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# DNA-ploidy Abnormalities Are a Reflection of the Metastatic Potential of Malignant Melanoma

## *Microfluorometric DNA Analysis*

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**Using DAPI (4′,6-diamidino-2-phenylindole)-DNA microfluorometry, we examined the nuclear DNA-ploidy abnormalities of 15 primary malignant melanomas and their 20 corresponding metastases. They all presented the aneuploid DNA histographic pattern. When the DNA index value was calculated as the reflection of DNA-ploidy abnormalities, it was found to be significantly higher in the metastases ($2.07 \pm 0.50$) than in the primary tumors ($1.76 \pm 0.50$) ($p < 0.01$). Sixteen (80%) of the metastatic tumors had a higher DNA index value than their primary tumors, whereas the remaining four (20%) had a lower value. The difference in the DNA index values between the primary and metastatic tumors did not correlate to any other conventional prognostic variables (e.g. histologic type, level, and thickness). When we added 15 non-metastatic melanomas to the above 15 primary melanomas and evaluated the predictors for metastasis using multivariate stepwise logistic regression analysis, the DNA index value of the primary melanomas was found to be the most reliable risk factor. These results suggest that primary melanoma cell populations, having high DNA index values, are usually responsible for subsequent metastasis, and that hence, DNA-ploidy abnormalities of primary melanomas are likely to provide useful information for patient potential with regard to metastasis. *Key word: metastasis.***

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Clinical and/or pathologic features like the site of the primary tumor, tumor thickness, level of invasion and histologic type have been known to be good markers for predicting malignant melanoma patient prognosis (1, 2). Malignant melanoma is also known to metastasize during its early stage of tumor progression, and there have been many reports which describe metastasis as being a critical determinant of patient survival (1, 2).

Since DNA-ploidy abnormalities have been reported to reflect the biological behavior of human neoplasms (3–7), primary and metastatic melanomas have been examined in terms of DNA-ploidy for the purpose of characterization of these two conditions during the tumor progression (6, 8–10). However, these investigations, which usually employ flow cytometry, have not yet yielded a convincing difference between these two conditions. In this regard, we have recently demonstrated that DNA-ploidy abnormalities measured by using DAPI (4′,6-diamidino-2-phenylindole)-DNA microfluorometry are quite sensitive to ascertaining the biological behavior and/or malignant potential of pigmentary neoplasms (11–13).

In the present study, we therefore investigated the nuclear DNA-ploidy of primary and metastatic melanomas, using DAPI-DNA microfluorometry, and evaluated the significance of any difference arising between the two conditions. We further examined which is the most reliable predictor for metastasis among clinical and/or pathologic prognostic factors including DNA-ploidy abnormalities of primary melanomas, since such an attempt has not previously been made.

## MATERIALS AND METHODS

### *Materials*

In order to compare the DNA-ploidy abnormalities between primary and metastatic melanomas, we included in this study 15 patients (11 males and 4 females; mean age, 56.5 years; range, 37–79 years), who had developed primary malignant melanoma, and 20 of their corresponding regional and/or distant metastases. Eight primary melanomas were acral lentiginous, 4 were superficial-spreading, and the other 3 were nodular. Twelve patients presented regional lymph node metastases, while 2 patients had metastatic tumors, in the brain in one and in the lung in the other. One patient exhibited multiple metastatic lesions (regional lymph node, brain, lung, kidney, adrenal gland, and intestine). In 11 patients, primary and metastatic tumors were simultaneously found at the time of the first visit and examination and were removed using the conventional surgical method. In the other 3 patients, metastases were found and removed after 30–76 months of the initial surgical therapy. The other patient died from systemic metastases and was autopsied 32 months after the initial surgical therapy.

These 15 primary melanomas and corresponding metastases were measured in terms of the DNA-ploidy abnormalities. The difference in DNA-ploidy abnormalities between the two tumors (we describe it hereafter as the "difference") was compared with patient sex and age, primary tumor histologic type (14), level of invasion (15), tumor thickness, and metastatic organ.

In order to examine further the correlation between metastases and several prognostic factors, we added another 15 patients (11 males and 4 females; mean age, 61.9 years; range, 20–86 years), who had not yet developed metastases during $74.4 \pm 29.7$ (34 to 142) months of follow-up, to the above-mentioned patients. Their primary tumors were acral lentiginous melanomas in 7, superficial-spreading melanomas in 4, lentigo maligna melanomas in 2, and mucosal melanomas in 2 patients. The 30 patients were divided into two groups – 11 metastatic and 19 non-metastatic patients – according to the evidence of having or not having metastasis at the time of their first visit and examination. The 19 non-metastatic patients included 4 patients who developed metastasis later and the newly added 15 patients.

All 30 patients were measured in terms of the DNA-ploidy abnormalities of their primary melanoma. Histologic type, level of invasion, and tumor thickness were also determined in the 30 primary melanomas. The 30 patients' age, sex, and treatment history and the interval between the probable time of the disease onset and the patient's first visit to our hospital (we describe it as the "interval") were included for analysis. Treatment history was determined based on whether the patient had undergone incisional biopsy or inadequate excision prior to the patient's first visit to our hospital.



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*Measurement of nuclear DNA-ploidy*

The nuclear DNA-ploidy was measured using the method previously mentioned (16). Briefly, paraffin-embedded specimens were cut into 50-μm-thick units and deparaffinized. Subsequently, the central part of the tumor mass was trimmed from the thick sections and then loosened by rinsing in Ringer-Lock's solution containing 0.05% collagenase (type IV, Sigma) for a day at 37°C. The loosened specimens were ultrasonified (Sonifier 185E, Branson, Danbury, Connecticut) to obtain cell suspensions. The isolated cells were then washed with salt solution, transferred to glass slides, and fixed with 100% methanol. Nuclear DNA was then stained with DAPI and observed with an Olympus-OSP1 microfluorometer (Olympus, Tokyo, Japan). The intensity of fluorescence was recorded and analyzed using a personal computer combined with the microfluorometer. Observations were made on 100 to 300 intact nuclei from a single specimen to obtain a DNA distribution histogram. Stromal lymphocytes were used as controls to obtain the normal diploid DNA-ploidy.

*Analysis of DNA distribution histogram and DNA-ploidy abnormalities*

The diploid histogram pattern was defined as that which exhibited a single fraction of cells containing diploid DNA with or without a small fraction of cells containing hyperdiploid and/or tetraploid DNA. The aneuploid cell fraction was defined as a peak in the histogram that differed by more than 10% from the nearest euploid peak. The aneuploid pattern was then defined as that which had an apparent aneuploid cell fraction or fractions. To evaluate DNA-ploidy as a simple value, a DNA index was calculated by dividing the mean value of the DNA content of all the tumor cells measured in a specimen by the value of the diploid DNA content of the same specimen.

*Statistics*

The Wilcoxon matched-pairs test was used to statistically analyze the DNA index values for these tumors. Using the Kruskal-Wallis test, we evaluated the relationship between the differences, patient sex and age, primary tumor histologic type, level of invasion, tumor thickness, and metastatic organs.

The relationship between metastasis and eight other variables (patient age and sex, interval, treatment history, histologic type, level of invasion, tumor thickness, and DNA index value) was analyzed by the Mann-Whitney test for quantitative variables (age, interval, thickness, and DNA index) and $\chi^2$ test for qualitative variables (sex, treatment history, type and level). A multivariate stepwise logistic regression analysis was then performed to determine the most reliable factor for predicting metastases at the time of the patient's first visit. The analyses were performed using the SAS statistical programs.

## RESULTS

All 15 primary melanomas and their corresponding 20 metastatic melanomas presented the aneuploid DNA histographic pattern. The additional 15 primary melanomas, which had not metastasized during 34 months of follow-up, also presented the aneuploid DNA histographic pattern. The DNA histographic patterns of primary and metastatic melanomas of a representative patient are exemplified in Fig. 1.

DNA index values were significantly lower in the 15 primary melanomas (mean value $\pm$ S.D.: $1.76 \pm 0.50$; range: 1.18–2.69) than in the corresponding 20 metastatic melanomas (mean value $\pm$ S.D.: $2.07 \pm 0.50$; range: 1.47–3.57) ($p < 0.01$) (Fig. 2). Sixteen (80%) of the 20 metastatic tumors (including all 6 metastases of the autopsied patient) presented higher DNA index values than their corresponding primary tumors (Fig. 2). The other 4 (20%) metastatic tumors exhibited lower DNA index values than did their primary tumors. The difference ranged from $-0.54$ to 1.78 (mean value $\pm$ S.D.: $0.40 \pm 0.51$).

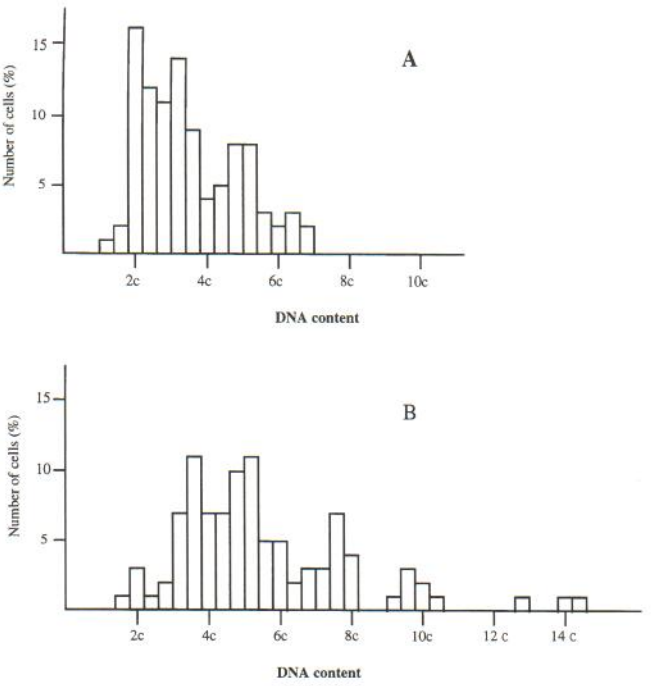


*Fig. 1.* DNA distribution histograms of a representative patient. A: primary tumor; DNA index = 2.07. B: metastatic tumor; DNA index = 2.96.

No significant relationship was found between the difference and the other variables of sex, age, histologic type, level, thickness, and metastatic organ.

Among the combined 30 primary melanomas, the metastasizing 11 ($1.97 \pm 0.56$) showed significantly higher DNA index values than did the non-metastasizing 19 ($1.49 \pm 0.27$) ($p < 0.01$). Tumor thickness of the former ($6.25 \pm 3.74$ mm) was also significantly higher than that of the latter ($3.82 \pm 3.03$ mm) ($p < 0.05$). Other variables (patient's age and sex, interval, treatment history, histologic type, and level of invasion) showed no significant relationship to the metastatic or non-metastatic evidence. A stepwise logistic regression analysis was then performed by removing the least important variable at respective steps until the remaining variables were all significantly important at the $p < 0.05$ level. The DNA index value was the only remaining variable at the step of final selection. When the DNA index value of the primary melanoma was greater by 0.5 or 1.0, the relative risk for patient metastasis was 4.09 or 16.7 times higher (Wald $\chi^2$ value was 5.64).

## DISCUSSION

We have demonstrated in this study that DNA-ploidy abnormalities are significantly greater in metastatic melanomas than their corresponding primary melanomas and that the DNA-ploidy abnormalities of primary melanomas are an important risk factor for subsequent metastasis.

Greater DNA-ploidy abnormalities have been reported by using the cytophotometric method in metastatic melanomas than in primary melanomas (3). However, several recently conducted image cytometric investigations have demonstrated that primary and metastatic melanomas present different DNA

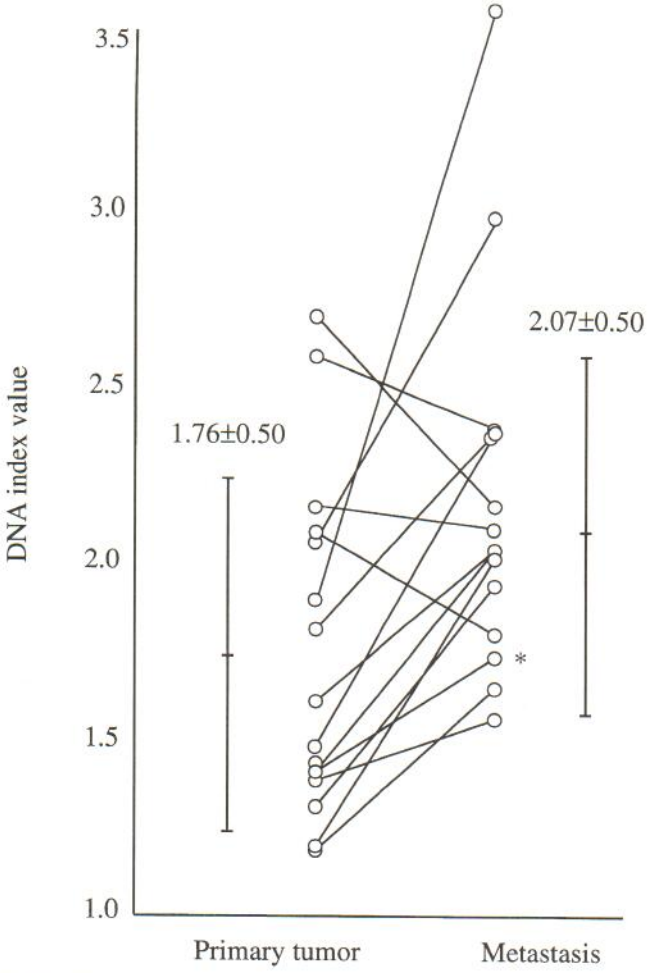


*Fig. 2.* DNA index values of primary melanomas and their corresponding metastases. ○ indicates the DNA index value of primary tumor or metastasis. The primary tumor and its corresponding metastasis are connected by a *solid line*. The *asterisk* indicates the mean value of the DNA index values of six metastases of an autopsied patient. The mean value and standard deviation are shown in the bar.

distribution patterns without any apparent significant relationship (9, 10), which does not support the above report.

Our previous studies demonstrated that DNA-ploidy measured by the DAPI-DNA microfluorometric method, especially when it is quantified as a DNA index value, is sensitive in evaluating the biological behavior of pigmentary neoplasms. It is an aid for differentiating Spitz nevus from melanoma (11, 12), for providing a cytological basis for the histologic marker of the malignant potential of melanoma (17), and even for predicting melanoma patients' prognosis (13). Since in this method the greater DNA-ploidy abnormalities are usually a reflection of greater malignant potential, the significantly higher DNA index value in metastatic melanomas than in their corresponding primary tumors, which has been demonstrated in the present study, suggests that the former have greater malignant potential than the latter.

The question naturally arises as to why there are usually greater DNA-ploidy abnormalities in metastatic melanomas and why the reverse is sometimes the case. Although the answer remains unclear, the population of cells exhibiting greater DNA-ploidy abnormalities, which probably constitutes the heterogenous DNA-ploidy profile of primary melanomas in the present and other reported studies (6–8), may usually have a propensity to metastasize and sometimes not. Several adenocarcinomas like breast cancer (18–20) and thyroid cancer (21) have been reported to present similar DNA-ploidy abnormalities between primary and corresponding metastatic tumors. The significant differences between primary and metastatic melanomas in this study are possibly associated with the characteristics of the tumor's biological behavior, especially its highly metastatic potential.

The multivariate logistic regression analysis showed the DNA-ploidy abnormalities of primary melanomas to be the most important and independent risk factor for subsequent metastases. The greater the DNA index value is the more likely it is that the primary tumor has a metastatic potential. Our result is supported, at least in part, by a previous investigation (3) which reported that primary melanomas with DNA-aneuploidy had a higher metastasis rate than those with DNA-diploidy. We have already reported that the DNA-ploidy abnormalities represent a sensitive and good predictor for melanoma patient prognosis (13). Since metastases are critical to patient prognosis of not only melanomas but a range of malignant neoplasms (22), DNA-ploidy abnormalities probably reflect melanoma patient prognosis by way of reflecting the metastatic potential of primary tumors. Thus, the examination of the DNA-ploidy abnormalities by employing the DAPI-DNA microfluorometric method is likely to provide useful information for subsequent metastasis and even melanoma patient prognosis.

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