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"Stealth" Melanoma Cells in Histology-Negative Sentinel Lymph Nodes

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Abstract

A proportion of patients who develop regional and distant recurrences of melanoma after a pathologically negative sentinel lymph node (SN) biopsy are reported to have enhanced signals for melanoma associated mRNA when sensitive molecular approaches such as RT-PCR are used to evaluate their SN tissue. The significance of these findings remains controversial because the cellular source of the augmented signals cannot be known because the nodal tissue is destroyed during preparation for RT-PCR. Nonetheless it is claimed that the source of the augmented signal is covert metastatic melanoma cells. To determine whether there are histologically occult metastases in SN and whether there are sources of augmentable melanoma-associated mRNA other than melanoma cells we applied the reverse transcriptase *in situ* polymerase chain reaction (RT *in situ* PCR) to formalin-fixed paraffin-embedded nodal tissue. This approach amplifies small amounts of melanoma-associated mRNA and permits identification of the cells that express that mRNA. Cells containing MART-1 mRNA were detected in 6/21 SN (29%) and 2/16 NSN (13%) that were tumor-negative on hematoxylin and eosin and immunohistochemical assessment for S-100, MART-1 and HMB-45. In patients with microscopic evidence of melanoma in their SN, MART-1 mRNA positive cells were identified in 2/7 NSN (29%) that were histologically tumor-free. MART-1 mRNA positive cells were also detected in tumor negative SN sections from 6/7 (86%) nodes that had tumor present in areas of the node not represented in the studied sections. Some cells that expressed MART-1 mRNA that was diffusely distributed in the cytoplasm appeared to be melanoma cells while others resembled macrophages. The latter cells expressed augmented mRNA on granules that were intermixed with melanin granules. In other cases MART-1 mRNA positive macrophage-like cells contained nuclei and nucleoli more typical of melanoma cells and may represent the macrophage-melanoma hybrids that have been previously

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reported. Combination of RT *in situ* PCR for MART-1 mRNA and immunohistochemistry for CD68 revealed that CD68 was co-localized in some cells that expressed MART-1 mRNA.

Some lymph nodes that are tumor negative by histology and immunohistochemistry contain cells that express mRNA for MART-1. Some of these cells may be interpreted as “stealth” melanoma cells in which, despite the presence of MART-1 mRNA there is an absence of immunohistochemically detectable MART-1 protein. Other cells that contain MART-1 mRNA are clearly not melanoma cells or may represent melanoma hybrids. These findings should be taken into account when interpreting and applying the results of RT-PCR analysis of nodal (and other) tissues.

Keywords

MART-1; macrophage; melanoma; metastasis; micrometastasis; sentinel lymph node

Introduction

Malignant melanomas have a high potential for metastasis and usually spread first to the regional lymph nodes. Lymphatic mapping and sentinel node (SN) biopsy (46) are recently developed procedures that are widely used in the management of melanoma patients. SN evaluation permits accurate staging of patients and determines the need for additional nodal surgery. Patients who have a melanoma-positive SN currently undergo complete dissection of the affected lymph node basin (completion lymphadenectomy), while patients with a histologically negative SN receive no additional nodal surgery. Approximately 20% of patients with a primary melanoma ≥1 mm in thickness have metastatic tumor in microscopically assessed (hematoxylin and eosin (H&E) and immunohistochemistry) sections from the SN (44, 45). Accurate determination of SN tumor status requires evaluation of multiple sections and immunohistochemistry for multiple melanocyte lineage-associated antigens (14). However, a portion of patients assessed as having a metastasis-negative SN on the basis of H&E histology and immunohistology subsequently develop regional (and distant) recurrences. Such patients have been claimed to reflect false-negative sentinel node biopsies. The false negative rate of SN (the number of false negative SN divided by the sum of the number of positive SN and that of false negative SN) has been reported to be 11-18% (7, 13, 39, 45, 58). False-negativity has been attributed to technical problems associated with identification of the true SN by nuclear medicine and surgery and errors in tissue sampling and interpretation during pathological assessment (34). Anomalies of tumor biology and variation in the timing and routing of spread of tumor cells from the primary site to the SN (34, 47) may also explain some “false negative” SN. Molecular analyses including the reverse transcriptase polymerase chain reaction (RT-PCR) has been reported to detect enhanced signal for melanoma-associated mRNA that may indicate micrometastases (3, 5, 22, 27, 44). This has been considered to indicate the presence of truly occult melanoma cells that are difficult to detect microscopically or missed by the relatively limited sampling that is used to evaluate SN in most pathology protocols. It is, however, also possible that the technique may be too sensitive, augmenting signals for mRNA in cells other than melanoma cells and providing results that are thus “false positive”. Evaluation of the results of RT-PCR studies is particularly challenging as the cellular source of the augmented mRNA signals cannot be directly determined in the RT-PCR approach since the tissue is disrupted during the preparative stages of the technique.

The reverse transcriptase *in situ* polymerase chain reaction (RT *in situ* PCR) amplifies and visualizes small amounts of specific mRNA and allows identification of the nature of the cells that contain the amplified mRNA. We have successfully modified the RT *in situ* PCR

approach to detect melanoma-associated genes in melanoma cell lines (26, 40) and formalin-fixed paraffin-embedded tissues (25, 31). mRNA expression reflecting the presence of melanoma-associated genes can be detected microscopically in lymph nodes containing metastases by this technique (31). To detect cells expressing MART-1 (melanoma associated antigen recognized by T cells-1: *MLANA*) mRNA, including histologically occult metastatic melanoma cells, we applied the RT *in situ* PCR to SN and non-sentinel nodes (NSN) that were tumor-negative by standard histopathological and immunohistochemical evaluations.

Materials and methods

Fifty one tissue blocks from lymph nodes (28 SN, 23 NSN) from patients with malignant melanoma were retrieved from the surgical pathology archives of UCLA and evaluated with Institutional Review Board permission. Patients had been treated by sentinel lymph node biopsy and completion lymphadenectomy if the SN contained metastatic tumor. Pathological examination of sentinel lymph node biopsy was performed by using published criteria (14). H&E and immunohistochemically stained (S-100 protein, HMB-45, MART-1) slides from all lymph nodes were re-reviewed to confirm the presence or absence of metastases (AJC and DRW). Microscopically melanoma-free SN (n=21) and NSN (n=16) were further evaluated. Histologically tumor-negative NSN from patients with tumor-positive SN were also evaluated (n=7). Tumor-free microscopic sections from tumor-positive SN were identified and evaluated (n=7). As a negative control, we examined 5 tumor-free axillary lymph nodes from patients with breast cancer (3 SN and 2 NSN).

RT *in situ* PCR

Pretreatment—Formalin-fixed, paraffin-embedded tissue sections (4μm thickness) were de-paraffinized and hydrated through xylene and a series of graded ethanols. The slide-mounted tissue sections were digested with trypsin (0.2-0.4 mg/ml) in phosphate-buffered saline (PBS) for periods that ranged from 30 to 60 min at 37°C (Roche Diagnostics, Mannheim, Germany) for optimization, followed by digestion with RNase-free DNase I (EPICENTRE Biotechnologies, Madison, WI) in PBS at 37°C overnight.

One step RT-PCR—Single step RT *in situ* PCR approach was performed using a technique based on descriptions in recent articles (2, 31, 32, 48). The Gene Amp EZ r*Tth* (recombinant *Thermus thermophilus*) RNA PCR Kit (Applied Biosystems, Foster City, CA) was employed. The oligonucleotide primer sequences (Invitrogen, Carlsbad, CA) for MART-1 mRNA were derived from a previous paper (55). The forward primer, 5'-CACGGCCACTCTTACACCAC-3' and the reverse primer, 5'-GGAGCATTGGGAACCACAGG-3' yielded a product of 254 bp (Table 1). Each slide was loaded with the following mixture: 15μl of 5× EZ r*Tth* buffer, 9μl of $Mn(OAc)_2$, 2.4μl each of dATP/dCTT/dGTT/dTTP, 3μl of r*Tth* DNA polymerase (Applied Biosystems), 0.9μl digoxigenin-11-dUTP, 2.4μl of 2% bovine serum albumin (BSA), 1.3μl of RNase inhibitor (Roche), 2.3μl of each primer, and 30.7μl diethylpyrocarbonate (DEPC)-treated water. The slides were covered with HybriWell sealing covers (Research Products International, Mount Prospect, IL) and placed on the Gene Amp In Situ PCR System 1000 thermal cycler (PerkinElmer, Foster City, CA). cDNA syntheses were performed at 60°C for 30 min. After initial denaturation at 94°C for 3 min, the cDNA was amplified by 30 cycles of annealing at 55°C for 1 min and denaturation at 94°C for 30 sec. After RT-PCR, the slides were washed in 0.1× saline-sodium citrate with 2% BSA at 60°C for 15 min and rinsed in Tris-buffered saline (TBS) three times for 5 min.

Immunodetection of RT-PCR products—Following incubation with an alkaline phosphatase–conjugated anti-digoxigenin antibody (Roche; 1:200) for 30 min, the RT-PCR product was developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP; Zymed Laboratories, South San Francisco, CA) for 15 min. After chromogen development, selected slides were stained by eosin.

Controls—Negative control preparations were prepared by: 1) omission of the primer pair for MART-1; 2) omission of the *Tth* DNA polymerase. Sections of metastatic melanomas in lymph nodes served as a positive control. Formalin-fixed paraffin-embedded sections of cultured human melanoma cells (M7, M14 and M24; courtesy of Dr Donald L. Morton, John Wayne Cancer Institute, Santa Monica, CA), expressing MART-1 mRNA (31), also served as a positive control.

Double labeling using RT *in situ* PCR for MART-1 mRNA and immunohistochemistry for CD68

Double staining using RT *in situ* PCR for MART-1 mRNA and immunohistochemistry for CD68 antigen was employed on 6 representative MART-1 mRNA-positive cases. After the RT *in situ* PCR products were developed with NBT/BCIP, immunohistochemistry for CD68 was performed. Endogenous peroxidase activity was blocked by exposure to 3% hydrogen peroxide for 30 min. Heat-induced epitope retrieval was performed by boiling in citrate buffer, pH 6.0 for 30 min. Monoclonal antibody for CD68 (clone: KP-1; Dako, Glostrup, Denmark) was diluted in PBS at 1:20. Sections were incubated with the primary antibody overnight at 4°C. The subsequent development of antibody-bridge labeling was made by the streptavidin-biotin-peroxidase method using biotinylated anti-mouse antibody (Vector Laboratories, Burlingame, CA) and streptavidin horseradish peroxidase (Zymed Laboratories). The sections were then reacted in with aminoethyl carbazole chromogen (AEC; Zymed Laboratories)

Results

Cells containing MART-1 mRNA were detected in 6/21 SN (29%) and 2/16 NSN (13%) that were tumor-negative on examination of H&E and immunohistochemistry stained slides. In cases with a positive SN, MART-1 mRNA positive cells were found in 2/7 NSN (29%) that were histologically negative. MART-1 mRNA positive cells were also detected in microscopically tumor-free areas of 6/7 (86%) tumor-positive SN (Table 2).

Scattered, single isolated cells in the nodal parenchyma that contained diffuse cytoplasmic MART-1 mRNA appeared to be (stealth) melanoma cells-melanoma cells that despite containing MART-1 mRNA did not show immunohistochemically detectable MART-1 protein (Figure 1A-C). With knowledge of the location of the RT *in situ* PCR positive cells, review of the original H&E slides allowed identification of melanoma cells in the areas of the node remote from the RT *in situ* PCR positive cells (Figure 1D&E). On the basis of size, nuclear characteristics and scattered melanin these cells were consistent with melanoma cells. Some MART-1 mRNA positive cells contained microdispersed melanin granules, a pattern that is typical of melanogenic cells, but lacked a nucleus which would have permitted determination of their malignant or benign status by H&E (Figure 1F). In other cases, MART-1 mRNA-positive cells were associated with small clusters of macrophage-like cells that contained nuclei and nucleoli regarded as more typical of melanoma cells as well as the fine micro-dispersed melanin granules typical of melanogenic cells (Figure 1G). These cells were adjacent to typical melanophages (Figure 1H) that had smaller, sometimes reniform nuclei, inconspicuous nucleoli and abundant coarse brown (aggregated) pigmented granules. Typical melanophages did not express MART-1 mRNA. Yet other MART-1

mRNA-expressing cells, singly dispersed and scattered in the nodal parenchyma, had typical macrophage morphology with coarse brown pigmented granules (compound melanosomes) characteristic of melanophages (Figure 2A&B). Immunohistochemistry on the corresponding SN/NSN tissue sections revealed that these MART-1 mRNA-expressing cells were negative for melanocytic markers except in 2 cases where positive signals for both HMB-45 and MART-1 were detected. In these two cases, singly dispersed and isolated immunoreactive cells that were HMB-45 and MART-1 and positive, with non-atypical nuclei and coarse melanin granules, were readily identified as melanophages (Figure 2C&D). Single isolated cells that were positive for MART-1 mRNA were detected in the corresponding RT *in situ* PCR slides.

There was one example of nodal nevus in the examined lymph nodes, but the nevus cells were not detected by RT *in situ* PCR for MART-1 mRNA.

Double staining for MART-1 mRNA by RT *in situ* PCR and CD68 antigen by immunohistochemistry showed that 40% of the cells that contained MART-1 mRNA co-expressed MART-1 mRNA and CD68. Cells coexpressing MART-1 mRNA and CD68 were identified in areas where there were abundant CD68-positive cells. Cells expressing MART-1 mRNA but not CD68 were co-localized in the same area as CD68-positive cells and cells that coexpressed MART-1 mRNA and CD68 (Figure 2E&F). As NBT/BCIP (blue/purple) and ACE (red) were used as chromogens, positive staining was clearly distinguishable from brown cytoplasmic melanin.

None of the five lymph nodes (SN and NSN) from patients with breast cancer showed positivity for MART-1 mRNA or MART-1 antigen (Figure 2G&H). None of these lymph nodes showed inclusions of melanocytic nevus cells.

Discussion

Identification of melanoma cells in H&E stained sections of sentinel nodes is usually straightforward when they form masses or clusters of tumor cells. However, early metastases may be seen as single melanoma cells, making their detection in H&E sections more challenging as such cells can show a considerable range of morphological variation, and are often amelanotic. Lightly pigmented or unpigmented melanoma cells may closely simulate sinus and other histiocytes that are often abundant in lymph nodes. Extended histopathological search for small metastatic deposits by serial sectioning and immunohistochemistry reportedly detects occult melanoma cells in up to 32% of SN (39). Micrometastases may be missed if the wrong part of a node is examined or if tumor cells do not express melanoma-associated markers detectable by immunohistochemistry.

There are reports of patients with an intermediate prognosis who have an enhanced signal for mRNA for melanoma-associated genes detected by RT-PCR, in nodes that showed no evidence of melanoma cells on microscopic evaluation (44). The RT *in situ* PCR technique is valuable in basic research and clinical science and permits detection of mRNA in specifically identifiable cells and tissues. In this study, we identified MART-1 mRNA-positive cells in SN and NSN in which prior search for cells expressing MART-1 protein by immunohistochemistry was negative. Some of these cells are truly occult melanoma cells that despite containing MART-1 mRNA do not have detectable cytoplasmic MART-1 protein. Such “stealth” melanoma cells were also detected in microscopically tumor-free NSN from patients with a tumor-positive SN When a completion lymph node dissection (CLND) is performed for patients with a positive SN, tumor is detected in the NSN of 10 to 20% of patients (21, 24, 43, 57). We showed that MART-1 mRNA-positive cells were also present in microscopically negative NSN. The frequency of tumor-positive NSN may

increase when nodal tissues are evaluated by sensitive molecular analyses (4). The biological significance of nodes found to contain tumor by special and extended evaluation remains unknown. Equally the biological potential and significance of “stealth” melanoma cells remains to be determined.

Human tumors express antigens recognized by autologous T lymphocytes. Tumour progression is often accompanied by alterations in the expression of such “differentiation” antigens, in some instances leading to tumor escape from immunosurveillance. Although MART-1 is detectable in most melanomas and is considered a suitable target for immunomodulation therapy, metastatic melanomas often shows heterogeneous expression of MART-1 (15, 28, 33). Melanocyte-specific microphthalmia-associated transcription factor (Mitf-M) regulates the expression of melanocyte-associated genes, and downregulation of Mitf-M results in the loss or reduced expression of melanoma-associated antigens in melanoma cells (16, 36). Loss of melanoma-associated antigens may also be induced by soluble factors produced by melanoma cells (37), including oncostatin M (19). Thus, when attempting to detect micrometastases in SNs by molecular methods, attention should be paid to the possibility of downregulation of genes for tumor-associated antigen. The use of multiple melanoma markers can significantly improve the specificity of molecular testing (65), but conversely may affect sensitivity, because alteration of melanoma-associated antigens has implications for tumor growth and metastasis and these melanoma-associated antigens are downregulated in metastatic melanoma cells.

Alternative cellular sources for mRNA for melanoma-associated genes in lymph nodes include macrophages that have phagocytosed materials from melanoma cells. MART-1 mRNA was detected in macrophages (melanophages) containing intracellular granules of melanin. Co-localization of CD68 and the RT *in situ* PCR enhanced signal for MART-1 mRNA was found, further supporting the view that these cells are macrophages. Melanophage accumulation is typically observed at the primary site in the early stage of melanoma evolution. Single isolated melanophages and groups of melanophages forming small granulomas often mimic micrometastasis in regional lymph nodes. Large irregularly shaped intracellular granules of pigment, comprised of aggregated melanosomes are morphological characteristics that help distinguish melanophages from melanoma cells where, in most instances, the melanosomes are small, regular in size and shape and diffusely dispersed. Electron microscopic examination has revealed that melanophages contain partially or fully melanized melanosomes that may be located within phagosomes. Melanosomes can also be found in dermal melanophages in postinflammatory hyperpigmentation (42, 63) or in tumor cells in cutaneous neoplasms other than melanoma such as pigmented squamous cell carcinoma and pigmented basal cell carcinoma (63). Melanosomes often exhibit immunoreactivity for HMB-45 (56), and HMB-45-positive melanophages have been demonstrated by immunoelectron microscopy. On the other hand, MART-1 is not localized to melanosomes but it is located in electron-lucent cytoplasmic vacuoles and vesicles in melanocytes (64). Our study revealed that the MART-1 epitope is also be detectable in melanophages, possibly indicating phagocytosed materials retained in their cytoplasm. However, we can not entirely exclude the possibility that the highly sensitive molecular biology technique that we employed may augment acquired nucleotides in the cytoplasm of macrophages that have phagocytosed melanoma cell components. MART-1- or HMB-45-positive melanophages may be difficult to differentiate from melanoma cells on light microscopy.

Melanoma-macrophage hybridization is another possible explanation for cells that express characteristics of both melanoma cells and macrophages. Until recently, the hypothesis that fusion of cancer cells and normal cells may facilitate metastasis has received limited attention. It has been suggested that cancer cells may spontaneously fuse with host cells to

produce hybrids (17, 18, 35, 49, 50). Evidence in support of cancer cell fusion with macrophages or other bone marrow-derived cells has been reported from studies of animal models of human cancer (6, 9-11, 49, 52, 53, 59). Hybrid cells formed from macrophages and melanoma cells may possess the malignant potential of the melanoma cells and the migratory phenotype of the macrophages. Such hybrid cells contain coarse intracellular melanin granules of the type typically seen in melanophages (53). In the tumor microenvironment, macrophages often constitute a major component of the tumor stroma. Macrophages are conventionally regarded as promoters of tumor progression (68) and are recruited to melanoma by expression of chemokines including CCL2 (MCP-1). However, it is difficult to further track the underlying macrophages and demonstrate hybridization with tumor cells in human tissues. A recent study reported that epithelial cancers arise by transdifferentiation of bone marrow stem cells, a process that did not involve hybridization since these cells were diploid without evidence of fusion of donor and recipient cells (23). Transdifferentiation of bone marrow stem cells to cancer cells may be another possible explanation of tumor dormancy and cancer cell metastasis.

In contrast, melanoma cells, particularly metastatic melanoma cells may exhibit an aberrant immunophenotype and may express many of the known lysosomal and phagocytic markers, such as CD68, α1 antitrypsin and α1 chymotrypsin that are typical of macrophages. They also frequently show immunoreactivity with macrophage markers including, MAC387 and HAM56 (1, 20, 38, 51). Some melanoma cells express MHC class II molecules that are normally present on antigen presenting cells and activated T cells. Metastatic melanoma cells may show macrophage-like phagocytic activities that involve cytoplasmic vesicles (41). Macrophage-like properties have also been observed in gliomas (29), which can preferentially express melanoma-associated antigens such as gp100, MART-1, tyrosinase, TRP-2, MAGE-A1 and MAGE-A3 (12, 54, 67). It remains unclear how melanoma cells develop aberrant macrophage-like immunophenotypes. The possibility that they are the result of melanoma-macrophage hybridization is clearly worthy of further consideration.

Another pitfall of the use of molecular biology techniques is that the “tumor-associated” genes used for detection of melanoma cells by RT-PCR are not absolutely specific to melanoma cells. These genes are mostly genes for melanocyte-lineage antigens that are also expressed by normal melanocytes and benign nevus cells. Nevus cells in lymph nodes represent a well known potential diagnostic pitfall in the RT-PCR analysis (61, 66). Histologically, nodal nevi appear as aggregates of nevus cells in the lymph node capsule and trabeculae, and are a potential source of confusion with metastatic melanoma. Discrimination of benign nevocytes from metastatic melanoma requires close consideration of cytologic features of the cells which are negative or at most faintly positive for HMB-45 (8).

Though infrequent, benign inclusions in lymph nodes represent the presence of heterologous elements not normally found in lymph nodes. These include salivary inclusions in peri-parotid lymph nodes, thyroid inclusions in cervical lymph nodes, mammary gland inclusions in axillary lymph nodes, or endometrial glandular inclusions in abdominal and pelvic lymph nodes (30, 60, 62). The occurrence of such inclusions provides an additional potential pitfall in the use of molecular biology to detect cancer metastases.

The prognostic significance of histology-negative RT-PCR-positive sentinel nodes remains debatable. There are no objective data to support or contradict complete lymph node dissection for histology-negative, molecular-positive sentinel nodes. A definitive answer must await completion of MSLT-2, a multicenter randomized trial in the United States, Europe and Australia (44). Although the clinical importance of the lymphatic system is well recognized, the actual molecular mechanisms involved remain poorly understood. As part of

the elucidation of these mechanisms improvements in the techniques and criteria involved in the detection of (early) lymph node metastasis of melanoma will be of great importance. These investigations will determine the clinical significance of “stealth” melanoma cells and the other MART-1 mRNA positive cells.

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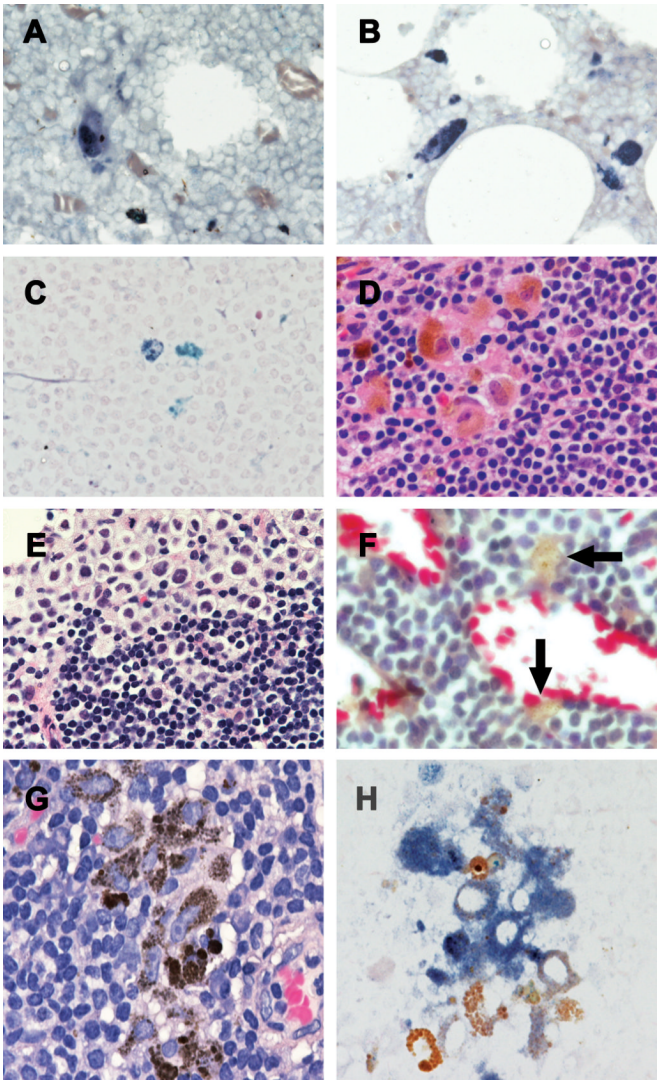

Figure 1.

"Stealth" melanoma cells identified by enhanced signal for MART-1 mRNA detected by RT *in situ* PCR (blue/purple color) (Panels A-C). In some original H&E slides, melanoma cells were present in areas separate from the RT *in situ* PCR-detected "stealth" melanoma cells (Panels D&E). Some MART-1 mRNA positive cells contained microdispersed melanin granules, a pattern that is typical of melanogenic cells, but lacked a nucleus which would have permitted determination of their malignant or benign status by H&E (Panel F). Small clusters of macrophage-like cells containing nuclei and nucleoli more typical of melanoma cells and microdispersed melanin granules (Panel G). These cells show positivity for MART-1 mRNA by the RT *in situ* PCR (blue/purple color) (Panel H). MART-1 mRNA is not seen in adjacent melanophages with coarse brown pigmented granules.

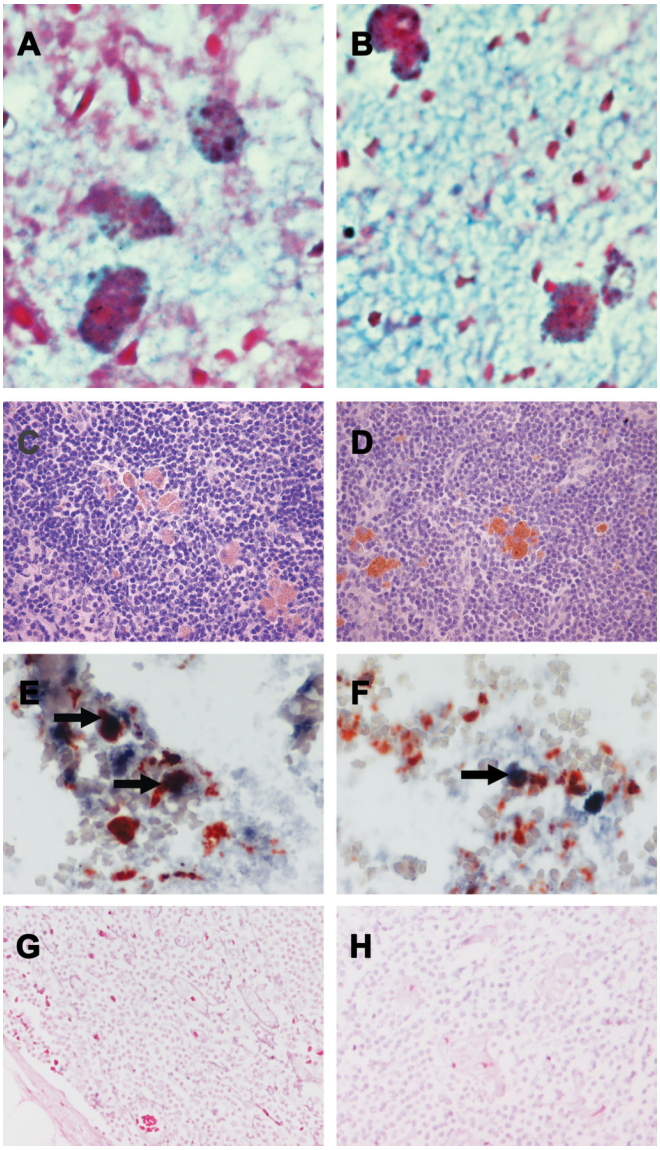

Figure 2.
MART-1 mRNA (blue/purple color) is seen by RT *in situ* PCR in melanophages with coarse pigment granules (Panels A&B). Immunohistochemistry for MART-1 shows a weakly positive signal in scattered large cells with “normal” nuclei and coarse melanin granules, consistent with melanophages (Panel C). Immunohistochemistry for HMB-45 shows a positive signal in scattered large cells, consistent with melanophages (Panel D). Double staining of MART-1 mRNA (blue/purple color) and CD68 antigen (red color) shows that some cells co-express both MART-1 mRNA and CD68 (arrow), while MART-1 mRNA and CD68 are separately expressed by other cells. (Panels E&F). Sentinel node (Panel G) and NSN (Panel H) from a breast cancer patient are negative for MART-1 mRNA.

Table 1

Primers used for MART-1 mRNA detection.

Target	Gene	Sequences		Amplicon size
MART-1	*MLANA*	Forward	5’-CACGGCCACTCTTACACCAC-3’	254 bp
		Reverse	5’-GGAGCATTGGGAACCACAGG-3’	

Table 2

MART-1 mRNA positivity in histology-negative SN and NSN

		MART-1 mRNA positive/total	%
SN	(SN: histology-negative)	6/21	29%
NSN	(SN, NSN: histology-negative)	2/16	13%
NSN	(SN: histology-positive, NSN: histology-negative)	2/7	29%
SN	(SN: histology-positive; Microscopically tumor-free areas were examined.)	6/7	86%