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Interphase cytogenetics of melanocytic neoplasms: numerical aberrations of chromosomes can be detected in interphase nuclei using centromeric DNA probes

This study shows that fluorescence *in situ* hybridization (FISH) to thin sections cut from paraffin-embedded material can be used to distinguish between groups of melanocytic neoplasms and thus may be useful as an investigational and diagnostic tool. FISH with a probe for a repeated, alpha satellite sequence specific to chromosome 17 was used to investigate the chromosomal composition of dysplastic (or Clark's nevus) and Spitz's nevi and malignant melanomas. Hybridization was to thin (~6 μm) sections cut from paraffin blocks. The number of signals per nucleus in normal diploid cells is expected to be less than 2 since the sections are thinner than one nuclear diameter. Keratinocytes and lymphocytes in these same sections showed 1–2 signals per nucleus with a mean of 1.2. Dysplastic nevi showed 1–4 hybridization signals per nucleus with a mean of 1.5. Spitz's nevi showed 1–2 signals per nucleus with a mean of 1.3. Melanomas showed 1–6 signals per nucleus with a mean of 2.1. We were thus able to use FISH to demonstrate differences in chromosome numbers between groups of benign and malignant melanocytic neoplasms. Technical improvements in the near future can be expected to result in more precise estimates of chromosomal number.

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Solid human tumors including melanocytic neoplasms are believed to progress toward metastatic disease through accumulation of multiple genetic aberrations (1). Some aberrations may alter the cellular phenotype and thus play a causative role in tumor progression. Others are likely to be random numerical or structural aberrations that result from the genetic instability that seems to be a hallmark of essentially all advanced solid tumors. We focus in this paper on the possibility that analysis of genetic heterogeneity resulting from genetic instability may allow discrimination between benign conditions such as dysplastic (Clark's) and Spitz's nevi and malignant melanoma, with the former appearing essentially diploid and the latter showing an increased fraction of cells with abnormally high chro-

Table 1. Hybridization signals observed for malignant melanoma, Clark's and Spitz's nevi and normal epidermal cells and lymphocytes in paraffin sections after hybridization with a probe for a pericentromeric repeated sequence on chromosome 17.

		Spots per nucleus							
Diagnosis	n	0	1	2	3	4	5	6	S/N
NM, Level IV	209	17	90	79	26	4	2	1	1.71
ALM, Level V	200	22	54	76	28	28	2	0	2.06
ALM, Level IV	106	14	12	48	19	10	1	2	2.09
ALM, Level IV	200	21	51	66	37	20	3	2	2.01
SSM, Level II	200	7	39	85	41	25	3	1	2.27
NM, Level II	200	8	40	92	46	9	4	1	2.12
SSM, Level II	199	5	57	75	43	9	7	3	2.14
NM, Level IV	200	8	40	92	46	9	4	1	2.12
Total	1514	102	383	613	286	114	26	11	2.06
% of total		6.7	25.3	40.5	18.9	7.5	1.7	0.7	
DN	202	16	101	75	10	0	0	0	1.39
DN	193	4	83	98	8	0	0	0	1.57
DN	173	8	86	69	9	1	0	0	1.47
DN	193	3	86	93	11	0	0	0	1.58
Total	761	31	356	335	38	1	0	0	1.50
% of total		4.1	46.8	44.0	5.0	0.1	0	0	
SN	210	24	111	75	0	0	0	0	1.24
SN	208	23	121	64	0	0	0	0	1.20
SN	208	16	118	73	1	0	0	0	1.28
SN	194	15	79	99	1	0	0	0	1.44
Total	820	78	429	311	3	0	0	0	1.29
% of total		9.5	52.3	37.9	0.2	0	0	0	
Keratinocytes	828	207	358	252	0	0	0	0	1.04
%		25	43.1	30	0	0	0	0	
Lymphocytes	827	127	379	321	0	0	0	0	1.23
%		16.4	45.8	38.8	0	0	0	0	

MM = Malignant melanoma; DN = Dysplastic (Clark's) nevus; SN = Spitz's nevus; NM = Nodular melanoma; ALM = Acral lentiginous melanoma; SSM = Superficial spreading melanoma.

mosome copy number resulting from genetic instability during tumor growth. Detection of genetic aberrations in these cells has been addressed previously through analysis of cellular DNA content using flow cytometry and image analysis (2). These studies suffered from limited sensitivity to small changes in DNA content or to the presence of highly abnormal, low frequency subpopulations.

Fluorescence *in situ* hybridization (FISH) with chromosome-specific probes seems to be a more promising approach since it has been shown to have high sensitivity for detection of altered chromosome copy number (3, 4) even when present in cells at low frequency. We describe here use of a modification of the hybridization procedure of Hopman et al. (5) for analysis of ~6-μm thick sections cut from paraffin-embedded Clark's and Spitz's nevi and from malignant melanoma. We show that FISH using a probe for the pericentromeric region of chromosome 17 reliably generated more hybridization signals (both average number and range) in malignant melanoma than in Clark's or Spitz's nevi.

Material and methods

Clinical material

Samples analyzed included (a) 8 primary cutaneous melanomas (3 nodular, 3 acral lentiginous, 2 superficial spreading types) received in the Department of Clinical Pathology, Iwate Medical University, fixed in 20% unbuffered formalin for 3 days and embedded in paraffin; (b) 4 compound'dysplastic nevi mounted in paraffin blocks from the archives of the dermatopathology section of the University of California, San Francisco (UCSF); and (c) 4 Spitz's nevi in paraffin blocks, also from the UCSF archives. The dysplastic nevi were compound lesions in which junctional nests extended beyond the lateral confines of a papillary dermal component, with bridging of adjacent rete ridges, fibroplasia of the papillary dermis, and sparse lymphocytic infiltrates; i.e. they showed the architectural features of dysplastic nevi and fulfilled Ackerman's criteria for Clark's nevus (6). They were not diagnosed on the basis of cytologic atypia of melanocytes, nor was such atypia discernible. The Spitz's nevi were classic compound examples and did not have exceptional nuclear pleomorphism. The histopathologic diagnoses of the 16 cases are shown in Table 1.

Tissue preparation

Six-μm thick sections were cut from paraffin embedded material and mounted on poly-L-lysine coated slides. Sections were deparaffinized two times for 20 min in xylene and rehydrated by incubation in 100%, 90% and 70% ethanol (v/v) for 5 min each. The slides were rinsed in distilled water and then digested in a pepsin solution (0.5% (5 mg/ml) pepsin/0.1N HCl) for 15–20 min at 37°C. This reaction was stopped by rinsing with PBS twice, and the sections were then fixed in 0.1% paraformaldehyde/PBS for 10 min. Finally, the slides were washed twice in saturated sodium citrate (SSC) for 10 min and dipped in 50% formamide/2xSSC.

In-situ hybridization

FISH was performed using a probe specific for a pericentromeric repetitive DNA sequence on chromosome 17 (cK17.10; 7). The probe was labeled using nick translation (Bionick labeling system, BRL, Gaithersburg, MD) with digoxigenin-11-dUTP (Boehringer-Mannheim Corporation, Indianapolis, IN). Hybridization was accomplished us-

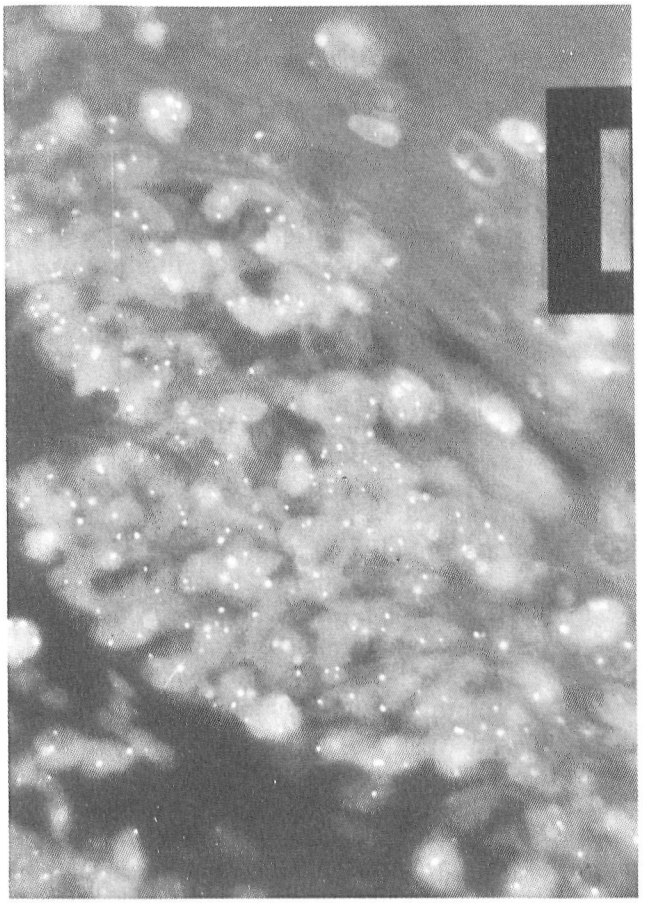

Fig. 1. FISH to cells of malignant melanoma adjacent to the epidermis. Probes were detected using fluorescein and appeared yellow. Nuclei were counterstained with propidium iodide and appeared red. Two or more hybridization signals are apparent in many neoplastic cells. Adjacent keratinocytes show only 1 or 2 signals. (40×).

ing 8 µl of master mixture (5.5 ml formamide, 1 g dextran sulfate, and 0.5 ml 20 × SSC, pH 7.0), 1 µl of sonicated herring sperm DNA (Sigma, St. Louis, MO) and 1 µl of labeled probe DNA. This mixture was denatured in a water bath at 74°C for 5 min and immediately placed on ice. Pretreated slides were denatured in 70% formamide/2xSSC at 74°C for 10 min, dehydrated in an ethanol series and air dried. Ten µl hybridization mixture was then added for each square centimeter of the tissue section, covered by a coverslip and sealed with rubber cement. The hybridization was incubated at 37°C overnight. Slides were washed at 42°C three times for 10 min each in 50% formamide/2xSSC and then at 42°C twice for 10 min in 2xSSC. Finally the slides were washed at room temperature for 10 min in 4xSSC.

Hybridized probe was detected immunochemically. Each slide was treated for 5 min with 50 µl 4xSSC/1% bovine serum albumin under a coverslip and then for 40 min with 50 µl FITC-conjugated sheep anti-digoxigenin (Boehringer-Mannheim), 2 µg/ml Fab fragments in PNM, washed three times at room temperature for 10 min in 4xSSC and once in PN for 10 min. The signal was further increased by treating the slides for 5 min with 50 µl PNM under coverslips and 50 µl rabbit anti-sheep IgG conjugated with FITC (Jackson Immunological Inc., West Grove, PA) 320 mg/ml in PNM. The slides were then washed three times in PNM and counterstained with propidium iodide (PI) in antifade (10 mg p-phenylene diamine HCl in 10 ml Dulbecco's PBS) or 4′-6-diamidino-2-phenyl-indole (DAPI) in antifade.

Evaluation of FISH signals

FISH signals were counted under 100× magnification using a Zeiss fluorescent microscope. One-hundred to 200 nuclei were counted in dermal neoplastic cells from melanoma and Spitz's nevus, and both junctional and dermal melanocyte nuclei were counted in dysplastic (Clark's) nevi. Counting was performed using criteria proposed by Hopman and colleagues (8). Specifically: a) only distinct isolated nuclei were counted, b) fluorescent signals were

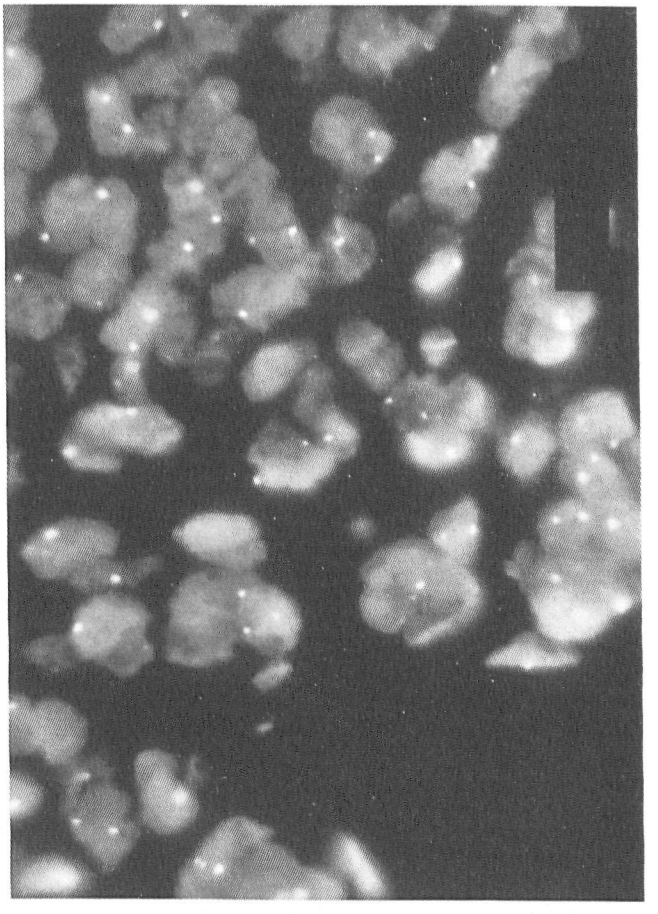

Fig. 2. FISH to melanocytes of a dysplastic or Clark's nevus. Probe detection and counterstaining as described in Fig. 1. Almost all cells show 1 or 2 hybridization signals. One melanocyte shows 3 signals. (×100).

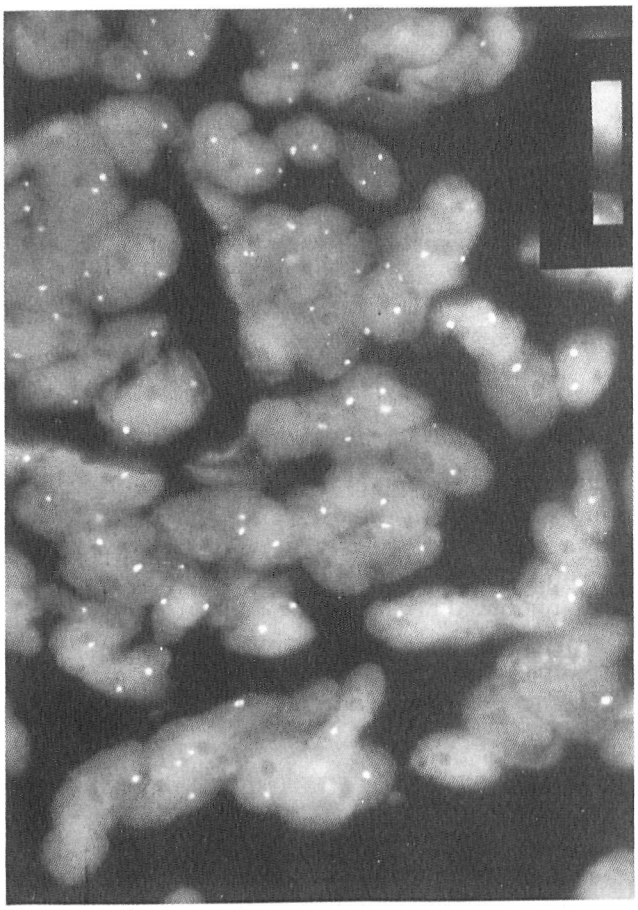

Fig. 3. FISH to a Spitz's nevus. Most cells show 1 or 2 signals. (×100).

scored as true hybridization events only if they were approximately the same size and intensity as those in adjacent cells, and c) paired signals were scored as single events. Lymphocyte and epidermal keratinocyte nuclei in the same sections were used as internal controls.

Results

Representative fluorescence photomicrographs of hybridization to melanoma cells, Clark's nevus, and Spitz's nevus are shown in Figs. 1, 2 and 3; respectively. Figs. 4 and 5 show hybridization to normal epidermal keratinocytes and lymphocytes; respectively. The results of quantitative hybridization signal analysis are shown and summarized in Table 1 for these tissues. In general, the number of spots/nucleus observed for the malignant melanoma was higher, both in mean number of events per nucleus and in range than for Clark's and Spitz's nevi. Specifically, the means (±1 std dev) for malignant melanoma, Clark's nevi and Spitz's nevi were 2.06 (±0.16), 1.50 (±0.09) and 1.28 (±0.11), respectively. The average number of hybridization domains for control epidermal keratinocytes and lymphocytes were 1.04 and 1.23 respectively. The mean number of hybridization domains for the malignant melanomas was significantly different than the mean for either Clark's nevi ($p = 7\times10^{-5}$) or Spitz's nevi using the two-tailed t-test assuming unequal variance ($p = 7\times10^{-6}$). The means for the Clark's and Spitz's nevi were not significantly different ($p = 0.02$). The means for all of three neoplasms were different than those for the normal epidermal nuclei and the lymphocytes.

Discussion

This study describes an analysis of chromosome copy number heterogeneity in a range of melan-

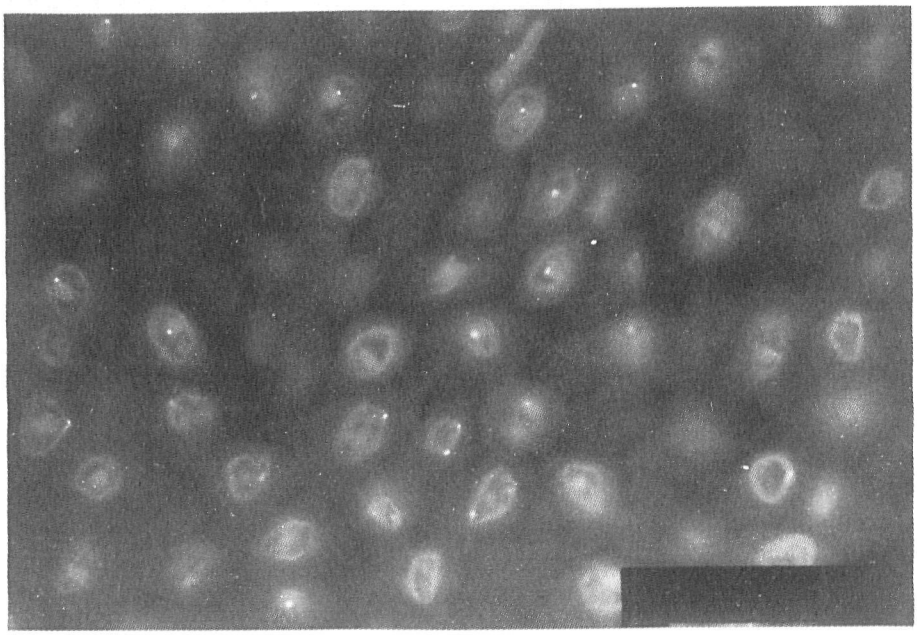

Fig. 4. FISH to normal keratinocytes. Most cells show 1 or 2 signals. (×100).

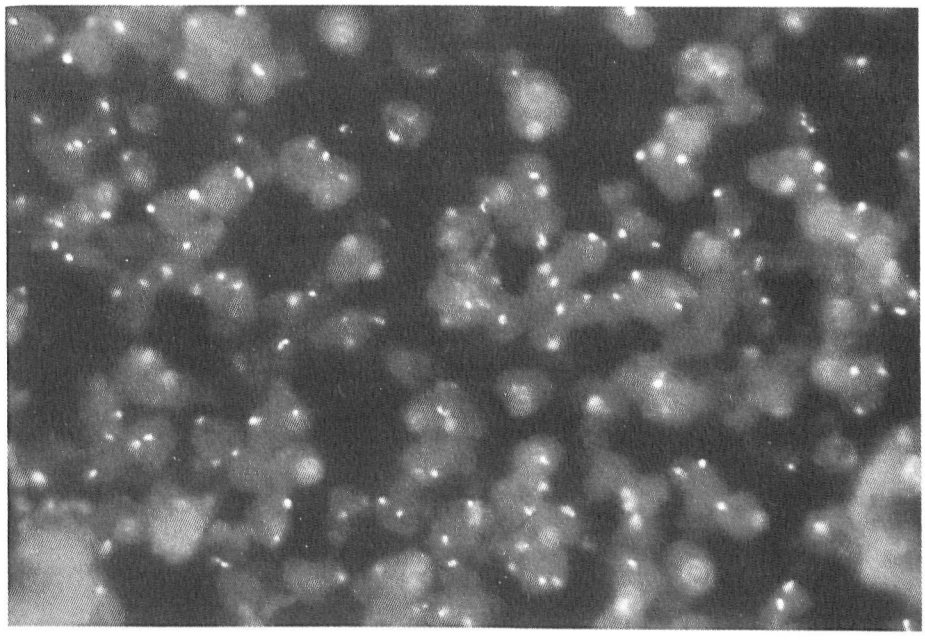

Fig. 5. FISH to normal lymphocytes. Most cells show 1 or 2 signals. (×100).

ocytic neoplasms. This was accomplished using FISH with a probe to a pericentromeric region on chromosome 17 to thin sections cut from routinely processed, paraffin-embedded tissue. We used this probe because it was highly specific and resulted in strong signals in interphase nuclei. Thus, we expected that the number of hybridization signals/nucleus would indicate the degree of genetic heterogeneity in the target tissue. Our presumption was that heterogeneity is a hallmark of malignancy and that malignant melanoma would show greater heterogeneity (and as a result a higher average copy number) than normal tissues or Clark's or Spitz's nevi. We did not anticipate that chromosome 17 was particularly important in the progression of melanoma.

Both the mean number of hybridization signals and the range in the number of signals was larger for malignant melanoma than for Spitz's nevi or for Clark's nevi or for normal cells in the same sections. This finding suggests that FISH may be beneficial for discrimination between malignant melanoma and Clark's and Spitz's nevus in cases that appear morphologically similar. This difference probably results from the fact that malignant melanoma is more unstable genetically than the other melanocytic neoplasms. However, we cannot conclude from these studies that Clark's and Spitz's nevi are not aneuploid or that they do not contain rare aneuploid cells since our studies were conducted using 6-μm thick sections. Many melanocytes in each of the classes of lesion that we analyzed had nuclei whose diameters exceed 6 μm. Thus, the number of spots per nucleus was often less than two (the expected copy number of chromosome 17 in diploid cells). The nuclei of Spitz's nevi are particularly large for those of a benign melanocytic neoplasm, and this may account for our finding that the mean spots per nucleus was lower in the nuclei for Spitz's nevi than for Clark's nevi, as proportionately less of the nucleus and fewer chromosomes are included in each section. Whether these findings will survive the analysis of nevi of either type with striking cytologic atypia awaits further analysis, as the group of dysplastic (Clark's) nevi were diagnosed on the basis of architectural and not cytologic criteria, and cells with extreme nuclear pleomorphism were systematically ignored during analysis of Spitz's nevi since these nuclei could not be distinguished from overlapping nuclei in these thin sections. Our study sheds no light on the question of whether dysplastic nevi are neoplasms with biological features intermediate between "common" nevi and malignant melanoma. We did not analyze a group of such specimens.

What can dermatopathologists expect from FISH in comparison with other cytometric methods for evaluating melanocytic neoplasms? It is similar to image analysis cytometry, in that either whole cells from disaggregated specimens or tissue sections can be examined. It has an advantage over image analysis or flow cytometry for ploidy analysis in that cells that have high DNA content because they are in S-phase can be recognized by apposition of the spots in FISH, and so do not result in an overestimate of stem-line DNA content. The chromosome 17 probe used in this study (and those specific for many other chromosomes) produces strong, distinctive signals, so that chromosome copy number can be readily assessed. FISH also allows analysis of low-frequency cell subpopulations identified by histologic analysis of adjacent sections. Current limitations

include the overly frequent failure of the sections to adhere throughout hybridization (dermal collagen sticks to even specially coated slides only with difficulty) and the labor involved in counting signals and nuclei. The assay also is complicated by the loss of nuclear material during thin sectioning so that accurate assessment of chromosome copy number is impossible. Progress is being made on elimination of these technical difficulties, and accurate analysis of much thicker sections may be possible in the near future (C. Thompson, personal communication).

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