRE11, DNA-PK, Rad50, Nbs1). The maximum level of unrepaired damage is observed approximately 30 min after radiation.<sup>1,2</sup>

In this paper, we suggest that assessment of  $\gamma$ -H2AX levels after irradiation could serve as a marker of sensitivity to radiation damage in normal tissue, enabling the estimation of the individual damage repair potential. Peripheral blood lymphocytes (PBL) could serve as effective and easily accessible surrogates of normal tissue. Previous studies have focused on lymphocytes irradiated *in vitro*, which fails to account for the tissue microenvironment and comorbidities. *In vivo* analysis using samples of fresh blood taken from patients immediately after irradiation offers a more reliable way to assess H2AX phosphorylation.<sup>3,4</sup> In the future, predictive factors showing high individual regenerative potential could allow for patient selection for escalated radiotherapy. Additionally, prediction of higher risk for acute and late effects would enable the clinician to intensify supportive care, *e.g.* nutrition and hydration, to maintain as good quality of life (QoL) and survival as possible.

The main objective of the study was to examine the clinical usefulness of assessing radiation ef-

fects based on the  $\gamma$ -H2AX evaluation in the PBL. The secondary goal was to assess the changes of  $\gamma$ -H2AX expression in PBL according to the severity of radiation-induced mucositis (M) during radiotherapy or chemoradiation. Mucositis is evaluated in clinic using many scales, most widely used are Common Terminology Criteria for Adverse Events v.4 (CTCAE) and European Organisation for Research and Treatment of Cancer/Radiation Therapy Oncology Group (EORTC/RTOG) scales.<sup>6,7</sup>

Table 1 presents grading of toxicity, using oral mucositis as an example, with two most commonly used scales.

## Patients and method

In this prospective study, we included patients with head and neck cancers treated with postoperative or definitive radiotherapy or chemoradiation. Patients treated with palliative intent were excluded from the study. The study was approved by the Ethics Committee of University of Medical Sciences in Poznan (No723/14) and written informed consent was provided by all the participants. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

## Treatment

Patients were treated with adjuvant radiotherapy or chemoradiation to 60-66 Gy, if the known features of risk of relapse were present in pathology report.

Patients with inoperable tumors were treated mostly with concomitant chemoradiation to dose 70 Gy (fractions of 2 Gy once daily, 5 times a week) concurrently with cisplatin 40 mg/m<sup>2</sup> once a week

TABLE 1 Grading of radiation-induced oral mucositis according to CTCAE v4 and EORTC/RTOG scales.<sup>6,7</sup>

Mucositis (oral)	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
CTCAE v4	Asymptomatic/mild symptoms, intervention not indicated	Moderate pain not interfering with oral intake, modified diet indicated	Severe pain interfering with oral intake	Life-threatening consequences, urgent intervention indicated	Death
EORTC/ RTOG	Irritation, may experience slight pain, not requiring analgesic	Patchy mucositis that may produce inflammatory serosanquinitis discharge, may experience moderate pain requiring analgesia	Confluent, fibrinous mucositis, may include severe pain requiring narcotic	Ulceration, haemorrhage or necrosis	N/A

TABLE 2. Demographics, staging and treatment information of studied patients

Characteristics		Total N = 50 (%)	M 0-2 N = 35 (%)	M 3 N = 15 (%)	p-value
<b>Sex</b>	Male	44 (88)	30 (85.7)	14 (93.3)	0.44
	Female	6 (12)	5 (14.2)	1 (6.6)	
<b>Primary site</b>	Parotid	5 (10)	5 (14.3)	0 (0)	0.27
	Oral cavity	11 (22)	7 (20)	4 (26.7)	
	Larynx	14 (28)	10 (28.6)	4 (26.7)	
	Hypopharynx	4 (8)	4 (11.4)	0 (0)	
	Oropharynx	12 (24)	6 (17.1)	6 (40)	
	CUP	2 (4)	1 (2.8)	1 (6.7)	
	Nasalcavity/paranasal sinuses	2 (4)	2 (5.7)	0 (0)	
<b>T classification</b>	T1	4 (8)	2 (5.7)	2 (13.3)	0.49
	T2	17 (34)	11 (31.4)	6 (40)	
	T3	8 (16)	7 (20)	1 (6.7)	
	T4	19 (38)	14 (40)	5 (33.3)	
	Tx	2 (4)	1 (2.8)	1 (6.7)	
<b>N classification</b>	N0	26 (52)	19 (54.3)	7 (46.7)	0.25
	N1	4 (8)	4 (11.4)	0 (0)	
	N2	20 (40)	12 (34.3)	8 (53.3)	
<b>Surgery</b>	Yes	28 (56)	22 (62.85)	6 (40)	0.13
	No	22 (44)	13 (37.1)	9 (60)	
<b>Concomitant chemotherapy</b>	Yes	33 (66)	26 (74.3)	7 (46.7)	0.06
	No	17 (34)	9 (25.7)	8 (53.3)	
<b>Comorbidities</b>	Yes	34 (68)	21 (60)	13 (86.7)	0.06
	No	16 (32)	14 (40)	2 (13.3)	
<b>Selected comorbidities</b>	Diabetes type II	6 (12)	5 (14.3)	1 (6.7)	0.09
	Hypertension	17 (34)	12 (34.3)	5 (33.3)	
	Ischemic heart disease	11 (22)	4 (11.1)	7 (46.7)	

CUP = cancer unknown primary; M = mucositis; grade 0-2 or grade 3 (of mucositis).

No grade 4 mucositis was observed. The statistical differences between groups were assessed with the use of Chi-square test.

or 100 mg/m<sup>2</sup> every three weeks of treatment, on days 1, 21, and 42 of radiotherapy (fraction 1, 16, 31). In every patient mucositis was evaluated once a week, independently, by radiation oncologist and head and neck surgeon, using the Common Terminology Criteria for Adverse Events version 4 (CTCAE v.4) and the Radiation Therapy Oncology Group (RTOG) scales.<sup>6,7</sup> For the purpose of this study, grading results based on the CTCAE scale were employed because it is more commonly used in clinic. The maximum score observed during treatment was used for correlation with  $\gamma$ -H2AX level.

### Blood samples and flow cytometry analysis

The level of  $\gamma$ -H2AX was analyzed in PBL. The first blood sample was collected before treatment, to measure basic level of  $\gamma$ -H2AX in every patient. The second sample was taken 45 minutes after the first fraction and then once a week, 45 minutes after radiotherapy fraction. The sampling time was

selected according to observation from a pilot study, showing that the level of  $\gamma$ -H2AX in PBL was highest 45 min after irradiation. In patients treated with concurrent chemotherapy, the blood sample was taken the day after chemotherapy. The collected blood samples were stabilized by Transfix (Cytomark, UK) administration according to manufacturer protocol. H2AX phosphorylation levels in the leucocytes were examined by flow cytometry according to manufacturer protocol (Apoptosis, DNA Damage, and Cell Proliferation Kit, BD, USA). Briefly, isolated leucocytes (centrifuging 1200g at 20°C for 20 min) were fixed with BD Cytofix/Cytoperm Fixation/Permeabilization Solution for 30 min at room temperature. Subsequently, cells were incubated with BD Cytofix/Cytoperm Plus Permeabilization Buffer for 10 min at 4°C, and re-fixed, with BD Cytofix/Cytoperm Fixation/Permeabilization Solution (5 min, at room temperature). Next, the cells were incubated with Alexa Fluor 647 Mouse Anti-H2AX antibody (pSer139). Staining with isotype control (APC Mouse IgG2b  $\kappa$  Isotype Control; Becton Dickinson, USA) was per-

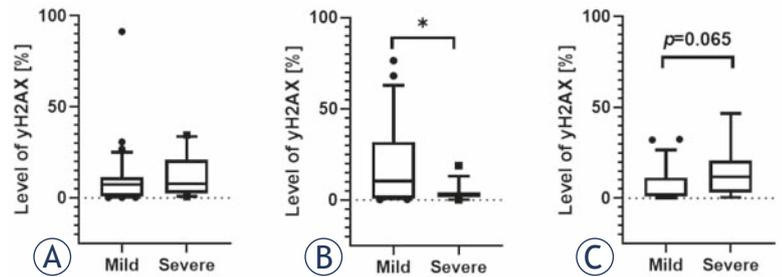
formed to assess the threshold of positive staining. Data acquisition was performed using a BD Accuri C6 Plus flow cytometer (BD Biosciences, USA), and analyzed using FlowJo software (FlowJo, LLC, USA).

### Statistical analysis

Statistical analysis was performed using STATISTICA (StatSoft, Inc. USA version 12, 2014). The differences between groups were assessed with the use of Chi-square test (Table 2). Two-sided Welch's t-test, and two-way ANOVA were applied to assess the statistical significance of differences between  $\gamma$ -H2AX levels. The result were evaluated at  $\alpha = 0.05$  significance level.

## Results

Fifty patients with head and neck cancers treated with adjuvant or primary radiotherapy or chemoradiation were included in the study. Mild (grade 1) to moderate (grade 2) mucositis was observed in 35 patients and severe (grade 3) in 15. No cases grade 4 mucositis were observed. No patient was lost from follow up. Median follow-up was 51 months (range, 2–60 months), median overall survival (OS) for whole group reached 51 months. For patients with mild and severe mucositis median OS was 50.5 and 55 months respectively. We did not observe any unexpectedly severe acute reaction *i.e.*, necrosis in the study. Mean radiotherapy dose for neck lymph nodes was 52 Gy (range 50–60 Gy) and mean total volume of irradiated tissue (Planning Target Volume, PTV) was 750.8 cm<sup>3</sup> (range 121.0–



**FIGURE 1.** Level of  $\gamma$ -H2AX in peripheral blood lymphocytes before radiotherapy (A); in week 7 (B); week 2 (C) of treatment. Mild = grade 1 or 2 mucositis; Severe = grade 3. No grade 4 mucositis was observed.

1022.0). Table 2 shows demographics, staging and treatment details of the patients.

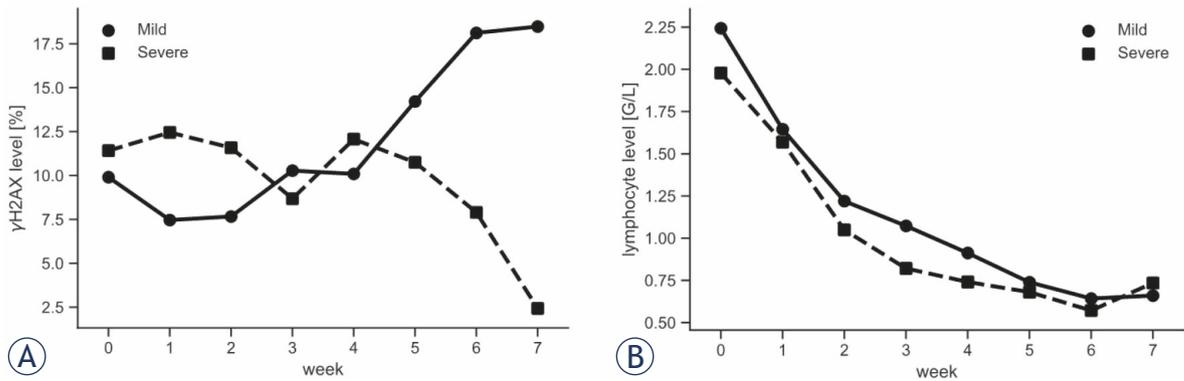
The difference in basic level of  $\gamma$ -H2AX between the groups with mild and severe mucositis was statistically insignificant ( $p = 0.25$ ) (Figure 1A). However, we observed a significantly lower level of H2AX phosphorylation in lymphocytes in week 7 ( $p = 0.011$ ) in the group with severe mucositis in comparison to the mild one (Figure 1B).

Interestingly, we noticed an opposite statistical trend in week 2 ( $p = 0.065$ ) (Figure 1C). Analyzing the kinetics of  $\gamma$ -H2AX during treatment, we observed a statistically significant difference ( $p = 0.0088$ ) between groups with mild and severe mucositis (Figure 2A, Table 3, Table S1 in Supplementary material). Between treatment weeks 4 and 7, levels of  $\gamma$ -H2AX decreased significantly in the group with severe mucositis and increased in patients with mild side effects. The observed difference is not caused by the expected decreasing of peripheral lymphocytes level during radiotherapy which was similar in both groups (Figure 2B).

**TABLE 3.** Level of  $\gamma$ -H2AX in peripheral blood lymphocytes before and during treatment

Week of radiotherapy	Mild mucositis		Severe mucositis		No. of samples	p-value
	Mean	SD	Mean	SD		
Before	10.64	16.38	11.40	11.71	48	0.868
Week 1	8.48	8.99	11.24	10.78	48	0.347
Week 2	7.22	9.86	13.27	11.94	48	0.065
Week 3	9.45	11.46	12.44	12.16	48	0.403
Week 4	10.81	14.10	11.87	15.46	48	0.812
Week 5	14.95	19.88	11.97	19.60	47	0.624
Week 6	18.25	20.96	8.90	15.62	47	0.121
Week 7	20.50	23.13	3.81	4.78	39	0.011*
Week 8	13.23	10.82	5.46	8.83	8	0.268

SD = standard deviation



**FIGURE 2.** Kinetic of  $\gamma$ -H2AX level in peripheral blood lymphocytes (A). Level of Peripheral Blood Lymphocytes during treatment (B). Mild = grade 1 or 2 mucositis; severe = grade 3. No grade 4 mucositis was observed.

Further analysis showed no statistical differences in  $\gamma$ -H2AX levels neither between the groups treated with concomitant treatment and radiotherapy alone nor between groups with and without common comorbidities like hypertension or diabetes, and habits like smoking. Furthermore, we did not notice correlation between  $\gamma$ -H2AX levels and survival, staging or tumor primary site.

## Discussion

The aim of this study was to test the usefulness of  $\gamma$ -H2AX as a clinically feasible predictor of severity of acute side effects caused by radiotherapy *i.e.*, mucositis. Such predictor would be of great value during the planning of treatment and supportive care and in selecting potential over-reactors.

$\gamma$ -H2AX is a marker of DNA damage and kinetics of repair. Thus, residual  $\gamma$ -H2AX can be valuable for evaluation of radiosensitivity and accumulation of unrepaired DNA damage. Due to difficulties in obtaining mucosa or skin cells during radiotherapy for *in vivo* studies, especially in the presence of acute mucositis, we used peripheral blood lymphocytes as a surrogate of normal cells, including skin and mucosa.<sup>8</sup>

To assess  $\gamma$ -H2AX levels, we employed flow cytometry. Although sensitivity of flow cytometry has been questioned by Beaton *et al.* in study attempting to identify radiation-sensitive patients with prostate cancer, the method is fast, accurate and easy to apply in daily clinical setting.<sup>9</sup>

The only significant difference in  $\gamma$ -H2AX levels between patients with mild and severe mucositis was found in the seventh week of radiotherapy. Our results are similar to findings of Vasireddy *et al.* and Li *et al.*, who suggested that the formation

of  $\gamma$ -H2AX foci measured *in vivo* was not significantly different between patients with severe and mild mucositis.<sup>4,10</sup> Li analyzed the formation of  $\gamma$ -H2AX, both *in vitro* and *in vivo*, and although the correlation was seen in *in vitro* settings, there was no similar correlation in *in vivo* study.

Other published reports analysing H2AX phosphorylation during acute phase of radiotherapy side effects are conflicting. Fleckenstein *et al.* did not find a direct correlation between mucositis and H2AX phosphorylation. However, according to Burton *et al.*, the residual  $\gamma$ -H2AX levels are higher in over reactors' PBLs *in vitro* at 24 hours after fraction or *in vivo*, after 3–6 hours.<sup>3,11,12</sup> Goutham *et al.* observed increasing residual DSB with increasing severity of the reaction but they did not find a clear correlation with grade of mucositis.

Many publications assess  $\gamma$ -H2AX formation and its 24-hours kinetics after one dose of irradiation, *in vivo* or *in vitro*. For example, Li *et al.* suggested that only a single fraction of irradiation as high as 8 Gy *in vitro* induces different levels of  $\gamma$ -H2AX between patients with severe and mild mucositis.<sup>4</sup> Patients included in our study were irradiated with 2 Gy fraction according to the standard protocol for head and neck radical treatment, to dose 66-70 Gy (33-35 fractions). Thus, conclusions from previous experiments with one fraction may not hold, especially at the end of fractionated treatment. Formation of  $\gamma$ -H2AX after multiple therapeutic fractions separated by more than 12 hours is still a subject of debate.<sup>14,15</sup>

We also did not find significant differences in baseline  $\gamma$ -H2AX levels between the individual patients before start of radiotherapy or chemoradiation. The mean baseline level of  $\gamma$ -H2AX was higher for patients with severe mucositis but observed difference was not statistically significant.

An interesting finding from our study is a rapid decrease in  $\gamma$ -H2AX levels after fourth week of therapy in group with severe mucositis in contrast to mild mucositis group, where an increase was observed. This phenomenon can be explained by ineffective DNA damage repair system (DDR) responsible for recognition and repair of DSB in most sensitive patients and changes in the conformation of chromatin after irradiation. Such changes can affect DSB signaling after the next fraction, resulting in lower phosphorylation of H2AX or lower detection of phosphorylated histones.<sup>15</sup> However, this phenomenon has been observed when the next fraction was applied 5–6 hours following the first. So far little is known about repair processes and  $\gamma$ -H2AX formation when the time between fractions is longer than 12 hours. Bouquet *et al.* showed that  $\gamma$ -H2AX levels reflect DSB repair activity only at doses of cytotoxic agent or irradiation producing less than 100-150 DSBs breaks per genome.<sup>16</sup>

Finally, we did not find  $\gamma$ -H2AX levels to be predictive for severe risk of mucositis before or early during treatment. Kinetics of  $\gamma$ -H2AX during treatment could potentially allow for such discrimination by evaluation of changes in subsequent  $\gamma$ -H2AX levels after 4 weeks of therapy. To our knowledge, this is the only study evaluating the kinetics of  $\gamma$ -H2AX during 7-week treatment.

In our study, we also considered selected, patient-related factors with potential impact on severity of mucositis like comorbidities, age and staging. Smoking was not included in the analysis as it was very challenging to obtain reliable information about smoking during treatment. We did not find any correlation between  $\gamma$ -H2AX levels and studied factors, as well as cisplatin use or radiotherapy setting. Similarly to the Werbrouck *et al.*, we did not observe any relationship between  $\gamma$ -H2AX levels during treatment and grade of late toxicity like skin fibrosis.<sup>17</sup>

The limitations of current *in vivo* studies are small sample sizes and heterogeneity of evaluated reactions, from acute mucositis to chronic reactions, like ulceration and fatigue. Moreover, different scales of evaluation of side effects are commonly used, making comparison between studies very difficult.<sup>3</sup> Standardization of evaluation and reporting of acute and late effects would be of a value not only for patients' benefit but also for the future studies and audits to prevent potential over-reaction due to errors in irradiation.<sup>13</sup>

In this study, we tested the PBL of relatively large group of patients during radiotherapy and chemoradiation. In line with other studies, we were

not able to prove that assessment  $\gamma$ -H2AX with flow cytometry is a method ready to be used clinically for identification of patients with high risk for mucositis before or early during treatment.<sup>9,11</sup> The probable reason for this is the multifactorial clinical and radiobiological background of acute mucositis, which depends among others on site of disease, total dose, dose per fraction, irradiated volume, comorbidities, concomitant chemoradiation, hydration, nutrition, and repair potential of normal tissue.<sup>5</sup> Furthermore, circulating PBLs receive variable doses of radiation and highly damaged cells might be eliminated from circulation, potentially biasing the results.

## Conclusions

Based on the results presented, it is not yet possible to predict the severity of mucositis induced by radiation using  $\gamma$ -H2AX. However, preliminary results indicating significant differences in kinetics of  $\gamma$ -H2AX levels between groups after fourth week of treatment encourage further studies.

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