Radiotherapy and Oncology 101 (2011) 39–45

Contents lists available at ScienceDirect

Radiotherapy and Oncology

journal homepage: www.thegreenjournal.com

DNA damage

Constitutive expression of γ-H2AX has prognostic relevance in triple negative breast cancer

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ARTICLE INFO

Article history:
Received 1 July 2011
Accepted 9 July 2011
Available online 15 August 2011

Keywords:
γ-H2AX
Breast neoplasms
53BP1
DNA damage

ABSTRACT

Background and purpose: Constitutive γ-H2AX expression might indicate disruption of the DNA damage repair pathway, genomic instability, or shortened telomeric ends. Here, we quantified expression of endogenous γ-H2AX and its downstream factor 53BP1 in a large number of breast cancer cell lines ($n = 54$) and a node-negative breast cancer cohort that had not received adjuvant systemic treatment ($n = 122$).

Materials and methods: Formalin fixed paraffin embedded breast cancer cell lines and tumors were immunohistochemically analyzed for γ-H2AX and 53BP1 expression, and related to cell line, patient and tumor characteristics and to disease progression.

Results: In breast cancer cell lines, γ-H2AX positivity was associated with the triple negative/basal like subgroup ($p = 0.005$), and with *BRCA1* ($p = 0.011$) or *p53* ($p = 0.053$) mutations. Specifically in triple negative breast cancer patients a high number of γ-H2AX foci indicated a significantly worse prognosis ($p = 0.006$ for triple negative vs. $p = 0.417$ for estrogen receptor (ER), progesterone receptor (PR) or HER2 positive patients). A similar association with disease progression was found for 53BP1. In a multivariate analysis with tumor size, grade, and triple negativity, only the interaction between triple negativity and γ-H2AX remained significant ($p = 0.002$, Hazard Ratio = 6.77, 95% CI = 2.07–22.2).

Conclusions: Constitutive γ-H2AX and 53BP1 staining reveals a subset of patients with triple negative breast tumors that have a significantly poorer prognosis.

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The DNA damage repair pathway is activated upon damage to DNA caused by a plethora of causes such as ionizing radiation, hypoxia, reactive oxygen species, and certain chemicals, as well as replicational or transcriptional errors [1]. Several types of damage can occur, including double stranded breaks (DSBs) in which both DNA strands have been cleaved. If left unrepaired, DSBs are lethal for the cell [2,3]. One of the first events in reaction to activation of the DNA damage repair pathway is phosphorylation of histone H2AX on serine 139 on each side of the break, yielding γ-H2AX foci [4–7]. γ-H2AX recruits other factors such as 53BP1, BRCA1, MDC1, and the MRE11-RAD50-NBS1 (MRN) complex to sites of damage [8–12]. Unrepaired DSBs, indicated by retention of irradiation induced γ-H2AX foci, have predictive value in tumors as a biomarker for sensitivity to radiotherapy [13].

Constitutive, endogenous γ-H2AX foci are rare in normal primary human cells and tissues [14]. However, tumor cells show different degrees of constitutive phosphorylated H2AX in the absence of exogenously induced DSBs [14]. Cell lines with more endogenous foci exhibit more chromosomal instability than those with fewer endogenous foci [15]. Colocalization of endogenous foci with other DNA repair factors (e.g. 53BP1, MRN-complex) seems to indicate that actual DNA repair is taking place at these sites [16,17]. Endogenous expression of γ-H2AX has not only been observed in tumor cell lines, but also in cancer tissues and even in their precursor lesions, suggesting a role for activated DNA damage repair in tumorigenesis [18,19]. Data from recent studies indicate that the endogenous expression of DNA damage response factors may also be associated with damaged, shortened telomeres [20–22] or hypoxia [23].

Recently, an association between γ-H2AX expression, and *BRCA1* mutation status and triple negativity has been found in a cohort of breast cancers [24]. Whether endogenous expression of γ-H2AX is consequential in breast cancer has, to the best of our knowledge, not been described. In the current study the constitu-

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0167-8140
doi:10.1016/j.radonc.2011.07.009

tive expression of γ-H2AX and its downstream factor 53BP1 were examined in breast cancer cell lines and breast cancer tissues and correlated with clinicopathological parameters and outcome.

Materials and methods

Tumor arrays

A breast cancer cell microarray of 54 breast cancer cell lines in triplicate was constructed as described previously [25]. A tumor microarray was constructed from formalin fixed paraffin embedded breast tumors [26]. Tumor cores were marked on hematoxylin and eosin-stained slides by a pathologist. Two millimeter diameter punches were made with a Tissue-Tek Quick Ray puncher (Sakura Inc.) and embedded in fresh paraffin.

Patients

Breast cancer patients were selected from a cohort surgically treated between January 1991 and December 1996 that had no axillary lymph node invasion, received no adjuvant systemic treatment, and had at least 5 years follow up or an earlier recurrence during follow-up [26]. By enriching for triple negative tumors, in order to obtain approximately equal sized groups of breast cancer subtypes, the selection criteria led to a patient cohort ($n = 122$) with 25% ER positive, 13% PR positive, 30% HER2-positive, and 44% triple negative patients. As approved by the institutional review board and according to national law, coded tumor tissues were used.

Immunohistochemical staining of formalin fixed paraffin embedded tissue sections

Sections were stained as described previously [27] with minor modifications. For γ-H2AX sections were rinsed in Tris Buffered Saline (pH 7.4), and normal donkey serum was diluted in 1% BSA in TBS with 0.2% Triton X-100 (Sigma–Aldrich, St. Louis, MO, USA). The primary antibodies used were rabbit anti-phospho-H2AX (#2577, Cell Signaling Technology, Danvers, MA, USA) diluted 1:500 in TBS with 1% BSA and rabbit anti-53BP1 (210-419-R050, Alexis Biochemicals Corporation, San Diego, CA, USA) diluted 1:5000. The secondary antibody was biotin-conjugated donkey anti-rabbit IgG (711-066-152, Jackson) diluted 1:400 in TBS (γ-H2AX) or PBS (53BP1).

Image acquisition, scoring procedure, and statistical analysis

All images were captured with a Leica DM 6000 microscope using IP-lab imaging software (Scanalytics Inc., Fairfax, VA, USA). Stained sections were viewed at a magnification of 200× and manually scored by at least two independent researchers (A.N and S.K. or P.S.) according to γ-H2AX or 53BP1 staining intensity, where 0 indicates no staining present, 1 is light staining, 2 is moderate staining, and 3 intense staining. Figs. 2 and 4 of the results section show examples of the different staining intensities found.

Statistical analyses

Statistical analyses were performed using SPSS 16.0 software (SPSS Benelux BV, Gorinchem, The Netherlands). Differences in staining intensities between patient subgroups were analyzed with χ^2 tests and/or Spearman rank correlation tests. Disease progression was defined as time between primary surgery and any recurrence (local or regional) or distant metastasis. Death by any cause was treated as censoring event in Kaplan–Meier survival curves with log-rank testing of differences in disease progression between patient subgroups. Multivariate analyses were performed with Cox Proportional Hazard modeling with backward selection of variables based on the Likelihood Ratio. A $p < 0.05$ was considered statistically significant.

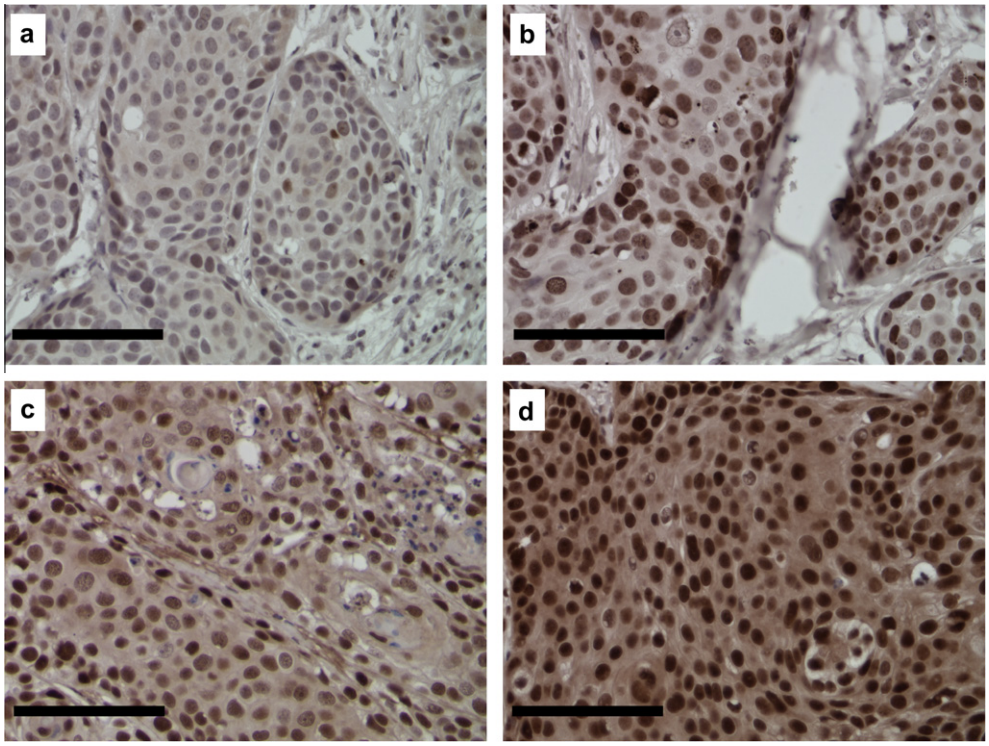

Fig. 1. Antibody validation for γ-H2AX (a and b) and 53BP1 (c and d) in irradiated SCCNij3 xenografts. Pre-irradiation a limited number of foci are present (a and c). Twenty-four hours after irradiation with 10 Gy foci are apparent (b and d). Original magnification: 400×, scale bar is 100 μm.

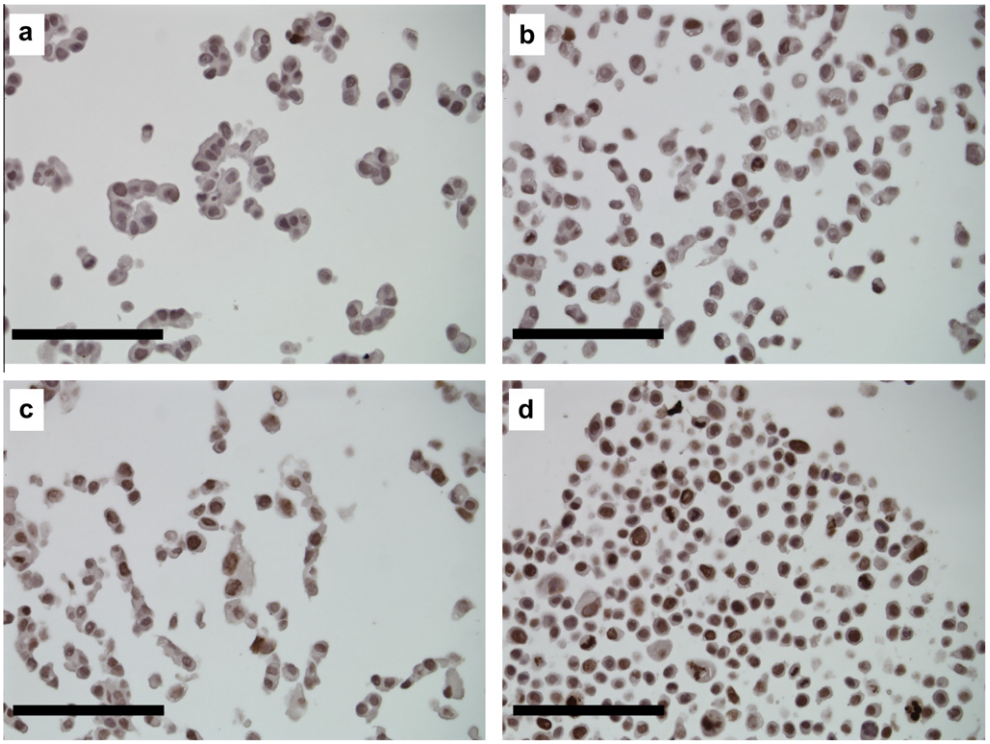

Fig. 2. Different staining intensities of γ-H2AX in breast cancer cell lines, ranging from 0 to 3 (a–d). Depicted cell lines are: MDA-MB-175VII (a), EVSA-T (b), BT-20 (c), and MDA-MB-435s (d). Original magnification: 400×, scale bar is 100 μm.

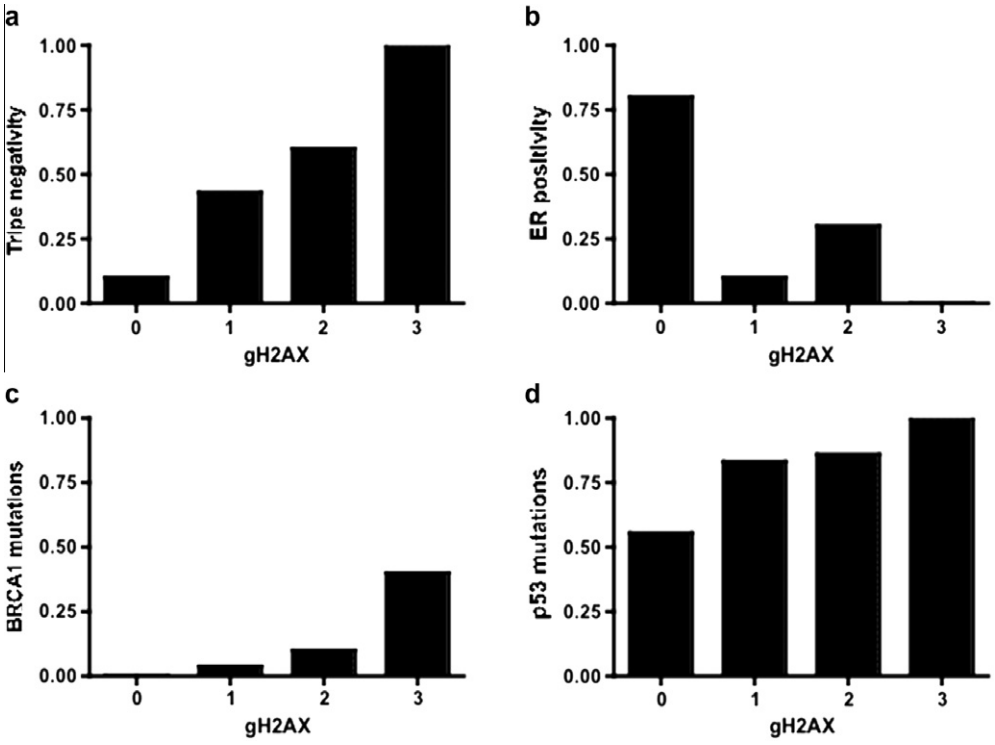

Fig. 3. Differences in cell lines with different γ-H2AX staining intensities and characteristics as fraction of cell lines positive for: (a) triple negativity ($p = 0.005$), (b) estrogen receptor ($p = 0.001$), (c) *BRCA* mutations ($p = 0.011$), and (d) *p53* mutations ($p = 0.053$).

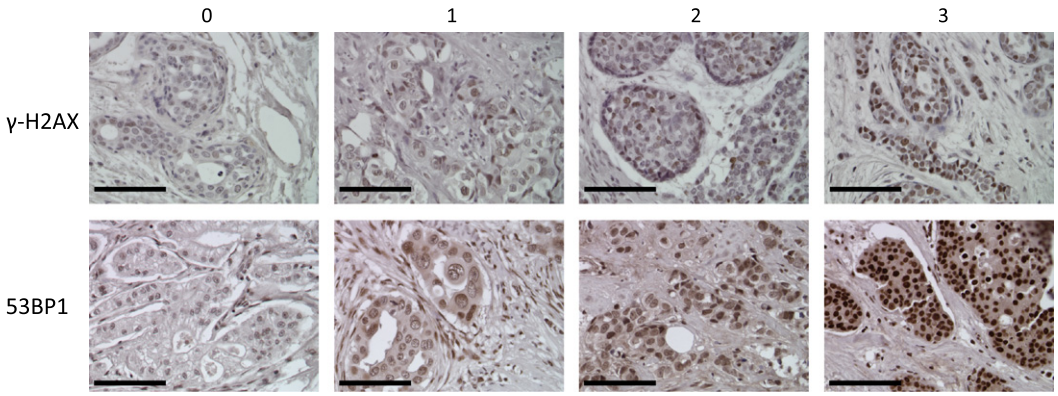

Fig. 4. Differences in γ-H2AX and 53BP1 expression in breast tumors. Typical examples of tumors with scores from 0 to 3 are shown. Original magnification: 400×, scale bar is 100 μm.

Table 1
Patient characteristics of low versus high γ-H2AX expression in triple negative and any receptor positive breast cancer patients.

		Triple negative			Any receptor positive		
γ-H2AX		Low	High	*p*	Low	High	*p*
Total *n*		30	12		39	15	
Mean age		57	51	0.341	59	49	0.011
pT	1	13	7	0.648	18	11	0.359
	2	14	5		19	4	
	3	2	0		2	0	
	4	1	0		0	0	
pN	0	30	12	–	39	15	–
	1	0	0		0	0	
M	0	30	12	–	39	15	–
	1	0	0		0	0	
Menopausal state	Pre	10	5	0.611	6	7	0.023
	Post	20	7		33	8	
Grade	I	0	0	0.783	1	1	0.717
	II	4	2		10	4	
	III	18	8		18	8	
	Unknown	8	2		10	2	
Tumor type	Ductal	23	11	0.136	31	13	0.867
	Lobular	2	0		4	1	
	Other	5	1		4	1	
Surgery	Mastectomy	17	1	0.021	19	5	0.263
	Lumpectomy	13	11		20	10	
Adjuvant radiotherapy	No	18	1	0.003	17	3	0.155
	Yes	12	11		22	12	
ER	Negative	30	12	–	18	9	0.202
	Positive	0	0		21	6	
PR	Negative	30	12	–	28	12	0.449
	Positive	0	0		11	3	
HER2	Negative	30	12	–	17	4	0.119
	Positive	0	0		22	11	

Results

γ-H2AX and 53BP1 antibody validation

The γ-H2AX and 53BP1 antibodies used in this study were validated on irradiated xenografted head and neck squamous cell carcinoma tissues. Formalin fixed paraffin embedded SCCNij3 tumors were available from previous studies [28,29]. γ-H2AX and 53BP1 staining on normal tissue of the breast, kidney, liver and lung showed no noteworthy expression. After a dose of 10 Gy the SCCNij3 tumors showed increased γ-H2AX and 53BP1 foci formation (Fig. 1), indicating that the antibody and staining procedures were valid.

Endogenous γ-H2AX expression in a large panel of breast cancer cell lines

A microarray containing cores of 54 different breast cancer cell lines, embedded in triplicate, was stained for γ-H2AX and subsequently scored. Examples of different staining intensities are shown in Fig. 2. Scores were correlated with known characteristics

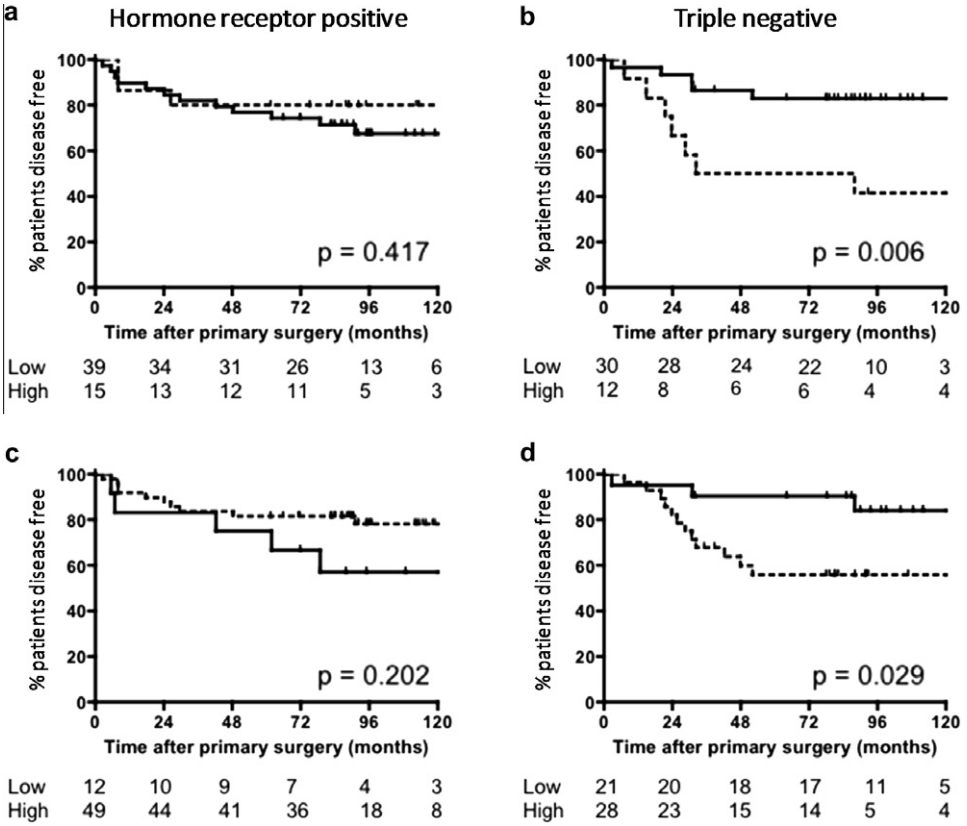

Fig. 5. Association of γ-H2AX and 53BP1 with disease progression. (a) No association of low γ-H2AX (black line) or high γ-H2AX (dotted line) staining intensity in receptor (ER, PR, and/or HER2) positive tumors was found ($n = 54$) but in triple negative tumors ($n = 42$) high γ-H2AX leads to a significantly shorter disease free interval ($p = 0.006$, b). (c) No association of low 53BP1 (black line) or high 53BP1 (dotted line) was found in receptor positives ($n = 61$), but in triple negatives ($n = 49$) high 53BP1 leads to a decrease in disease free interval ($p = 0.029$, d). Patient numbers between γ-H2AX and 53BP1 may differ due to loss of patient samples during the staining procedure.

of the cell lines [25]. γ-H2AX positivity was associated with the triple negative/basal like subgroup ($p = 0.005$), estrogen receptor negativity ($p = 0.001$) and with *BRCA1* ($p = 0.011$) or *p53* ($p = 0.053$) mutations (Fig. 3).

Expression of γ-H2AX and 53BP1 in breast tumors

Next, the expression of γ-H2AX and 53BP1 was analyzed in a heterogeneous group of 122 non-adjuvantly treated patients with breast cancers. The range of staining intensities for both markers is shown in Fig. 4. The γ-H2AX staining was found to be negative in 35% of the cases and highly positive in 8% of the cases. No significant correlation was found between γ-H2AX expression and hormone receptor status. In addition, 53BP1 was stained on consecutive sections of the breast cancer tissue microarray. Similar to γ-H2AX, different patterns of staining intensity of 53BP1 were observed (see Fig. 4). Scores for γ-H2AX and 53BP1 showed a strong correlation ($p < 0.001$), although 53BP1 showed a higher number of positive tumors: 14% of the cases were negative and 32% of the cases were scored as strongly positive. In the receptor positive group young age ($p = 0.011$) and premenopausal status ($p = 0.023$) were associated with stronger γ-H2AX staining (Table 1). In the triple negative patients, stronger γ-H2AX staining was found in patients eventually treated with lumpectomy vs mastectomy ($p = 0.021$) and with (post-surgical) adjuvant radiotherapy ($p = 0.003$).

Expression of γ-H2AX and 53BP1 has prognostic relevance in triple negative breast cancers

Next, the relation between γ-H2AX and disease progression was evaluated. Due to the small sample size, staining scores of 0 and 1 as well as 2 and 3 were taken together for this purpose. For the group as a whole, no significant associations with prognosis were found. However, within the group of triple negative tumors a highly significant association with disease progression was found ($p = 0.006$, Fig. 5a and b), which was absent in the hormone receptor positive group. Similar results were found for 53BP1 ($p = 0.029$, Fig. 5c and d).

Within the triple negative breast cancers, a multivariate analysis with surgery and radiotherapy, both with which γ-H2AX was associated (Table 1), revealed that γ-H2AX remained a significant factor ($p = 0.010$; Hazard Ratio (HR) = 4.06, 95% CI = 1.40–11.77). Thus, the prognostic relevance of γ-H2AX in triple negative breast cancers was independent of its association with these factors.

Next, we performed a multivariate Cox Regression analysis including prognostically relevant parameters, i.e. tumor size, histological grade, and tumor type (triple negative, steroid hormone, and/or HER2 positive). All patients were node negative and did not receive adjuvant systemic treatment, thus these parameters were not entered in the model. In addition, an interaction variable for triple negativity and γ-H2AX was entered in the model, which would indicate whether the prognostic value of γ-H2AX was different in these tumors. Only this interaction between triple negativity and γ-H2AX remained significantly associated with disease progression ($p = 0.002$, HR = 6.77, 95% CI = 2.07–22.2) after correction for tumor size, grade, and triple negativity.

Discussion

Here, we found that constitutive γ-H2AX expression is higher in triple negative, and in *BRCA1* and *p53* mutated breast cancer cell lines. In addition, endogenous γ-H2AX and 53BP1 expression in breast cancer tissue was evaluated and correlated with

clinicopathological parameters and outcome in a human breast cancer patient cohort. It has been reported that triple negative breast cancers display more endogenous γ-H2AX expression [24] and that this subset of cancers has a higher incidence of carrying aberrations in components of the DNA damage repair pathway [24,30]. In the current study a correlation between γ-H2AX expression and triple negativity was found in a group of breast cancer cell lines, but this association could not be confirmed in breast cancer patients. Further, basal like and/or triple negative tumors are known to have a larger number of *BRCA1* mutation carriers, which previously have been associated with a higher occurrence of γ-H2AX positivity [24]. The *BRCA*-status of the patients included in the current study, however, is unknown. Nevertheless, we did find that in breast cancer patients high endogenous γ-H2AX or 53BP1 expression revealed a subset of triple negative tumors with poor prognosis. Our results seem to indicate that triple negative and/or basal like breast tumors represent a fundamentally different subgroup of tumors where apparently endogenous γ-H2AX expression has relevance for disease progression, although this should be validated in independent, unselected larger breast cancer patient cohorts.

Several studies have attempted to elucidate the exact identity of the endogenous γ-H2AX foci observed in many different cancerous lesions and whether they are associated with actual DNA damage. Recruitment of other downstream DNA damage repair factors to these foci has led to the idea that actual DNA repair is attempted at these sites [16,17]. One recent explanation for the occurrence of endogenous γ-H2AX in cancers is the association with telomeres, protective structures which form the chromosome ends in eukaryotes. Duplication of DNA results in shortened telomeres after every cell cycle. Shortening of telomeres and activation of telomerase, an enzyme necessary for telomere lengthening, occurs frequently in (pre-)cancerous lesions [31–35]. Normally, telomere shortening is a signal for replication arrest and replicative senescence. In the absence of a telomeric structure, chromosome ends are highly unstable and are prone either to degradation, merging with other chromosomes (genomic instability) or DNA double stranded breaks [20,21]. Nakamura et al. showed that a considerable number of endogenous γ-H2AX foci do not represent actual double stranded breaks but are in fact uncapped telomeres [22]. Thus, telomere dysfunction can lead to DNA damage and phosphorylation of H2AX at these sites [36,37]. In addition, telomere associated chromosomal rearrangements may lead to a tumor phenotype with the associated immortality and unlimited replicative potential [38]. However, at the moment it is unclear what endogenous γ-H2AX and 53BP1 expression represents in our patients. The mechanism behind the occurrence of these foci and the relation with disease progression and/or treatment sensitivity remain to be elucidated.

In conclusion, in the current breast cancer cohort an association was found between expression of the DNA damage repair factors γ-H2AX and 53BP1 and disease progression in triple negative patients. Independent validation of this finding is necessary in larger unselected breast cancer patient cohorts, and further studies in breast cancers from germline *BRCA1* mutation carriers and by correlating γ-H2AX expression directly to telomere length should be performed to determine the nature of these foci.

Conflict of interest statement

The authors made no disclosures.

Acknowledgment

The authors would like to thank Mrs. Irene Otte-Holler (Department of Pathology, Radboud University Nijmegen Medical Centre) for her help with optimization of staining protocols.

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