

# $\gamma$ -H2AX expression detected by immunohistochemistry correlates with prognosis in early operable non-small cell lung cancer

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**Background:** Phosphorylation of the H2AX histone is an early indicator of DNA double-strand breaks and of the resulting DNA damage response. In the present study, we assessed the expression and prognostic significance of  $\gamma$ -H2AX in a cohort of 96 patients with operable non-small cell lung carcinoma.

**Methods:** Ninety-six paraffin-embedded specimens of non-small cell lung cancer patients were examined. All patients underwent radical thoracic surgery of primary tumor (lobectomy or pneumonectomy) and regional lymph node dissection.  $\gamma$ -H2AX expression was assessed by standard immunohistochemistry. Follow-up was available for all patients; mean duration of follow-up was 27.50  $\pm$  14.07 months (range 0.2–57 months, median 24 months).

**Results:** Sixty-three patients (65.2%) died during the follow-up period. The mean survival time was 32.2  $\pm$  1.9 months (95% confidence interval [CI]: 28.5–35.8 months; median 30.0 months); 1-, 2- and 3-year survival rates were 86.5%  $\pm$  3.5%, 57.3%  $\pm$  5.1%, and 37.1%  $\pm$  5.4%, respectively. Low  $\gamma$ -H2AX expression was associated with a significantly better survival as compared with those having high  $\gamma$ -H2AX expression (35.3 months for low  $\gamma$ -H2AX expression versus 23.2 months for high  $\gamma$ -H2AX expression,  $P = 0.009$ ; hazard ratio [HR] 1.95, 95% CI: 1.15–3.30). Further investigation with multivariate Cox proportional hazards regression analysis revealed that high expression of  $\gamma$ -H2AX remained an independent prognostic factor of shorter overall survival (HR 2.15, 95% CI: 1.22–3.79,  $P = 0.026$ ). A combined p53/ $\gamma$ -H2AX analysis was performed, and we found that the p53 low/ $\gamma$ -H2AX low phenotype was associated with significantly better survival compared with all other phenotypes.

**Conclusion:** Our study is the first to demonstrate that expression of  $\gamma$ -H2AX detected by immunohistochemistry may represent an independent prognostic indicator of overall survival in patients with non-small cell lung cancer. Further studies are needed to confirm our results.

**Keywords:** H2AX, DNA damage response, non-small cell lung cancer, p53, prognosis

## Introduction

Lung cancer is the most common cause of cancer mortality worldwide for both men and women, causing approximately 1.2 million deaths per year.<sup>1</sup> In the United States, there will be an estimated 221,000 new cases of lung cancer and 157,000 deaths in 2011.<sup>2</sup> The traditional evaluation of prognosis in non-small cell lung carcinoma (NSCLC) has relied, as in most other malignant tumors, on the stage of disease at the time of clinical presentation. Other factors currently commonly considered include performance status, weight loss, and presence or absence of symptoms at diagnosis, as well as time-honored pathologic parameters, eg, tumor size, tumor differentiation, and histologic subtype.<sup>3</sup> However, advances in molecular biology have provided important insights into other potentially significant prognostic biomarkers during the last decade.<sup>4</sup>

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Phosphorylation of the H2AX histone is an early indicator of DNA double-strand breaks (DSB) and of the resulting response (DNA damage response [DDR]).<sup>5</sup> H2AX is a histone H2A variant that constitutes 2%–25% of mammalian histone H2A depending on the organism and cell type.<sup>5</sup> As a result of DNA DSBs in eukaryotic cells, the serine amino acid at position 139 of the H2AX proteins is phosphorylated in response to DNA damage.<sup>5,6</sup> Moreover, the occurrence of a DSB is generally accompanied by the formation of hundreds of histone  $\gamma$ -H2AX (H2AXS139PO4 in humans) molecules in the chromatin flanking the DSB site.<sup>7</sup> Both these events can be detected by means of fluorescent-tagged specific antibodies, thus revealing the number of DSB foci within a nucleus and providing valuable information of the DNA damage-response status of the cell.<sup>8,9</sup> Detection of  $\gamma$ -H2AX foci has been used as a biomarker for aging and cancer, as a biosimeter for drug development and radiation exposure, and for clinical trials for cancer chemo- and radiotherapy.<sup>6,10</sup> Furthermore, emerging uses for  $\gamma$ -H2AX include detection of toxic environmental agents and of chronic inflammation.<sup>11</sup>

The aim of the present study was to assess the expression of  $\gamma$ -H2AX in a cohort of 96 consecutive patients with NSCLC and correlate its expression with survival and other clinicopathological parameters. This is, to the best of our knowledge, the first study correlating  $\gamma$ -H2AX expression with survival in patients with NSCLC.

## Materials and methods

### Study subjects

Paraffin-embedded specimens from 96 consecutive patients with NSCLC were examined. All patients underwent radical thoracic surgery of primary tumor (lobectomy or pneumonectomy), together with regional lymph-node dissection between January 2002 and December 2005 at the Cardiac Surgery Department of Evaggelismos Hospital. Histology reports were issued according to World Health Organization criteria.<sup>12</sup> Staging was performed according to the seventh edition of *TNM in Lung Cancer*.<sup>13</sup> Institutional review board approval was obtained to use archived material for research purposes. Patient survival was calculated from the day of surgery until death, in months.

### Tissue microarrays

Representative areas from each tumor were chosen in hematoxylin and eosin-stained sections; these areas were subsequently punched out from donor paraffin blocks, in order to construct tissue microarrays (TMAs), using a manual tissue arrayer from Chemicon

International Inc (Temecula, CA) (model ATA-100). One to five 2 mm-wide tissue cores from each tumor were chosen. All cases were distributed over eight TMA recipient paraffin blocks, which were incubated at 56°C for 5 minutes, in order for recipient and donor paraffin to stick together.

### Immunohistochemistry

Immunostaining of TMAs was performed on 3  $\mu$ m-thick sections, deparaffinized in xylene and hydrated in a series of graded alcohol dilutions. Slides were boiled in a microwave at 650 W for 20 minutes, immersed in a high-pH target-retrieval solution, (K8004; Dako, Glostrup, Denmark) and subsequently cooled at room temperature for 20 minutes. Endogenous peroxidase activity was blocked by means of the peroxidase-blocking reagent S2001 (K5007; Dako). Immunohistochemistry was performed using the Dako Autostainer Plus device. Sections were incubated with primary antibodies against Ki67 (mouse monoclonal, clone MIB-1; DAKO, 1:100 dilution), cleaved caspase-3 (Asp175) (rabbit monoclonal, clone 5A1 [cell signaling], 1:100 dilution), p53 (mouse monoclonal, clone DO-7; Dako, 1:50 dilution) and phosphohistone H2AX (Ser139) ( $\gamma$ -H2AX) (rabbit monoclonal, clone 20E3 [cell signaling], 1:100 dilution). Slides were then incubated with Envision/HRP for 30 minutes, following which the antigen–antibody complex was visualized using DAB as chromogen for 10 minutes. All sections were lightly counterstained with hematoxylin prior to mounting. All series included both positive (ie, tissues known to express the relevant antigen) and negative (ie, duplicate sections processed as above, apart from omitting incubation with the primary antibody solution) controls.

Immunostained slides were evaluated by estimating the percentage of positive cells per TMA punch. If more than one punch was used from a case, the average percentage was calculated.

For the evaluation of immunoreactivity in tumor cells, a dichotomised scoring system was used as follows: high p53 mutant-protein expression if >20% of tumor cells demonstrated nuclear staining,<sup>14</sup> high Ki-67 labeling index if >30% tumor cells were stained,<sup>15</sup> high caspase-3 expression if nuclear or cytoplasmic staining was present in >3.22% of tumor cells according to the 75th percentile; high  $\gamma$ -H2AX expression if >21.85% of tumor cells demonstrated nuclear staining according to the 75th percentile.

### Statistical analysis

Statistical analysis of the data was performed using SPSS version 19.0 (IBM, Armonk, NY). The Chi-square test was used to evaluate any potential association between the

molecular expression and the clinicopathological parameters; odds ratios (ORs) and their 95% confidence intervals (CIs) were calculated by means of simple logistic regression analysis as the measure of the above associations. Multivariate stepwise logistic regression analysis was performed to explore the independent effect of the clinicopathological parameters on the molecular expression. Patients' overall survival (OS) was measured in months from the day of surgery until death. Survival rates were calculated with the Kaplan–Meier method, and the statistical difference between survival curves was determined with the log-rank test. Multivariate Cox proportional hazards regression analysis, using a backward-selection approach, was performed to explore the independent effect of variables on survival. All tests were two-tailed, and statistical significance was considered to be  $P < 0.05$ .

## Results

The study included tumors from 96 patients (77 males and 19 females), aged between 36 and 80 years (median age, 66 years). In this cohort, 81 (84.4%) patients were smokers, and 17 (17.7%) patients had  $FEV_1 < 70\%$ . Regarding histology, 42 (43.8%) tumors were adenocarcinomas and 42 (43.8%) were squamous cell carcinomas, while eight (8%) were large-cell carcinomas and four (4%) were undifferentiated carcinomas. Clinicopathological data are summarized in Table 1.

High expression of all antigens assessed was found as follows:  $\gamma$ -H2AX in 25 (26.0%) cases, p53 in 55 (57.3%) cases, caspase-3 in 24 (25.0%) cases, and Ki67 in 29 (30.2%) cases.

## Correlation between immunohistochemical profile of tumors and clinical data

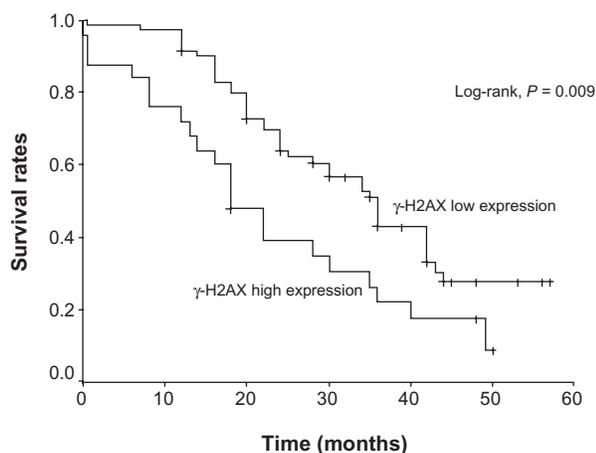
High levels of  $\gamma$ -H2AX were significantly correlated with T3 tumors (44.4% vs 21.8%,  $P = 0.048$ ; OR 2.87, 95% CI: 1.00–8.40), disease stage III/IV (42.4% vs 17.5%,  $P = 0.008$ ; OR 3.48, 95% CI: 1.35–8.99), and lymphatic infiltration (42.9% vs 21.3%,  $P = 0.047$ ; OR 2.77, 95% CI: 1.00–7.71). No significant association was found between  $\gamma$ -H2AX expression patterns and caspase 3 ( $P = 0.081$ ), p53 ( $P = 0.208$ ), N stage ( $P = 0.075$ ), and Ki67 percentage ( $P = 0.081$ ).

Follow-up was available for all patients; mean duration of follow-up was  $27.50 \pm 14.07$  months (range 0.2–57 months, median 24 months). Sixty-three patients (65.2%) died during follow-up. The mean survival time was  $32.2 \pm 1.9$  months

**Table 1** Clinicopathologic characteristics of patients included in the study

Variables	n	%
Total number of patients	96	
Age (years)		
Mean $\pm$ standard deviation	65.64 $\pm$ 7.23	
<65	36	37.5
$\geq 65$	60	62.5
Sex		
Male	77	80.2
Female	19	19.8
Histology		
Adenocarcinoma	42	43.8
Squamous cell carcinoma	42	43.8
Large-cell carcinoma	8	8.3
Undifferentiated carcinoma	4	4.1
Grade		
Low	25	26
Medium/high	71	74
T status		
T1	13	13.5
T2	65	67.7
T3	18	18.8
Regional lymph-node status		
N0	50	52.1
N1	27	28.1
N2	19	19.8
Stage		
I	41	42.7
II	22	22.9
III	31	32.3
IV	2	2.1
Ploidy		
Diploidy	78	81.3
Aneuploidy	18	18.7
Smoking history		
Yes	81	84.4
No	15	15.6
Adjuvant treatment		
Chemotherapy-adjuvant	72	75.0
Radiotherapy-adjuvant	41	42.7
Blood-vessel infiltration	41	42.7
Lymphatic infiltration	21	21.9
High expression of p53	55	57.3
High expression of caspase-3	24	25.0
High expression of $\gamma$ -H2AX	25	26.0
High Ki67 labeling index	29	30.2

(95% CI: 28.5–35.8 months, median 30.0 months); 1-, 2-, 3-, and 4-year survival rates were  $86.5\% \pm 3.5\%$ ,  $57.3\% \pm 5.1\%$ ,  $37.1\% \pm 5.4\%$ , and  $25.4\% \pm 5.2\%$ , respectively. A significant association between high  $\gamma$ -H2AX levels and a shorter survival was detected (23.2 months vs 35.3 months,  $P = 0.009$ ; hazard ratio [HR] 1.95, 95% CI: 1.15–3.30) (Figure 1). Other factors significantly associated with overall survival are listed in Table 2.



**Figure 1** Overall survival of patients with non-small cell lung carcinoma in relation to  $\gamma$ -H2AX expression.

Further investigation with multivariate Cox proportional hazards regression analysis revealed that squamous cell carcinomas (HR 1.95, 95% CI: 1.22–3.79;  $P = 0.016$ ), N2 (HR 3.80, 95% CI: 2.01–7.17;  $P < 0.001$ ), low-grade tumors (HR 2.10, 95% CI: 1.21–3.65,  $P = 0.009$ ), high expression of p53 (HR 1.74, 95% CI: 1.01–3.00,  $P = 0.047$ ) and high  $\gamma$ -H2AX expression (HR 2.15, 95% CI: 1.22–3.79;  $P = 0.026$ ) remained independent prognostic factors of a shorter OS.

### Combined p53 and $\gamma$ -H2AX analysis

Tumors were divided into three groups according to expression of p53 and  $\gamma$ -H2AX. Group A ( $n = 33$ ) tumors had both p53 and  $\gamma$ -H2AX low expression levels; group B ( $n = 46$ ) tumors had either p53 high/ $\gamma$ -H2AX low or p53 low/ $\gamma$ -H2AX high expression levels; group C ( $n = 17$ ) tumors had both p53 and  $\gamma$ -H2AX high expression levels. A significant association between different p53/ $\gamma$ -H2AX patterns with survival was observed with the p53 low/ $\gamma$ -H2AX low pattern having a statistically better outcome compared with all the other combinations ( $P = 0.001$ ) (Figure 2A and B).

### Discussion

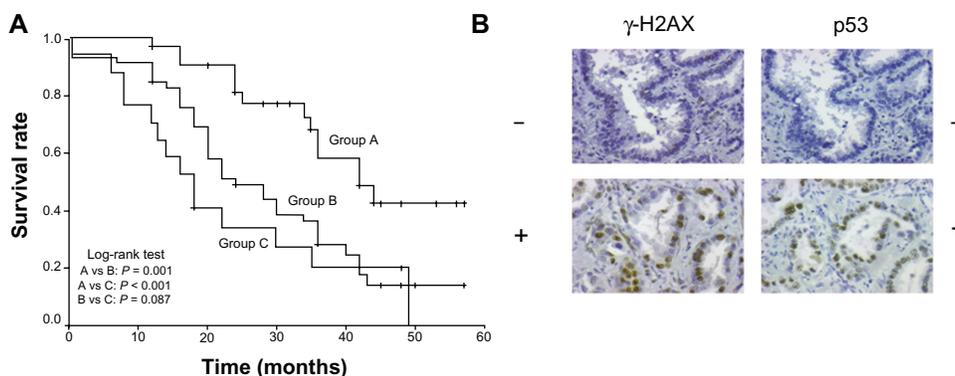
Before genomic instability and malignant conversion, human cells activate a DDR network in correlation with DNA damage. In later stages of DDR, the  $\gamma$ -H2AX foci become larger, and presumably a higher concentration of repair proteins can be measured. The mechanism that explains our results regarding H2AX and p53 should be interpreted under the light of the following mechanism that connects the DDR with p53: the response to DNA damage results in either cell-cycle arrest to allow the lesions to be repaired or apoptosis; p53 is essential in both pathways. Specifically, in cell-cycle arrest at the  $G_1$  phase, p53 enhances p21 transcription, which in turn inhibits cyclin-dependent kinase activity. This prevents

**Table 2** Survival analysis according to clinicopathologic and histologic parameters

Variables	Mean survival time (95% CI), months	Survival rate (%)		P-value
		2-year	3-year	
Age				0.377
<65 years	30.2 (24.4–36.0)	50.0	33.9	
$\geq 65$ years	33.3 (28.6–37.9)	62.1	39.1	
Sex				0.019
Male	30.0 (26.0–33.9)	52.0	30.0	
Female	41.5 (33.7–49.3)	79.0	65.1	
Histology				0.008
Adenocarcinoma	37.2 (31.8–42.5)	73.1	46.0	
Squamous cell carcinoma	28.1 (23.8–32.4)	49.0	32.1	
Large-cell and undifferentiated carcinomas	24.6 (13.3–35.9)	33.3	25.0	
T				0.146
T1–T2	33.6 (29.6–37.6)	63.5	40.0	
T3	20.0 (16.0–24.0)	29.6	23.7	
N				<0.001
N0–N1	35.6 (31.5–39.7)	66.7	46.5	
N2	18.4 (14.4–22.4)	18.4	0.0	
Stage				<0.001
I–II	37.9 (33.7–42.1)	74.1	51.0	
III–IV	19.9 (15.8–24.0)	23.8	7.9	
Grade				0.005
Low	23.3 (17.6–28.9)	36.0	20.6	
Medium/high	35.2 (31.0–39.4)	65.0	43.0	
p53				0.003
High	38.7 (33.2–44.2)	74.8	51.2	
Low	27.1 (22.7–31.6)	44.1	26.4	
Caspase 3				0.650
Low	32.5 (28.2–36.8)	58.7	39.3	
High	30.7 (23.8–37.6)	52.9	29.8	
$\gamma$ -H2AX				0.009
Low	35.3 (31.2–39.4)	63.8	42.8	
High	23.2 (16.8–29.6)	39.3	21.8	
Ki67 labeling index				0.018
Low	35.0 (30.5–39.4)	64.9	44.9	
High	26.4 (20.5–32.3)	39.7	19.8	
Blood-vessel infiltration				0.104
No	34.7 (29.9–39.6)	62.6	41.1	
Yes	27.5 (22.9–32.1)	50.3	31.4	
Lymphatic infiltration				0.009
No	34.7 (30.6–38.7)	62.7	40.7	
Yes	22.5 (16.0–29.0)	38.1	23.8	
Chemotherapy-adjuvant				0.440
No	34.0 (25.1–42.9)	57.6	46.2	
Yes	31.8 (27.9–35.7)	57.1	34.4	
Radiotherapy-adjuvant				<0.001
No	37.5 (32.9–42.2)	74.3	45.7	
Yes	23.5 (19.2–27.9)	33.1	24.0	

**Abbreviation:** CI, confidence interval.

pRb from depressing E2F1, inhibiting progression from  $G_1$  to S phase.<sup>16,17</sup> Another way in which p21 leads to  $G_1$  arrest is by binding to proliferating cell nuclear antigen and inhibiting its function in replication.<sup>18</sup> The loss of p21 results in replication defects and may lead to cell death after treatment



**Figure 2 (A)** Overall survival of patients with non-small cell lung carcinoma in relation to coexpression of p53 and  $\gamma$ -H2AX. Group A: p53 and  $\gamma$ -H2AX low-expression tumors. Group B: p53 high-/ $\gamma$ -H2AX low- or p53 low-/ $\gamma$ -H2AX high-expression tumors. Group C: p53 and  $\gamma$ -H2AX high-expression tumors. **(B)** Representative serial sections of adenocarcinomas immunostained for  $\gamma$ -H2AX and p53.

with anticancer drugs.<sup>19,20</sup> Progression to the S phase after p53/p21-mediated arrest is achieved by the proteasome-dependent degradation of p21;<sup>21</sup> p21 is a known inhibitor of apoptosis, and its degradation can lead to cell death.<sup>19,22</sup> Moreover, it has been shown to inhibit caspase-3, which is a crucial component of apoptotic cell death and therefore it was selected to be measured in our study.<sup>23</sup>  $\gamma$ -H2AX levels may reflect endogenous genomic instability in tissues. Increasing staining levels of  $\gamma$ -H2AX in our study were associated with unfavorable risk factors like tumor stage, tumor grade, lymphatic infiltration, and shortened OS, respectively. The present study is the first to demonstrate a significant association between high  $\gamma$ -H2AX expression levels and decreased OS in patients with NSCLC. Additionally, tumors having both low  $\gamma$ -H2AX levels and low p53 expression levels were statistically associated with a better OS in comparison to patients that had both high  $\gamma$ -H2AX and high p53 expression levels: we cannot suggest a plausible explanation for this association. However, low  $\gamma$ -H2AX expression levels may be indicative of a slowly proliferating, less aggressive tumor phenotype with good prognostic features. Such tumors possibly provide enough time to the DNA repair machinery, which thus requires low H2AX levels. On the contrary, high  $\gamma$ -H2AX expression levels may indicate a more aggressive, highly proliferating tumor phenotype accumulating massive DNA defects and thus having a worse prognosis.

In the combined p53/ $\gamma$ -H2AX analysis, we found that the p53 low/ $\gamma$ -H2AX low phenotype was associated with a significantly better outcome compared with the other expression patterns. This finding is consistent with the previously reported roles of both proteins. Immunohistochemical detection of p53 is mostly indicative of the presence of the mutant, more stable p53 protein, associated with a rather defective DNA repair mechanism.<sup>24,25</sup> On the contrary, absence of p53 immunohistochemical detection is – most of the time – relevant to the presence of the

wild-type p53 protein; hence the association of low p53/low  $\gamma$ -H2AX expression pattern with a better overall survival. This theory is in accordance with the model of Halazonetis et al, who propose a barrier to tumorigenesis predominated by the apoptotic index and characterized by low levels of activation of the DDR system at normal tissues, a peak at precancerous lesions and sustained high levels at tumors.<sup>26</sup> The link between p53 and  $\gamma$ -H2AX has already been proposed by Brunner et al showing that increased expression levels of  $\gamma$ -H2AX are associated with bad prognostic features in endometrial carcinomas.<sup>27</sup> Nagelkerke et al also found that in triple-negative breast carcinomas, a high number of  $\gamma$ -H2AX foci indicated a significantly worse prognosis.<sup>28</sup> The observed associations between high  $\gamma$ -H2AX expression and parameters, such as tumor size, stage, lymphatic invasion, and prognosis, in our study are in keeping with those reported in these studies.<sup>24,25</sup> The correlation between OS and other tumor parameters such as stage, Ki67, and p53 are also in keeping with those already demonstrated by other investigators in NSCLC and are not commented upon.<sup>29,30</sup>

In conclusion, our study is the first to demonstrate that overexpression of  $\gamma$ -H2AX may represent an independent prognostic indicator of worse OS in patients with non-small cell lung carcinoma. Further studies in a larger cohort of patients are needed to confirm our results and establish a prognostic role for this protein, which represents a major component of the DNA repair machinery.

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the results, developed the tables and figures, and wrote the manuscript. PGF participated in the design and supervision of the study. PH identified eligible patients, and provided the cohort of patients for the analysis. PGF, MK, IGP, EP, and PK issued pathological reports of the surgical specimens, assessed immunostained sections, and developed the figures. GT did the statistical analyses. DB and EC made corrections and supervised the study. SK participated in the interpretation of the results, corrected the manuscript and supervised the study.

## Disclosure

The authors report no conflicts of interest in this work.

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