

## SHORT REPORTS

### Flow Cytometric DNA Analysis of Primary Cutaneous Malignant Melanoma

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**DNA-ploidy of primary malignant melanoma was retrospectively compared by flow cytometry with sections of paraffin-embedded blocks, among tumours at different histopathological stages (levels) and among tumours and their peripheral lesion. In none of 10 melanomas at levels I – II or 10 melanocytic nevi was an aberrant DNA pattern found. In contrast, 8 of 18 melanomas at levels III – V including 6 of 9 melanomas at level V, showed a non-diploid (aneuploid) DNA pattern. However, even in these non-diploid melanomas the peripheral area showed a diploid pattern where tumour cell invasion was slight, similar to the level I – II melanomas. These results indicate that DNA-aneuploidy in melanoma is closely associated with the lesional advancement, and reflect a poor prognosis for patients with melanoma at level III or higher. Key words: DNA-ploidy; Prognosis.**

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Non-diploid DNA (DNA-aneuploidy) as a malignancy-specific cell marker was studied in a variety of malignant tumours (1-3). Recently, quantitative DNA flow cytometry has been applied to this investigation (4, 5 among others), and abnormal DNA content in human malignancies was established. Hedley et al. (6) developed a method for analysing the cellular DNA content of paraffin-embedded materials by flow cytometry. This method made retrospective analysis possible (7-10). Von Roen et al. (11) found that the DNA aneuploidy of malignant melanoma was closely related with the lesional depth of and prognosis for patients. The present investigation was aimed at reconfirming what DNA-ploidy of melanoma is correlated with, and to ascertain whether this examination could be applied to the analysis of patients with cutaneous melanoma.

## MATERIALS AND METHODS

### Subjects

Primary malignant melanomas surgically resected from 28 patients (12 males and 16 females) who visited our clinic between 1979 and 1989 and showed no metastasis by the time of surgery, were used for study. The mean age of the patients was 55 (range 18-80) years. The material consisted of 3 lentigo maligna melanomas, 11 superficial spreading melanomas, 12 acral lentiginous melanomas and 2 nodular melanomas. Histopathological staging was performed using the histologic classification system developed by Clark et al. (12). In 12 patients, the tumour area was further compared with that of its peripheral-flat, or less pigmented lesion, which was separated with scissors from the tumour area, in part before specimen-fixation, in part at the preparation of the paraffin sections. As a control, a skin lesion from each of randomly selected 10 patients with melanocytic nevus (4 males and 6 females) was similarly studied. The mean age of controls was 24 (range 7-61) years.

### Sample preparation

All specimens for histology had been fixed in 10% formalin and embedded in paraffin wax by a routine method. Preparation for flow cytometry was done by a modified method of Hedley et al. (6). Six or 7 leaves of a 30 µm thick section from a specimen were dewaxed with three changes of xylene for 10 min each at room temperature, and rehydrated with 100, 95, and then 70% ethanol for 10 min each time at room temperature. Then the sections were washed in distilled water and digested with 0.5% pepsin (Sigma) in 0.9% NaCl at pH 1.5 for 30 min at 37°C with intermittent vortex mixing. The cells were stained with 50 µg/ml of propidium iodide (Sigma) in calcium- and magnesium-free, phosphate-buffered saline containing 1 mg/ml RNase A type I-A (Sigma) for 30 min at room temperature, then filtered through nylon mesh to remove aggregates. At the same time, 5-µm-thick sections for hematoxylin and eosin staining were prepared to confirm histological findings of the sections used for DNA analysis.

### Flow cytometry

The relative DNA content of at least 10,000 nuclei per sample was measured by flow cytometry (Fluorescence Activated Cell Sorter IV, Becton Dickinson) with an argon laser operating 400 mW at 488 nm for excitation and a 625/635 nm bandpass filter for the emission wavelength. All samples contained inflammatory and stromal cells which served as an internal diploid standard (11). A sample showing a single peak ( $G_0/G_1$  phase) often followed by a minor peak ( $G_2 + M$  phase) was classified as diploid, while a

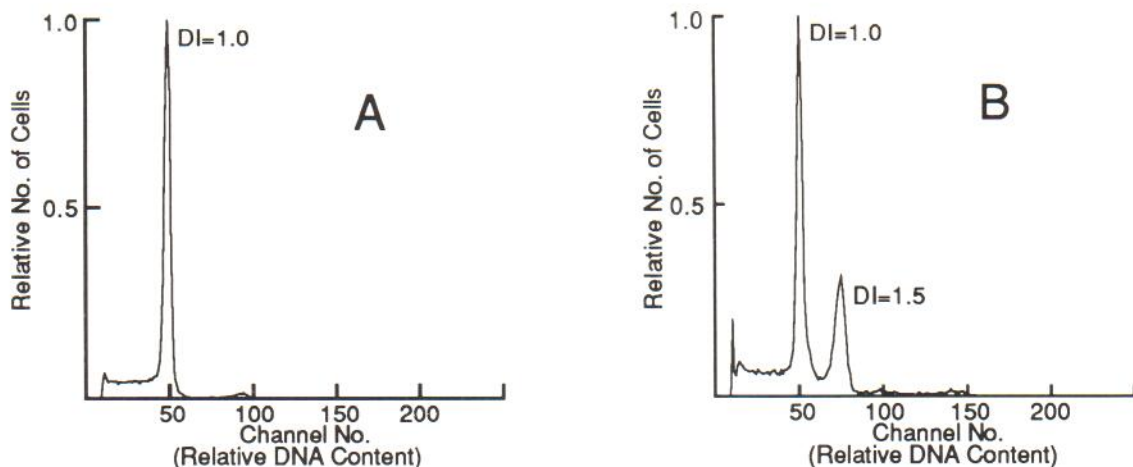


Fig. 1. (A) Flow cytometric DNA examination of melanocytic nevus, showing diploid pattern (DI = 1.0, CV = 3.7%, SPF = 4.3%). (B) Malignant melanoma (level III) showing non-diploid DNA pattern (DI = 1.5, CV = 3.4%, SPF = 18.7%). DI, DNA index; CV, coefficient of variation; SPF, S-phase fraction.

sample with another  $G_0/G_1$  peak containing 10% or more of total nuclei was classified as non-diploid. The DNA index for the non-diploid population was calculated as the quotient of the channel of the non-diploid peak divided by the channel of the diploid peak. A coefficient of variation (CV) for the diploid peak was calculated for each sample with the built in software of the Fluorescence Activated Cell Sorter IV and the data was stored on a computer (FACST<sup>TM</sup> DT-PC, Fujisawa Pharmaceutical). Additionally, the S-phase fraction (SPF) was calculated using the rectangular histogram analysis method described by Baisch et al. (13). In the non-diploid DNA pattern, SPF was calculated on non-diploid cells. Furthermore, samples of a CV over 8.0% with excess debris or samples with an overlapping or unidentified small stemline were excluded from SPF analysis. Statistical analysis was done by Student's *t*-test and  $X^2$ -determination.

## RESULTS

Each sample examined had a definite diploid  $G_0/G_1$  peak (Fig. 1A). The coefficient of variation of the diploid  $G_0/G_1$  peak was  $4.3 \pm 1.3$  ( $M \pm SD$ ) in malignant melanomas, and  $4.1 \pm 0.7$  in melanocytic nevi. Although all melanocytic nevi showed the diploid pattern alone, 8 (29%) of 28 primary cutaneous melanomas additionally exhibited one or more minor  $G_0/G_1$  peak (Fig. 1B). DNA-aneuploidy was correlated with the grade of tumour cell invasion in the skin (Table I). A non-diploid pattern was observed exclusively in melanomas at level III or higher, and the incidence of DNA-aneuploidy in level V melanomas was significantly higher than that of the other melanomas at lower levels ( $p < 0.01$ ). Furthermore, the similarity of aneuploidy within a

Table I. DNA-aneuploidy related with the degree of melanoma cell invasion in non-metastatic, cutaneous malignant melanoma,  $n=28$

Melanoma (tumour area)				Peripheral lesion		
(Level)	Diploid	Non-diploid	Recurrence <sup>a</sup>	No.	Diploid	Non-diploid
I	$n=2$ 2	0	0	NE		
II	$n=8$ 8	0	0	3	3	0
III	$n=8$ 6	2	1	4 (2)	3 (1)	1 (1)
IV	$n=1$ 1	0	0	NE		
V	$n=9$ 3	6	3	5 (4)	4 (3)	1 (1)

Parenthesis indicate non-diploid melanoma.

NE = not examined.

<sup>a</sup> Follow-up months (mean: 67; range: 17–149).

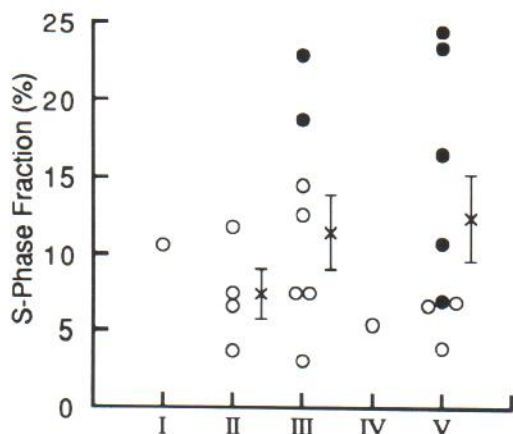


Fig. 2. Correlation between S-phase fraction and level in melanoma. Bars represent mean  $\pm$  SEM.  $\circ$ , diploid melanomas;  $\bullet$ , non-diploid melanomas.

melanoma was confirmed in three specimens examined, by comparing results between specimens further divided into two pieces. Nevertheless, the peripheral lesion of the non-diploid melanomas showed a diploid pattern in four of six specimens examined. The peripheral aneuploidy was observed in the other two peripheral lesions where there was a deep tumour cell invasion, similarly to the level III melanoma. On the other hand, the peripheral lesions of the diploid melanoma examined were all diploid. Additionally, DNA-ploidy was not significantly associated with the patient's age, sex, clinical duration or clinical type although tumour size expressed as the maximum diameter was significantly greater in the non-diploid than in the diploid melanomas ( $p < 0.05$ ). SPF was determined in all of 10 melanocytic nevi and 21 of 28 melanomas. SPF of melanomas ( $11.1 \pm 6.6\%$ ,  $M \pm SD$ ) was significantly higher than that of melanocytic nevi ( $4.4 \pm 1.6$ ) ( $p < 0.01$ ). Further, SPF of non-diploid melanomas (7 samples,  $17.6 \pm 6.7$ ) was significantly high as compared with diploid melanomas (14 samples,  $7.8 \pm 3.4$ ) ( $p < 0.01$ ). In addition, in 5 melanomas examined, SPF ( $10.7 \pm 6.5$ ) of the tumours was statistically higher than that ( $5.9 \pm 3.8$ ) of the peripheral lesions ( $p < 0.05$ , at paired  $t$ -test). However, there was no significant correlation between levels of melanoma and SPF (Fig. 2).

## DISCUSSION

The present study reconfirmed that the cellular DNA content can be analysed by flow cytometry with paraffin-embedded blocks. Using this method,

Von Roen et al. (11) obtained clearly interpretable DNA histograms in 92% of the cases examined, and in the present study the DNA pattern was identified in all of the samples examined. The non-diploid rate of the present melanomas (tumour area) was 29% – similar data to those reported by Von Roen et al. By comparing DNA content, not only in tumours at different levels but also in the tumour area and peripheral lesions in individuals, this study also showed that DNA-aneuploidy was closely associated with advanced skin lesion. Two possibilities could be considered: 1) clonal change secondary to the development of the lesion, and 2) manifestation of non-diploid cells with a high invasive activity in the advanced lesion. There is evidence that patients with cutaneous melanoma at a high level have a poor prognosis compared with those with melanoma at a lower level (14). The frequent aneuploidy appearance of melanomas at level III or higher may indicate poor prognosis for patients with these melanomas. In fact, a preliminary follow-up study of the present patients demonstrates a tumour recurrence occurring exclusively in patients with melanomas at level III or higher (Table I). DNA analysis by flow cytometry may therefore be useful for detecting a severe melanoma lesion. Besides, SPF was not correlated with the grade of melanoma cell invasion at the present study, although SPF was associated with the malignant change of melanocytes, as shown in breast carcinoma (15, 16).

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