OBSERVATION

Circulating Benign Nevus Cells Detected by ISET Technique

Warning for Melanoma Molecular Diagnosis

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Background: The notion that only malignant melanoma cells circulate and diffuse is shared by oncologists and pathologists. Isolation by size of epithelial tumor cells (ISET) allows the identification of circulating tumor cells by filtration according to size.

Observations: During a study of identification of circulating melanoma cells using ISET, blood samples from a 69-year-old man with an atypical melanocytic lesion on his back were evaluated. Binucleated and multinucleated cells that fulfilled the criteria for circulating tumor cells were found. The morphological features were similar to those of the excised skin tissue specimen, and the patient was subsequently diagnosed as having a congenital melanocytic nevus. *BRAF* (V600E)-mutated DNA was detected in both plasma and formalin-fixed tissue specimens, and the blood samples demonstrated an increase in tyrosinase messenger RNA levels.

Conclusion: The finding that benign nevus cells may circulate in blood brings into question the value of tyrosinase or other melanocytic markers as a molecular surrogate for circulating melanoma cells.

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ALTHOUGH THE MECHANISM by which nevus cells reach lymph nodes is still unclear, one possibility is that nevus cells enter the dermal lymphatics and travel via lymphatics to regional lymph nodes. This process, referred to as *benign metastasis theory*, has been supported, in rare instances, by the histologic observation of nevus cells invading dermal lymphatic vessels in tissue sections

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of melanocytic nevi.[1] However, to our knowledge, there has been no report of migration of benign nevus cells from draining lymph nodes to the peripheral blood circulation. Therefore, the passage of nevus cells to the peripheral circulation occurs via a hitherto undefined pathway.

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REPORT OF A CASE

A 69-year-old white man presented with a recently changing cutaneous pigmented lesion on his back. He stated that the lesion, which had been present since birth, grew slowly in size. He denied a history of blistering sunburns over the course of his life, and he did not report any history of melanoma or other cancers. He had no other clinically or dermoscopically suspicious pigmented lesions. On clinical examination, the lesion was round (about 21 mm in its long axis) and consisted of a darker, flat, black-brownish central area and a peripheral light brown surrounding area with irregular and asymmetrical borders (**Figure 1**A). Dermoscopic evaluation of the lesion revealed the presence of a slightly thin peripheral pigmented network, asymmetrical in shape, together with a regressive central black-blue-whitish area with punctiform vessels and rare blue globular structures (Figure 1B). The clinical and dermoscopic findings were suggestive of an atypical melanocytic lesion, and a diagnosis of melanoma arising on a congenital melanocytic nevus could not be ruled out. Therefore, the lesion was removed by excisional biopsy and examined histologically.

METHODS

The patient was enrolled in a study to identify melanoma cells in the peripheral blood by isolation by size of epithelial tumor cells (ISET Device; Metagenex, Paris, France). ISET, a simple, fast, and direct method for identifying circulating tumor cells, allows the separation of tumor cells from peripheral blood leukocytes with filtration by size.[2-4] It is also a sensitive method as it detects one single tumor cell in 1 mL of blood. The main advantage of ISET over other methods for the identification of circulating tumor cells is that morphological studies of the isolated cells, including melanoma cells, can be performed, as we have recently demonstrated.[5,6] After written, informed, and institutional review board–approved consent was obtained, the patient underwent peripheral blood sampling. Three sequential blood samples in triplicate were obtained, one before surgery and the other two postoperatively, 15 days and 6 months after surgery. The first blood sample (5-10 mL) was collected in an EDTA tube and filtered within 4 hours using ISET with a filtration block containing an 8-µm-diameter porous membrane, as previously described.[2]

Subsequent histopathologic examination showed dermal proliferation of small monomorphous melanocytes (**Figure 2**A). In the superficial dermis, highly pigmented melanocytes were arranged in a bandlike pattern (Figure 2B and C), while in the mid and deep reticular dermis, melanocytes were observed in single file between collagen bundles along epithelial structures of adnexa and in an angiocentric distribution. Numerous floretlike, multinucleated melanocytes were present (Figure 2D). Based on these findings, the diagnosis of congenital melanocytic nevus of the superficial type was suggested. The patient did not receive any other treatment. His postoperative course was free of complications, and he was clinically disease free at his last follow-up, 2 years after surgery.

Cells isolated by ISET were first stained with hematoxylin-eosin and submitted to cytopathologic evaluation. Isolated circulating cells, larger than 11 µm, were retained in the ISET filter in the second and third postoperative blood samples (8 cells/7 mL and 1 cell/10 mL of blood, respectively), whereas no cell was found in the preoperative blood sample. The identified cells fulfilled the criteria for circulating tumor cells, including (1) cell size 16 µm or larger, (2) nucleocytoplasmic ratio of 50% or greater, (3) irregular nuclear shape or multinucleated cells, (4) hyperchromatic nucleus, and (5) basophilic cytoplasm. Binucleated (**Figure 3**A) and floretlike multinucleated cells were observed (Figure 3B and D). In the second postoperative sample, numerous naked nuclei were detected. The morphological features of the circulating cells were similar to those of the nevus cells that were detected within the excised skin tissue specimen.

For the second sample, the patient's whole blood sample was collected in a PAXgene tube (PreAnalytiX GmbH, Hombrechtikon, Switzerland), submitted to total RNA extraction (PAXgene Blood RNA Kit; PreAnalytiX GmbH), and analyzed by real-time reverse transcriptase–polymerase chain reaction for tyrosinase messenger RNA (mRNA) expression using a predeveloped Taqman Gene Expression Assay (Hs00165976_m1; Applied Biosystems Inc, Foster City, California). The third EDTA blood sample was collected to isolate free circulating DNA that was extracted from 500 µL of the patient's plasma sample using the QIAamp DSP Virus Kit (Qiagen, Hilden, Germany). DNA was also extracted from 1 formalin-fixed, paraffin-embedded tissue sample. A real-time polymerase chain reaction assay was performed to quantify the percentage of DNA bearing the *BRAF* (V600E) mutation in plasma DNA using the following primers and probe (forward primer 5′-AAA ATA GGT GAT TTT GGT CTA GCT ACA GA-3′; reverse primer 5′-GAC AAC TGT TCA AAC TGA TGG-3′; dual-labeled locked nucleic acid probe 5′-FAM-T[+C]GAGA[+T]TT[+C][+T][+C]TG[+T]AG[+C]T-BHQ1-3′) (Sigma, St Louis, Missouri). Tyrosinase mRNA showed positive levels in the second and third postoperative blood samples (13.5 and 20.2 SK-MEL-28 cell equivalents per milliliter of blood, respectively). *BRAF* (V600E)-mutated DNA was detected in all samples (5%, 4%, and 8% corresponding to 0.83, 0.45, and 0.13 ng, respectively, of mutated DNA per milliliter of plasma) (**Figure 4**). The presence of the *BRAF* (V600E) mutation was confirmed in the DNA extracted from the formalin-fixed tissue specimen as well as by high-resolution melting analysis.[7]

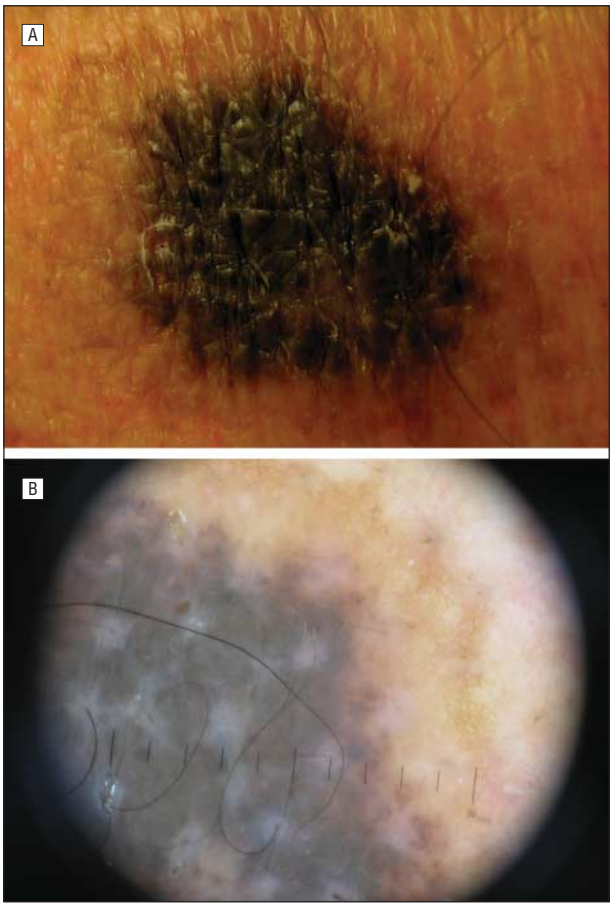

Figure 1. Clinical (A) and dermoscopic (B) findings. A, Clinical examination demonstrated a cutaneous pigmented round-shaped lesion consisting of a darker black-brownish central area with a peripheral light-brown surrounding area with irregular and asymmetrical borders. B, Dermoscopy revealed the presence of a slightly thin peripheral pigmented network, asymmetrical in shape, and a regressive central black-blue-whitish area with punctiform vessels and rare blue globular structures.

COMMENT

To our knowledge, this is the first time that circulating benign nevus cells have been identified in peripheral blood. It seems that benign nevus cells detached from the primary tumor and reached the peripheral blood. This conclusion is supported by the simultaneous observation of ISET-isolated cells, which were morphologically identical to those seen in the excised tissue specimen, and high levels of circulating tyrosinase mRNA and *BRAF* (V600E)-mutated DNA. The presence of benign nevus

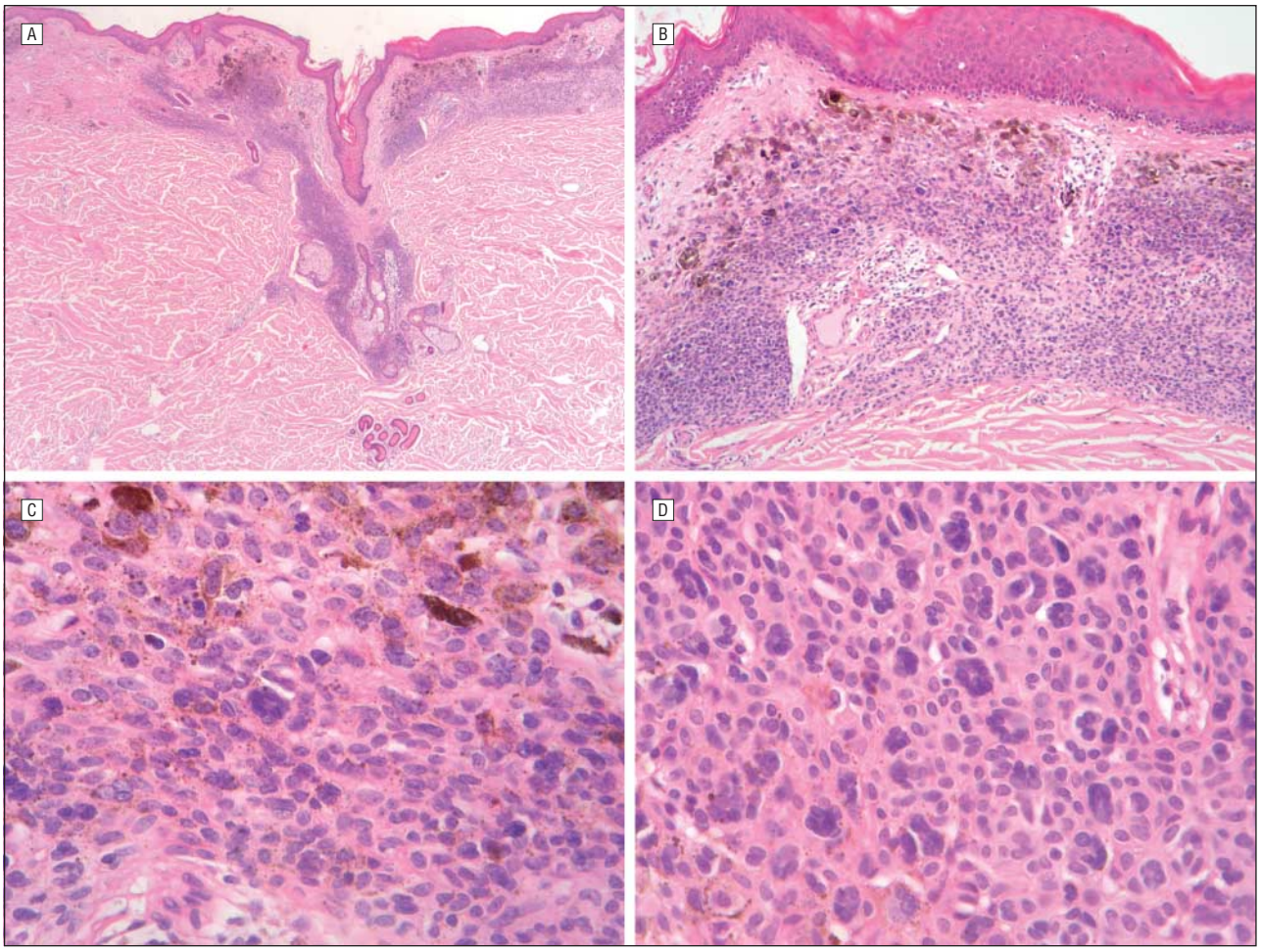

Figure 2. Histopathologic features. Histopathologic examination revealed a congenital melanocytic nevus of the superficial type characterized by a dermal proliferation of melanocytes showing adnexocentricity (A), highly pigmented melanocytes in a bandlike pattern in the superficial dermis (B), small monomorphous melanocytes among collagen bundles (C), and floretlike, multinucleated melanocytes (D) (hematoxylin-eosin, original magnification ×10 [A], ×20 [B], ×20 [C], and ×40 [D]).

cells within lymph nodes is a known incidental finding.[8] The overall frequency of nevus cell aggregates in lymph nodes is highly variable, ranging from 0.33% to 7.3% in lymph nodes removed for nonmelanoma cancers[9] to as much as 22% in melanomas.[10] In this case, circulating nevus cells were found after, but not before, surgery, and the number of cells was greater shortly after surgery than it was 6 months after surgery. This finding supports the general hypothesis that surgical procedures contribute to tumor cell shedding and their passage into circulation. Indeed, intraoperative tumor manipulations at surgery for breast, colorectal, and prostatic cancers have been found to induce tumor cell dissemination.[11,12] Although it is not known whether circulating tumor cells are proliferating or quiescent (dormant), have the ability to self-renew (the hallmark of cancer stem cells), and can survive long term, dispersed cells that are not encased in a tissue may quickly undergo apoptosis owing to a process called *anoikis*.[13] In the third sample (obtained 6 months after surgery), we also observed an increased number of naked nuclei, which could be indicative of nonviable cells. This observation suggests that cell bloodstream diffusion from benign lesions is a self-limiting phenomenon with no further consequences.

However, another possibility should be carefully considered. A proportion of patients with melanoma have a high number of melanocytic nevi (an important risk factor for melanoma) and not infrequently undergo excision of multiple pigmented lesions (melanoma and melanocytic nevi). Present findings advance the hypothesis that circulating tumor cells may derive from both sources. More importantly, we would like to draw attention to the present observation that benign nevus cells may be present in the bloodstream of an otherwise healthy subject. The notion that only malignant melanoma cells circulate and diffuse is shared by oncologists and pathologists. The present observation should alert oncologists to the potential pitfall that may occur during real-time monitoring of the efficacy of systemic adjuvant therapies in patients with metastatic melanoma through molecular analysis of mRNA tyrosinase as a surrogate for circulating melanoma cells. Because selective transcripts for malignant melanocytes are not available at this time, reverse transcriptase–polymerase chain reaction–based molecular analyses alone cannot reliably distin-

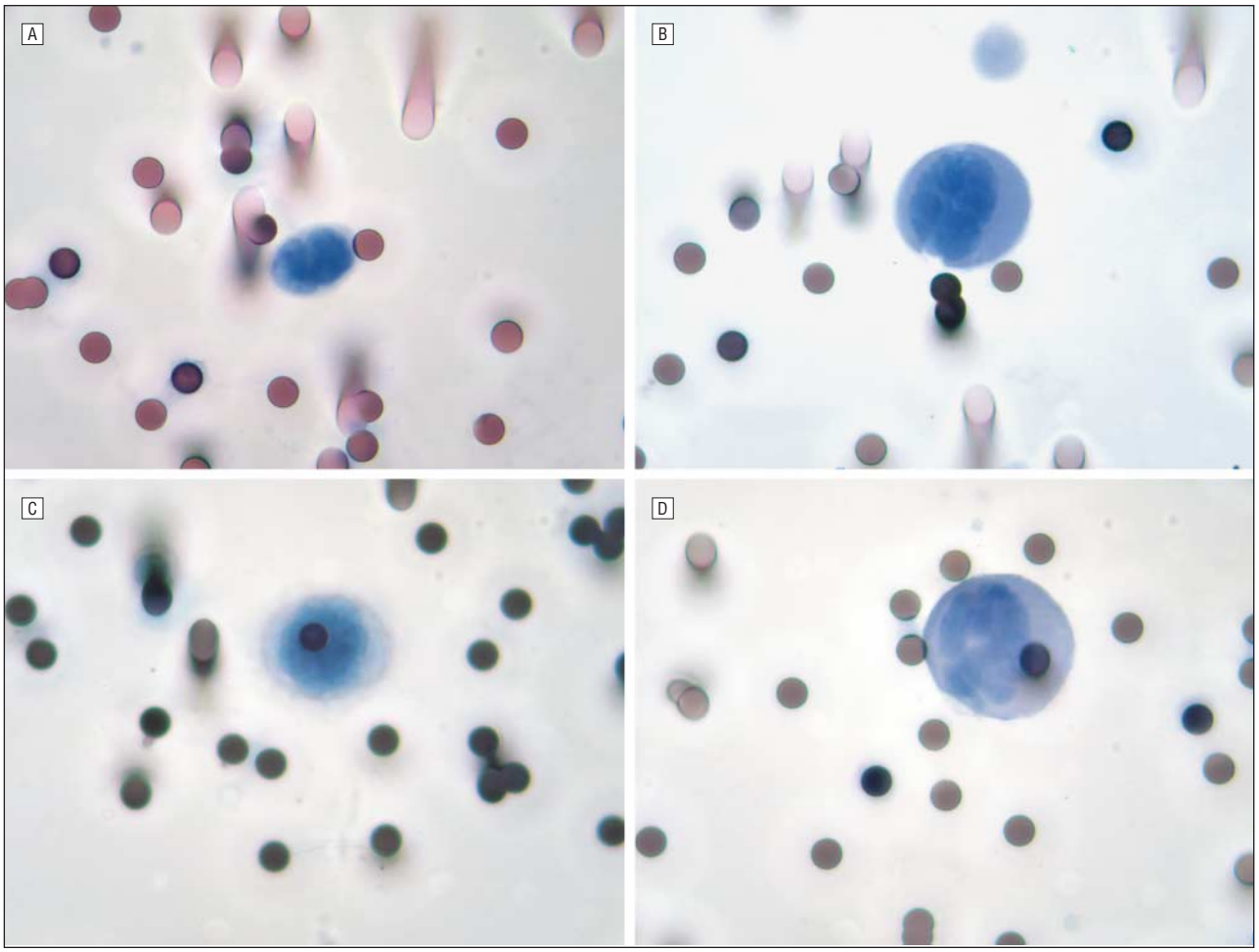

Figure 3. Cytopathologic evaluation of isolated circulating cells retained in the ISET (isolation by size of epithelial tumor cells) filter. Binucleated (A) and floretlike, multinucleated (B-D) cells fulfilling the criteria for circulating tumor cells ([1] cell size ≥16 µm, [2] nucleocytoplasmic ratio ≥50%, [3] irregular nuclear shape or multinucleated cells, [4] hyperchromatic nucleus, and [5] basophilic cytoplasm) (hematoxylin-eosin, original magnification ×63).

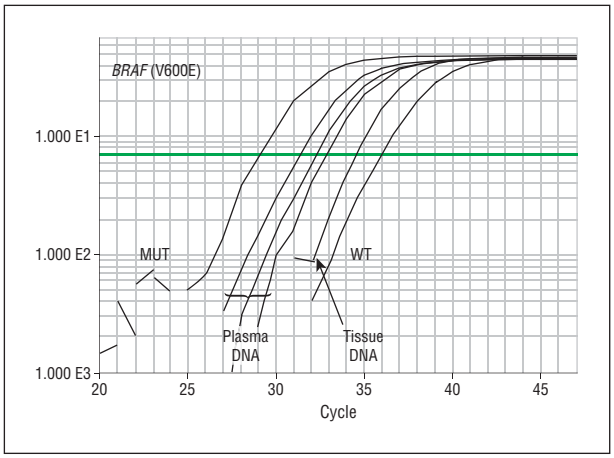

Figure 4. Detection of *BRAF* (V600E)-mutated (MUT) DNA. A real-time polymerase chain reaction (PCR) assay was performed to quantify the percentage of DNA bearing the *BRAF* (V600E) mutation in plasma DNA in all samples (5%, 4%, and 8% corresponding to 0.83, 0.45, and 0.13 ng, respectively, of MUT DNA per milliliter of plasma). WT indicates wild-type.

guish between benign nevus cells and melanoma cells. Therefore, circulating nevus cells can contribute to a false-positive score, which could explain why some patients with melanoma and high blood mRNA tyrosinase levels do not have recurrences.

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The Best of the Best

Top-Accessed Article: Photoaging and Chronological Skin Aging

Fisher GJ, Kang S, Varani J, Bata-Csorgo Z, Wan Y, Datta S, Voorhees JJ. Mechanisms of photoaging and chronological skin aging. *Arch Dermatol*. 2002;138(11):1462-1470.

This review article summarizes how UV radiation reduces the procollagen synthesis and makes the collagen fibril disorganized in the human skin. Also, the authors explain the roles of the partially degraded collagen fragments and the resultant reduced mechanical tension on fibroblasts in the basal procollagen synthesis in photoaged and chronologically aged skin, as well as how retinoids repair and prevent skin aging. In today's world, every woman and man, regardless of age, is interested in maintaining young and healthy skin. To develop truly effective cosmeceutical products and anti–skin-aging drugs, an understanding of the molecular mechanism of skin aging and photoaging is essential; this is provided very nicely by this article.

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