Mutations and Copy Number Increase of HRAS in Spitz Nevi with Distinctive Histopathological Features

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Spitz nevus is a benign melanocytic neoplasm that can be difficult or impossible to histologically distinguish from melanoma. We have recently described copy number increases of chromosome 11p in a subset of Spitz nevi. To study the molecular and histological features of this group, we studied 102 Spitz nevi for 11p copy number increases using fluorescence in situ hybridization (FISH) on tissue arrays. Copy number increases of at least threefold were found in 12 cases (11.8%) and involved the HRAS gene on chromosome 11p. Sequence analysis of HRAS showed frequent oncogenic mutations in cases with copy number increase (8/12 or 67%), contrasting with rare HRAS mutations in cases with normal HRAS copy numbers (1/21 or 5%, P < 0.0001). Tumors with 11p copy number increases were larger, predominantly intradermal, had marked desmoplasia, characteristic cytological features, and had an infiltrating growth pattern. Proliferation rates in the majority of these cases were low to absent. HRAS activation by either mutation or copy number increase alone could explain several of the histological features that overlap with those of melanoma. We speculate that HRAS activation in the absence of co-operating additional genetic alterations drives the partially transformed melanocytes of these Spitz nevi into senescence or a stable growth arrest. Although there is no data suggesting that Spitz nevi with HRAS activation are at risk for progression to melanoma, future studies are warranted to assess their biological behavior more accurately. (Am J Pathol 2000, 157:967–972)

Materials and Methods

Selection of Cases

Paraffin blocks of Spitz nevi were retrieved randomly from the archives of the Dermatopathology section of the Departments of Dermatology and Pathology at the University of California, San Francisco. Specifically, we performed a computer search of the database of the Dermatopathology Section with the following criteria: all cases from 1/1/98 to 12/31/98 that were assigned a main diagnosis of one of the 30 different descriptive variants of Spitz nevus that are used in our laboratory. Cases sent in as slide in consultation were excluded to avoid a bias toward unusual Spitz nevi. The request yielded 144 cases from which blocks were available.

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(967)
In addition to these cases, 22 cases of the Department of Dermatology, University of Würzburg, Germany were included in the study. These cases had originally been retrieved for comparative genomic hybridization and only included Spitz nevi with at least 1 mm thickness.

**Assembly of Tissue Arrays**

Tissue arrays were constructed according to Kononen et al. In brief, a tissue arraying instrument (Beecher Instruments, Silver Spring, MD) was used to punch 0.8 mm biopsy cores of the most cellular areas of the nevi. The biopsy cores were arrayed in recipient paraffin blocks, according to the manufacturer’s instructions. Multiple sections of 6 μm thickness were cut with a microtome using an adhesive-coated tape sectioning system (Instrumedics, Hackensack, NJ). H&E sections were used for the histological examination of the biopsy cores. Only cases with at least one area with a cohesive population of neoplastic melanocytes were included in the analysis.

**FISH to Formalin-Fixed Tissue Microarray Sections**

Dual-color FISH was carried out on tissue sections of the array as described previously. We used a BAC clone (RMC11B022) that contained HRAS for the detection of copy number increases of chromosome 11p, and a reference P1 clone (RMC11P008) for the q arm of chromosome 11. Probes were labeled with Cy3 (Amersham, Arlington Heights, IL) or with digoxigenin (Boehringer Mannheim, Indianapolis, IN) by nick-translation. Tissue sections were deparaffinized, hydrated, and pretreated for 2 to 4 minutes in 1mol/L sodium thiocyanate at 80°C, for 4 to 8 minutes. After dehydration, sections were denatured in 70% formamide, 17% gel loading dye) (PE Applied Biosystems, Mannheim, Indianapolis, IN) diluted in 4 mg/ml pepsin in 0.2 N HCl at 37°C for 4–8 minutes. The sequencing products were purified using a Sepharose 6 fast Track kit (Amersham) by gel filtration.

**DNA Sequence Analysis**

DNA was extracted from 30-μm sections from which the tumor-bearing areas were dissected manually with a scalpel under a dissecting microscope. Two to three sections were collected in a 0.5 ml tube and after washing with xylene and ethanol were incubated at 55°C with 0.4 mg/ml proteinase K (Life Technologies, Inc.) in PCR buffer (Perkin Elmer) containing 0.5% Tween 20 for three days. Fresh proteinase K was added every 24 hours to a final concentration of 0.4 mg/mL.

HRAS codon 12 primers were 5'-AGAGGAGACCCGT-TAGGAGGA-3' (forward) and 5'-CGCTAGGCTCACCT-TATAGTG-3' (reverse) and codon 61 primers were 5'-CTGCAAGATTCTACCAGGA-3' and 5'-CTTGGGTGTT-GTTGATGGCA-3'. PCR was carried out in a Gene Amp PCR System 9700 Thermal Cycler (Perkin Elmer) in 25 μL reaction volumes. Each PCR reaction contained 3.5 mmol/L MgCl2, 0.2 mmol/L dNTP, 0.625 U Taq gold polymerase (Perkin Elmer), 1X PCR Buffer II, 0.5 μmol/L each of forward and reverse primer, and 50 to 300 ng of genomic DNA. PCR cycling conditions were as follows: 95°C for 15 minutes followed by 35 cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 60 seconds, and a final hold at 72°C for 10 minutes.

Before sequencing, PCR products were purified using the PCR product pre-sequencing kit (Amersham) to remove excess primers and nucleotides. Fluorescent DNA sequencing was carried out using Big Dye terminator sequencing chemistry (PE Applied Biosystems). Briefly, 30 to 50 ng of purified PCR product and 3.2 pmol of sequencing primer were used for sequencing in a 15 μL reaction according to the manufacturer’s instructions. The sequencing products were purified using a Sephadex G50 column, dried in a vacuum concentrator and resuspended in 3 μl of gel loading buffer (83% deionized formamide, 17% gel loading dye) (PE Applied Biosystems). 0.5 μl of the sample was then loaded on a denaturing sequence gel on an ABI automated DNA sequencer. All samples were sequenced in both forward and reverse directions to confirm the presence or absence of mutations. Data were analyzed using the Sequencer software (Gene Codes, Ann Arbor, MI).

**Immunohistochemistry**

Proliferation was assessed using an antibody against Ki-67 (Mib-1, Beckman Coulter, Fullerton, CA, dilution 1:500) according to the manufacturer’s instructions.

**Statistical Analysis**

χ² tests were used to calculate the associations of the histological criteria and the mutations of HRAS with copy number increases of chromosome 11p. The Bonferroni adjustment was used to correct for multiple comparisons made and P < 0.01 was regarded as statistically significant.
Results

FISH Analysis of Chromosome 11p Copy Number using Tissue Arrays

High-quality hybridizations of cases in which tumor cells could be definitively identified were obtained from 102 cases. This yield of 61.4% is relatively low compared to arrays that we have constructed from melanomas. Most of the cases that could not be analyzed were very small Spitz nevi that consisted only of single cells or small nests of junctional melanocytes so that the neoplastic melanocytes could not be reliably recognized in the array. 39 cases (38.2%) were from male and 61 (59.8%) from female patients, in two cases the gender was not known. The mean age was 30.0 years. 52 (51%) of the cases had features of the pigmented spindle cell nevus variant of Spitz (PSCN).

The hybridization efficiency could be assessed by counting the hybridization signals in normal epidermal keratinocytes, which were present in many of the biopsies. The average copy numbers for test and reference probes in normal keratinocytes were 1.7 and 1.6, respectively. Hybridizations were analyzed of three separate sections of the array, and counts from two or more sections were available for 47 (46.1%) cases. In 45 (95.7%) of these, the result of the separate counts were identical, in one case a definitive copy number increase was seen in one analysis, and was not found in the cells present in the other section. Copy number increases were only scored if more than 30% of the tumor cells had at least threefold increased signals of 11p when compared to the reference probe on 11q. According to these criteria, copy number increase of 11p was found in 12 (11.8%) cases (Figure 1E). The average thickness of cases with copy number increases was significantly greater than the thickness of cases with normal copy number of 11p (1.1 mm versus 0.6 mm, \( P = 0.01 \)). The frequency of copy number increases within the randomly retrieved set of cases was 6/84 (7.1%), whereas of the 18 cases that had been selected for thickness, 6 (33.3%) showed copy number increases of chromosome 11p.

Mutations of HRAS

The HRAS gene is located at the tip of chromosome 11p at 11p15.5 and is a candidate oncogene targeted by the copy number increase found in Spitz nevi. Oncogenic mutations of HRAS typically involve codons 12 or 13 in exon 1 and codon 61 in exon 2. We obtained genomic sequences of exons 1 and 2 of HRAS of 9 cases in which FISH detected an copy number increase of chromosome 11p, the three remaining cases with 11p copy number increase were not informative. In addition, we obtained sequences of 13 cases in which FISH showed normal copy numbers of chromosome 11p. Five of nine cases (56%) with 11p copy number increase had HRAS mutations, significantly more (\( P = 0.002 \)) than in the cases with normal 11p copy numbers, in which only one (8%) had a mutation. Three mutations were 61Gln→Leu (Figure 1F), two 61Gln→Arg, and one 12Gly→Arg.

Additionally, we sequenced HRAS in 11 Spitz nevi used for our previous analysis using comparative

Figure 1. Histological features associated with copy number increase of 11p (A–D). A: Compound, predominantly intradermal Spitz nevus. B: Melanocytes with ample amphophilic cytoplasm and vesicular nuclei with prominent nucleoli between haphazardly arranged collagen fibers. C: Melanocytes with pleomorphic nuclei singly and in clusters between thickened collagen fibers. D: Cells are surrounded by a fine eosinophilic rim (arrowheads and inset). E: Dual-color FISH with a probe containing HRAS (green signals) and a reference probe for chromosome 11q (red signals) showing a central nest of melanocytes with multiple green signals surrounded by normal cells. F: Sequence profile of a portion of exon 2 of HRAS showing a A→T mutation of codon 61 (boxed).
genomic hybridization (CGH). We found HRAS mutations in all three cases (100%) in which CGH detected increased copies of chromosome 11p. All of these involved codon 61; two cases had a transition of glutamine to arginine, and the other to leucine. The seven cases in which CGH found normal copy numbers of chromosome 11p had wild-type sequences of both exons of HRAS.

Of the total of 33 Spitz nevi in which HRAS was sequenced, 8/12 cases (67%) with amplified 11p had HRAS mutations, significantly more (P < 0.0001) than in the cases with normal copies of chromosome 11p (1/21 or 5%).

Table 1. Histological Features Associated with Copy Number Increase of Chromosome 11p in Spitz Nevi

<table>
<thead>
<tr>
<th>Histological feature</th>
<th>n</th>
<th>11p Amp</th>
<th>11p Normal</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single cells between collagen bundles</td>
<td>17 (22.5%)</td>
<td>5 (41.6%)</td>
<td>12 (13.3%)</td>
<td>0.014305</td>
</tr>
<tr>
<td>Desmoplasia</td>
<td>26 (25.5%)</td>
<td>8 (66.7%)</td>
<td>18 (20.0%)</td>
<td>0.000552</td>
</tr>
<tr>
<td>Vesicular nuclei</td>
<td>36 (35.3%)</td>
<td>10 (83.3%)</td>
<td>26 (28.9%)</td>
<td>0.000210</td>
</tr>
<tr>
<td>Ample eosinophilic cytoplasm</td>
<td>26 (25.5%)</td>
<td>8 (66.7%)</td>
<td>18 (20.0%)</td>
<td>0.000493</td>
</tr>
<tr>
<td>Eosinophilic membranes</td>
<td>8 (7.8%)</td>
<td>5 (41.6%)</td>
<td>3 (3.3%)</td>
<td>0.000004</td>
</tr>
<tr>
<td>Marked nuclear pleomorphism</td>
<td>30 (29.4%)</td>
<td>10 (83.3%)</td>
<td>20 (22.2%)</td>
<td>0.000015</td>
</tr>
</tbody>
</table>

Percentages in the n column refer to the total number of 102 cases. The other percentages refer to the number of cases in the respective column. The P value was calculated using the χ² test on 2-way tables of the respective histological feature and the copy number increase of 11p.

Based on the height of the peaks of the electropherograms the approximate mutant to wild type allelic ratios were analyzed. Three cases with 11p copy number increase had mutant to wild type allelic ratios of 2 (Figure 1F), three cases had ratios of 1, and in two cases the ratios were 0.7 and 0.5. In the single case with normal copy number of 11p and a mutated HRAS the allelic ratio was 0.3. These findings indicate that in the majority of cases the 11p copy number increase is associated with an increased dosage of mutated HRAS. A ratio smaller than 1 in the two cases with 11p copy number increase could be explained by a considerable contamination of the DNA from normal cells or by concomitant copy number increase of the wild type allele.

Discussion

The present study shows that copy number increases of chromosome 11p in Spitz nevi are associated with particular histological features and with mutations of the HRAS gene, which maps to the distal end of chromosome 11p. The frequent mutations of HRAS in cases with 11p copy number increase, together with infrequent mutations in cases with normal copies of chromosome 11p make it likely that mutated HRAS is the target of selection forces of the process leading to increasing copy numbers. Amplification of wild-type HRAS is sufficient to transform sensitive cells. Therefore, the fact that some cases with copy number increase of 11p had no detectable HRAS mutations does not necessarily imply that it is not the gene targeted by the copy number increase. Furthermore, some mutations might have remained undetected because of contamination by normal cells in the microdissected tissue. In keeping with this possibility, we note that all three cases with increased copies of chro-
mosome 11p detected by CGH\textsuperscript{11} had detectable mutations. To detect chromosomal copy number changes by CGH, the DNA contributed by normal cells should be below 50%. However, we cannot rule out that additional genes residing on chromosome 11p drive the copy number increase.

HRAS belongs to the family of ras genes, which contains the two additional members KRAS and NRAS.\textsuperscript{15} NRAS mutations are found in about 25% of primary cutaneous melanomas, (see\textsuperscript{17} for review). Interestingly, HRAS itself is rarely mutated in melanoma.\textsuperscript{18,19} Mutations of NRAS are also described in congenital melanocytic nevi,\textsuperscript{20} which, depending on their size, are at increased risk to progress to melanoma.\textsuperscript{21} In contrast, Spitz nevus is not regarded as a precursor of melanoma. There are no reports of melanomas arising in a pre-existing Spitz nevus. However, this occurrence could be difficult to detect or prove because of the histopathological similarities between Spitz nevus and melanoma. Follow-up over 6 to 8 years of the cases with 11p copy number increase from our initial study\textsuperscript{11} that we report here to have HRAS mutations showed no evidence of metastasis. If Spitz nevus with 11p copy number increases or HRAS mutations represented a precursor to melanoma, one would also expect a higher frequency of HRAS mutations or amplifications in melanoma in general, which is not the case. To date we have studied over 150 melanomas by CGH analysis and have only found a single case with an amplification of HRAS.\textsuperscript{22} It was an acral melanoma and the change involved only a small fraction of the distal chromosome 11p, different from the findings in Spitz nevi, in which the entire p-arm is gained.\textsuperscript{11} These findings indicate that the presence of HRAS mutations and/or copy number increases in a subset of Spitz nevus does not point toward a highly increased risk of progression to melanoma. However, future studies are necessary to assess the behavior of Spitz nevi with HRAS activation more accurately.

Significant amplification (>10 fold) of ras genes has been observed in a variety of human tumors including bladder carcinoma,\textsuperscript{23} gastric carcinoma,\textsuperscript{24} and ovarian carcinoma.\textsuperscript{25,26} However, the overall incidence of ras gene amplification in human neoplasia has been estimated to be less than 1%.\textsuperscript{16,27}

It is well established that cancer is not caused by a mutation in a single gene, but requires genetic alterations affecting several pathways.\textsuperscript{28} Ras genes are on their own insufficient to induce tumorigenesis but need cooperating oncogenes or inactivation of p53 or p16 to transform normal cells.\textsuperscript{15,29} This is illustrated in neoplasms of the colon, where hyperplastic polyps, which only rarely progress to cancer, frequently have mutations of KRAS. In contrast, dysplastic polyps with a high risk of progression initially acquire mutations of APC which are followed by ras mutations.\textsuperscript{30} These findings indicate that not only the type of genes whose functions are altered is important, but also the order in which these alterations occur. The HRAS activation in Spitz nevi may be analogous to the KRAS activation in hyperplastic polyps. It could result in incompletely transformed melanocytes that share several features with melanoma cells but have a limited proliferative capacity. Albino and co-workers reported that transduction of normal human melanocytes with a murine retrovirus carrying a mutated HRAS results in anchorage independence and altered morphology, not tumorigenicity.\textsuperscript{31} However, when cultured for several months the cells became tumorigenic. This was accompanied by a marked alteration of the chromosomal complement similar to melanoma. The initial phenotype of this model has some similarities to Spitz nevus, however our data together with the view of Spitz nevus as stable lesions indicate that they seem somehow to be protected from acquiring subsequent genetic changes that might make them progress to melanoma. This could be due to senescence induced by exhausted telomeres and a permanent G1 arrest. This would be consistent with our finding of a very low to absent proliferation rate in the tumors with a mutated or amplified HRAS gene. Expression of activated ras in normal cells induces a senescence-like state by up-regulation of p16 and p53.\textsuperscript{32} Spitz nevi have to undergo multiple rounds of cell division to reach their average size, making it likely that control over entry into S-phase is at least temporarily altered. In addition, it is likely that the melanocytes of Spitz nevi are resistant to pro-apoptotic signals that result from signaling imbalances provoked by oncogene activation,\textsuperscript{33} since their apoptotic rate is low.\textsuperscript{34} In fact, loss of heterozygosity at chromosome 9p21 has been demonstrated in a minority of Spitz nevi,\textsuperscript{35} and we have observed two Spitz nevi in which CGH found loss of chromosome 9p occurring with copy number increase of 11p (data not shown). Chromosome 9p21 is the location of INK4A, which encodes the p16 and p14\textsuperscript{ARF} proteins that are important components of the G1 checkpoint and p53 dependent apoptotic pathway, respectively.\textsuperscript{36} However, in the vast majority of Spitz nevi there seems to be no obvious loss at this locus. Future, more detailed studies are needed to clarify the status of the INK4A locus in Spitz nevus.

The major histological criteria for differentiation of Spitz nevus from melanoma include overall lesional symmetry, predominance of nests of melanocytes over single cells at the dermo-epidermal junction, maturation of cells, absence of significant pagetoid spread or the confinement of that change to the center of the lesion, and the presence of Kamino bodies.\textsuperscript{37} Using these criteria and others, the majority of Spitz nevi can be distinguished reliably from melanoma. Diagnostic uncertainty rises if the cases of Spitz nevus in which the histology deviates by one or more criteria from the "stylized depiction" of Spitz nevus.\textsuperscript{3} The Spitz nevi with 11p copy number increases were significantly thicker, larger in diameter, and were associated with distinct histological features. Typically, they were predominantly intradermal with a pronounced desmoplastic stromal reaction. The cells were larger and had pleomorphic nuclei. They had in common a pattern of infiltrating growth at their base, characterized by extensive zones of single cells situated between collagen bundles with fine eosinophilic rims surrounding their cytoplasm. Activation of the ras pathway has been shown to cause an infiltrating growth pattern in breast cancer\textsuperscript{38} and recently ras has been linked to the regulation of cell size.\textsuperscript{39} It is thus conceivable that the histopathological
findings associated with 11p copy number increase are a consequence of the activation of the ras pathway. Our data further strengthens the notion that Spitz nevus shares several of the hallmarks of cancer\textsuperscript{28} with melanoma. We believe that the major difference that separates Spitz nevus from melanoma is genomic stability.\textsuperscript{11,12} In contrast to the limited chromosomal alterations found in Spitz nevi, CGH profiles of the majority of primary melanomas indicate pronounced genomic instability.\textsuperscript{12,22} Evidence is emerging that genomic instability is genetically controlled.\textsuperscript{20} If the control over genomic stability is not altered in time, the probability of acquiring the necessary number of genetic events for full transformation is minimal.\textsuperscript{41} The partially transformed cell that results from the arrest that would make subsequent changes unlikely. As genomic instability in melanoma is expressed on the chromosomal level, the determination of chromosomal aberrations could represent a helpful diagnostic tool in Spitz nevi that share overlapping features with melanoma.

Acknowledgment

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References