

Increased micronucleus count predicts malignant behavior in pleural effusion fluid

Tuba Dilay KÖKENEK ÜNAL^{1*}, İpek ÇOBAN²

¹Department of Pathology, Faculty of Health Sciences, Kayseri Research and Training Hospital, Kayseri, Turkey

²Department of Pathology, Gayrettepe Florence Nightingale Hospital, İstanbul, Turkey

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Background/aim: Micronucleus (MN) frequency is used as a biomarker of chromosomal damage, genome instability, and cancer risk. The aim of this study was to evaluate the diagnostic usefulness of MN frequency to differentiate between malignant and benign pleural effusion samples.

Materials and methods: Retrospectively, 78 pleural fluid cytology samples (including 20 cases of benign reactive mesothelial cells, 22 cases of suspicious cytology, and 36 cases of malignant cytology) were examined. The number of micronucleated cells in 1000 well-preserved cells was counted. Statistical tests were performed to compare the study groups. Receiver operating characteristics (ROC) curve analysis was performed to suggest a cut-off value for predicting malignant behavior.

Results: We evaluated a total of 78 cases of pleural effusion cytology. The number of micronucleated cells was significantly higher in cases with malignant outcome compared to cases with benign outcome. We observed that malignant samples had more micronucleated cells than suspicious ones, and suspicious cases had more micronucleated cells than reactive ones. There was a significant difference among all study groups. In addition, the frequency of MN-containing cells in suspicious cases correlates well with their outcomes.

Conclusion: The results of this study reveal that there is an absolute, consistent, and proportional relationship between MN counts and malignancy in cytological samples of pleural effusions. MN scoring may be a helpful diagnostic tool for distinguishing malignant effusions from benign ones, and may be used as an adjunct tool to predict malignant behavior in challenging cases.

Key words: Cytology, malignant cytology, malignant mesothelioma, micronucleus, pleural effusion

1. Introduction

Micronucleus (MN) is a small extranuclear body found in the cytoplasm formed during cell division by various mechanisms (1,2). MN formation can be observed in healthy individuals due to several causes such as radiation, drugs, chemicals, chemo- or radiotherapy, chronic inflammation, and metabolic, infectious, or genetic diseases (3). Besides these, it may be a way for tumor cells to survive in inconvenient circumstances by overcoming the destabilization of chromosomes in the DNA amplification process (4). MN formation is an indicator of chromosomal breakage and instability, and can be easily identified by light microscopy (5). The predictive value of MN formation in carcinogenesis has been investigated by several studies, which have indicated that the presence and frequency of MN can be used as a biomarker of genome instability, chromosomal damage, and cancer risk (2,6,7).

Evaluation of pleural effusion cytology and pleural biopsy specimens are among the most controversial topics in histopathology. Differentiation between malignant and

reactive mesothelial cells remains challenging not only in cytological samples, but also in pleural biopsies, even among expert pathologists (8,9). Invasion remains the best marker of malignancy for biopsies (10). Recently, depending on the molecular basis, detection of deletion of p16 by fluorescence in situ hybridization and loss of BAP1 by immunohistochemistry emerged as two important reliable markers for discriminating malignant from benign mesothelial proliferation, both in cytological specimens and biopsies (11). These are highly specific markers, yet their sensitivity is much lower (11,12). Despite the well-documented cytological characteristics of malignant mesothelioma, several ancillary techniques, and newly described molecular markers (13), differential diagnosis can be challenging in routine practice. On the other hand, the immunohistochemical staining panel, recommended by the International Mesothelioma Interest Group and World Health Organization, is used to distinguish metastatic carcinoma from mesothelial proliferation, in addition to well-described morphological features (10,14–16).

* Correspondence: tubadilay@gmail.com

In the present study, we evaluated the presence and frequency of MN count in conventionally prepared pleural effusion smears. We hypothesized that this chromosomal instability marker, which is easily detectable by light microscopy, can help us in differential diagnosis. Furthermore, it may be used as an adjunct tool for separating malignant from benign cells of pleural effusions. To the best of our knowledge, this is the first study in the literature that reveals MN scoring in pleural effusions, including in suspicious cases.

2. Materials and methods

This cross-sectional study was conducted following institutional review board approval (ID: 20102016/57-21). The cases with pleural effusions were retrospectively selected among surgical pathology reports, signed out between 2011 and 2016 from the archives of the Pathology Department, Kayseri Training and Research Hospital. Cases with malignant and suspicious cytological diagnosis and histopathological correlation were included in the study. Stained slides were deidentified and reviewed by two pathologists. Cases with only uniform agreement on both sample types were included in the study. Hypocellular samples and slides full of degenerated cells or staining artifacts were exempt. Finally, a total of 78 cytological cases were included. Specifically, 36 cases of malignant cytology ((16 cases of malignant mesothelioma (MM), 20 cases of adenocarcinoma (AC) originating from lung (10), breast (6) and ovary (4)), 22 cases of suspicious cytology, and 20 cases of reactive mesothelial cells were selected and reviewed for this study.

Cytological evaluation was performed according to the established morphological criteria and findings of routine immunohistochemical staining. Cases were classified into three main groups as benign reactive mesothelial proliferation (n = 20), suspicious for malignancy (n = 22), and malignant (n = 36) ((further subdivided into two groups as AC (n = 20) and MM (n = 16)). All patients with reactive mesothelial cells had neither history of malignancy nor any suspected laboratory or radiological findings at the time of diagnosis, as well as an average of 2 years follow-up. In suspicious cases, occasional mild to moderate cytological and structural atypia was observed, and the epithelial origin of cells was excluded by immunohistochemical methods. However, MM could not be excluded. By succeeding pleural biopsies, 12 cases of suspicious cytology were diagnosed as MM and 10 cases of suspicious cytology were diagnosed as reactive proliferation.

Alcohol-fixed and air-dried smears as well as cell blocks were prepared from pleural effusions and were evaluated. Conventionally prepared and May-Grünwald-Giemsa (MGG) stained slides were used for MN counting.

2.1. Evaluation and quantification of MN

Each cytology slide was reviewed under 400× and/or 600× magnification. Well-preserved mesothelial cells in benign cases and tumor cells in malignant cases were investigated for the presence and frequency of MN. The number of micronucleus-containing cells was counted per 1000 cells. In the review process, scoring of MN took approximately 25 min per case.

For the evaluation of MN, we used the following pre-established criteria: a diameter smaller than 1/3 of the main nucleus, round/oval shape with regular contours, same staining intensity with the main nucleus, and no connection to the main nucleus (3,17) (Figure).

We avoided degenerated cells and did not evaluate cells without cytoplasm. Additionally, with careful observation, we eliminated possible mimickers such as apoptotic fragments, superimposed lymphocytes, and staining artifacts, as mentioned in the literature (3).

2.2. Statistical analysis

Statistical analyses were performed using IBM SPSS 22.0. Numerical variables were calculated by medians (min-max) and means with SDs for different diagnostic groups. The Kolmogorov-Smirnov test was used to examine whether the numerical variables showed normal distribution or not. The Levene test was used to assess the equality of variances. The Mann-Whitney U test was performed to compare the number of cells with MN between benign and malignant groups, and the Kruskal-Wallis test was used for the comparison among groups. The Siegel Castellan test was performed for pairwise comparison of different groups. The post hoc power test was performed to test whether it has adequate power or not. Receiver operating characteristics (ROC) curve analysis was used to detect the best cut-off values to indicate malignancy with the highest sensitivity and specificity sum. Positive and negative predictive values were calculated. $P < 0.01$ was regarded as statistically significant.

3. Results

From the total series of 78 cases, 48 cases had a malignant outcome and 30 cases had a benign outcome. Malignant cases included both malignant mesothelioma (n = 28) and adenocarcinoma (n = 20). Twelve cases of MM were cytologically diagnosed as suspicious. The age of the patients ranged from 22 to 92. There were 44 male and 34 female patients.

When we compared our groups, we found that the MN score was significantly higher in malignant cases than in benign ones; the mean value of the MN count was 24 in malignant samples and only 6 in benign samples ($P < 0.001$, with a power of 0.99, Table 1). A comparison between cases with reactive mesothelial cells and cases with suspicious mesothelial proliferation revealed a

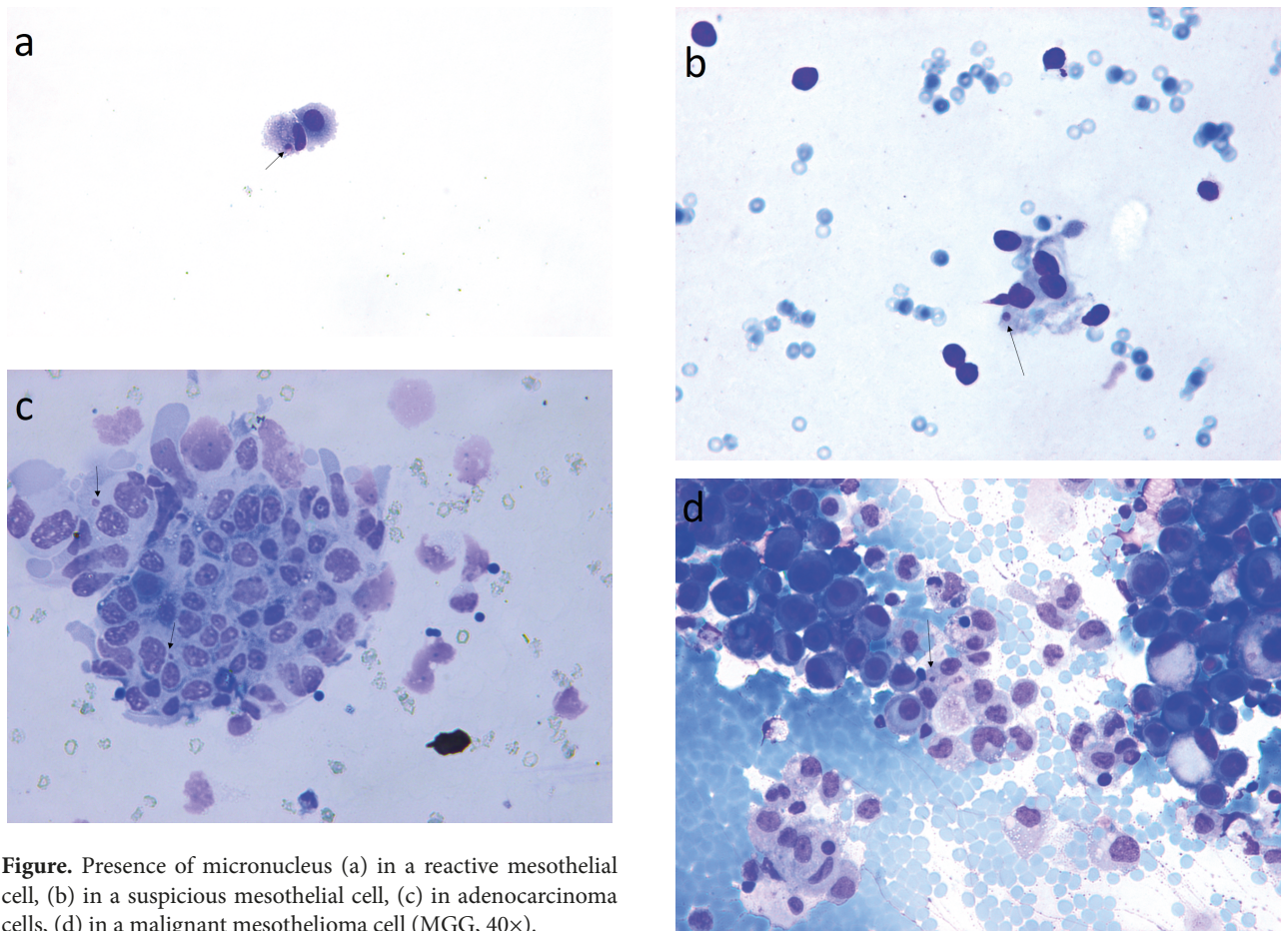


Figure. Presence of micronucleus (a) in a reactive mesothelial cell, (b) in a suspicious mesothelial cell, (c) in adenocarcinoma cells, (d) in a malignant mesothelioma cell (MGG, 40×).

Table 1. MN-containing cells in pleural fluid cytology of malignant and benign cases.

	Cases with benign outcome (n = 30)	Cases with malignant outcome (n = 48)	P
Number of MN-containing cells	6.4 ± 2.6 6 [1-10]	24.8 ± 10.3 24 [11-60]	<0.001*

MN: micronucleus, *: significant.

significant difference in MN count. Furthermore, our study showed that there is a significant difference between suspicious and malignant cases ($P < 0.001$, Table 2). In suspicious cytological samples, we observed that the cases with a malignant outcome had more micronucleated cells compared to cases with a benign outcome. The difference was statistically significant ($P < 0.001$, with a power of 0.99, Table 3). On the other hand, we observed that AC cases had a slightly higher number of micronucleated cells than MM cases. However, when we compared MM to AC cases, the number of MN-containing cells did not differ significantly ($P = 0.172$, Table 4).

Moreover, by using a ROC curve analysis, we revealed a cut-off of 11 for the MN count, giving a sensitivity of 1.0 and a specificity of 1.0 for detecting malignant cases ($AUC = 1.0$). While all the malignant outcome cases had ≥ 11 MN count, the highest MN count in only one-benign-outcome cases was 10.

The statistical analysis did not exhibit any relation between MN scores, age, and sex.

4. Discussion

In this retrospective cross-sectional study, we found that malignant and suspicious cytological samples have a higher

Table 2. Frequency of micronucleated cells in benign, suspicious, and malignant cytological samples.

	Benign (n = 20)	Suspicious (n = 22)	Malignant (n = 36)	P
Number of MN-containing cells	5.8 ± 2.9 5 [1-10]	16.3 ± 8 16 [6-28]	25.2 ± 11.5 25 [11-60]	<0.001*

MN: micronucleus, *: significant.

Table 3. Micronucleus frequency in suspicious cytological samples with different outcomes.

	Suspicious cytology (n = 22)		
	With benign outcome (n = 10)	With malignant outcome (n = 12)	P
Number of MN containing cells	8.2 ± 2.3 8 [6-12]	23.2 ± 5.2 24 [16-28]	<0.001*

MN: micronucleus, *: significant.

Table 4. MN counts in cytological slides in cases with a malignant outcome.

		<i>Histopathological diagnosis</i>			
		MM	AC	P	Total
		Number of MN; mean ± SD; median [min-max]			
<i>Cytological diagnosis</i>	Suspicious cytology with malignant outcome	n = 12 23.2 ± 5.2 24 [16-28]	n = 0	0.172	12
	Malignant cytology	n = 16 21.7 ± 5.0 21 [14-28]	n = 20 29.0 ± 14.1 25 [11-60]		36
Total		28	20		48

MN: micronucleus; MM: malignant mesothelioma; AC: adenocarcinoma.

MN score than benign pleural effusion samples consisting of reactive mesothelial proliferation. We noted that malignant cases had a higher MN count than suspicious cases, and suspicious cases had a higher MN count than benign cases. The difference among all study groups was significant ($P < 0.001$). In addition, micronucleus scoring in suspicious cases correlates well with their outcome. We concluded that MN scoring is a simple and easy-to-use method for the evaluation of pleural effusions, especially in challenging cases. It can be used as an adjunct tool, because an increased number of cells containing MN have

a good diagnostic ability to discriminate between benign and malignant cases.

MN is a small round/oval nucleus not connected to the main nucleus. It contains whole chromosome or chromosomal fragment (17,18). Potential mechanisms that lead to MN formation include chromosomal breakage, dysfunction of mitotic apparatus, and defect in mitotic spindles (3,19). MN can be formed in healthy individuals, and the presence of micronucleated cells can indicate initial stages of nuclear damage for any reason. Consistently with the literature, but to a lesser extent,

cases with benign reactive mesothelial cells in our study showed micronucleus formation. This nuclear feature is a nuclear envelope alteration indicating chromosomal instability, and it is included in the list of cytological criteria of malignancy (20) and high frequency of MN formation can be a clue for malignancy (21). Accordingly, in the present study, we counted a considerably high number of micronucleated cells in malignant cases.

Under the light microscope, MN formation is a clearly visible, but often overlooked cytological finding. It is not even mentioned in pathological examination, in contrast to other nuclear alterations such as moldings, inclusions, grooves, nuclear shape irregularities, koilocytes, and chromatin texture [20]. However, it is an important indicator of genetically damaged cells and is found more frequently in malignant cells. Surprisingly, there are limited studies in the literature evaluating its diagnostic usefulness from a cytopathological point of view. In the present study, we evaluated the presence and frequency of MN in pleural fluid cytology. The limited studies in this field clearly pointed out that neoplastic lesions had high MN counts compared to their benign counterparts (22–24). Consistently with the literature, we found that all the cytological smears with a malignant outcome had a significantly higher MN count compared to benign ones ($P < 0.001$, Table 1). According to the literature, in the fine needle aspirates of breast lesions, ductal carcinoma cells had significantly higher MN counts than cells of fibroadenoma (25,26). Likewise, in cervical smears, cases of high-grade intraepithelial lesions or invasive carcinoma had more micronucleated cells than cases of reactive changes (27). Furthermore, the presence of MN was noted in malignant thyroid aspirates (28,29). It is found that the smears of papillary thyroid carcinoma include a significantly higher number of micronucleated cells than smears of follicular nodular disease (29). Besides fine needle aspiration smears, the frequency of MN helped to detect atypical cells in urine samples and ascitic effusion, indicating malignancy (24,30,31). Moreover, our findings are consistent with the findings of studies on the frequency of MN in exfoliative, fine needle aspiration, and effusion smears (22,25–29,30–34).

Pleural effusion cytology is the first important step in the diagnosis of malignancy, especially malignant mesothelioma. Although cases with prominent benign or malignant morphology are far from a diagnostic challenge, suspicious cases are often problematic. There is no definite method for differential diagnosis (10). The most important part of this study was exploring the role of MN scoring in suspicious cases. We observed that suspicious cases had significantly higher MN frequency than reactive cases and lower MN frequency than malignant cases ($P < 0.001$). These findings are

compatible with the results of other studies, which indicate that MN scoring is correlated to the grade of malignancy in breast aspirations and cervical smears (22,27,32). In addition, we found that MN counts in these suspicious cytological samples are correlated well with their final diagnoses ($P < 0.001$, with a power of 0.99). As a limitation to this study, we had a limited number of suspicious cases, but our results showed clearly that malignant cells have invariably increased MN frequency, and MN scoring was completely successful in indicating malignancy.

Accepting that in daily practice MN counting in every case might be time-consuming, we consider that it might be used in challenging cases. A high MN count can guide pathologists in deciding whether mesothelial proliferation consists of benign or malignant cells or not, in addition to other findings.

Moreover, in this study we investigated the role of MN scoring in differentiating MM from AC. We found higher MN counts in cases of AC than in MM, and ROC analysis revealed a cut-off value of 29 for MN count to distinguish these cases. However, there was no statistically significant difference between AC and MM. In this study, we examined a limited number of cases, which might result from low sample size. We hope that further extended studies may clarify this point.

Finally, when considering all malignant-outcome and benign-outcome cases, we suggest a working cut-off value of ≥ 11 for an MN count with 100% sensitivity and specificity for detecting malignant cases. A recently published study found a cut-off value of >5 for malignant cases; however, it examined fewer cases than ours and did not evaluate any suspicious ones (35). These studies are initial and limited, and we think that further and larger studies are required to attain an absolute cut-off value for MN scoring.

In conclusion, this is the first study in the literature that indicates the importance of the frequency of MN-containing cells in suspicious cytological preparations of pleural effusion, which predicts malignant behavior in these samples. Based on previous literature and the promising results of our study, it is clear that there is an absolute, consistent, and proportional relationship between MN counts and malignancy in cytological samples of pleural effusions. This finding is very important, because discrimination of malignant mesothelial cells from benign reactive cells may be difficult, or even impossible, in cytological differential diagnosis. Therefore, we aimed to reveal the potential diagnostic usefulness of MN in pleural effusion samples. The question of usability of this nuclear feature in the routine cytological examination of pleural specimens needs to be addressed via further studies.

References

- Iarmarcovai G, Ceppi M, Botta A, Orsière T, Bonassi S. Micronuclei frequency in peripheral blood lymphocytes of cancer patients: a meta-analysis. *Mutat Res* 2008; 659: 274-283.
- Fenech M, Holland N, Chang WP, Zeiger E, Bonassi S. The HUMAN MicroNucleus Project—an international collaborative study on the use of the micronucleus technique for measuring DNA damage in humans. *Mutat Res* 1999; 428: 271-283.
- Samanta S, Dey P. Micronucleus and its applications. *Diagn Cytopathol* 2011; 40: 84-90.
- Stopper H, Muller SO. Micronuclei as a biological endpoint for genotoxicity: a mini review. *Toxicol In Vitro* 1997; 11: 661-667.
- Fisher AH. Diagnostic pathology of the nuclear envelope in human cancers. In: Schirmer EC and de las Heras JJ, editors. *Cancer Biology and the Nuclear Envelope*. New York, NY, USA: Springer, 2014. pp. 49-75.
- Mateuca R, Lombaert N, Aka PV, Decordier I, Kirsch-Volders M. Chromosomal changes: induction, detection methods and applicability in human monitoring. *Biochimie* 2006; 88: 1515-1531.
- Bonassi S, El-Zein R, Bolognesi C, Fenech M. Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies. *Mutagenesis* 2011; 26: 93-100.
- Cagle PT, Churg A. Differential diagnosis of benign and malignant mesothelial proliferations on pleural biopsies. *Arch Pathol Lab Med* 2005; 129: 1421-1427.
- Churg A, Galateau-Salle F. The separation of benign and malignant mesothelial proliferations. *Arch Pathol Lab Med* 2012; 136: 1217-1226.
- Galateau-Salle F, Churg A, Roggli V, Travis WD. The 2015 World Health Organization classification of tumors of the pleura: advances since the 2004 classification. *J Thorac Oncol* 2016; 11: 142-154.
- Churg A, Sheffield BS, Galateau-Salle F. New markers for separating benign from malignant mesothelial proliferations: are we there yet? *Arch Pathol Lab Med* 2016; 140: 318-321.
- Walts AE, Hiroshima K, McGregor SM, Wu D, Husain AN, Marchevsky AM. BAP1 Immunostain and CDKN2A (p16) FISH analysis: clinical applicability for the diagnosis of malignant mesothelioma in effusions. *Diagn Cytopathol* 2016; 44: 599-606.
- Whitaker D. The cytology of malignant mesothelioma. *Cytopathology* 2000; 11: 139-151.
- Pereira TC, Saad RS, Liu Y, Silverman JF. The diagnosis of malignancy in effusion cytology: a pattern recognition approach. *Adv Anat Pathol* 2006; 13: 174-184.
- Su XY, Li GD, Liu WP, Xie B, Jiang YH. Cytological differential diagnosis among adenocarcinoma, epithelial mesothelioma, and reactive mesothelial cells in serous effusions by immunocytochemistry. *Diagn Cytopathol* 2011; 39: 900-908.
- Husain AN, Colby T, Ordóñez N, Krausz T, Attanoos R, Beasley MB, Borczuk AC, Butnor K, Cagle PT, Chirieac LR et al. Guidelines for pathologic diagnosis of malignant mesothelioma: 2012 update of the consensus statement from the International Mesothelioma Interest Group. *Arch Pathol Lab Med* 2013; 137: 647-667.
- Fenech M, Kirsch-Volders M, Natarajan AT, Surrallés J, Crott JW, Parry J, Norppa H, Eastmond DA, Tucker JD, Thomas P. Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. *Mutagenesis* 2011; 26: 125-132.
- Bhatia A, Kumar Y. Cancer cell micronucleus: an update on clinical and diagnostic applications. *APMIS* 2014; 121: 569-581.
- Crasta K, Ganem NJ, Dagher R, Lantermann AB, Ivanova EV, Pan Y, Nezi L, Protopopov A, Chowdhury D, Pellman D. DNA breaks and chromosome pulverization from errors in mitosis. *Nature* 2012; 482: 53-58.
- Fischer AH, Zhao C, Li QK, Gustafson KS, Eltoum IE, Tambouret R, Benstein B, Savaloja LC, Kulesza P. Cytologic criteria of malignancy. *J Cell Biochem* 2010; 110: 795-811.
- Luzhna L, Kathiria P, Kovalchuk O. Micronuclei in genotoxicity assessment: from genetics to epigenetics and beyond. *Front Genet* 2013; 4: 131.
- Shi YH, Wang BW, Tuokan T, Li QZ, Zhang YJ. Association between micronucleus frequency and cervical intraepithelial neoplasia grade in Thinprep cytological test and its significance. *Int J Clin Exp Pathol* 2015; 8: 8426-8432.
- Hemalatha A, Suresh TN, Harendara Kumar ML. Micronuclei in breast aspirates. Is scoring them helpful? *J Cancer Res Ther* 2014; 10: 309-311.
- Kaur J, Dey P. Micronucleus to distinguish adenocarcinoma from reactive mesothelial cell in effusion fluid. *Diagn Cytopathol* 2009; 38: 177-179.
- Samanta S, Dey P, Nijhawan R. The role of micronucleus scoring in fine needle aspirates of ductal carcinoma of the breast. *Cytopathology* 2010; 22: 111-114.
- Goel S, Bhatia A, Dey P. Spontaneously occurring micronuclei in infiltrating ductal carcinoma of breast: a potential biomarker for aggressive phenotype detection? *Diagn Cytopathol* 2011; 41: 296-302.
- Samanta S, Dey P, Nijhawan R. Micronucleus in cervical intraepithelial lesions and carcinoma. *Acta Cytol* 2010; 55: 42-47.
- Nilsson G. Micronuclei studied in fine needle goitre aspirates. *Acta Pathol Microbiol Scand A* 1978; 86: 201-204.
- Kokenek-Unal TD, Coban I. Micronuclei and nuclear buds: highlighting overlooked indicators of chromosomal damage in thyroid aspiration smears. *Diagn Cytopathol* 2017; 45: 673-680.

30. Arora SK, Dey P, Saikia UN. Micronucleus in atypical urothelial cells. *Diagn Cytopathol* 2010; 38: 811-813.
31. Tyagi R, Dey P, Uppal R, Rajwanshi A. Analysis of morphological markers of chromosomal instability in ascitic fluid. *Diagn Cytopathol* 2015; 43: 855-858.
32. Samanta S, Dey P, Gupta N, Mouleeswaran KS, Nijhawan R. Micronucleus in atypical squamous cell of undetermined significance. *Diagn Cytopathol* 2011; 39: 242-244.
33. Verma S, Dey P. Correlation of morphological markers of chromosomal instability in fine needle aspiration cytology with grade of breast cancer. *Cytopathology* 2013; 25: 259-263.
34. Wen CH, Lin CH, Tsao SC, Su YC, Tsai MH, Chai CY. Micronucleus scoring in liver fine needle aspiration cytology. *Cytopathology* 2013; 24: 391-395.
35. Ganesan N, Phansalkar MD, Ambrose MM, Varghese RG. Validating micronucleus score in effusion fluids. *J Cytol* 2017; 34: 193-196.