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A DNA-flow Cytometric Analysis of Trichilemmal Carcinoma, Proliferating Trichilemmal Cyst and Trichilemmal Cyst

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DNA-flow cytometric analyses were performed on paraffin-embedded sections of trichilemmal carcinoma, proliferating trichilemmal cyst, and trichilemmal cyst. Nine out of 10 cases of trichilemmal carcinoma and 2 out of 5 cases of proliferating trichilemmal cyst showed aneuploidy. No aneuploidy was detected in 6 cases of benign trichilemmal cyst. The proliferating index (percentage of cells that exceeded the diploid peak (G0/G1) plus 4 standard deviations) of trichilemmal carcinoma, proliferating trichilemmal cyst, and trichilemmal cysts was 43.5 ± 3.8%, 32.5 ± 7.5%, and 23.1 ± 2.0%, respectively. In one out of the 6 cases of trichilemmal carcinoma that were large enough for us to investigate regional differences, DNA ploidy heterogeneity was detected. The incidence of aneuploidy of trichilemmal carcinoma was significantly higher than that of squamous cell carcinoma (14%), previously described by us using the same method. ***Key word: aneuploidy.***

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Flow cytometry permits a rapid analysis of thousands of cells from individual lesions and thus makes feasible the study of a series of cases from paraffin-embedded archival material. Although image analysis may allow identification of small aneuploid or near aneuploid populations (1) that may be below the limit of resolution of flow cytometry, the flow cytometric study permits significantly better resolution by counting more than 100 times as many cells per tumor sample (2).

Analyses have been performed on various skin tumors (squamous cell carcinoma (SCC), Bowen's disease, melanoma, Merkel cell carcinoma) (3–6). Usually the presence of aneuploidy is assumed to be a criterion for malignancy, although aneuploidy may be detected in histologically benign adenomas or premalignant lesions, as derived from for instance colon (7).

We applied flow cytometric analyses to three typical trichilemmal tumors: trichilemmal carcinoma, proliferating trichilemmal cyst (PTC) and benign trichilemmal cyst.

As described below, trichilemmal carcinoma was characterized by a high incidence of DNA aneuploidy, which was also observed in 40% of histologically "benign" PTCs.

No aneuploidy was detected in benign trichilemmal cyst. The incidence of DNA aneuploidy of trichilemmal carcinoma was higher than that of SCC reported by us using the same method (8).

MATERIAL AND METHODS

Subjects

Ten cases of trichilemmal carcinoma, 5 cases of PTC and 6 cases of trichilemmal cysts were examined. These were found in histological record samples in the Department of Dermatology, Asahikawa Medical College, taken over the period 1977–1992. The clinical data of these cases are summarized in Table I. Trichilemmal carcinoma was defined as multilobulated, invaginating epithelial tumor composed of highly anaplastic cells with features of trichilemmal keratinization. PTC was defined as a well demarcated, lobulated, and anastomosing cystic tumor with trichilemmal keratinization. Although PTC may show a slight degree of nuclear atypia, it was not so marked, and the whole tumor was characterized by sharp circumscription. Thus highly anaplastic more invasive malignant proliferating tumor was included in the trichilemmal carcinoma. Trichilemmal cyst was defined as a cystic tumor with trichilemmal keratinization with no evidence of cellular atypia.

The specimens had been fixed with 10% formalin, processed routinely, and embedded in paraffin.

Sample preparation

The extraction of cells from paraffin-embedded tissues was performed according to the method described by Hedley et al. (9), with minor modifications. Three or four 50-μm sections were cut from paraffin blocks with a microtome. The sections were trimmed under a stereomicroscope to cut off the normal tissue from the tumor. The sections were then placed in (10 × 76 mm) glass tubes, and rehydrated through 3 ml each of xylene (2 × 10 min), 100% ethanol (2 × 10 min), 95% ethanol (1 × 10 min), 70% ethanol (1 × 10 min), 50% ethanol (1 × 10 min) and left in 10 ml distilled water overnight. The tissue was washed twice in phosphate-buffered saline (PBS) (pH 7.4) and treated for 60 min at 37°C with 1.5 ml of 0.5% pepsin in 0.9% NaCl adjusted to pH 1.5 with HCl. After the incubation, each section was pipetted vigorously and filtered through a metal mesh (# 150). Nuclei samples were then centrifuged at 500 × g for 5 min. The pellet was resuspended in 1 ml of propidium iodide (0.5 mg/ml) and RNase (1500 U/ml) and treated in the dark for 30 min at 4°C. Each nuclei suspension was filtered through 37 μm nylon mesh just before the flow cytometric analyses.

Flow cytometry

The relative DNA content of 10^4 nuclei was analyzed by a CS-20 cell sorter (Showa Denko Co, Tokyo) equipped with an argon ion laser excited at 488 nm. It was calibrated by fluorescent beads so that the coefficient of variation was less than 2%. Histograms were evaluated by one of us (YH) without the information of histologic diagnosis. Specimens with a coefficient of variation value greater than 10% were automatically discarded as uninterpretable. Aneuploidy was defined as the presence of an abnormal G_1 peak that was distinct from the G_1 diploid peak.

The proliferation index of tumor cells (% > 4 SD) was defined as the percentage of cells that exceeded the diploid peak (G0/G1) plus 4 standard deviations (SD). This was equivalent to the percentage of cells of S + G_2M phase according to the analysis of Dean & Jett (10).

Forward light scatter, which indicates cell size (11), was also analyzed. A two-parameter plot of nuclear DNA and forward light scatter of tumor cells was performed on each sample to resolve diploid-aneuploid mixtures of cells. This was especially helpful in separating the aneuploid cell populations that overlapped the DNA histogram of the normal diploid population (Fig. 1a, b). Statistical analyses were performed with the Student's *t*-test (unpaired analyses) and chi-squared tests.

RESULTS

All samples showed a definite dipioid G0/G1 peak. In addition, nine out of 10 cases of trichilemmal carcinoma showed aneu-

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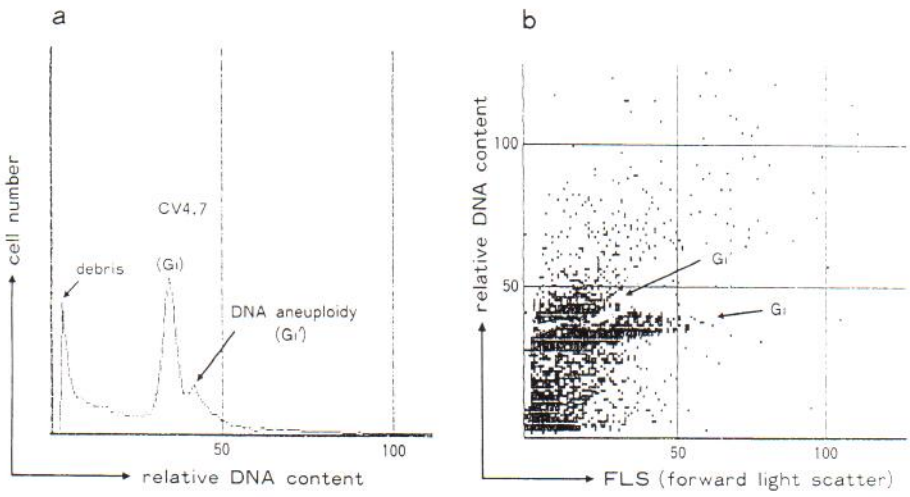

Fig. 1. A two-parameter plot of nuclear DNA (*a*) and forward light scatter (*b*) of tumor cells to resolve diploid-aneuploid mixtures of cells. (TC Case 8).

ploid DNA histograms, whereas 2 of 5 cases of PTC showed aneuploidy. No aneuploidy was detected in 6 cases of trichilemmal cyst.

The proliferating index of trichilemmal carcinoma, PTC, and trichilemmal cyst was 43.5 ± 3.8%, 32.5 ± 7.5%, 23.1 ± 2.0%, respectively. The proliferating index of trichilemmal carcinoma was significantiy higher than that of trichilemmal cyst.

DNA ploidy was studied in various sites of the trichilemmal carcinomas that were large enough for us to investigate regional differences. DNA heteroploidy was detected in one of the 6 cases examined.

DISCUSSION

Our results indicate that nine out of 10 cases of trichilemmal carcinomas from paraffin-embedded archives showed DNA aneuploidy. This was higher than the incidence of aneuploidy of SCC (1/7 = 14%) reported by us using the same preparation (8). It has been known that the incidence of DNA aneuploidy from paraffin-embedded sections is less than that from fresh samples. This is related to higher coefficient of variation values in the former preparation, resulting in less sensitivity in detecting near diploid aneuploidy. In this context, the incidence of aneuploidy in SCC previously reported by us may be an underestimation (8, 12). Despite this flaw in the methodology, trichilemmal carcinoma was shown to be characterized by a high incidence of

Table I. *Clinical data*

		Age	Sex	Location	Size	Local recurrence	Lymph node metastasis	Course	(Follow-up time)
Case	1	47	F	forehead	10×10 mm	(–)	(–)	alive	(16 years)
	2	66	F	scalp	8×10 mm	(–)	(–)	alive	(13 years)
	3	70	F	scalp	18×13 mm	(–)	(–)	alive	(8 years)
	4	40	M	scalp	15×15 mm	(+)	(–)	alive	(5 years)
	5	55	F	scalp	27×20 mm	(–)	(–)	alive	(4 years)
Case	1	83	M	hand	30×25 mm	(–)	(–)	alive	(5 years)
	2	72	F	buttock	13×11 mm	(+)	(–)	alive	(5 years)
	3	91	F	forehead	20×20 mm	(–)	(–)	died of unrelated cause	(2 years)
	4	60	M	axillae	20×20 mm	(–)	(–)	alive	(4 years)
	5	70	M	scrotum	12×4.5×4.5 cm	(–)	(+)	died 2 months later, respiratory failure	
	6	73	F	thigh	35×37 mm	(–)	(+)	alive	(4 years)
	7	86	F	scalp	14×14 mm	(–)	(–)	died of unrelated cause	(3 years)
	8	61	M	scalp	6×6×3.5 cm	(–)	(+)	died 7 months later, systemic metastasis	
	9	75	M	abdomen	10×12 cm	(–)	(–)	alive	(2 years)
	10	67	M	shin	1.5×1 cm	(–)	(–)	alive	(1 year)
Case	1	61	M	scalp	1×1 cm	(–)	(–)	alive	(3 years)
	2	65	F	scalp	5×6 mm	(–)	(–)	alive	(3 years)
	3	48	F	wrist	6×6 mm	(–)	(–)	alive	(4 years)
	4	17	F	face	1×1 cm	(–)	(–)	alive	(6 years)
	5	52	F	back	6×6 mm	(–)	(–)	alive	(7 years)
	6	30	F	hand	5×5 mm	(–)	(–)	alive	(7 years)

+: present, –: not present.

DNA aneuploidy. Trichilemmal carcinoma behaves more anaplastically than SCC on the basis of flow cytometric analysis.

Proliferating index is another property measured by DNA content flow cytometric study. As might be expected, the proliferating index of trichilemmal carcinoma was significantly higher than that of trichilemmal cyst. PTC showed intermediate proliferating index values.

PTC has been considered to be essentially a benign tumor even in the presence of a slight degree of nuclear atypia. In the present study, DNA aneuploidy was detected in 2 out of 5 cases of PTCs, suggesting its pre-malignant nature. There are several case reports of PTC showing carcinomatous transformation with distant metastases (13–17). The 2 PTC cases with aneuploidy have been followed for 8 and 4 years, with no evidence of recurrences. Newton et al. (12) reported DNA aneuploidy in vulvar lichen sclerosis et atrophicus, which is another pre-malignant condition.

It has been suggested that the presence of DNA aneuploidy indicates a poor prognosis in various malignancies (12, 18–20). This is inconsistent with our results, since the lymphnode metastasis of trichilemmal carcinoma has been reported to be relatively low. In SCC of the upper digestive tract, DNA aneuploidy correlates with a favorable prognosis, as compared with diploid tumors (21). Consistent with the results of Newton et al., the incidence of aneuploidy in Bowen's disease was much higher than that of SCC in our study (data not shown).

Newton et al. (12) also reported that keratoacanthoma shows a high incidence of DNA aneuploidy, similar to SCC (48% and 58%, respectively). Although the incidence of aneuploidy in SCC is considerably higher than that of our previous results, it is interesting to note that the incidence of aneuploidy in Bowen's disease (94%) is much higher than that of SCC.

All these results suggest that DNA aneuploidy does not strongly correlate with the prognosis in epithelial skin tumors. The reason for this is still unknown. We speculate that DNA aneuploidy may be reversible in some instances, and is, *per se*, not sufficient to assert a cell population to be malignant in the clinical sense. It is evident that many other factors besides DNA aneuploidy govern the prognosis of epithelial skin tumors, as suggested by Koss et al. (21).

Malignant tumors may exhibit a mixed cell population with different DNA ploidies. Francis et al. (22) reported that the presence of DNA ploidy heterogeneity correlates with a poor prognosis in lung cancer. In our series, too, the only case with DNA ploidy heterogeneity showed a rapid aggressive course (Table I).

Our results indicate that flow cytometric analyses are effectively performed on trichilemmal tumors. Comparison of the data with other types of epithelial skin tumors, however, did not show a significant correlation between the clinical course and the incidence of aneuploidy.

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