ORIGINAL ARTICLES

Baseline γH2AX foci, 53BP1 values and late morbidity after definitive radio-chemotherapy in head and neck carcinoma patients

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Received: June 29, 2016 **Accepted:** September 22, 2016 **Online Published:** September 29, 2016
DOI: 10.5430/jst.v7n1p11 **URL:** http://dx.doi.org/10.5430/jst.v7n1p11

ABSTRACT

Purpose: Can double strand breaks in peripheral lymphocytes measured as baseline γH2AX and 53BP1 values be used as a signal for reduced DNA repair capacity and an increased late radiation morbidity?
Methods and materials: Of 357 patients, homogenously treated with a definitive radio-chemotherapy, 11 patients without any relevant comorbidity concerning baseline DNA double strand breaks, a follow up of more than 3.5 years and different levels of late morbidity were selected. γH2AX and 53BP1 foci were analyzed, using the fully automated AKLIDES® system (Medipan). For foci detection, images of different z-planes throughout the nucleus were obtained. For further evaluation mean focus number/cell as well as percentage of γH2AX/ 53BP1 focus-positive cells were used.
Results: Patients with late toxicity levels 0°, I° and III° showed median numbers of γH2AX foci per peripheral mononuclear cell of 0.26, 0.34 and 0.73 respectively. The corresponding median values of 53BP1 foci per cell were 0.13, 0.06 and 0.40, respectively and concordant percentages of γH2AX positive cells for the 3 different late toxicity groups were 10.85, 14.30 and 30.67 as well as of 53BP1 positive cells 8.5, 5.7 and 25.37, respectively. Despite the wide interindividual range of all values, patients with severe late morbidity showed increased values for γH2AX as well as 53BP1 foci compared to the other groups, whereas no differences between groups with no or mild toxicity were seen.
Conclusion: The determination of baseline γH2AX foci in peripheral blood mononuclear cells could be suited as a marker for late morbidity after definitive radio-chemotherapy of head and neck carcinoma. Blood sampling at the time of treatment *i.e.* moment of origin of the late effect should be more effective rather than a retrospective study. To address this question a prospective trial with a larger cohort of patients and a long term follow up is needed.

Key Words: Head and neck carcinoma, Late morbidity, γH2AX, 53BP1

1. INTRODUCTION

The repair capacity of radiation induced DNA double strand breaks (DSB) is one of the main factors for the wide spread of interindividual differences in radiosensitivity of various tumours as well as normal tissues.[1–3]

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An early response to DNA DSB in cells is the phosphorylation of the histone subunit H2AX in the vicinity of DNA damage sites into γH2AX and can be visualized as distinct foci by immunofluorescence microscopy.[4,5] γH2AX plays a central role in the cellular DNA damage response as unrepaired DNA breaks lead to cell cycle arrest or pause which allows time for DNA repair.[6] The formation of γH2AX foci is linked to the recruitment of the p53 binding protein 53BP1 to the DNA damage sites, regulating cellular DNA repair pathways. During repair of DSB, the level of foci declines over time.[5,7] The presence of persistent γH2AX foci indicates impaired DNA repair. The quantification of γH2AX foci has been suggested to be a sensitive biomarker for radiation induced DNA DSB in human cells[8] and has been described to be well suited for identifying patients with DNA repair deficiencies.[9] Residual γH2AX foci are linked to an incomplete repair of DNA DSB in tumour cells and can be used to predict an increased local tumour control after radiotherapy as well as lethal DNA damage in normal tissue cells.[7,8,10,11] The aim of this study was to determine, if residual DSB in peripheral lymphocytes measured as baseline γH2AX and 53BP1 values, could be used as a signal for reduced DNA repair capacity and to indicate late radiation morbidity. To answer this question we investigated a cohort of patients, showing different late toxicity rates after a homogenous treatment and a long term follow up.

2. MATERIAL AND METHODS

2.1 Patients

Three hundred and fifty-seven patients with a squamous cell carcinoma of head and neck have been definitive treated with a radio-chemotherapy without any prior resection in our department since 2001. The diagnostics, treatment as well as acute and late toxicity data were prospectively registered. Of 140 patients with a follow up of more than 3.5 years after radio-chemotherapy 11 patients without any relevant comorbidity concerning baseline DNA double strand breaks (Diabetics, Obesity, chronic inflammatory disease) with different levels of late morbidity were selected for this investigation. Patient characteristics are shown in Table 1.

2.2 Treatment

The definitive treatment of these patients consisted of an intensity modulated (IMRT) or volumetric modulated (VMAT, rapic arc®) radiotherapy with a single dose of 2 Gy, 6 times / week and a total dose of 72 Gy in 6 weeks for the macroscopic tumor and involved lymph nodes in a dose painting technique. The remaining potentially microscopic involved treatment volume got a single dose of 1.83 Gy and a total dose of 66 Gy in a simultaneous integrated boost technique. The simultaneous chemotherapy consisted of cisplatinum 75 mg/m^2, day 1 and 5-FU 800 mg/m^2, day 1-5 in a continuous infusion, week 1, 4 and 7. All patients underwent simultaneous examinations of the head and neck surgeon and the radio oncologist after therapy every 3 months.

Table 1. Patients' characteristics

Characteristics	Number
Male	11
Female	0
Oropharynx	1
Floor of the mouth	3
Larynx	3
Hypopharynx	4
T-stage	
T1	2
T2	5
T3	1
T4	3
N-stage	
N0	3
N1	2
N2a	0
N2b	3
N2c	3
N3	0

2.3 Blood sampling and γH2AX measuring

Peripheral blood cell isolation and slide preparation

Blood samples were obtained by venipuncture using BD Vacutainer CPT Cell Preparation Tubes. Tubes were centrifuged at 1,500 g for 20 minutes at room temperature. The PBMC fraction was washed and resuspended to a concentration of 2×10^6 cells/ml in phosphate buffered saline (PBS). 50 μl cell suspension was pipetted onto silanized glass slides and cells were allowed to settle for 10 minutes before 2% paraformaldehyde (PFA) fixative was added into the drop for another 15 minutes. Subsequently, slides were washed in PBS and stored in dry conditions at 4°C until further processing.

2.4 Immunocytochemistry

Slides were washed in PBS and permeabilized with 0.2% Triton X-100 for 5 minutes on ice. Subsequently, slides were incubated with PBS containing 1% bovine serum albumin (BSA).

For coimmunostaining anti-phosphohistone H2AX mouse monoclonal antibody (γH2AX, Millipore) and anti-53BP1 rabbit polyclonal antibody (Novus Biologicals) were diluted 1:200 in 1% BSA/PBS and slides were incubated for 1 hour

at room temperature. After a further washing step, 1:500 diluted Alexa Fluor 488 goat anti-mouse IgG (Invitrogen GmbH) and Alexa Fluor 647 goat anti-rabbit IgG (Invitrogen GmbH) were added for 1 hour at room temperature. After a final washing step with PBS, cells were covered with 4,6'-diamidino-2-phenylindole (DAPI) containing mounting medium.

2.5 Analysis of γH2AX and 53BP1 foci

γH2AX and 53BP1 foci were analyzed using the fully automated AKLIDES® system (Medipan GmbH) allowing an automated evaluation of fluorescence microscope images, as described previously.[12,13] For each well at least 100 cells were selected randomly for analysis. Cell aggregates, granulocytes, and the majority of monocytes as well as cells exhibiting a pan-stained nucleus and cells with a γH2AX signal comprising 70% of the DAPI signal were excluded from the analysis. For foci detection, images of different z-planes throughout the nucleus were obtained. Objects were classified as foci when focus size and intensity reached the defined threshold[14] as well as when sub-nuclear foci had a minimum diameter of 0.2 μm, a maximum diameter of 1.2 μm and a minimum intensity of 70 gray values on a 8 bit gray scale (see Figure 1). For further evaluation of the results the following parameters were used: mean focus number/cell as well as percentage of γH2AX/53BP1 focus-positive cells.

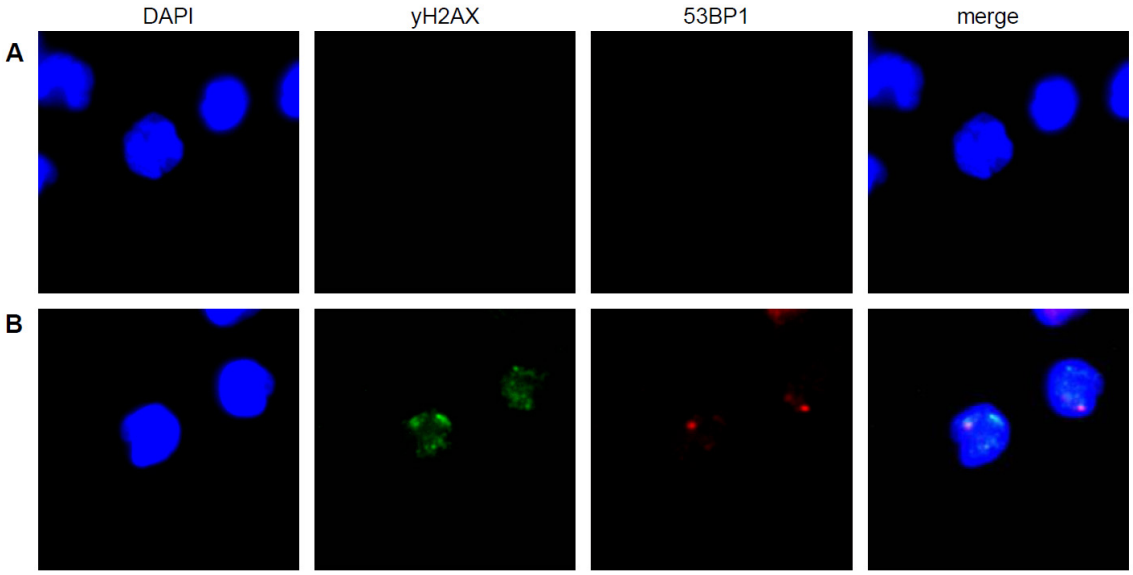

Figure 1. Images taken by the AKLIDES system after γH2AX (green) and 53BP1 (red) immunofluorescence staining as well as DAPI counterstaining (blue) of patient lymphocytes. Representative images are shown of A) a patient without and B) a patient with severe late morbidity.

Table 2. Median γH2AX and 53BP1 foci per cell, median number of γH2AX and 53BP1 cells and 53BP1/γH2AX rate according to late toxicity

	Late toxicity group	Foci per cell		Positive cells		γH2AX/53BP1
		γH2AX	53BP1	γH2AX	53BP1	ratio
Pat. 1	0°	2.03	1.03	58.98	55.85	50.76
Pat. 2	0°	0.14	0.03	9.77	3.17	24.30
Pat. 3	0°	0.08	0.11	1.52	5.72	141.06
Pat. 4	0°	0.37	0.14	11.92	11.37	36.33
Pat. 5	I°	0.13	0.02	9.56	1.89	14.23
Pat. 6	I°	0.34	0.11	18.57	10.21	31.24
Pat. 7	I°	0.09	0.05	6.79	3.20	52.63
Pat. 8	I°	0.66	0.33	19.85	19.84	49.39
Pat. 9	I°	0.38	0.06	14.30	5.70	15.02
Pat. 10	III°	1.10	0.64	41.46	36.72	57.66
Pat. 11	III°	0.35	0.15	19.88	14.01	42.66

2.6 Late morbidity

Fibrosis of the bilateral neck region and teleangiectasia was used for the determination of late toxicity. It was categorized according the common toxicity criteria and the Lent-Soma Scale.[12] 4 patients had no late side effects, 5 patients mild and 2 patients severe side effects.

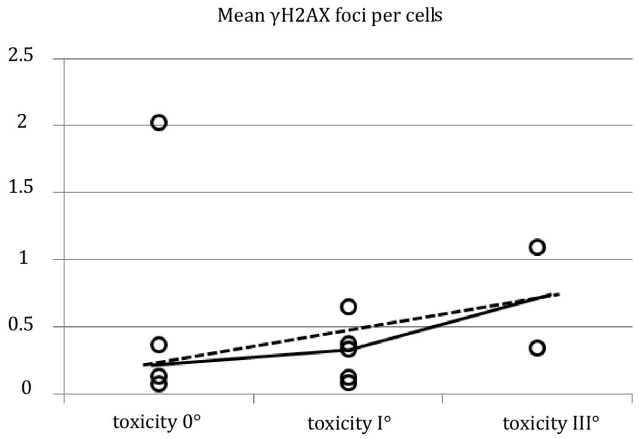

Figure 2. The mean of γH2AX foci per cell according to late toxicity of the individual patients

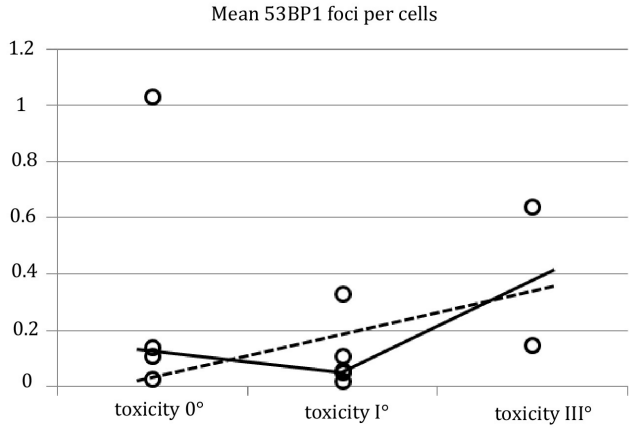

Figure 3. The mean of 53BP1 foci per cell according to late toxicity of the individual patients

3. Results

Patients with late toxicity of 0°, I° and III° showed the following median numbers of γH2AX foci per peripheral mononuclear cell: 0.26, 0.34 and 0.73 respectively. The corresponding median values of 53BP1 foci per cell were 0.13, 0.06 and 0.40, respectively. The concordant percentage of γH2AX positive cells for the 3 different late toxicity groups were 10.85, 14.30 and 30.67 as well as of 53BP1 positive cells 8.5, 5.7 and 25.37, respectively. The individual values per patient are shown in Table 2 and Figures 2-5. Despite the wide interindividual range of all values, patients with severe morbidity showed increased values for γH2AX as well as 53BP1 foci compared to the other groups. There was no difference in γH2AX foci between group 0° and I°. However, we could detect a slight decrease in 53BP1 foci in group I° compared to group 0°.

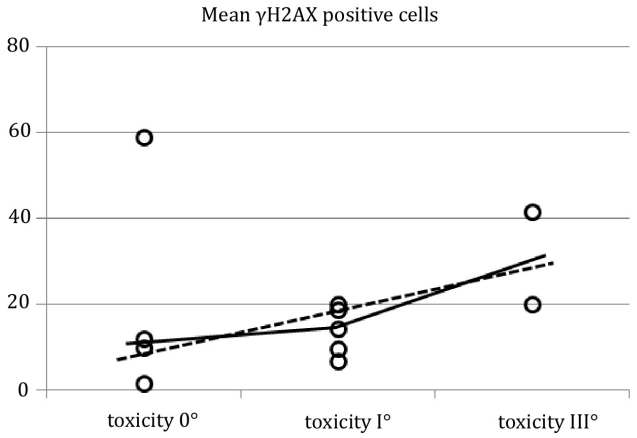

Figure 4. The number of γH2AX of positive cells according to late toxicity

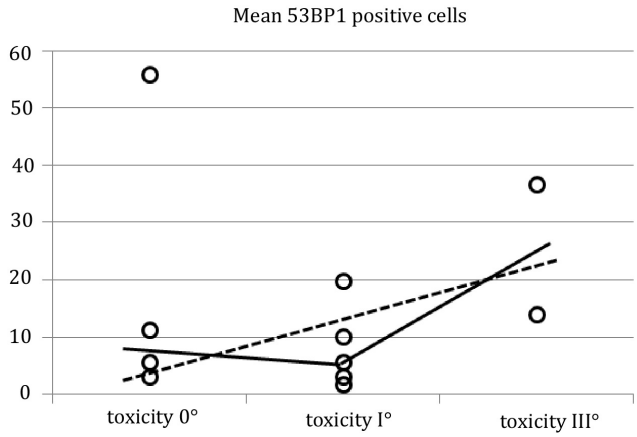

Figure 5. The number of 53BP1 of positive cells according to late toxicity

4. Discussion

As shown in Table 2 there is a wide range of individual differences concerning γH2AX and 53BP1 foci numbers in the homogenous toxicity groups. However, the median numbers for the different toxicity groups showed differences between the groups despite the low number of patients. As such, we found an increased mean number of γH2AX and 53BP1 foci in the two patients with severe late morbidity compared to the two other groups. Goutham *et al.* published comparable

results of increased values in overreactors with acute toxicity.[13] The determination of γH2AX foci in our analysis was a couple of years after irradiation, whereas the time of determination of γH2AX foci in acute toxicity was during the same time as irradiation.

Our results could be influenced by some additional effects: Despite the homogenous treatment schedule the dose distribution could be vary between patients according to the of lymph nodes, the proportion of fat tissue in the neck region and the distance of the gross tumour volume to the skin surface leading to different high dose volumes as well as skin doses of the neck. Consequential late effects, *e.g.* the protracted wound healing of severe acute reactions, depending not only on DNA repair mechanisms as well as mechanical impairment of the skin could be influence late skin morbidity, The individual radiosensitivity can change over time with patient's age and environmental conditions.[15]

Despite these uncertainties, we found an increased mean number of γH2AX and 53BP1 foci in the two patients with severe late morbidity compared to the two groups with no or mild late toxicity.

5. Conclusions

The determination of baseline γH2AX foci in peripheral blood mononuclear cells could be suited as a marker for late morbidity after definitive radio-chemotherapy of head and neck carcinoma. Blood sampling at the time of treatment *i.e.* moment of origin of the late effect should be more effective rather than a retrospective study. To address this question a prospective study with a larger cohort of patients and a long term follow up is needed.

Conflicts of Interest Disclosure

The authors declare that there is no conflict of interest statement.

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