oxidant radioprotectors MPG[8] and *Ocimum* leaf extract[9]. Thus, *Phyllanthus niruri* could be a promising candidate for nontoxic radiation protection and, therefore, needs to be studied in more detail.

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Application of FISH-MN technique to probe micronuclei formation in normal, transformed and malignant cells using alpha satellite pan centromeric DNA

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A micronucleus assay employing biotinylated alpha pan centromeric DNA probe was used in the present study. The study sample consisted of 4 normal, 2 transformed and 9 malignant cell lines of human origin. The frequency of centromere positive micronuclei (c + MN) was found to decrease from normal (67%) to transformed (64.9%) to cancer cells (59%). The c + MN/c–MN ratio from normal to malignant cells was found to vary significantly ($P < 0.01$), while the signal ratio showed absence of any correlation ($P > 0.05$). These results demonstrate the usefulness of combining FISH technique and micronuclei assay to trace the mechanism of micronucleus formation in cells of diverse histopathological origin.

THE micronucleus test provides a quick and easy method of evaluating chromosomal damage. Formation of micronuclei (MN) can occur either from lagging chromosomes or due to their loss from the cell during division. The MN assay has been used on a variety of tissues including blood lymphocytes[1,2], fibroblasts[3], mouse splenocytes[4], bone marrow[5] and tumour cells[6,7]. Although the routine MN assay is used for determining the extent of genomic damage, it suffers from one serious drawback, i.e. it does not uncover the nature of damage. The problem becomes more relevant in the light of findings that indicate preferential chromosome integration in micronuclei[8,9]. To overcome these limitations, fluorescence *in situ* hybridization technique (FISH) has been successfully used in the past. FISH assay has been employed for visualizing the whole chromosomes[8,10], kinetochores[3,5,11], centromeres[12–17] and telomeres[18] in the micronuclei. By using this technique it is also possible to distinguish between clastogenic and aneugenic activity of chemicals or physical agents[4,19,20]. It provides a convenient way of analysing chromosome segregation in interphase cells or detecting amplified genes in tumour cells[6]. Thus, problems which flaw the routine staining techniques are overcome using this method. Recently, flow cytometry has been used in combination with FISH technique to make it more useful[10,21,22].

Although many FISH-MN studies have been conducted in the past, most of the work has been carried out in lymphocytes and cell lines. To our knowledge, no report exists on the FISH-MN status in cell lines exhibiting different levels of malignancy. To address this question, the present study was carried out on normal, transformed and malignant human cell lines.

Established human cell lines representing normal fibroblasts (HMB, FRS, TUB, SV350 and SV371), transformed fibroblasts (WI-38 VA 13, GM) and cancer cells, i.e. melanoma (MeWo, Be11), head and neck cancer (4197, 4451), glioblastoma (U87-155), cancer of rectum (RKO) and colon (SW 480) were considered. Cells were grown in RPMI 1640 medium enriched with 10% (v/v) foetal calf serum (pH 7.2) at 37°C in an atmosphere of 5% CO_2 and 95% air mixture. Before harvesting, cells were trypsinized at 37°C under sterile conditions and subcultured for 24 h at 1×10^6 cells/ml concentration. At the termination of culture, cells were fixed in methanol/acetic acid (3:1) and slides were prepared. The FISH staining was carried out as per previously described procedures[23]. Briefly, cells were first treated with RNase, followed by pepsin treatment and post fixation in a solution of phosphate buffered saline, magnesium chloride and formaldehyde for 10 min.

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Slides were rehydrated in the descending alcohol grade (70%, 90%, 100%, 100%) at room temperature. Cellular DNA was denatured in a solution of formamide and 20 × SSC (ratio 9:1, pH 7.0), at 75°C. Cells were hybridized overnight with the centromeric DNA probe (Oncor, USA) at 37°C in the humidity chamber. Post hybridization washes were performed in a solution of formamide, 20 × SSC (pH 7.0) and water (ratio 5:1:4) for 15 min. Hybridization between probe and target DNA was detected by subjecting the cells to three rounds of antibody reactions with FITC-avidin, goat antiavidin and FITC-avidin, respectively. Finally, slides were mounted in DABCO antifade-Propidium Iodide (PI) solution and stored in dark at 4°C. At least 1000 PI stained cells were counted to record the overall frequency of micronuclei. In comparison, one hundred FISH stained cells/sample were analysed. Cells having more than four micronuclei were excluded from the study to eliminate structures resulting from karyorrhexis and budding[24]. A new parameter, 'signal ratio', was introduced to quantitate the transmission frequency of centric fragments/whole chromosomes into the micronuclei. The signal ratio was calculated as the total number of signals/total number of c + MN. All microscopic studies were performed at a magnification of 1000. Depending upon the presence of fluorescent signal, micronuclei were scored as centromere positive (c + MN) and centromere negative (c–MN). Further, their ratio (i.e. c + MN/c–MN) was also determined.

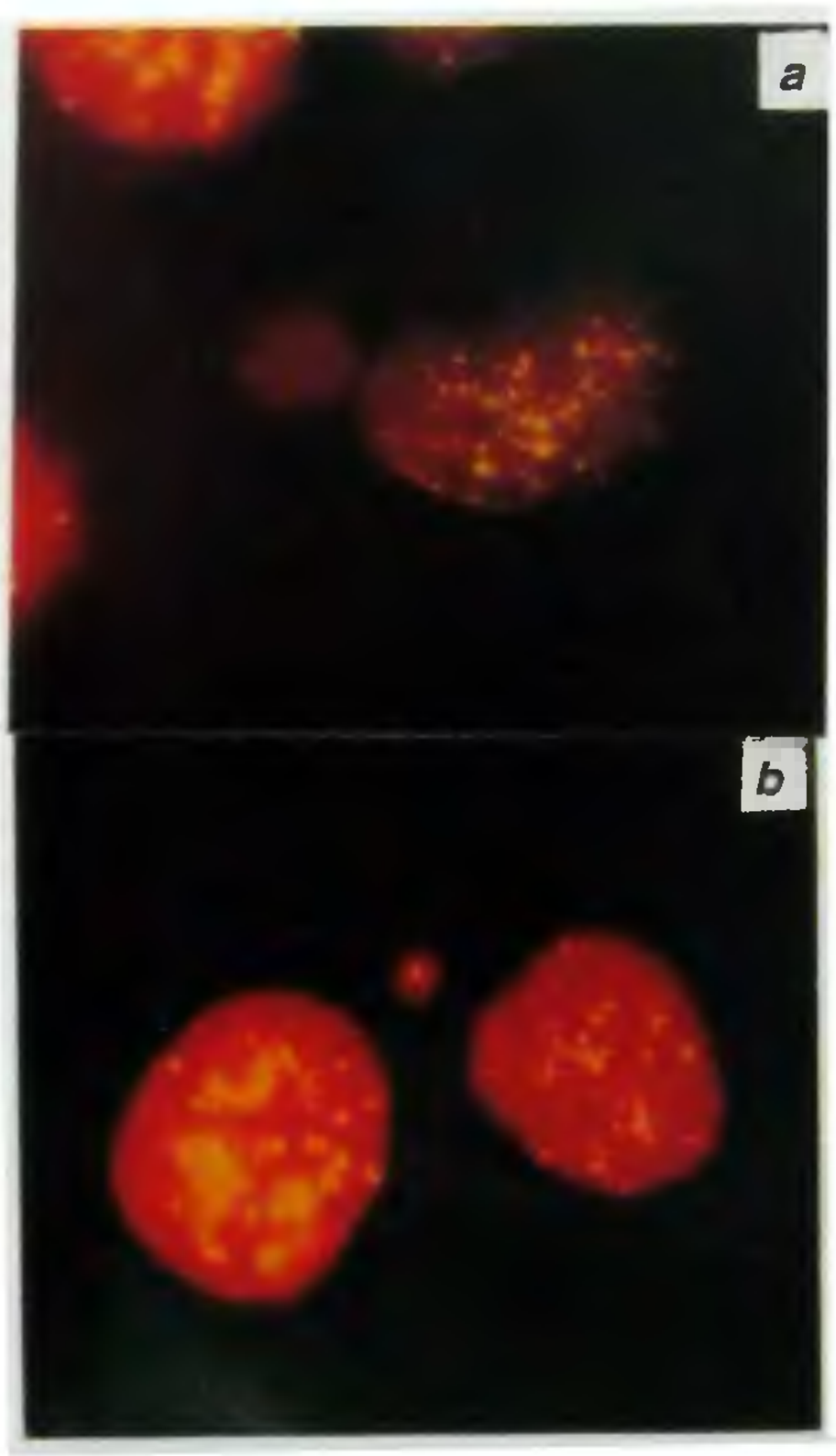

Figure 1. *a*, Centromere negative micronuclei (c–MN); *b*, Centromere positive micronuclei (c + MN).

Table 1. Summary of FISH data

Cell line	% MN frequency	FISH stained cells: Total MN	c + MN	c– MN	Ratio (c+/c–)	Total signals	Signal ratio
Normal							
HMB	2.2	100	64	36	1.78	121	1.89
SV350	1.9	100	69	31	2.23	85	1.24
SV 371	1.4	100	71	29	2.45	91	1.29
FRS	1.0	100	64	36	1.78	115	1.79
Transformed							
WI38VA13	2.7	115	70	45	1.56	99	1.41
GM	2.2	100	69	31	2.23	89	1.29
Cancer							
Melanoma							
MeWo	3.1	117	63	54	1.17	79	1.25
Bell	8.6	100	56	44	1.27	104	1.85
Head and neck cancer							
4197	4.1	100	58	42	1.38	84	1.44
4451	2.4	106	61	45	1.36	97	1.59
Colon carcinoma							
RKO	0.7	100	59	41	1.44	77	1.30
SW480	2.7	100	52	48	1.08	91	1.75
Glioblastoma							
U87-155	1.7	100	65	35	1.86	96	1.47

The specificity of the FISH technique in detecting centromeres in micronuclei is shown in Figure 1. Summarized results have been presented in Table 1. The background frequency of micronuclei was found to be more in cancer cells (3.3%) than in transformed cells (2.4%) and in normal fibroblasts (1.6%). The frequency of c + MN showed a gradual decrease from normal cells (mean 67%, range 64–71%) to transformed cells (mean 64.9%, range 60.8–69%) to cancer cells (59%, range 52–65%). However, the signal ratio was found to be randomly distributed among different groups ($P > 0.05$). On an average, one c + MN/cell showed 13% decrease from normal to cancer cells. In comparison, two to four c + MN/cell showed approximately 50% increase between normal and cancer cells, thus indicating the presence of 'heavier' micronuclei in malignant cells in comparison with the normal cells.

Our most interesting results were obtained on comparing the c + MN and c – MN among different subgroups (Figure 2). The c + MN/c – MN ratio showed a progressive decrease from normal to transformed to cancer cells ($P < 0.01$), thus indicating a possible *pattern* in the formation of micronuclei. It seems that micronuclei forma-

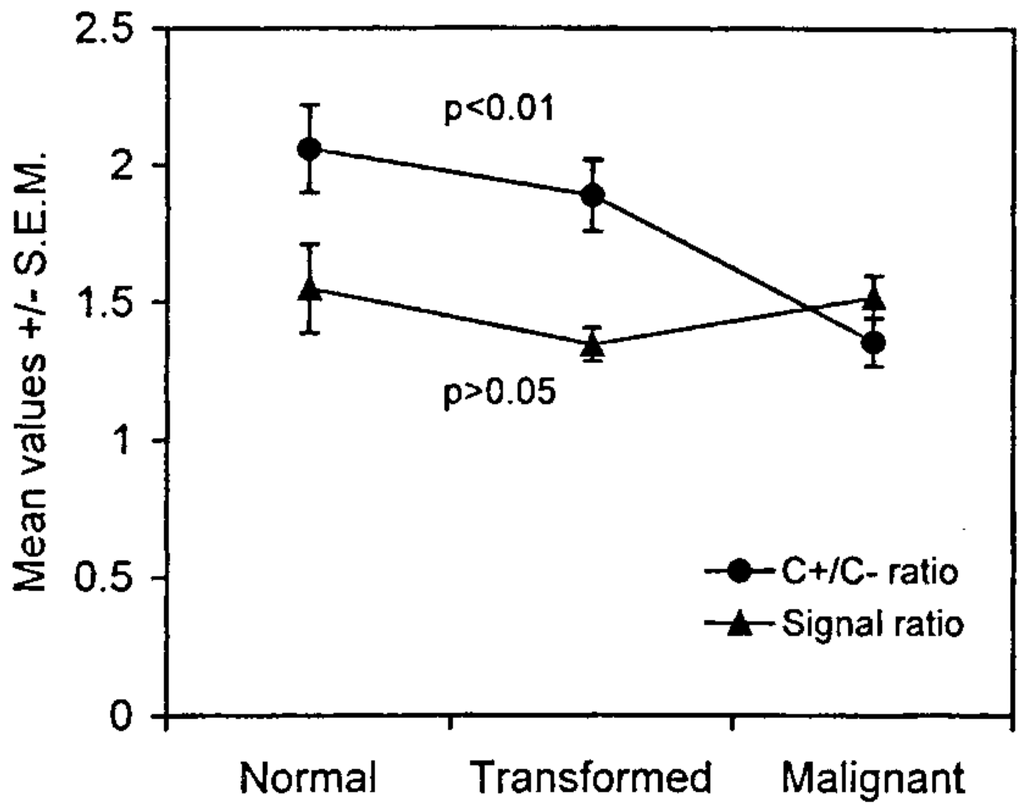

Figure 2. Mean frequency of c + MN/c – MN and signal ratio.

tion in malignant cells involves greater participation of acentric fragments, in contrast to the normal and transformed cells that exhibit more number of whole chromosomes/centric fragments.

Although routine MN assay has found a wide acceptance in radiation and environmental biology, FISH approaches are slowly taking over the traditional methods because they allow better identification of mechanisms by which micronuclei originate. The application of FISH technology is especially useful in determining the composition of micronuclei, thereby helping to evaluate the aneugenic potential of physical and chemical agents[25–27]. Micronuclei may contain entire chromosomes or acentric fragments[28], but since all the chromosomes are not equally sensitive to DNA breaking agents[9] it is essential to know which chromosomes contribute more frequently in the formation of micronuclei. Recently[8,29] researchers have found greater participation of chromosomes 2 and 7 in the micronuclei formation than was expected by chance alone.

It is possible that the cell cycle phase may have a role in the transmission of chromosomes to micronuclei. Cells exposed to radiation[30], especially those in the G1 phase, have been found to exhibit higher c + MN than cells in the G2 phase at the time of exposure[31]. It would be interesting to know which chromosomes are micronucleated more often in cells of specific histopathological status. We have attempted to show that (1) by determining the centromere positivity of micronuclei insight can be gained into the nature of DNA damaging agents (clastogenicity versus aneugenicity), and (2) genomic composition of micronuclei may be tissue influenced. The present study indicates a possible diagnostic application of FISH studies for differentiating cell types of different histological backgrounds. However, since only two transformed cell lines were available for investigation, the present findings need to be carefully interpreted.

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