

Expression of γ H2AX may help in defining a genetically more stable subtype of infiltrating ductal carcinoma of breast

Alka Bhatia, Pranab Dey*, Shubham Goel & Gurpreet Singh**

*Departments of Experimental Medicine & Biotechnology, *Cytopathology & **General Surgery, Postgraduate Institute of Medical Education & Research, Chandigarh, India*

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Background & objectives: Gamma H2AX, a marker of DNA double stranded breaks (DSB) has been found to be over expressed in various tumours. The objective of the present work was to study the expression of γ H2AX in infiltrating ductal carcinoma (IDC) and fibroadenoma (FA) cases and to associate the expression in IDC with cytomorphological features and DNA ploidy.

Methods: The expression of γ H2AX was studied in fine needle aspirates of 16 cases of IDC and 15 FA cases. The expression in IDC was correlated with the cytological grade, apoptotic (AI) and mitotic indices (MI) and ploidy status.

Results: A high γ H2AX expression was noted in IDC as compared to FA. Amongst the IDC cases the γ H2AX was found to be significantly over expressed in DNA diploid IDC cases as compared to the aneuploid ones.

Interpretation & conclusions: The study suggests a role of γ H2AX in breast carcinogenesis which needs to be explored further. Moreover, the γ H2AX expression together with ploidy status may serve as a means of assigning the patients of IDC to a better prognostic category irrespective of the cytomorphological parameters.

Key words Apoptosis - cytology - fibroadenoma - γ H2AX - grading - IDC - ploidy

In eukaryotic cells, the role of histone proteins in organization of chromatin is well-recognized. H2AX is a histone variant belonging to H2A family which comprises 2-10 per cent of the H2A complement in the mammalian tissues¹. H2AX is activated when DNA molecules inside the cell are broken by external factors such as radiation or sometimes spontaneously as in tumours². H2AX becomes phosphorylated at serine 139 residue in chromatin surrounding DNA double-strand breaks (DSBs) to form Gamma H2AX

(γ H2AX)¹. The phosphorylation process is rapid and extensive, covering large regions of the chromosome adjacent to each break and producing foci that can be visualized microscopically after antibody labelling^{3,1}. γ H2AX is proposed to concentrate repair factors at sites of DNA damage and maintain genomic stability⁴. Animal experiments have shown increased incidence of genomic instability and tumour formation in case of partial or complete loss of H2AX gene⁵. Therefore, H2AX has been proposed to act as a tumour suppressor

in concert with other tumour suppressors like p53. The soluble H2AX, that is, the non-chromatin-associated H2AX has been found to sensitize cells with DNA damage to undergo apoptosis⁶. The DNA damage response pathway is activated early in the process of carcinogenesis even before activation of p53⁷. Hence it has been proposed that antibody to γ H2AX can be used to detect precancerous lesions or as a biomarker for cancer detection at the metastasis site^{8,9}.

It is now well known that genetic factors play an important role in development of invasive breast cancer¹⁰. A sequential accumulation of genetic alterations involving activation of oncogenes and by inactivation of several tumour suppressor genes has been found in this malignancy¹⁰. More recent studies have suggested that mechanisms and pathways regulating DNA damage and repair may play a mutator role in driving breast cancer pathogenesis¹¹. Though preliminary work has shown increased expression of γ H2AX in tumours of breast, melanoma, prostate, and urinary bladder, information is limited¹²⁻¹⁴. Moreover, a systematic correlation with other significant parameters like grade, apoptosis, mitosis and ploidy status has not been studied before in breast cancer. The present work was therefore, undertaken to study the expression of γ H2AX in infiltrating ductal carcinoma (IDC) and fibroadenoma (FA) cases and to relate the expression in IDC with cytomorphological features and DNA ploidy.

Material & Methods

This prospective study involved 53 patients with a palpable breast lump coming to Fine Needle Aspiration Cytology (FNAC) clinic of department of Cytopathology or Surgery, Postgraduate Institute of Medical Education & Research, Chandigarh, a tertiary care hospital between May 2009 and March 2011. The work was carried out after obtaining ethical clearance by Institute's ethics review committee and after an informed written consent was obtained from all the patients.

Only fresh cases without any history of breast cancer in the family were included in the study. Those on any kind of treatment for breast disease were excluded. Fine needle aspirate was collected by the standard protocol of breast FNAC and was utilized for making the cytology smears and for performing flow cytometry. The smears were stained with Giemsa for cytological diagnosis and grading as per the grading system proposed by Robinson *et al*¹⁵. Apoptotic and

mitotic indices (AI & MI) were determined by counting 1000 tumour cells and calculating the percentage in each case of IDC. For studying the γ H2AX expression, cell suspension of the aspirate was prepared in phosphate buffered saline (PBS) pH 7.4. The cell concentration (2×10^6) was checked by counting the number of cells using a haemocytometer. After fixation in 1 per cent formaldehyde- PBS (pH 7.4) and permeabilization in cold ethanol, immunostaining was carried out by incubating the cells with 200 μ l mouse monoclonal FITC conjugated anti-phosphohistone γ H2AX antibody (1:500 dilution, Biologend, San Diego, USA). The stained cells were then analysed under flow cytometer (BD FACS Calibur, 2002, USA) using BD cell questTM pro data software. The instrument was calibrated using Calibrite beads. Approximately 10,000 events were collected from each sample for analysis. The ploidy status and S-phase fraction (SPF) was determined by flow cytometry after incubation with propidium iodide (PI). Statistical analysis of the data was carried out using SPSS programme (version 16) IBM, USA using independent t test, ANOVA test and Pearson's correlation analysis.

Results

Out of 53 cases included in the study, a cytological diagnosis of IDC was offered in 21 cases and 17 cases were reported as fibroadenoma (FA). The age range of patients was 19-76 yr, median age being 38 yr. The age was between 35-76 yr (median 40.5 yr) for IDC and 19-65 yr (median 28 yr) for FA. The lump size ranged from 0.5-10 cm with a mean of 3.4 cm. The mean lump size was greater (4.02 cm) for malignancy than in FA (2.58 cm).

γ H2AX Expression in FA and IDC: γ H2AX expression was analysed in 16 cases of IDC and 15 cases of FA. The mean and median γ H2AX expression was found to be higher in IDC (28.30 and 20.35%, respectively) and lower in FA (17.07 & 6.68%, respectively) (Fig. 1). However, the difference in γ H2AX expression in malignant and benign cases was not significant.

Morphological parameters in IDC

Apoptotic and mitotic index: The apoptosis was counted morphologically in the Giemsa stained smears in 21 cases of IDC. The AI was expressed as percentage of apoptotic cells. The apoptotic cells were identified by cellular shrinkage, chromatin condensation and formation of apoptotic bodies. The AI was variable in IDC with percentage of apoptotic cells varying from

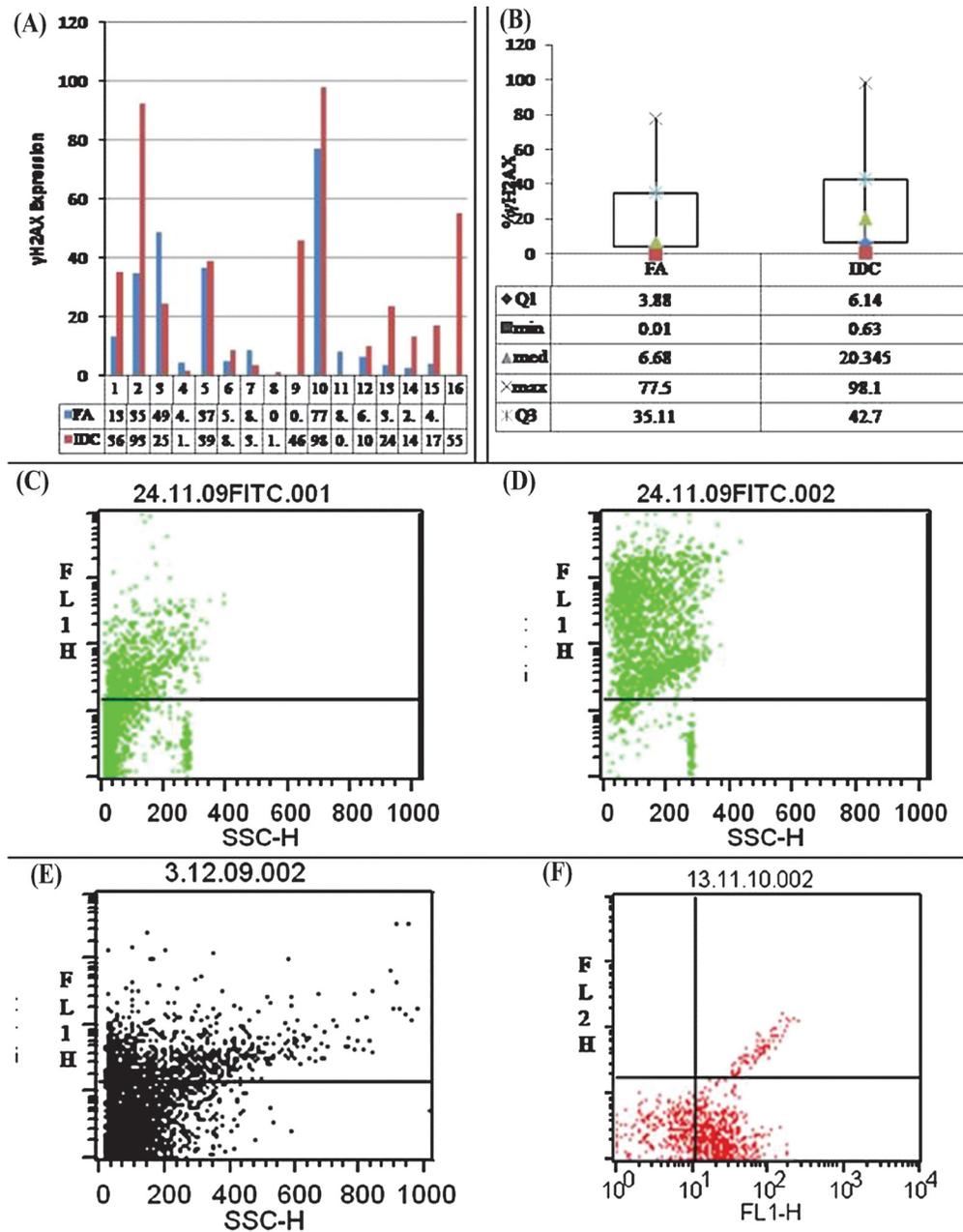


Fig. 1. (A) Percentage γ H2AX expression in fibroadenoma (FA) and infiltrating ductal carcinoma (IDC). (B) Box plot diagram showing the difference in median, minimum and maximum values of % γ H2AX expression in FA & IDC. (C, D) % γ H2AX expression (35.52 & 92.67%) in two cases of IDC seen as FITC labelled population in FL1-H channel. (E, F) Low and high % γ H2AX expression (13.41 and 77.15%) in 2 cases of FA seen as FITC labelled population in FL1-H channel.

0.5 to 35 with a median of 4.5 (Table). The mitotic figures were also counted and expressed as percentage as in case of AI. The MI ranged from 0-8 with a median of 2.65 (Fig. 1).

Cytological grading of IDC cases: Cytological grading was performed on all 21 fresh cases of IDC. The lesions

with small cells exhibiting slight pleomorphism and many gland-like structures were classified as grade 1, those with moderate atypia and pleomorphism and 10-75 per cent gland formation as grade 2, whereas those with large cell size, marked pleomorphism, prominent nucleoli and minimal gland formation were categorized

as grade 3 (Fig. 2A-C). There were two cases of grade 1, 13 cases of grade 2 and 6 cases of grade 3 IDC (Table).

Ploidy status in IDC: The ploidy status was analysed in 21 cases of IDC. The DNA histogram with an additional G0-G1 peak and a DNA content of $<2N$ or $>2N$ were classified as aneuploid and an SPF (S-phase fraction) of >10 per cent was considered to be high. In all, 13 (61.90%) cases showed aneuploidy, eight hyperdiploid aneuploidy, four hypodiploid aneuploidy and one case showed both hyper- as well as hypodiploid peak. Eight cases (38.1%) were diploid, of whom three cases showed raised SPF as well.

Correlation of cytological grades with AI and MI: The mean values of both AI and MI showed an increase with increasing grades of the IDC (Fig. 2D). On statistical analysis, the difference in AI between grade 3 and the remaining two grades was found to be

significant ($P<0.05$). The difference in the MI of the three groups was not significant. Correlation of AI with MI in IDC cases also showed a proportional increase in both the parameters which was statistically significant (Pearson's correlation, $r = .009$, $P<0.05$).

Correlation of γ H2AX expression with grade, AI, MI and ploidy status in IDC: Gamma H2AX expression in different grades of IDC showed a considerable overlap amongst individual cases belonging to each grade (Table). Mean γ H2AX was found to be highest in grade 3 (46.30%) as compared to the lower grades (11.76% in grade 2 and 23.41% in grade 1, respectively) (Fig. 2E). The difference between the three groups was not significant. No significant correlation was observed between the MI and AI and γ H2AX expression.

Correlation of ploidy status with γ H2AX expression in IDC showed a higher expression in diploid IDC ($n=4$, Mean = 61.83%) especially in those with diploidy

Table. Expression of γ H2AX in infiltrating ductal carcinoma of breast and its correlation with clinical and morphological parameters and DNA ploidy

Sr no	Age (yr)	Laterality	Lump size (cm)	Cytology grade	AI	MI	Ploidy	γ H2AX %
1	41	Rt	2.5	3	12	2	A	35.52
2	49	Rt	2	3	8	5	D \uparrow SPF	92.67
3	45	Rt	4	2	8	3	A	24.53
4	42	Lt	4	2	1	4	A	1.88
5	35	Lt	10	3	25	2.5	A	39.21
6	38	Rt	3	3	3	8	A	8.79
7	38	Lt	8	2	3	5	A	3.49
8	40	Rt	5	2	3.7	2.8	D	1.23
9	40	Lt	4	1	1.5	NMS	A	46.19
10	37	Rt	2	2	4.5	0.1	D \uparrow SPF	98.10
11	50	Rt	2.5	1	2.8	0.4	A	0.63
12	56	Rt	2	2	0.5	0.5	A	10.17
13	37	Rt	7	2	18	2	A	23.59
14	32	Rt	4	3	35	0.3	D	55.3
15	43	Rt	5	2	4	3.5	A	13.6
16	38	Lt	2	2	3.4	4.8	A	17.1
17	34	Lt	2	2	5	NMS	D	—
18	52	Rt	6	2	6	NMS	D	—
19	48	Rt	6	3	12	2.5	D	—
20	65	Lt	4	2	3	1	A	—
21	35	Rt	1	2	9.2	3.4	D \uparrow SPF	—

Rt, right; Lt, left; IDC, infiltrating ductal carcinoma; AI, apoptotic index; MI, mitotic index; A, aneuploidy; D, diploid, SPF, S-phase fraction; NMS, no mitosis seen

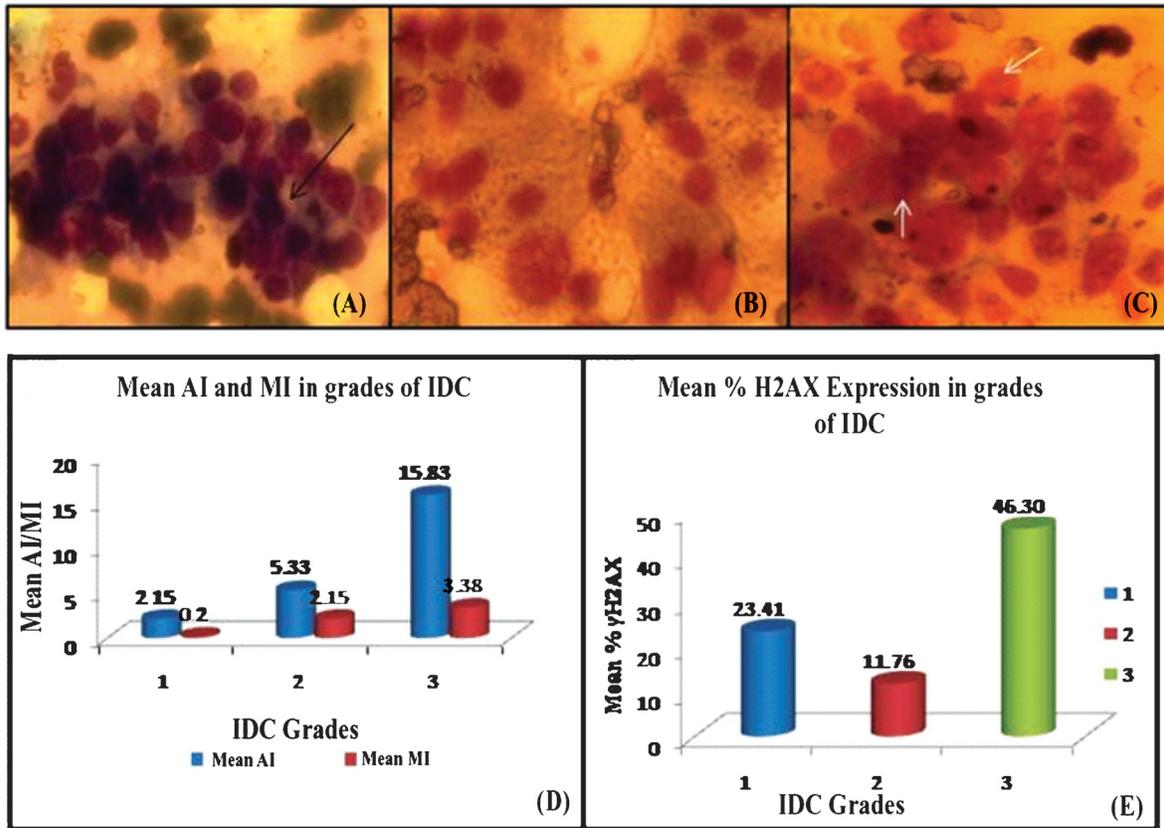


Fig. 2. (A, B, C) Microphotographs showing cytological grading of IDC in FNAC smears. 2A shows grade 1 cancer with, cohesive cells, minimal pleomorphism and gland like structures (arrow black); 2B shows grade 2 cancer with moderate degree of pleomorphism and cellular discohesion; 2C shows grade 3 cancer with poorly differentiated cells exhibiting marked nuclear pleomorphism and nucleolar prominence (arrow white) (Giemsa staining) (D) Increased mean values of both AI and MI with increase in cytological grade of cancer (E) % γ H2AX expression in different grades of IDC. Note the much higher expression in grade 3 as compared to the other grades of IDC.

and high SPF (n=2, 92.67 & 98.10 respectively) as compared to the aneuploid cases (n = 12, Mean = 18.73%). Statistical analysis using independent t-test also showed the difference in mean values for aneuploid and diploid cases to be significant ($P < 0.05$) (Fig. 3). The mean AI in DNA diploid cases was 12.8 as against a value of 6.9 for the aneuploid ones.

Discussion

The DNA DSB is a serious lesion that can initiate chromosomal instability (CIN), ultimately leading to cancer¹⁶. DNA DSBs caused by exposure to DNA damaging agents initiate phosphorylation of histone H2AX to form γ H2AX, which is considered a surrogate marker of DSBs¹⁷. In addition to being a cause of cancer, DSB induction is paradoxically an effective treatment for cancer. Many therapeutic agents act by introducing sufficient DSBs into cancer cells to activate the cell death pathways. Therefore, γ H2AX

expression is generally used to assess the response to chemoradiotherapy¹⁸.

In the present study, FNAC samples from IDC (untreated) and FA patients were analysed for the presence of spontaneous γ H2AX foci. The mean for the two groups showed a higher value for IDC although statistically insignificant. Similar results were observed in the study by Wasco *et al*¹⁹ who compared the difference in expression of γ H2AX in nevi and melanoma cases. This may be due to the complex mechanism by which DSBs and their repair occurs. Moreover, the detection of γ H2AX foci by the antibody requires conglomeration of several foci at the break site in the absence of which a focus may not be detected²⁰. Cheung *et al*¹⁴ in their study on 60 cases of low grade urothelial carcinoma found that γ H2AX positivity was associated with a lower rate of tumour recurrence. However, in this study cases of recurrent cancer have

not been included. Wasco and Pu²¹ in their study on metastatic renal cell carcinoma (RCC) concluded that γ H2AX in conjunction with other antibodies may be used to detect RCC metastasis, however, in their study also a patchy staining of normal tubules was observed with γ H2AX antibody²¹.

The overexpression of γ H2AX in IDC as opposed to FA indicates its possible role in breast carcinogenesis.

A previous report also confirms the presence of copy number alterations of *H2AFX* gene in sporadic breast cancers²². However, in view of presence of overlap amongst the FA and IDC cases in our study, the potential clinical utility of detection of γ H2AX remains uncertain. Perhaps a larger series may help to highlight better the intricacies of γ H2AX expression in benign and malignant lesions of the breast.

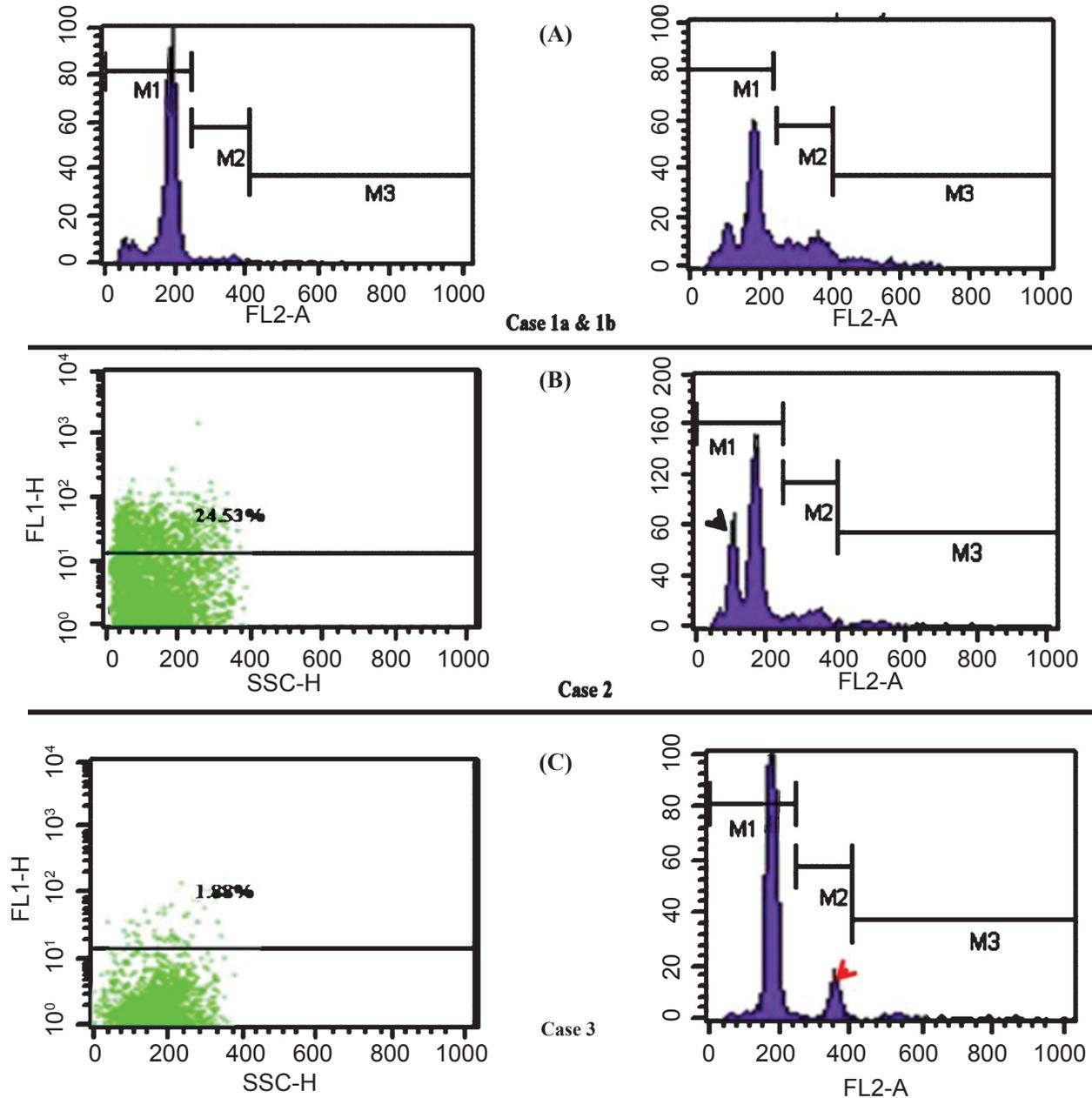


Fig. 3. (A) Case 1a: DNA diploid histogram of case of FA. Corresponding γ H2AX expression is shown in Fig. 1E; Case 1b: DNA diploidy with high SPF in DNA histogram of a case of IDC. Corresponding high γ H2AX expression is shown in Fig. 1D (B) Case 2: A case of IDC showing hypodiploid aneuploidy (black arrowhead) with 24.53% population showing γ H2AX expression (C) Case 3: A case of IDC showing small aneuploid peak and low (1.88%) γ H2AX expression (red arrowhead).

DNA ploidy has been considered as an important factor for estimating the degree of CIN which is supposed to be reflected in the form of tumour aggressiveness. Our results showed an increased expression of γ H2AX in diploid IDC, more so, in diploidy with high SPF. Hau *et al*²³ showed that polyploidy was associated with increased expression of γ H2AX as compared to the diploidy. However, their study mainly included tetraploid cases rather than aneuploid ones²³. Although many studies have analysed the significance of ploidy status in breast cancer, there is still controversy regarding its exact significance, with many studies showing an adverse outcome in DNA aneuploid than in diploid carcinomas^{24,25}. The increased γ H2AX expression in diploid IDCs in the present study may explain the more genetically stable nature of diploid than aneuploid IDCs⁵. Moreover, the DNA diploid cases had a higher mean AI than those with aneuploidy thereby further suggesting lower aggressiveness. Therefore, the assessment of γ H2AX expression in conjunction with ploidy status in IDC patients may provide a means for assigning them to a genetically more stable and hence a prognostically more favourable group. However, in view of only four cases of DNA diploid IDC in our study these observations need to be confirmed on more number of diploid IDC cases.

Earlier studies on apoptosis and γ H2AX expression in malignant cell lines have shown that γ H2AX formation is an early chromatin modification following initiation of DNA fragmentation during apoptosis¹. Solier *et al*²⁷ reported that a coordinated histone phosphorylation signature comprising γ H2AX together with PS14-H2B and possibly PY142-H2AX exists for apoptosis and this signature together with the γ H2AX ring pattern at the nuclear periphery may provide a new feature to monitor and study apoptosis. However, most of the previous work on γ H2AX has been carried out on radiation treated cells or cells exposed to apoptogens^{26,27}. Spontaneous γ H2AX expression in malignant samples of fresh cases of IDC and a correlation with AI and MI has not been studied previously.

The grades of IDC when correlated with AI showed increased apoptosis in higher grade of IDC. This increase in apoptosis was accompanied by a similar increment in MI. This is in accordance to the previous study by Vakkala *et al*²⁸ on apoptosis in breast cancer which showed a positive correlation between apoptosis and MIB1 and concluded that increased apoptosis is

associated with a worse prognosis in both *de novo* as well as recurrent breast cancers.

In this work, majority of the patients presented in 30-40 years of their life. These trends indicate the increasing prevalence of IDC amongst the younger women in our set up. Also, majority of the patients presented with a higher grade (grade 2 or 3) disease. This may perhaps mean that most people come to seek medical help only when symptomatic, and on an average, most 'symptomatic' cancers are of higher grade. Also the disease was found to be more aggressive in the younger patients. Fredholm *et al*²⁹ observed that in younger women the cancer is characterized by a worse survival which may be accounted for by the late presentation and a more aggressive tumour biology. These findings point towards the need for creating awareness and for the introduction of screening programmes in 3rd and 4th decades for early detection and treatment of breast cancer in our country.

In conclusion, an increased presence of spontaneous γ H2AX foci in IDC as compared to FA suggests that DSBs and their repair mechanisms may be playing a crucial role in the aetiopathogenesis of IDC. A higher expression in diploid IDCs may explain the genetically stable nature of these lesions and may serve as a means of assigning the patients of IDC to a better prognostic category. In future, a larger study correlating the expression of γ H2AX and other DNA damage and repair molecules with parameters like axillary lymph node status, hormonal profile and other prognostic markers like Ki67, EGF receptors, Her2 expression may help to provide more relevant clinical information on the influence of DNA repair molecules in IDC progression.

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Reprint requests: Dr Alka Bhatia, Assistant Professor, Department of Experimental Medicine & Biotechnology
Postgraduate Institute of Medical Education & Research, Chandigarh 160 012, India
e-mail: alkabhatia@ymail.com