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Comparative study of phosphorylated histone H2AX expressions in the cervical cancer patients of pre- and post-neoadjuvant chemotherapy

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Summary

Objective: This study aimed to determine whether phosphorylation of histone H2AX (γH2AX) is a predictive marker for neoadjuvant chemotherapy patients of cervical cancer. *Materials and Methods:* The sections were divided into three sets. Set 1 consisted of 40 pre-treatment biopsies. Post-treatment tissues includes 38 patients in set 2 and 34 patients in set 3 who received cisplatin concurrent docetaxel treatment for one or two cycles, respectively. Formalin-fixed paraffin-embedded sections were analyzed after antigen retrieval and fluorescence antibody labeling for γH2AX staining. *Results:* The expressions of γH2AX in cervical cancer tissues of post-neoadjuvant chemotherapy decreased to 22.94 ± 14.02% and 23.68 ± 13.55% (one and two cycles treatment, respectively) compared to pre-neoadjuvant chemotherapy (28.29 ± 15.67%), however there was no significant difference for γH2AX expression between pre- and post-neoadjuvant chemotherapy patients (F=1.425, $p = 0.245$). There was no significant correlation between γH2AX expression and clinicopathologic parameters in patients of pre- and post-neoadjuvant chemotherapy. *Conclusions:* As a predictive marker for neoadjuvant chemotherapy of cervical cancer, more extensive research regarding γH2AX expression should be explored.

Key words: γH2AX; Cervical cancer; Neoadjuvant chemotherapy.

Introduction

DNA double-strand breaks (DSB) can arise from mistakes during DNA replication, from external agents, such as ionizing radiation or during genomic rearrangements. DSB can induce chromosomal aberrations that cause cells to malfunction, resulting in cell death or tumorigenesis [1, 2]. One of the earliest steps in the cellular response to DSB is the phosphorylation of histone H2AX at serine 139 (γH2AX), the site of γ-phosphorylation [3]. The number of γH2AX foci is a significant marker for DSB.

Invasive squamous cell carcinoma (ISCC) of the uterine cervix is one of the most frequent malignancies in women worldwide. Concurrent use of cisplatin with radiotherapy has become the standard of care in the treatment of patients with advanced cancer of the cervix [4, 5]. Prediction of response to treatment therefore requires a method that is sensitive to tumor response to both agents. Several papers have discussed the potential for γH2AX to serve as a predictive marker for cancer treatment [6-10].

Immunohistochemical analyses of γH2AX have been reported for human cancers of the urinary bladder, breast, lung, colon, and prostate [11-13]. It was also reported that γH2AX-positive cells are overexpressed in ISCC of uterine cervix and the cervical cancer of radiochemotherapy [14-16]. These results suggest that staining of γH2AX correlates with DNA damage checkpoint activation in malignant lesions and the process of tumor therapy. Therefore, the existence of γH2AX foci might be a useful and sensitive marker for cancer, especially for detecting the results of cancers therapy.

In ordered to test if the expression of γH2AX in ISCC of uterine cervix could be a more accurate indicator for neoadjuvant chemotherapy, the authors analyzed γH2AX expression in cervical cancer tissues of pre- and post-neoadjuvant chemotherapy followed by surgery. In addition to their primary goal: to assess for a relation between γH2AX expression and clinicopathologic characteristics, as another more important goal the expression of γH2AX in cervical cancer tissues of pre- and post- neoadjuvant chemotherapy was also analyzed for it's impact on response to chemotherapy.

Materials and Methods

Patients and tumor specimens

Formalin-fixed, paraffin-embedded sections were stained for γH2AX foci and were analyzed using visual scoring methods. Tumor incisional biopsies and surgical specimens, four-μm thick, were prepared from three patients sets. The first set of slides was

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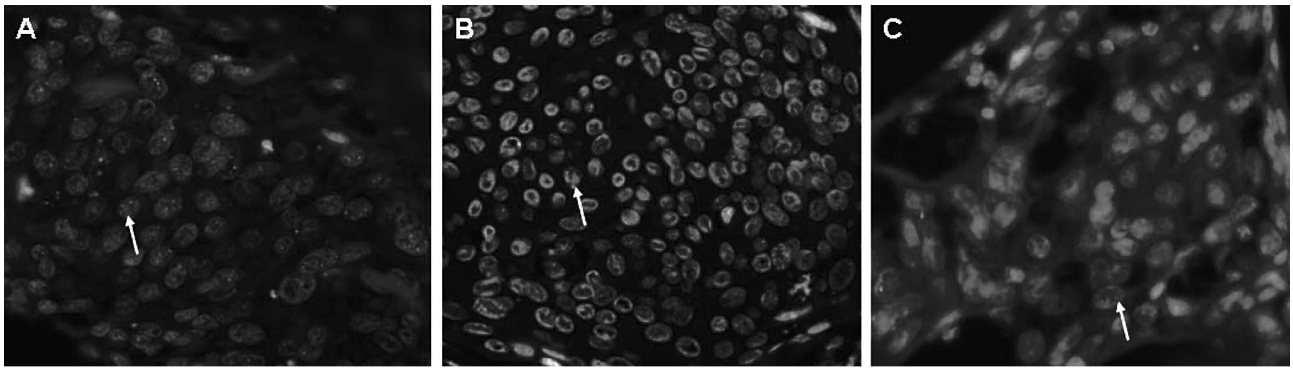

Figure 1. — Immunohistochemical analysis of γH2AX expression in cervical cancer tissues. (A) pre-neoadjuvant chemotherapy; (B) post-neoadjuvant chemotherapy (one cycle); (C) post-neoadjuvant chemotherapy (two cycles). Granular staining of γH2AX is found in the nucleus (magnification, ×400).

prepared from pre-chemotherapy incisional biopsies from 40 patients with cervical cancer. The second and third sets of slides were taken from 38 and 34 surgical specimens of cervical cancer patients who received the neoadjuvant chemotherapy. These patients received treatment of cisplatin (40 mg/m^2) concurrent docetaxel (75 mg/m^2) for one (set 1) or two cycles (set 2). Tumors ranged from FIGO Stage IB-IIIA, and all the tumors were squamous cell carcinomas (ISCC). The mean ages of the patients pre- and post- neoadjuvant chemotherapy were 48.87 and 48.27 years, respectively (range: from 21 to 78 and 24 to 66) for ISCC. All the slides were collected and prepared by the Pathology Department at the GanSu Tumor Hospital between 2012-2013, and the sections were kept at 4°C before dewaxing and staining.

Immunohistochemistry analysis of γH2AX

From each patient, one representative tumor block, including the tumor center and invasive front was examined by immunohistochemistry. In cases of large, late-stage tumors, different sections were examined to include representative areas of the tumor center, as well as of the lateral and deep tumor invasive fronts.

The same paraffin-embedded tissues as those used for the original hematoxylin and eosin-stained sections were chosen for immunohistochemistry. Paraffin-embedded tumor tissue was prepared, and after dewaxing and rehydrating in graded alcohols, slides were immersed in high pH target retrieval solution in a 95°C water bath for 30 minutes. Slides were washed in TBS, blocked for ten minutes, and incubated with mouse anti-γH2AX antibody (1:500 dilution) for two hours. Slides were rinsed and incubated for one hour with Alexa Fluor 594-conjugated AffiniPure goat anti-mouse IgG antibody (1:200 dilution). To stain nuclei, slides were submersed in 0.05 μg/mL DAPI for five minutes, rinsed, and finally mounted with ten μL Fluorogard. Tumor sections were viewed using a fluorescent microscope, using ×10, ×40, and ×100 Plan Neofluor objectives. As this was considered a feasibility study, no specific procedures were adopted to ensure that images were obtained randomly throughout the section. Because sections rather than whole cells were scored, cells with one or more γH2AX foci were counted as positive. Efforts were made to score only tumor regions, and obvious regions of necrosis or areas of stroma were not included in the analysis. Foci results are presented as averages of scores for several high-power images. Digitized images (eight to 12 images containing 50-100 nuclei each) were scored by eye for the percentage of nuclei presenting γH2AX foci, and the results are presented as averages [14, 15, 17].

Statistical methods

Data are presented as means ± SD. The levels of γH2AX expression in cervical cancer tissues of pre- and post-neoadjuvant chemotherapy were analyzed by One-way ANOVA. Association between γH2AX expression levels and clinicopathologic parameter (ages, lymph node metastasis or TNM stage) were evaluated using t-test. Association between γH2AX expression level and tumor grade was evaluated by One-way ANOVA too. SPSS version 17.0 software was used for the statistical analysis. A p-value < 0.05 was considered statistically significant.

Results

High power images of the biopsies were analyzed visually for fraction of nuclei that were γH2AX positive, defined as one or more foci per nucleus. γH2AX yielded granular, nuclear staining. The nuclei were frequently immunoreactive without any pattern, even pyknotic nuclei of the cells associated with horn pearls displayed immunostaining. The reactivity was frequently detected in mitotic and apoptotic cells or karyorrhectic debris, respectively. Normal glandular epithelia remained unstained. In addition, nuclei of immature squamous metaplasia were decorated by the antibody used, as were lymphocytes. Normal glandular endocervical epithelia showed occasional nuclear staining. Cells with pannuclear γH2AX staining, consisting of hundreds of individual foci, were observed in these tumor sections. The classic imagination of γH2AX expression in biopsies of pre and post- chemotherapy are shown in Figure 1.

For the tumor biopsies of pre- neoadjuvant chemotherapy, the γH2AX positive cells ranged from 5% to 64%, and on average, only 28.29% of cells scored as γH2AX positive. The tissues taken from the patients after first cycle neoadjuvant chemotherapy showed a significant decrease in the percentage of nuclei with γH2AX foci on average 22.94% nuclei (range: 5–71%). A similar decrease was shown in tissues taken from the patients after second cycle neoadjuvant chemotherapy. There was 23.68% of the nuclei that scored as γH2AX positive with a range of 5% to 52%. However, there were no remarkable differences in γH2AX

Table 1. — *Immunohistochemical analysis of γH2AX expression in cervical cancer patients of pre- and post-neoadjuvant chemotherapy.*

	n	$\bar{x}$ ± SD	F	*p*
Pre-neoadjuvant chemotherapy	40	28.29±15.67	1.425	0.245
Post-neoadjuvant chemotherapy (one cycle)	38	22.94±14.02		
Post-neoadjuvant chemotherapy (two cycle)	34	23.68±13.55		

Table 2. — *Relations between γH2AX expression and clinicopathologic characteristics in cervical cancer patients of pre-neoadjuvant chemotherapy.*

Characteristics	γ-H2AX expression n	$\bar{x}$ ± SD	t/F	*p*
Age, years				
<50	21	26.76±13.77	t = -0.541	0.592
≥50	18	29.53±18.17		
Stages				
I	8	32.35±21.88	t = -0.816	0.419
II-III	32	27.27±13.98		
Tumor Grade				
Low	16	27.92±12.45		
Intermediate	14	28.69±16.62	F = 0.009	0.991
High	10	28.31±20.15		
Lymph node metastasis				
No	10	28.27±15.97	t = -0.015	0.988
Yes	30	28.34±15.56		

Table 3. — *Relations between γH2AX expression and clinicopathologic characteristics in cervical cancer patients of post-neoadjuvant chemotherapy.*

Characteristics	γ-H2AX expression n	$\bar{x}$ ± SD	t/F	*p*
Age, years				
<50	37	23.15±11.79	t = -0.297	0.768
≥50	35	24.11±15.66		
Stages				
I	8	20.36±12.69	t = -0.709	0.481
II-III	64	24.02±13.88		
Tumor Grade				
Low	27	21.63±10.79		
Intermediate	23	23.61±16,54	F = 0.171	0.843
High	17	23.67±13.90		
unknown	5			
Lymph node metastasis				
No	17	23.54±13.21	t = -0.080	0.937
Yes	55	23.85±15.68		

expression between pre- and post-neoadjuvant chemotherapy groups by using One-way ANOVA (F=1.425, p = 0.245, Table1).

The authors then analyzed the association between γH2AX staining and clinicopathologic parameters in cervical cancer tissues of pre- and post- neoadjuvant chemotherapy. γH2AX staining showed no significant association with ages, tumor grade, lymph node metastasis or TNM stage in the cervical cancer tissues of pre- and post-neoadjuvant chemotherapy groups (Tables 2, 3).

Discussion

Impaired radiochemotherapy responsiveness of tumor is a major clinical problem in several solid tumor types including cervical carcinoma. Activation of DNA damage repair networks is central in the molecular responses to radiochemotherapy, and within these networks the γH2AX is the significant marker of DNA damage and repair [18]. In order to detect the potential of γH2AX as a predictive marker for neoadjuvant chemotherapy patients, the expression of γH2AX in cervical cancer tissue of pre- and post- neoadjuvant chemotherapy were researched in the present study.

When the expression of γH2AX was investigated in cervical cancer tissues of pre- and post neoadjuvant chemotherapy, three different categories of immunoexpression was confirmed by statistical evaluation. The results of γH2AX expression in uterine cervix of pretreatment was 28.29%, similar to those previously using 47 pre-treatment cervical cancer biopsies [16]. What the present authors are more concerned with is the information provided by γH2AX expression on cervical cancer response to chemotherapy and the potential for γH2AX to serve as a predictive marker of cervical cancer treatment. The expression of γH2AX in cervical carcinoma pre- and post radiochemotherapy were analyzed [15,16]. In one research, eight patients received weekly cisplatin (40 mg/m2) to a maximum of five cycles and concurrent external beam pelvic radiation of 45 Gy in 25 fractions over five weeks. Biopsies were obtained before the day's radiation treatment. On average, only 25% of tumor nuclei exhibited one or more γH2AX foci before treatment and 74% after the start of treatment. In another research, 26 tumor biopsies taken before and 24 hours after the first fraction provided the opportunity to examine the retention of γH2AX foci in relation to local control and fraction size. Before treatment, 24% of cells contained γH2AX foci, 24 hours after exposure to the first fraction of 1.8–2.5 Gy, 38% contained foci. These results are in marked contrast to the present, in which cervical cancer of post-neoadjuvant chemotherapy showed a downregulation of γH2AX expression and may be explained by several factors. Thus, in the study of Bañuelos *et al.* [16] the biopsies were taken before the day's radiation, however the exact time point, such as the cycle number of chemotherapy before the biopsies were taken, was unclear. Moreover, Olive *et al.* [15] analyzed γH2AX expression at 24-hour post-radiation, that is, at ongoing radiation-induced cell cycle perturbation, while the present analysis was per-

formed at 21-31 days after first or second cycles of neoadjuvant chemotherapy. One potential mechanism that could explain the observed decrease in γH2AX expression is that tumor cells with high γH2AX expression or with capacity to induce γH2AX expression will be arrested either at G1 or at G2/M, whereas tumor cells with low γH2AX expression will progress through the cell cycle after treatment and will hereby constitute a larger proportion of the residual tumor. Another potential mechanism is that apoptosis occurred for many γH2AX staining cells because 56% of cells with pan-nuclear γH2AX staining were also positive for the apoptosis marker, activated caspase-3 [16]. In fact except γH2AX, the other downstream signaling components of DSB, such as p53, p21, and mdm-2 have been researched with the aim of predicting radiochemo-therapy response in cervical cancer [19, 20]. The results reported by Beskow *et al.* [19, 20] are partly in accordance with the present. In their research a decreased expression of p21 was shown in cervical cancer biopsies of radiotherapy, and suggesting downregulation of p21 associated with radioresistant. The expression of γH2AX is decreased in cervical cancer tissues of post neoadjuvant chemotherapy, however, there were no remarkable differences in γH2AX expression between pre- and post- neoadjuvant chemotherapy groups. Perhaps, much more samples or corresponding tumors of pre- and post- neoadjuvant chemotherapy should be used to analyzed γH2AX expression in the future research.

According to Brustmann *et al.*'s research, no statistical significance could be established for FIGO Stages I *vs* II in ISSC, because their study was limited to the low FIGO stages only [14]. Although the present authors chose 40 pre- and totally 72 post-neoadjuvant chemotherapy specimens with FIGO stages ranging from IB-IIIA, γH2AX staining showed no significant association with ages, tumor grade, lymph node metastasis or TNM stage in the uterine cervix

The present authors report that the expression of γH2AX showed no significant association with ages, tumor grade, lymph node metastasis or TNM stage in the uterine cervix. They also observed a decreased expression of γH2AX in cervical cancer tissues of post-neoadjuvant chemotherapy, although there were no remarkable differences. The present results suggest that one mechanism of chemotherapy resistance in cervical cancer perhaps can include down-regulation of γH2AX expression. However, the findings reported in this study need to be confirmed in larger materials.

Conclusion

The expression of γH2AX is not different between pre- and post-neoadjuvant chemotherapy patients. As a predictive marker for neoadjuvant chemotherapy of cervical cancer, more extensive research regarding γH2AX expression should be explored.

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