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CASE REPORT

Cytopathology and the Micronucleus Test in Monitoring Post-Radiotherapy Patients for Cervical Cancer

Cátia Padilha

*catialeitepadilha@gmail.com

ABSTRACT

The use of radiotherapy as a form of treatment for cervical cancer induces morphological changes both in neoplastic cells and in normal epithelial and stromal cells. These alterations represent a challenge for the identification of residual lesions, generating a scenario of complexity in the post-radiotherapy cytopathological evaluation. Therefore, the objective of this study was to comparatively analyze the cytopathological examination (Conventional Cytology X Liquid-Based Cytology) with the Micronucleus Test of post-radiotherapy patients for cervical cancer and to describe the genotoxicity of radiation. This is a cross-sectional, descriptive study whose population consisted of ten patients with cervical cancer undergoing radiotherapy at the Cancer Hospital II of the National Cancer Institute (INCA – Rio de Janeiro/RJ, Brazil). For analysis of the Micronucleus Test, a control group with ten patients, equivalent to the study group, was included. The results showed that the low quality of Conventional Cytology in cases of post-radiotherapy control of cervical cancer can be a barrier to the speed of diagnosis and identification of recurrent lesions. A relevant alternative is the use of Liquid-Based Cytology, which contributes positively to the quality of diagnosis in irradiated patients, mainly associated with the Micronucleus Test, but the adoption of Liquid-Based Cytology for mass screening may be hampered by the cost of the technique. On the other hand, the use of the Micronucleus Test, which proved to be significant in irradiated patients, is a low cost and easy analysis technique. Therefore, in view of the above, we propose, as a follow-up protocol for patients undergoing radiotherapy for cervical cancer, performing Liquid-Based Cytology associated with the Micronucleus Test, which can be extended to other types of tumors.

Keywords: Uterine Cervical Cancer. Radiotherapy. Cytopathology. Micronucleus. Cellular Genotoxicity.

Introduction

The current approach to cancer control is conceived as a continuous sequence of actions, starting with the management of exposure to risk agents, followed by early identification of the lesion, treatment and culminating in patient follow-up[1].

When the lesion is identified in advanced stages, it will lead to less effective and sometimes palliative treatments, this step will require monitoring throughout the survival phase and care at the end of life, for individuals who do not achieve cure or disease control[2].

Global statistical data show that cervical cancer represents the fourth most prevalent neoplasm among females, registering a projection of 604,000 new cases, which is equivalent to 6.5% of all cancer cases in the world[3].

In Brazil, the annual estimated number of new cases of cervical cancer, in the period from 2023 to 2025, amounts to 17,010. This figure translates into a potent risk of 15.38 cases per 100,000 women[4].

Despite advances in molecular techniques, the recommendation of the World Health Organization for the early detection of cervical cancer is to perform a Pap smear[5]. Brazilian guidelines recommend starting screening from the age of 25 in pregnant women or not, as long as they have started sexual activity[6].

In this sense, several authors have demonstrated that Liquid-Based Cytology (LBC) has superior sensitivity and specificity when compared to Conventional Cytology (CC) for cyto-oncotic diagnosis[7–9].

The adoption of the liquid-based technique offers the advantage of reducing unsatisfactory results due to the ability to filter cell debris and leukocyte exudates from the sample[10].

However, the low cost associated with CC maintains its relevance as the method of choice for the prevention of cervical cancer, with regard to mass screening, in view of its accessible and effective attributes[10–12].

Though, the delay in diagnosing cancer and its precursor lesions can result in more invasive and less effective therapeutic interventions, generating increased costs with hospitalizations and a high mortality rate[13,14]. Factors such as shyness, apprehension, access restrictions or lack of information contribute to early detection remaining on the margins of prevention strategies[15].

A significant proportion of patients diagnosed with malignant neoplasm of the uterine cervix who are referred for radiotherapy manifest the disease in advanced stages, a circumstance that contributes to high rates of recurrence at the local and regional level[16].

The use of radiotherapy in the treatment of cervical cancer induces morphological changes both in neoplastic cells and in normal epithelial and stromal cells[17] These changes constitute a challenge for the identification of the residual lesion, generating a scenario of complexity in the post-radiotherapy cytopathological evaluation[18].

Cytopathological analysis for the follow-up of patients after radiotherapy becomes more difficult due to the cellular actinic effects and the quality of the gynecological material,

since fibrosis and stenoses caused by radiation impair the collection of biological material and lead to a representative number of unsatisfactory samples[13,14,19].

In this context, the LBC significantly contributes to obtaining satisfactory samples without the interference of residues and with cellular representativeness due to the anatomical coverage of the gynecological regions, in relation to the CC, promoting the improvement of the quality in the diagnosis[8,20,21].

In order to improve sensitivity in screening for cervical cancer, other complementary methods have been described, we can highlight molecular tests for the identification of the high-risk Human Papillomavirus (HPV), recognized as an etiological factor of intraepithelial lesions and cervical cancer[2,22] and the micronucleus identification test[22,23].

The Micronucleus Test (MN) has been employed as an effective biomarker, demonstrating success particularly in populations at high risk of cancer, as it has proved to be a sensitive indicator of genetic damage[23].

Chromosomal damage often results from exposure of cells to various genotoxic agents, such as ionizing radiation, chemicals, or biological agents[24]. As a result, they are detected both numerically and structurally using the MN Test, offering an accessible approach to assessing epithelial lesions[25].

Therefore, the objective of this study was to comparatively analyze the Cytopathological Examination CC X LBC with the Test MN of post-radiotherapy patients for cervical cancer and to describe the genotoxicity of the radiation.

Material and Methods

This is a cross-sectional, descriptive study, where the population consisted of patients from the Hospital do Cancer II (HC II) of the Instituto Nacional do Cancer (INCA – Rio de Janeiro / RJ, Brazil), with cervical cancer who underwent the radiotherapy. The work approved by the Research Ethics Committee of the National Cancer Institute (CEP/INCA). It had the numbering of the Certificate of Ethical Appreciation Presentation / CAAE: 64287517. 0.0000.5274 and the Opinion: 2.104.514.

A Control Group (CG) was included for comparative analysis with the MN Test, quantitatively equivalent to the Study Group (SG). The CG volunteers were recruited by the main researcher, with the following inclusion criteria: annual frequency of performing a colpocytological exam (Papanicolaou), absence of intraepithelial lesions in all exams performed throughout their lives and agreement and signature of the Term of Free and Informed Consent (TCLE), as part of the ethical protocol. The collection was carried out by an experienced professional, a volunteer, in a private clinic, located in the city of Rio de Janeiro.

Identification and Collection of Information and Sample

SG patients were identified and recruited at the HCII gynecology outpatient clinic. Clinical and epidemiological data were collected from medical records, then the researcher responsible for the project individually interviewed the selected patients, explaining the scope of the study.

All collected data were cataloged in an instrument developed for this purpose, which

also contained a questionnaire aimed at understanding and perception of patients in relation to the disease and radiotherapy treatment, each interview took about 20 minutes.

After signing the informed consent form, the patients were referred for material collection by the head of the Gynecology Oncology Section, accompanied by the main researcher. Gynecological samples were collected in the following order: a smear was taken and immersed in fixative (70% alcohol) for Papanicolaou staining and CC was performed, which is the institution's standard procedure.

Then a second brush was used for collection and immersed in a conical bottom tube (Falcon) containing 5 ml of saline solution, for the micronucleus test (MN), which used Giemsa staining. Finally, a third sample was collected and immersed in liquid medium, together with the brush from the CC sample (first collection), to perform the LBC.

For the collection of the last sample directed to perform the LBC, "CellPreserv" material was used, which identifies the "Kolplast" liquid-based cytology method.

Through the analysis of the medical records, the treatment protocol was identified, which involved a combination of external radiotherapy: megavoltage at a dose of 45 to 50Gy, in 25 fractions, followed by intracavitary brachytherapy: high dose rate (HDR), with micro source of Iridium 192, with 3 insertions of 8Gy, with weekly intervals, totaling 80Gy.

Sample Processing

The smears for CC were sent to the Integrated and Technological Service in Cytopathology (SITEC) of the Division of Pathology (DIPAT) at INCA, as a standard procedure for monitoring patients after radiotherapy. The two other samples (for the MN Test and LBC) were processed and stained by the researcher responsible for the study, at DIPAT/INCA.

CC smears were stained by the Papanicolaou method and analyzed by the DIPAT pathologist (standard procedure of the institution), later reviewed by the main researcher. The LBC material was also stained by the Papanicolaou method and analyzed by the main researcher of the study. The material for the MN Test, both the CG and the GS, were stained using the Giemsa method and analyzed by the main researcher.

Statistical Analysis of Samples

In the project schedule, the period established for collection was six months (from January to July 2001). However, due to the COVID-19 pandemic, the period was postponed to the second half. At the time, considering the inclusion and exclusion criteria, it was possible to obtain a series of 10 patients in the GS and the equivalent number (n=10) of female volunteers for the CG.

The collected epidemiological and cytopathological data were tabulated in the Microsoft Excel program and the statistical tests were analyzed in the SPSS v20.3 program. To assess the validity of the micronucleus tests, sensitivity, specificity, positive and negative predictive value were calculated, considering the cytopathological test as standard.

Regarding sample size calculation, we aimed to estimate a minimum size that would be sufficient to achieve the target level of

precision in our estimates for a specific population parameter and we generally use the GPower tool, which is completely reliable in the scientific community.

However, it is important to note that, in the context of this study, non-parametric statistical tests were used due to the nature of the data and research hypotheses. On the other hand, we are also concerned with determining the level of statistical significance, which is often represented by the P value <0.05, through the desired effect sizes and analysis.

Therefore, to evaluate the number of micronucleated cells and micronuclei per cell, according to the results of the cytopathological tests, the "Student's t" test was calculated, being considered statistically significant, $p < 0.05$.

Results

All patients in the CG completed the radiotherapy protocol. Considering the main combinations and therapeutic approaches that are used for cervical cancer, we observed that most patients underwent radiotherapy concomitantly with chemotherapy (60%) and the others radiotherapy alone (40%). No patient underwent surgery associated or not with chemotherapy combined with radiotherapy.

The average age at diagnosis of cervical cancer was 45.4 years (ranged from 26 to 80 years) on the date of collection, an age variation of 52.2 years (ranged from 33 to 85 years) was observed.

At the time of the interview, when asked about the frequency of carrying out the preventive examination for cervical cancer (Papanicolaou), 30% of the patients (n=3) answered that they underwent it a year ago, 30% (n=3) underwent the examination two years ago, 10% (n=1) performed it more than 3 years ago, 10% (n=1) performed it 4 years ago, 10% (n=1) performed it 7 years ago, and 10% (n=1) did not did you remember when you did the last preventive.

Of the recruited patients, 70% (n=7) had a histological diagnosis of squamous cell carcinoma, and 30% (n=3) adenocarcinoma, with clinical staging: IB2 (n=1), IIB (n=6) and IIIB (n= 3). Half of the patients (n=5) had a family history of cancer, 50% were white and 50% brown. Regarding smoking habits, 20% were smokers and 10% former smokers, the others never smoked, 20% declared themselves alcoholics. The average number of sexual partners was 4 partners (ranged from 1 to 16 sexual partners). As for marital status, 40% were married, 20% single, 20% widowed and 20% in a consensual union.

Regarding the CC of the LBC, it is important to emphasize that the analysis was performed by two professionals, a cytopathologist (standard procedure for follow-up cases) and a main researcher, independently, without prior knowledge of the results (double-blind review), and there was no difference in diagnosis in 9 out of 10 cases. However, in 1 case (Patient # 4), the analysis using CC was unsatisfactory for cyto-oncotic evaluation because it was purulent and thick, but it was possible to make a quality diagnosis using the material processed in LBC, where neoplastic cells were not distributed. **(Figure 1)**

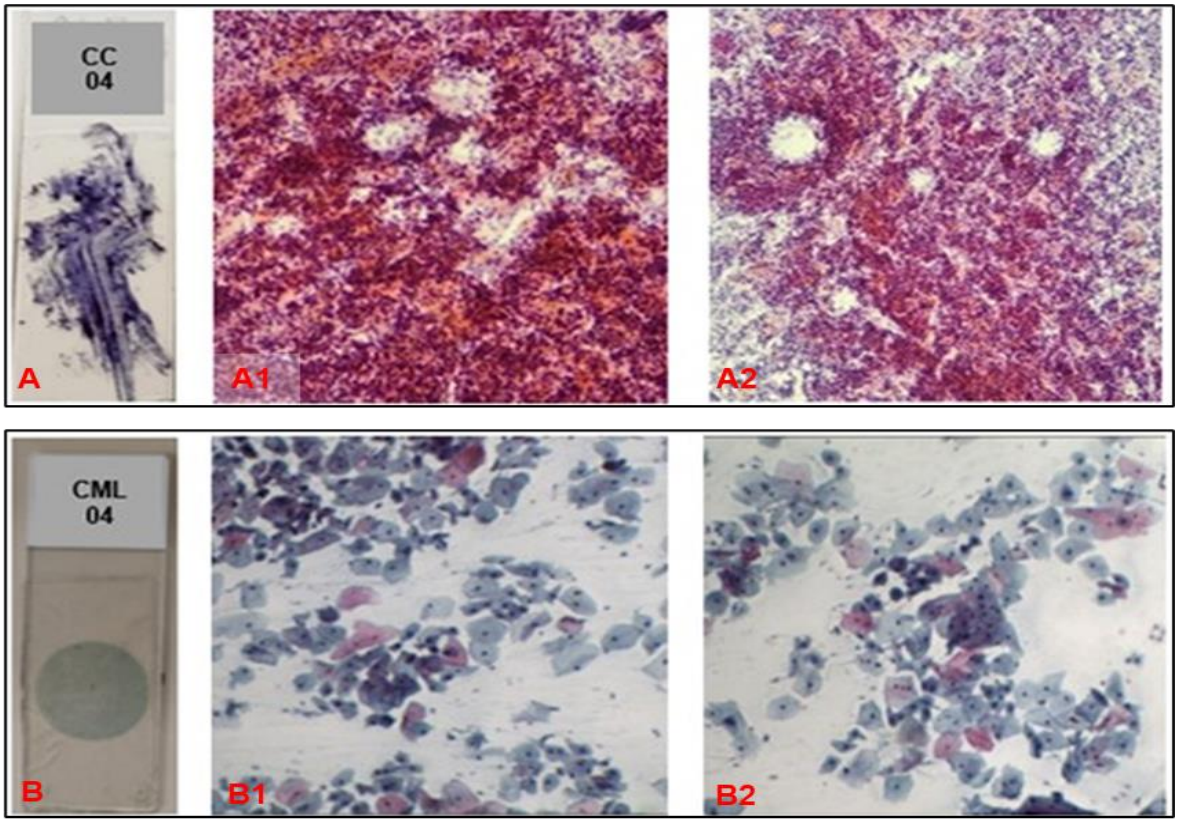


Figure 1 – Images of the smears and micrographs taken under an optical microscope (100x magnification) with material collected from the same patient, stained using the Papanicolaou method. A – CC smear and B – Liquid-Based Cytology slide, identified as CML (equivalent to the acronym LBC in Portuguese). Micrographs A1 and A2 demonstrate the presence of leukocyte infiltrate (purulent and thick smear), unsatisfactory for cyto-oncotic evaluation. However, in micrographs B1 and B2, squamous cells and rare polymorphonuclear leukocytes are observed in satisfactory material for diagnosis.

In both groups (CG and SG) all cases were negative for malignancy, in the GS benign cells were observed with alterations compatible with actinic/radiotherapy effects (nuclear and cytoplasmic enlargement, cellular gigantism, pleomorphism, cytoplasmic vacuolation, anisokaryosis, macro and multiple nucleoli, amphophilia, karyorrhexis, nuclear pyknosis, nuclear vacuolation, binucleation and multinucleation).

In the CG, the average age of women was 42.2 years, ranging from 36 to 52 years. None had a family history of cancer, 20% brown and 80% white; 20% were ex-smokers and 80% had never smoked, most declared themselves to be social drinkers (80%) and 20% do not use alcoholic beverages and all were married. For the evaluation of MN, 2,000 cells were analyzed per slide, considering only non-fragmented and non-overlapping cells. In the CG, 1 MN was observed in the slide of 2 women (20%) and only 1 binucleation.

In SG, MN were identified on all slides, with an average of 3 micronuclei per patient (ranging from 1 to 6). As for binucleations, only 1 slide was not found, however a multinucleated cell was identified in this slide. Multinucleations were present in 40% (n=4) of patients exposed to radiation (SG). **(Table 1)**

PATIENTS	CONTROL GROUP (CG)			STUDY GROUP (SG)		
	Nª MN	N° Bin	N° Multi	Nª MN	N° Bin	N° Multi
Patient #01	0	0	0	5	5	2
Patient #02	0	2	0	3	2	0
Patient #03	1	0	0	6	4	0
Patient #04	0	0	0	3	10	1
Patient #05	0	0	0	2	0	1
Patient #06	0	0	0	3	1	0
Patient #07	0	0	0	2	2	0
Patient #08	1	0	0	1	2	0
Patient #09	0	0	0	4	3	1
Patient #10	0	0	0	3	1	0

MN: micronuclei. Bin: binucleations. Multi: multinucleations.

Table 1 - Distribution of patients in the CG and those exposed to radiation (post-radiotherapy) SG, according to the MN Test.

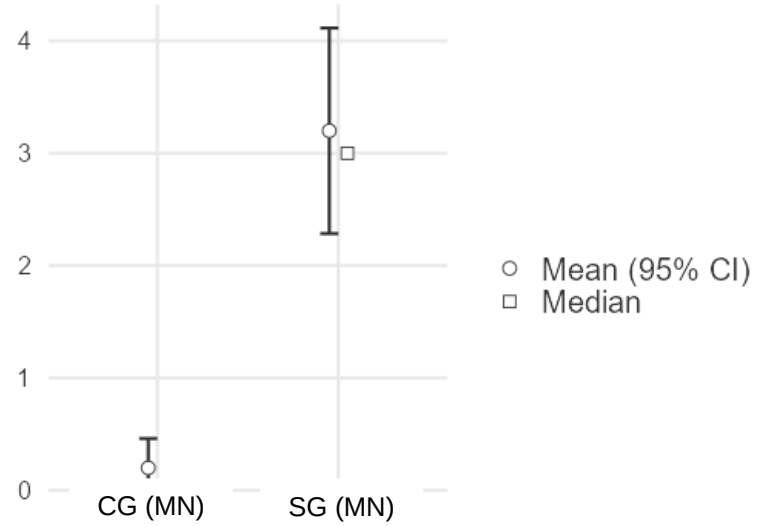
The comparative analysis between the CG and SG, considering the table above and based on the literature: The Design of Experiments (Fisher, 1935), revealed a significant difference. In this case, in particular, a non-parametric method was used, as it was a small sample. Thus, H0: CG = SG and Ha: CG ≠ SG were tested, and the Wilcoxon (W) test was used, which is equivalent to the paired t-test, that is, it tests a set of before and after values for the same person.

Therefore, regarding the MN analysis, "W" is significant, given that (W; p) = (0.00; 0.008). This value of p = 0.008 which is less than 5% (default represented by p = 0.05) which indicates the existence of a statistically significant difference between the previous and subsequent measurements. Table 2 shows the confidence intervals for the control group (CG), CI = [0.20 ± 0.42] and for the study group / exposed to radiation (SG), CI = [3.20 ± 1.48].

Descriptions	CG (MN)	SG (MN)
N	10	10
Mean	0.20	3.20
Median	0.00	3.00
Standard Deviation	0.42	1.48
25° Percentil	0.00	2.25
50° Percentil	0.00	3.00
75° Percentil	0.00	3.75

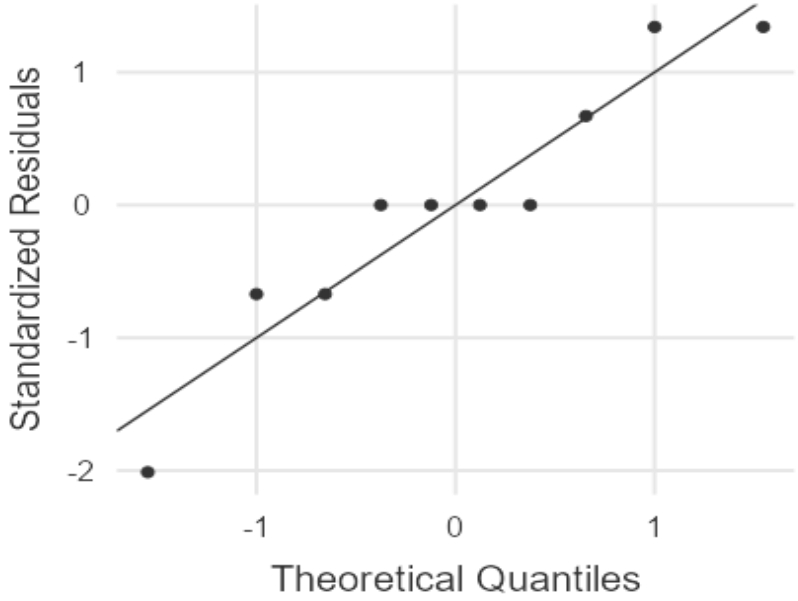
Table 2 - Descriptive statistics in relation to micronuclei: control group (CG) and study group / exposed group (SG).

Graph 1 shows the error bars for the CG and SG (exposed to radiation), it can be seen that the mean, median and confidence interval (CI) are constructed from the measurements descriptions for the MN Test.



Graph 1 - Distribution of error bars in relation to CG and SG, for the MN Test.

Graph 2 represents the quantile-quantile (QQ-Plot), constructed using the Jamovi tool. Whereas the QQ-Plot evaluates the normality of the data, it compares the quantiles of the probability data and, when the elements lie on top of the line, it shows that the data have an adequate normal distribution. However, an observation of deviation from the straight line suggests asymmetry in the data, thus justifying the choice of a non-parametric test.



Graph 2 – QQ-Plot with the distribution of the studied groups.

Figures 2 and 3, show the images of the slides referring to the MN Test of the ten patients exposed to radiation in the SG, in the first column the slides stained by the Giemsa method, in the second column microscopic images of the micronucleated cells (increase 400x) and in the third column microscopic images of binucleated and/or multinucleated cells (400x magnification).

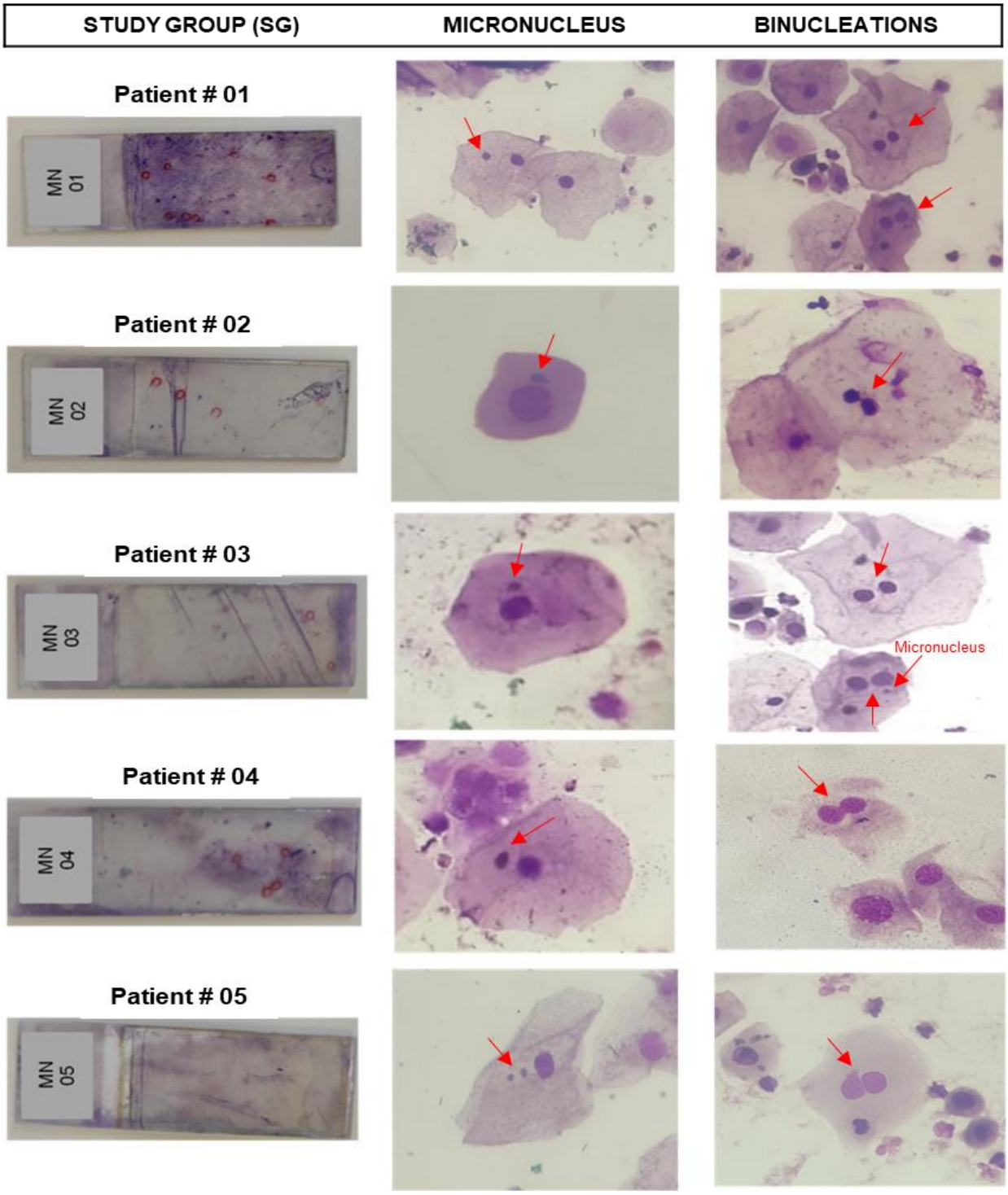


Figure 2 – Images of the slides of patients identified from #01 to #05 of the SG, the second column shows the micronuclei, and the third column shows binucleation, all structures are highlighted by the red arrows.

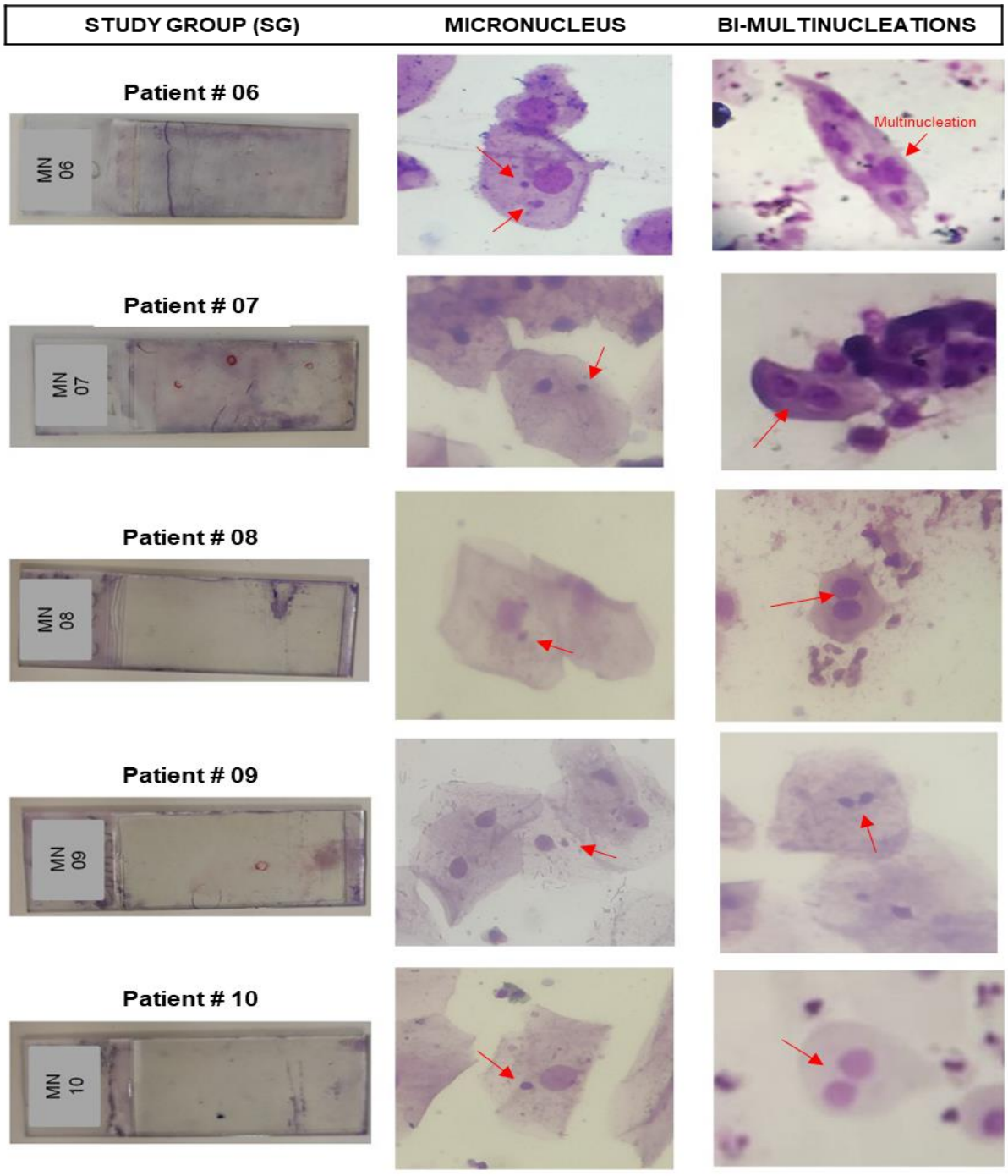


Figure 3 – Images of the slides of patients identified from #06 to #10 of the SG, the second column shows the micronuclei, and the third column shows the binucleation and one multinucleation, all structures are highlighted by the red arrows.

The analysis of binucleations revealed a higher frequency of these findings in patients from the SG compared to women from the CG. The Wilcoxon (W) test was applied to both groups. In the SG the "W" was significant (0.00; 0.014), that is, $p < 0.05$, on the other hand in the CG it was different because the value was $p = 0.089 > 0.05$, therefore it is relevant to consider the binucleation as a significant alteration in post-radiotherapy cytology.

Discussion

Several studies concerning patients undergoing radiotherapy for cervical cancer have shown an average age of patients of approximately 46.8 years,[13,26,27] which demonstrated proximity to the women in our SG (45.4 years), on the other hand, it presented a wide variation, covering ages between 26 and 80 years.

It is important to emphasize that 30% of them, at the time of the interview, reported that they did not follow the recommendation regarding the frequency of carrying out the preventive cytological test (Papanicolaou), in the public health system where only CC is performed. In Brazil, the guidance for screening for cervical cancer and its precursor lesions is to repeat the exam every three years, after two consecutive normal exams performed with an interval of one year[10,16].

In a study carried out by Rosa, et al. (2016)[28], showed that the sensitivity of CC is 54.0% and specificity 91.0%. However, several studies demonstrate that LBC can considerably increase the quality of the diagnosis, both in sensitivity and specificity, exceeding 87% and 99% respectively[7,20,29,30].

Despite the notable advantages offered by LBC and its relative simplicity in collecting and processing cervical samples, it is imperative to consider that CC retains its clinical utility significantly, both methods found effective in detecting cervical anomalies and cervical cancer, and the choice between them must be based on criteria that consider not only their technical characteristics, but also economic and logistical factors[31].

However, in cases of post-radiotherapy follow-up for cervical cancer, LBC appears as the most indicated approach where the quality and precision of the CC may be compromised due to anatomical and tissue changes[32]. Collecting representative samples can make a task more challenging in these scenarios, as benign changes resulting from irradiation, post-irradiation dysplasia, and the frequent presence of repair cells and active stromal cells in post-irradiation smears can cause diagnostic problems[18].

Even considering the size of our sample, it is important to emphasize that one of the CC samples was unsatisfactory for diagnosis due to being purulent and thick; however, it became satisfactory when high-quality LBC was used for cyto-oncotic analysis.

In a comparative study between CC and LBC of patients undergoing radiotherapy for cervical cancer, it was concluded that LBC had better performance compared to CC to detect recurrence of squamous cell carcinoma, its sensitivity, specificity and accuracy were significantly higher than the conventional method[21].

Zannoni, et al. (2008), demonstrated in their work, which included 50 women diagnosed with advanced cervical carcinoma undergoing radiotherapy, that residual neoplastic cells showed a broad pattern of cytoplasmic and nuclear changes that interfere with interpretation and diagnosis[33].

Indeed, radiation-induced changes can simulate recurrent malignant neoplasia in negative cases[34]. On the other hand, atypias in recurrent carcinoma cells can be interpreted as post-radiotherapy actinic alterations, in this case leading to underdiagnosis[13].

There is great concern about toxicity rates after radiotherapy and chemotherapy due to many controllable variables related to

treatment, such as exposure to ionizing radiation, which involves dose, fractionation, and sensitization of normal tissues, with this being of great concern. Detailed attention to all these factors is of great importance[35].

Research conducted by Nersesyan (2007) proposed that the analysis of micronucleus taxa in exfoliated cervical cells can serve as a supplementary parameter to assess the potential risk associated with cervical cancer. This approach is capable of enhancing both the sensitivity and specificity of cytology, potentially influencing diagnostic accuracy and the effectiveness of secondary prevention of cervical cancer[24].

Recent work demonstrated that May-Grünwalds-Giemsa staining and Papanicolaou staining obtained very similar results when it comes to counting micronuclei (MN) in cellular samples, demonstrating that both techniques are equally effective in evaluating genomic instability. Furthermore, this study also revealed that human papillomavirus plays a crucial role in the induction of MN, highlighting its potential as a marker of genotoxicity[36].

However, it is important to note that the Papanicolaou stain may present some staining artifacts and morphological findings, such as keratohyaline granules, which may be confused with the presence of MN, highlighting the need for careful and specific interpretation when using this technique in clinical and research contexts[23,34,37,38].

In another study, the authors make a critical analysis of the existing gaps in investigations aiming at a more significant expansion in the application of the MN Test. This commitment aims to establish a more solid base to allow the comparison of the results with the information presented by international researchers[25].

Our findings corroborate the unexplored importance of optimizing and aligning the use of the MN Test to achieve a more comprehensive and scientifically based comparative dimension.

In work that had as study population forty-nine patients with oral cancer who received radiotherapy (60 Gy/25 fractions/5 weeks), showed that the presence of MN was statistically significant, concluding that the test is useful to assess radiosensitivity[39].

Conclusion

The results of this study reinforce that LBC is the most adequate method for the cyto-oncotic diagnosis, considering the limitations associated with CC. This assertion is supported by the recurrent limitations attributed to CC, which have frequently been the object of notification of difficulty in diagnosis, leading to the need to repeat the exam. It is imperative to highlight that the wide use of LBC may come up against a substantial barrier related to cost, limiting its potential application in the screening of precursor neoplasms of cervical cancer. However, considering that the number of patients undergoing post-radiotherapy control is small, the use of LBC would have a positive impact, without burdening the system.

On the other hand, we should consider the MN Test as an important instrument, when associated with LBC, for the follow-up of post-radiotherapy patients for cervical cancer. The presence of MN acquires a crucial role here,

as an endogenous dosimeter sensitive in cells to the action of genotoxic agents. Furthermore, the detection of micronucleated cells appears not only as a direct reflection of the genotoxic impact of radiotherapy, but also as a perceptive indicator of the potential for the development of neoplastic conditions. Which reiterates the MN Test as a complementary strategy in post-radiotherapy control.

Final Considerations

The low quality of CC in cases of post-radiotherapy control of cervical cancer can be a barrier to the speed of diagnosis and identification of recurrent lesions, in these cases LBC contributes positively to the quality of diagnosis of irradiated patients, mainly associated with the test from MN.

The use of the MN Test in research and population studies with exposure to radiation and other genotoxic agents can increase knowledge of the carcinogenic potential of these effects in humans and contribute to the understanding of actinic changes, being a cheap technique and easy professional training.

In view of the above, we propose, as a follow-up protocol for patients undergoing radiotherapy for cervical cancer, performing LBC associated with the MN test, which can be extended to other types of tumors.

Conflict of Interest Statement:

None

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