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Molecular Cytogenetic Analysis of Spitz Nevi Shows Clear Differences to Melanoma

Boris C. Bastian, Ulrich Wesselmann, Dan Pinkel, and Philip E. LeBoit*

Cancer Genetics Program, Cancer Center, University of California San Francisco, San Francisco, California, U.S.A.; *Dermatopathology Section, Departments of Pathology and Dermatology, University of California San Francisco, San Francisco, U.S.A.

Spitz nevus is a benign neoplasm of melanocytes that can be difficult or impossible to distinguish from melanoma by clinical and histopathologic examination. We studied genomic DNA from 17 Spitz nevi by comparative genomic hybridization (CGH). Thirteen lesions showed no chromosomal aberrations, three cases had a gain involving the entire p-arm of chromosome 11, and one case showed a gain of chromosome 7q21-qter. Fluorescence *in situ* hybridization (FISH) on lesional tissue with a probe for the p-arm of chromosome 11 showed 6–10 p-arm signals per nucleus in those cases with a CGH-detected gain of chromosome 11p. One case with a normal CGH profile also showed increased copy number of 11p by FISH. Thus, the majority of Spitz nevi have a normal chromosomal complement at the level of CGH resolution; however some may contain gains, with 11p apparently being the most frequently involved location. These findings differ significantly from the previously reported changes in primary cutaneous melanoma, which show frequent deletions of chromosomes 9p (82%), 10q (63%), 6q (28%), and 8p (22%), as well as gains of chromosomes 7 (50%), 8 (34%), 6p (28%), 1q (25%) by CGH analysis. These clear differences in the location and frequencies of chromosomal aberrations in Spitz nevi and primary cutaneous melanomas could represent a basis for developing adjunctive techniques for refining accuracy in the difficult differential diagnosis of spitzoid melanocytic neoplasms. *Key words: comparative genomic hybridization/epitheloid and spindle cell pathology/genetics/skin neoplasms. J Invest Dermatol 113:1065–1069, 1999*

Spitz nevi are benign melanocytic neoplasms that can resemble melanoma on histopathologic examination. They were first described as 'juvenile melanoma" by Sophie Spitz in 1948 and were initially regarded as a subset of childhood melanoma that follows a benign course (Spitz, 1948). Spitz nevi account for about 1% of surgically removed melanocytic nevi (Casso *et al*, 1992).

Although in general the pathologic diagnosis of Spitz nevus can be reliably achieved by conventional histopathologic criteria, there is a subset of cases in which it is difficult or impossible to differentiate Spitz nevi from melanoma. Both Spitz nevi and melanoma can be composed of melanocytes with abundant cytoplasm and large nuclei. These nuclei can be pleomorphic and contain macronucleoli. Mitotic figures, sometimes numerous, occur in both neoplasms.

Misdiagnosis of Spitz nevus as melanoma and vice versa has been repeatedly reported (Okun, 1979; Goldes *et al*, 1984; Peters and Goellner, 1986). A retrospective study of 102 melanocytic tumors occurring in patients under age 17, which were originally diagnosed as melanoma, found that only 60 cases were classified as melanoma after re-examination by a panel of experts (Spatz *et al*, 1996). Forty-two lesions were re-classified as nevi, 22 of those as Spitz nevi. The hazard of mistaking a Spitz nevus for melanoma is that the patient may be subjected to needless surgery and adjunctive therapy, become unable to plan for the future, be psychologically traumatized, and have difficulties in obtaining health insurance. For obvious reasons the misdiagnosis of a melanoma as nevus can have even more dramatic sequela. The difficulties in differentiating Spitz nevi from melanoma and the consequences of underdiagnosis lead some authors to propose that Spitz nevi and melanoma represent a continuum of disease (Casso *et al*, 1992).

We recently reported on frequent losses of chromosomes 9 and 10 as well as gains of chromosomes 7, 8q, and 6p in primary cutaneous melanoma (Bastian *et al*, 1998). The purpose of this study was the characterization of genomic aberrations in Spitz nevi using comparative genomic hybridization (CGH) (Kallioniemi *et al*, 1992) as an unbiased genome scanning approach to identify potential differences to melanoma.

MATERIALS AND METHODS

Tumor material Formalin-fixed, paraffin-embedded tissue from Spitz nevi from 17 patients were retrieved from the archives of the Department of Dermatology (University Hospital, Würzburg, Germany) and the Dermatopathology Section, Departments of Pathology and Dermatology (University of California, San Francisco). We selected lesions in which the histopathologic diagnosis was unequivocal using widely agreed upon criteria, had an extensive and densely cellular dermal component that allowed the collection of mostly melanocytes (>50%), and had at most a sparse lymphocytic infiltrate, so that lymphocyte DNA would not obscure aberrations in the neoplastic cells.

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Reprint requests to: Dr. Boris C. Bastian, Cancer Genetics Program, Cancer Center, University of California San Francisco, Box 0808, San Francisco CA 94143–0808. Email: bastian@cc.ucsf.edu

Abbreviations: CGH, comparative genomic hybridization; FISH, fluorescence *in situ* hybridization.

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Table I. Clinical information of the Spitz nevi and aberrations found by CGH and FISH[a]

Case	Sex	Age	Aberrations	Clinical information	Site	Histology	Follow-up
S1	f	17.9	none	Since 1 y slowly enlarging 1.1 × 1.4 cm reddish papule with brown rim	Shin	compound with desmoplasia	FOD, 5.4 y
S2	m	31.0	none	Since one unchanged cherry pit-sized dome-shaped reddish nodule	Lower arm	dermal with desmoplasia	FOD, 3.4 y
S3	f	21.5	none	NA	Upper back	compound	FOD, 1.2 y
S4	f	7.2	none	Since 1 y cherry-pit-sized tumor	Knee	dermal with desmoplasia	FOD, 4.0 y
S5	f	23.8	Amp. 11p (FISH)	Since 1 y discretely enlarging	Knee	compound	FOD, 7.5 y
S6	m	7.4	none	Since 3 mo slowly growing lentil-sized, slightly keratotic, elevated	Ear helix	compound with desmoplasia	FOD, 9.3 y
S7	m	13.7	none	Since years 1.5 cm, raised, firm nodule	Lateral abdomen	compound	FOD, 2.6 y
S8	f	3.2	none	Since 1.5 y growth of a pigmented tumor	Cheek	PSCT	FOD, 6.9 y
S9	f	31.3	none	NA	Thigh	compound	FOD, 2.3 y
S11	f	3.0	Gain of 7q31-qter	NA	Inner thigh	PSCT	FOD, 8.1 y
S12	f	23.0	none	NA	Epigastrium	PSCT	FOD, 4.2 y
S13	f	45.8	Amp. of 11p	NA	Thorax	dermal with desmoplasia	FOD, 5.3 y
S14	f	10.8	none	Since 7 mo itching, reddish papule	Cheek	compound	NA
S15	f	26.7	none	NA	Shin	dermal	FOD, 1.2 y
S16	f	23.2	Amp. of 11p	1.5 y ago curettage of a lesion that arose 4 mo earlier. Lesion recurred 1 mo later and was again curetted. Recurred again after 2 mo and was excised. Now, after 2 mo, again recurring. Reddish, sharply circumscribed lentil-sized papule.	Tip of nose	compound with scar tissue	FOD, 7.3 y
S17	m	12.5	none	NA	Mid back	compound	NA
S18	m	11.3	Amp. of 11p	enlarging skin lesion for 3 mo	Calf	compound with desmoplasia	NA

[a]PSCT, pigmented spindle cell tumor; FOD, free of disease; NA, not available.

DNA preparation

Paraffin material Thirty micrometer sections were cut, with a 5 μm section for hematoxylin and eosin every five sections. The unstained 30 μm sections were placed on glass slides and an area of interest was microdissected without de-paraffinizing.

Microdissection was carried out manually under a dissecting microscope. Depending on the size of the tumor, 20–60 unstained sections were used and regions with a high density of tumor cells were separated from normal cells. The dissected tumor parts were collected in tubes and de-paraffinized by washing with xylene and ethanol. DNA extraction and labeling was performed as published by Isola *et al* (1994). Briefly, tissue was incubated until complete digestion (3 d) with proteinase K (Life Technologies, Gaithersburg, MD) in a 50 mM Tris pH 8.5, 1 mM EDTA, 0.5% Tween 20 buffer. DNA was extracted with phenol-chloroform-isoamylalcohol (25:24:1, vol/vol), precipitated with 7.5 M ammonium acetate and 100% ethanol, and resuspended in water. The amount of DNA obtained ranged from 2 to 12 μg.

Comparative genomic hybridization (CGH) and digital image analysis All tumors were measured both with the tumor DNA labeled with fluorescein-12-dUTP (Dupont, Boston, MA), and reference DNA with Texas red-5-dUTP (''standard'' labeling), and with the labeling reversed. Labeling was performed by Nick translation. Nick translation conditions were adjusted so that the mean probe fragment size after labeling ranged between 800 and 1500 bp. The hybridization mixture consisted of 200–1000 ng of labeled tumor DNA, 200 ng inversely labeled sex-matched normal human reference DNA from peripheral blood lymphocytes, and 25 μg human Cot-1 DNA (Life Technologies) dissolved in 10 μl hybridization buffer (50% formamide, 10% dextrane sulfate, and 2 × SSC, pH 7.0). Hybridization was carried out for 2–3 d at 37°C to normal metaphases (Kallioniemi *et al*, 1994). All samples were investigated with a single batch of metaphase slides. Slides were washed three times in a washing solution (50% formamide in 2 × SSC, pH) at 45°C, once in PN buffer (0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4, and 0.1% Nonidet P40, pH 8.0), and once in distilled water (both 10 min at room temperature). Slides were counterstained with 4,6-diamino-2-phenylindole in an antifade solution. Hybridization quality was evaluated as published previously (Bastian *et al*, 1998). Digital images were collected from five metaphases with a Photometrics CCD camera (Microimager 1400, Xillix Technologies, Vancouver, British Columbia, Canada) on a standard fluorescence microscope. The average tumor/reference fluorescence ratios along each chromosome were calculated with custom CGH analysis software. Measurements were made on at least four copies of each autosome.

Controls and threshold definitions Normal DNA and DNA from tumor cell lines with known aberrations were used as controls. Definition of aberrant regions was performed in two stages. First, thresholds levels were set at tumor:reference fluorescent ratios of 0.80 and 1.2 (Richter *et al*, 1997). Any region where the ratio exceeded these thresholds with either of the standard or reverse labeled hybridizations was called abnormal.

Becuase potential copy number changes that only occurred in a subset of cells would be reduced in magnitude, however, we also applied an additional, narrower threshold interval of 0.85 and 1.15. These values are based on a series of 15 comparisons of normal DNA samples, where the ratios never were outside the range of 0.9–1.1. Because the narrower thresholds have a greater chance of falsely indicating aberrations, we required ratio profiles for both the standard and the reverse labeled hybridizations to be outside the 0.85 and 1.15 interval for a region to be called abnormal.

Fluorescence *in situ* hybridization (FISH) Dual-color FISH was carried out on tissue sections of the cases in which tissue was left after CGH (14 of 17). A BAC clone and a P1 clone mapping to the short arm (RMC11B022 and RMC11P014) as well as a P1 clone mapping to the long arm (RMC11P008) of chromosome 11 were obtained from the resource of the laboratory. Probes were labeled by nick translation with Cy3 (Amersham, Arlington Heights, IL) or Digoxigenin (Boehringer Mannheim, Indianapolis IN). Six micrometer sections were mounted on positively charged glass slides (Fisher Scientific, Pittsburgh, PA), deparaffinized, and hydrated by decreasing strength ethanol. Sections were incubated for 2–4 min in 1 M sodium thiocyanate at 80°C, in 4 mg Pepsin per ml in 0.2 N HCl at 37°C for 4–8 min, dehydrated by increasing strength ethanol, and air-dried. Slides were denatured in 70% formamide, 2 × SSC pH 7.0 for 5 min at 72°C, and dehydrated again in a graded ethanol series. 2.5–25 ng of each of the labeled probes together with 20 μg Cot-1 DNA (Life Technologies) were dissolved in 10 μl hybridization buffer (50% formamide, 10% dextrane sulfate, and 2 × SSC, pH 7.0) and denatured for

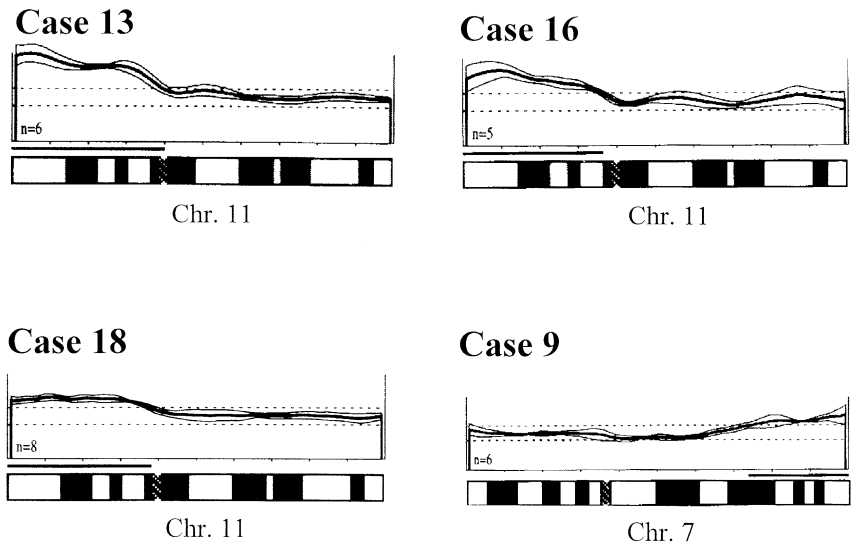

Figure 1. Average ratio profiles of fluorescence intensity of tumor *versus* reference DNA in the four Spitz nevi that had abnormal CGH profiles. The dotted lines indicate the 1.2 and 0.8 ratio thresholds that were used for defining aberrations. n indicates the number of chromosomes measured for the respective profile.

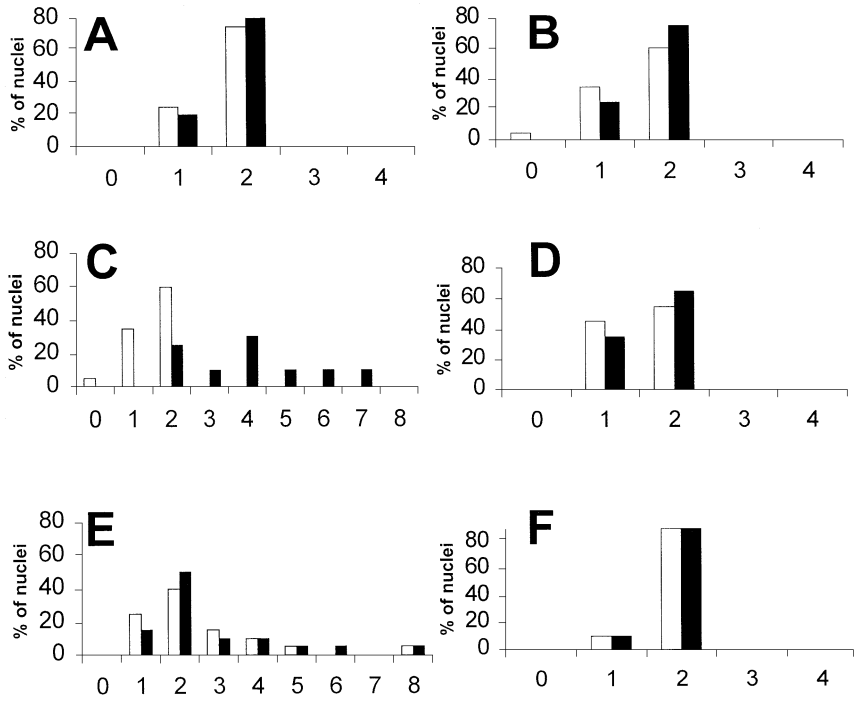

Figure 2. Frequency distribution of hybridization signals after dual-target hybridization of probe RMC11B022 for chromosome 11p and RMC11P008 for chromosome 11q. (*A*, *C*, *E*) Signal distribution in tumor cells; (*B*, *D*, *F*) signal distribution in keratinocytes of the corresponding lesions; *black bars*, chromosome 11p; *white bars*, chromosome 11q. Three cases of Spitz nevi are shown. Case 2 (*A*, *B*) showed no chromosomal aberrations by CGH, case 13 (*C*, *D*) had a gain of chromosome 11p by CGH, case 15 (*E*, *F*) did not show aberrations by CGH, it had a subpopulation of tumor cells with large nuclei.

10 min at 72°C. Hybridization was carried out for 48–72 h at 37°C. Slides were washed three times in washing solution (50% formamide in 2 × SSC, pH 7.0) at 45°C, once in 2 × SSC at 45°C, once in 2 × SSC at room temperature, and once in 0.1% Triton × 100 in 4 × SSC at room temperature. Subsequently, sections were incubated with 10% bovine serum albumin in 4 × SSC in a moist chamber at 37°C, and then with a FITC labeled antidigoxigenin antibody (Boehringer Mannheim, Indianapolis IN) diluted in 4 × SSC with 10% bovine serum albumin. Sections were counterstained with 4,6-diamino-2-phenylindole (Sigma) in an antifade solution.

Statistical analysis The two-tailed student's t test was used for the comparison of FISH signals for the locus of interest and the reference probe.

RESULTS

The clinical data of the cases are shown in **Table I.** Patient ages ranged from 3 to 45 y (mean 18 y). Follow-up was available from most patients. The follow-up time was 1.2–9 y (mean 4.9 y). All patients with available follow-up were free of disease by the end of the follow-up interval. In one case (case 16) two recurrences prior to the final excision of the lesion that entered the study occurred, possibly because the tumor was curetted twice. Re-cut sections of all cases represented typical Spitz nevi by histopathologic examination. Thirteen of the 17 tumors (76%) showed no DNA copy number changes by CGH. Three cases (18%) showed gain of the entire short arm of chromosome 11 as the sole abnormality. (**Fig 1**). One case showed gain of chromosome 7q21-qter as the only abnormality (**Fig 1**).

We performed FISH to tissue sections in order to study the histopathologic distribution of the recurrent gain on chromosome 11p and to find potential minor populations of cells with this aberration in the cases with normal CGH profiles. We selected a test probe that mapped to the distal part of chromosome 11p (11p15.5, BAC clone RMC11B022) and a reference probe mapping to chromosome 11q23 (P1 clone RMC11P008). In all experiments keratinocytes of the epidermis adjacent to the lesion

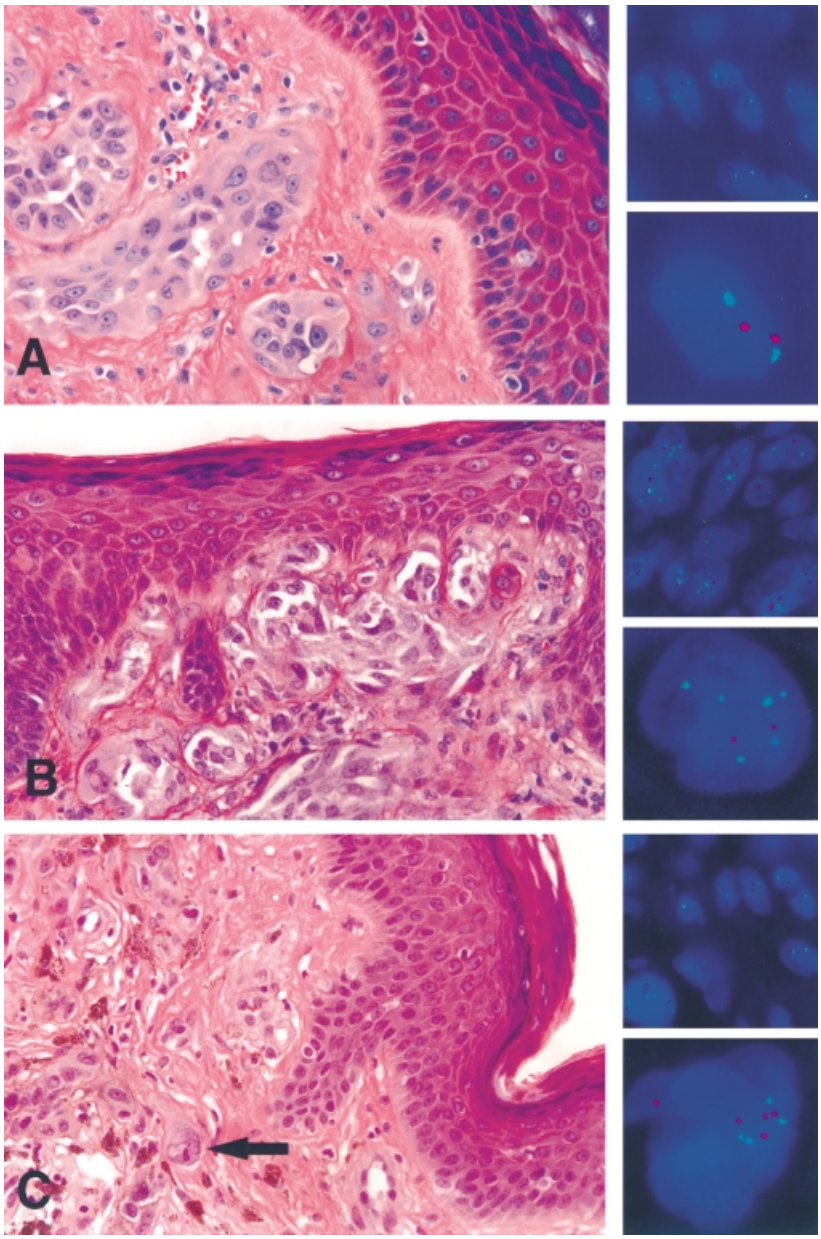

Figure 3. Histology and dual-target FISH with probe RMC11B022 for chromosome 11p and RMC11P008 for chromosome 11q. (*A*) Case 2, normal CGH measurement; (*B*) case 13, gain of chromosome 11p; (*C*) case 15, normal CGH measurement, FISH shows that infrequent cells with large nuclei (*arrow*) are polyploid; *green signals*, chromosome 11p; *red signals*, chromosome 11q. The cases correspond to the cases shown in **Fig 2**.

were used as internal controls. The nuclear signal counts in keratinocytes for the q-arm and the p-arm probe ranged from mean values of 1.6–1.9 and 1.7–1.9, respectively (**Fig 2*b***, ***d***, ***f***). The three cases that had a gain of chromosome 11p by CGH showed a mean of 3.5–5.3 signals with the p-arm probe compared with a mean of 1.5–2.1 counts for the q-arm probe (**Figs 2*c***, **3*b***). This difference was highly significant ($p < 0.00001$). The counts for the q-arm (control) probes were not statistically different from signal counts in keratinocytes (normal cells) of the respective lesions. The ratio of p-arm signals to q-arm signals in the cases with increased copies of chromosome 11p ranged from 1.8 to 3.0. The increased signal number of the p-arm probe was present in virtually every cell of each of the nevi. From the 14 tumors that had no gain of chromosome 11p by CGH, 12 could be studied by FISH. In the other two cases the paraffin blocks were exhausted. Of these 12 cases, 11 had no significant differences in signal distribution of the probes for p-arm and the q-arm of chromosome 11 (**Figs 2*a***, ***b***, **3*a***). One case (case 5) had 2.4 p-arm signals *versus* 1.9 q-arm signals, a difference that was statistically significant ($p = 0.01$). In two cases (cases 3 and 15) a subpopulation of cells was present that had increased numbers of both the q-arm and the p-arm signal (**Figs 2*e***, **3*c***). These cells mostly had considerably larger nuclei than the tumor cells with 1–2 signals, and are thus likely to be polyploid.

As illustrated in **Fig 1** the area of chromosome 11 that was found by CGH to be gained in three cases seems to be identical. The profiles of case 13 and case 16 suggest the highest increase of DNA copy number towards the p-telomere; however, the profiles of the CGH measurement in which the labeling was reversed showed a decrease of red:green fluorescence ratio toward the telomere (data not shown), indicating that the p-telomeric ratio increase is artifactual. To confirm this, we performed FISH with a different probe for the p-arm that mapped more proximally to 11p14 (RMC11P014). The number of signals in the nuclei of the tumor cells with this probe was similar to that found with the probe for 11p15 (data not shown).

We did not detect any histopathologic differences between the Spitz nevi that had a gain of 11p and those without this aberration.

DISCUSSION

Our study shows that the majority of Spitz nevi have a normal chromosomal complement, but that a subset may have abnormalities. The finding of an increased copy number of chromosome

11p in four of 17 lesions indicates that this aberration represents a recurrent change in Spitz nevi. It suggests that increased dosage of genes of chromosome 11p has relevance in the pathogenesis of this tumor. As the gained genomic fragment is large, additional studies are warranted to refine the extent of the region as a first step toward identifying the critical gene(s). It may well be that in the Spitz nevi without 11p gain, those genes, or the pathways they belong to, are activated by a different mechanism than increased gene copy number. A previous study found interstitial deletions of chromosome 9p in two of 27 Spitz (Healy *et al*, 1996). Chromosome 9p is frequently lost in melanoma but the authors showed that it is not exclusive to melanoma. Thus Spitz nevi is one of the many benign lesions that may contain genetic abnormalities at the chromosomal level such as lipoma and leiomyoma (Mitelman, 1994).

A gain of chromosome involving the entire short arm of chromosome 11 was not seen in any of the 120 primary melanomas we have analyzed by CGH (in part published in Bastian *et al*, 1998). Only one case of our series had a high-level amplification limited to the tip of chromosome 11p (band 11p15.5). Gains including 11p were also found in only two cases of the 239 published karyotypes, mostly from metastatic melanomas (Mitelman, 1994; Thompson *et al*, 1995). In these two cases the gain extended far on to the q-arm (Thompson *et al*, 1995).

It is notable that the chromosomal alterations most frequently found in primary melanomas are absent in Spitz nevi. In our study using CGH on 32 primary melanomas we found losses of chromosome 9p in 82%, chromosome 10 in 63%, and 6q in 28% of the cases (Bastian *et al*, 1998). Frequent gains in melanoma involved chromosome 7p (50%), 8q (34%), 6p (28%), and 1q (25%). None of these changes was found in our series of Spitz nevi. Thus it may be that the determination of copy number of other chromosomal regions such as 7p, 10q, 6p, and 1q, may prove to be helpful in the differential diagnosis of Spitz nevi and melanoma. The efficacy of such a test needs to be evaluated through the analysis of a larger set of tumors with the inclusion of cases that have conflicting histopathologic criteria but have known follow-up. This will permit determination of the sensitivity and specificity under clinically relevant conditions.

FISH measurements not only confirmed the CGH findings but also allowed some interesting insight into the ploidy and clonality of Spitz nevi. Because almost all cells in the nevi had two copies of the control locus on 11q by FISH and CGH showed no aberrant copy numbers for that locus, the large majority of the cells in these nevi are diploid, which is consistent with previous flow cytometry studies (Winokur *et al*, 1990). Two cases had a subpopulation of cells with large nuclei. Those cells with elevated copy number had elevated FISH signals for the two loci tested, indicating that the increased nuclear size is most likely due to polyploidy. Our data also show that Spitz nevi are probably comprised of a monoclonal population of melanocytes. This can be concluded from the three cases with a gained 11p, because the increased copy number of this chromosomal arm was present in all cells of the lesions

In summary, we demonstrated that in Spitz nevi (i) the majority of cases have a normal chromosomal complement at the level of CGH resolution, (ii) gains of chromosome 11p represent a recurrent aberration in a subset of lesions, (iii) Spitz nevi probably are clonal neoplasms, (iv) the majority of the melanocytes of a Spitz nevus are diploid with the exception of cells with large nuclei that can be polyploid, and (v) the clear differences in the location and frequencies of the cytogenetically detectable aberrations in Spitz nevi compared with those previously found in primary cutaneous melanoma could make CGH and FISH promising techniques for refining diagnostic accuracy of this difficult differential diagnosis.

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