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Short Communication

Molecular Test for the Detection of Tumor Cells in Blood and Sentinel Nodes of Melanoma Patients

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Lymph node metastasis dramatically decreases the 5-year survival of melanoma patients. The so-called sentinel node surgery offers a therapeutic approach to resect the first draining lymph node. This technique enables accurate staging of melanoma patients in an early stage of the disease. Detection of a sentinel node metastasis is a strong argument for local lymphadenectomy. To improve the detection of micrometastasis in sentinel nodes of melanoma patients, molecular biological techniques were used. The exclusively melanocyte-specific tyrosinase transcript was amplified by reverse transcription followed by polymerase chain reaction (RT-PCR). In sentinel node examination, the detection of tyrosinase-positive cells by RT-PCR was compared with routine immunohistochemistry. From 16 patients, a total of 28 lymph nodes were tested. The lymph nodes were derived after lymphadenectomy (4 patients) and after sentinel node resection (12 patients with melanoma stage I). By using RT-PCR we could detect 100 tumor cells in a background of 10^8 peripheral blood mononuclear cells. The negative controls were all negative for tyrosinase. Cryostat sections of lymph nodes for mRNA isolation were alternated with sections for immunohistochemistry. By using tyrosinase RT-PCR, we detected 6 additional positive sentinel nodes in patients with melanoma stage I. Furthermore, the tyrosinase RT-PCR enabled us to design a blood test for circulating melanoma cells. Therefore, mRNA was directly isolated from whole blood of 23 blood samples, of which 3 samples were positive for tyrosinase. The present study demonstrates the possibility of a simple and rapid blood test for melanoma patients that has not been available until now. Furthermore, the detection of micrometastasis in sentinel nodes by tyrosinase RT-PCR dramatically increases the accuracy of melanoma staging. (Am J Pathol 1996, 149:759–764)

Reverse transcription and polymerase chain reaction (RT-PCR) is used to amplify the tyrosinase gene as a melanoma-specific tumor marker. Tyrosinase is a melanin biosynthetic enzyme exclusively expressed in cutaneous melanocytes and melanoma cells. A molecular biological approach, based on the detection of tyrosinase, to detect circulating melanoma cells in peripheral blood by RT-PCR was described by Smith et al.[1] The advantage of PCR-based techniques in contrast with immunohistochemistry and flow cytometry is the extremely high sensitivity.[2] The specificity was improved by a modification of the nested primer set in this study.

The elective lymph node dissection (ELND) in the surgical management of melanoma patients is controversial, as the majority of dissected lymph nodes are reported to be tumor free.[3] Because of postop-

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erative complications of ELND, such as lymph edema and dysfunction of extremities, some surgeons adopt a defensive strategy with careful follow-up examinations and delayed therapeutic lymph node dissection if clinically detectable metastasis develops in the regional nodes. However, the survival rates after the ELND are significantly higher for patients with clinically occult metastasis compared with patients treated with LND for clinically detectable metastases.[4,5] This improved survival is presumably due to resection of metastatic disease at an early stage. Dissemination of cutaneous melanoma cells via the lymphatic basin is not a random event.[6] Therefore, Morton et al[7] introduced the sentinel node biopsy, which allows the identification of the first draining lymph node and offered an alternative in the surgical management of melanoma patients. If this sentinel node contains (micro)metastases, it seems logical and prudent to perform a therapeutic lymph node dissection. Until now, examination of these sentinel nodes was established by histological techniques using monoclonal antibodies (MAbs).

The present study evaluates a tyrosinase RT-PCR-based system to detect micrometastases in sentinel nodes of patients with melanomas and compares this with standard immunohistochemical techniques. In a prospective study, 28 lymph nodes from 16 patients were analyzed following a lymph node protocol. Cryostat sections for RT-PCR were alternated with cryostat sections for immunohistochemistry. Furthermore, this study enabled us to develop a rapid and simple blood test to detect circulating melanoma cells in the peripheral blood. A functional blood test for melanocytes could represent a powerful tool in the screening and follow-up of melanoma patients. Enhanced detection of micrometastasis as well as a blood test based on molecular techniques is now available. Its clinical applicability will be discussed.

Materials and Methods

Patients

Four patients diagnosed with malignant melanoma and clinically suspected for positive lymph nodes were undergoing an ELND. Twelve patients with melanoma stage I were selected for sentinel node resection. From these sixteen patients, 28 lymph nodes were resected. From eleven patients, blood samples (5 ml of venous blood) were taken before and after surgery and from one patient before surgery only.

Controls

As negative controls, we used 23 blood samples from healthy volunteers and 20 blood samples and 5 lymph nodes from patients with other diseases. Moreover, we tested tumor cell lines (prostate cancer cells LNCaP, DU-145, and PC-3 and breast cancer cells MDA-MB-231, BT549, and T47D) as well as normal tonsil tissue. As positive control, the Mewo melanoma cell line was used. All cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD). The cell lines were cultured under the conditions as described by the American Type Culture Collection.

RT-PCR

Total RNA was isolated with TRIzol reagent (Gibco, Life Technologies, Paisley, UK) according to the instructions of the manufacturer. cDNA synthesis was performed with Superscript and oligo-dT primers (Gibco) by using 3 μg of initial RNA and following the product protocol. The quality of cDNA synthesis was monitored with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (sense, 5′-GCATCCTGGGCTACACTGAGC-3′; antisense, 5′-GGTACATGACAAGGTGCGGC-3′), which resulted in a 368-bp product. Tyrosinase was specifically amplified with the outer primers described by Smith et al,[1] resulting in a 283-bp product. To improve specificity, the nested PCR was carried out with modified primers resulting in a 203-bp product: HTYR 1, 5′-TTGGCAGATTGTCTGTAGCC-3′ (outer, sense); HTYR 2, 5′-AGGCATTGTGCATGCTGCTT-3′ (outer, anti-sense); MHTYR 3, 5′-GTCTTTATGCAATGGAAC-3′ (modified nested, sense); MHTYR 4, 5′-TATCCCAGTAAGTGGACT-3′ (modified nested, anti-sense).

A 3-μl volume of cDNA was added to the PCR mixture containing 1X PCR buffer (Perkin Elmer, Norwalk, CT), 1.5 mmol/L MgCl (Perkin Elmer), 200 μmol dNTPs, 150 pmol each of HTYR 1 and HTYR 2, 2 U of *Taq* DNA polymerase (Perkin Elmer), and aqua dest in a total reaction mix of 45 μl. Thirty-five cycles were carried out (94°C for 60 seconds, 60°C for 60 seconds, and 72°C for 120 seconds) in a Perkin Elmer DNA Thermo Cycler 480. For re-amplification with modified nested primers, 0.05 μl of PCR product was used and again 35 cycles were carried out (94°C for 60 seconds, 55°C for 60 seconds, and 72°C for 120 seconds). All PCR products were analyzed by electrophoresis on 2% agarose gel, followed by ethidium bromide staining.

Identification of Tyrosinase RT-PCR Sensitivity

To test the sensitivity of the tyrosinase RT-PCR, serial dilution studies were performed by using Mewo melanoma cells in peripheral blood mononuclear cells (PBMCs). The cells were trypsinized with 0.25% trypsin and 0.01% EDTA at 37°C for 1 minute. The cells were counted and diluted to a concentration of 100 cells/ml. Sensitivity was determined by adding 1 ml of the tumor cell suspension in different concentrations of PBMCs (from 10^5 to 10^9 PBMC). Total RNA was isolated as described above, and after cDNA synthesis, amplification with GAPDH primers and with tyrosinase primers was performed. Tests were done in triplicate.

Blood Test

Isolation of mRNA was done with TRIzol LS reagent (Gibco) following the product protocol. TRIzol LS reagent is a new ready-to-use reagent for the isolation of total RNA from liquid samples without requiring red blood cell lysis. With this product it is possible to isolate RNA directly from whole blood in a procedure within 1 hour. RT-PCR was performed overnight, and analysis of the PCR products was done on an ethidium-bromide-stained gel.

Lymph Node Protocol

Lymph nodes were divided in two parts. One-half of each node was formalin fixed, paraffin embedded, and used for routine histological diagnosis with immunohistochemistry using a polyclonal Ab S-100 (Dako, Glostrup, Denmark) or anti-melanoma MAb HMB45 (IgG_1; Enzo Diagnostic, New York, NY). The other half was frozen and used for cryostat sections for immunohistochemistry and RT-PCR. Three series of cryostat sections were done, for which 6-μm cryostat sections for immunohistochemistry were alternated with five 20-μm sections for RNA isolation. Immunohistochemistry was performed by a three-step reaction using a mouse anti-melanoma MAb (HMB45, Enzo Diagnostic) or S-100 (Dako) as the first-step antibody. As the second step, a biotin-labeled horse anti-mouse antibody was used, followed by incubation with a streptavidin-labeled alkaline phosphatase. This was developed by an alkaline phosphatase reagent following a standard protocol.

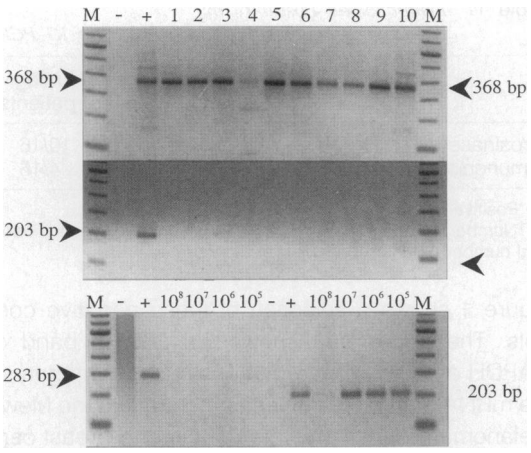

Figure 1. *Specificity and sensitivity of tyrosinase RT-PCR. The* **top panel** *shows the GAPDH control of some representive negative controls. The integrity of the mRNA samples is demonstrated by a single 368-bp band. The* **middle panel** *represents the tyrosinase RT-PCR of the same samples. After amplification and re-amplification, a single 203-bp band is visualized.* **Lane M,** *100-bp ladder;* **lane −,** *negative control;* **lane +,** *Mewo;* **lane 1,** *LNCaP;* **lane 2,** *PC-3,* **lane 3,** *DU-145;* **lane 4,** *BT-549;* **lane 5,** *T47D;* **lane 5,** *MB-MDA-231;* **lanes 7** *and* **8,** *control peripheral blood;* **lanes 9** *and* **10,** *control lymph nodes;* **lane M,** *100-bp ladder. The* **bottom panel** *shows the detection of 100 Mewo cells in different concentrations of PBMC to define the sensitivity of tyrosinase RT-PCR. The* **left half** *shows the primary amplification, resulting in a single 283-bp band.* **Lane M,** *100-bp ladder;* **lane −,** *negative control;* **lane +,** *Mewo cells (positive control); remaining* **lanes** *show the number of PBMCs mixed with 100 Mewo cells. The* **right half** *represents the results after re-amplification of the dilution study. The re-amplification generated a single 203-bp band.* **Lane −,** *negative control;* **lane +,** *Mewo cells (positive control); next four* **lanes** *show the number of PBMCs mixed with 100 Mewo cells;* **lane M,** *100-bp ladder.*

Results

Sensitivity and Specificity of Tyrosinase RT-PCR

As positive control, Mewo melanoma cells were used. Amplification with tyrosinase-specific outer primers (HTYR1 and HTYR2) resulted in a fragment of 283 bp, and re-amplification with modified nested primers (MTYR3 and MTYR4) resulted in a 203-bp fragment (Figure 1). Serial dilution studies were done to determine the sensitivity of the tyrosinase RT-PCR. After adding 100 Mewo cells to different concentrations of PBMCs, the tyrosinase RT-PCR was performed. Amplification with outer primers resulted in a slight positivity at a concentration of 10^5, 10^6, and 10^7 PBMCs (Figure 1). Re-amplification with nested primers generated a single band of 203 bp in a background of 10^5, 10^6, 10^7, and slightly in 10^8 PBMCs (Figure 1). It was not possible to detect 100 Mewo cells in a background of 10^9 PBMCs (data not shown). The specificity of the RT-PCR was tested by analyzing 6 different nonmelanocytic cancer cell lines, 5 lymph nodes, 1 tonsil, and 43 blood samples (healthy volunteers and prostate cancer patients).

Table 1. *Lymph Node Positivity by Immunohistochemistry and Tyrosinase RT-PCR*

	Positive LN*	Number of patients†
Tyrosinase RT-PCR	16/28	10/16
Immunohistochemistry	10/28	4/16

*Positive lymph nodes/total number of lymph nodes.
†Number of patients with one or more positive lymph nodes/total number of tested patients.

Figure 1 shows a selection of these negative controls. The upper part shows the 368-bp band of GAPDH amplification to demonstrate the integrity of the mRNA of these samples. In contrast to the Mewo melanoma cells, all prostate cancer and breast cancer cell lines were negative for specific amplification of tyrosinase (Figure 1). All 43 control peripheral blood samples were negative for tyrosinase RT-PCR. Figure 1 shows two representative samples (lanes 7 and 8). Other tissues, such as tonsil and control lymph nodes were also negative for tyrosinase RT-PCR (Figure 1, lanes 9 and 10).

Detection of Melanoma Cells in Lymph Nodes by RT-PCR Compared with Immunohistochemistry

A total of 28 lymph nodes derived from 16 patients were tested following a lymph node protocol. Routine histology of these 28 lymph node specimens revealed positivity in 10 lymph nodes from 4 patients (4/16 patients; Table 1). These 4 patients all had had a lymphadenectomy. The sentinel nodes from the 12 patients with malignant melanoma stage I were all negative by routine histology and immunohistochemical methods. RT-PCR detected 16 positive nodes in 10 different patients (10/16 patients) including the 10 nodes of the 4 patients with positive lymph nodes detected by immunohistochemistry (Figure 2). In the group of sentinel node biopsies, the RT-PCR detected 6 additional positive lymph nodes (Figure 2). Evaluation of the control sections, which were alternated with the sections for tyrosinase RT-PCR, demonstrated no microscopic melanoma cells. By serial sections of the additional 6 tyrosinase RT-PCR-positive nodes it was possible to detect a single anti-melanoma (HMB45)-positive cell by immunohistochemistry (Figure 3).

Tyrosinase RT-PCR-Based Blood Test for Melanoma Patients

To perform a tyrosinase RT-PCR with peripheral blood specimens from melanoma patients a new

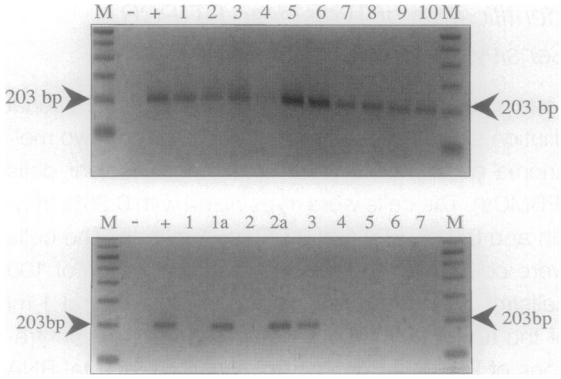

Figure 2. *Detection of melanocytes in lymph nodes and blood samples of patients with melanomas. The* **top panel** *represents the tyrosinase RT-PCR product after amplification and re-amplification. A single 203-bp product was visualized on an ethidium-bromide-stained gel.* **Lane M,** *100-bp ladder;* **lane −,** *negative control;* **lane +,** *Mewo cells (positive control);* **lanes 1** *to* **4,** *lymph nodes of melanoma patients after ELND;* **lanes 5** *to* **10,** *sentinel nodes of melanoma patients stage 1;* **lane M,** *100-bp ladder. The* **middle panel** *shows the tyrosinase RT-PCR results of blood samples of melanoma patients before and after sentinel node surgery. Only the PCR products after re-amplification are shown.* **Lane M,** *100-bp ladder;* **lane −,** *negative control;* **lane +,** *Mewo (positive control);* **lanes 1** *and* **1a,** *patient 1 before and after ELND, respectively;* **lanes 2** *and* **2a,** *patient 2 before and after ELND, respectively;* **lane 3,** *patient 3 before ELND;* **lanes 4,** *patient 4 before sentinel node biopsy;* **lane 5,** *patient 5 after sentinel node biopsy;* **lane 6,** *patient 6 after sentinel node biopsy;* **lane M,** *100-bp ladder.*

mRNA isolation method was used. We used TRIzol LS reagent on whole blood samples, without red cell lysis steps. RNA isolation with this technique compared with conventional methods was simple and could be performed within 1 hour. The amount of RNA was higher and was of a high quality. By using this improved technique the results of the blood test were revealed within 12 hours. We tested blood samples of 11 patients before and after surgery (22 samples) and from 1 patient only before surgery. After amplification and re-amplification, tyrosinase

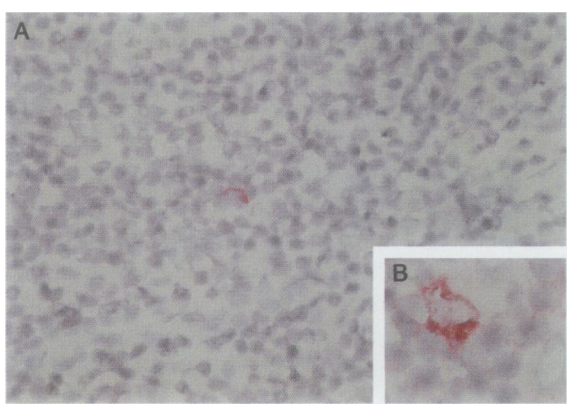

Figure 3. *Immunohistochemistry of tyrosinase RT-PCR-positive sentinel nodes. Serial sections of sentinel nodes, which were negative for routine histology, were stained with the immunoalkaline phosphatase technique by using anti-melanoma MAb (HMB45) as the first-step antibody. Two sentinel node specimens with an anti-melanoma-positive cell are shown. Original magnification, ×350 (***A***) and ×500 (***B***).*

mRNA was detected in three blood samples of patients having a lymphadenectomy. Figure 2 shows that in two cases the blood samples after surgery were positive. In one case the tyrosinase RT-PCR positivity was detected before surgery (Figure 2). The blood tests of other patients with RT-PCR-positive lymph nodes were negative for tyrosinase (Figure 2, patients 4 to 6).

Discussion

The malignant melanoma is a cutaneous malignancy that, once metastasized, has a poor prognosis.[5,8] Melanoma cells metastasize nonrandomly via the first draining local lymph node. Morton et al[7] introduced a technique that allows the mapping and identification of this first draining lymph node, the so-called sentinel node. If this lymph node is positive, a radical lymphadenectomy is indicated.[9] The patients with negative sentinel nodes have no benefit from this procedure, because if the sentinel node is negative, the remainder of the nodes are also expected to be negative.[6,9] Until now, the examination of these sentinel nodes was done by immunohistochemistry using, for example, MAb HMB45 or polyclonal S-100 antibodies. Introduction of the RT-PCR techniques in the detection of circulating tumor cells by Smith et al[1] represents a powerful and extremely sensitive tool in oncological diagnosis.

In this paper we demonstrate a molecular biological approach based on tyrosinase RT-PCR to detect micrometastasis in sentinel nodes and compare this method with the results of conventional immunohistochemical procedures. Furthermore, we developed a rapid and simple blood test to detect melanoma cells in the peripheral blood circulation. Therefore, we introduced a new and simple method to isolate mRNA directly from whole blood samples. Sentinel node and blood analysis was performed with RT-PCR, which includes the synthesis of cDNA with oligo-dT, followed by specific amplification with tyrosinase primers. Identification of the PCR product was performed after blotting and hybridization with an internal oligonucleotide (data not shown). To improve specificity, the nested primers (published by Smith et al[1]) were modified.

With this technique, following a lymph node protocol, we were able to detect 16 tyrosinase RT-PCR-positive lymph nodes. Compared with immunohistochemistry, we were able to detect 6 additional tyrosinase-positive sentinel nodes. Control sections proved that no metastases or micrometastases slipped through the routine histology by dividing the nodes. However, in some cases, serial sections of these additional RT-PCR-positive nodes demonstrated that it was possible to detect one single anti-melanoma-positive cell by using immunohistochemistry. Serial sections and immunohistochemistry in the routine diagnostics will increase sensitivity but will be extremely time consuming and expensive. The possibility to detect micrometastasis with tyrosinase RT-PCR will simplify this procedure and will be a powerful tool in the staging of melanoma patients. Because of the poor prognosis of lymph node positive melanoma patients, tyrosinase-positive sentinel nodes could be a clear indication for lymphadenectomy.

A tyrosinase RT-PCR-based blood test was developed by using only 5 ml of venous blood. Blood samples of melanoma patients stage I and II were tested. Simplifying the RNA isolation enabled us to get the results of this test within 12 hours with a maximal detection rate of 100 tumor cells in 10^8 PBMCs. A 5-ml volume of peripheral blood contains approximately 10^7 mononuclear cells. This implicates that a test with a potential detection rate of 1 tumor cell/10^6 PBMCs requires at least a 10-fold excess of melanoma cell load for tyrosinase positivity to be detected. Three patients showed tyrosinase positivity in their blood, one before and two after surgery; all of these patients had had a lymphadenectomy. This indicates that tyrosinase blood positivity occurs in a later stage of this malignancy if a sufficient tumor load is reached. Nevertheless, this blood test is the first molecular test that allows the detection of melanoma cells by using only 5 ml of peripheral blood. Battayani et al[10] and Wang et al[11] demonstrated that RT-PCR positivity in the circulation even in stage I melanoma patients has prognostic values. In this study we demonstrated that tyrosinase positivity in patients with lymph node metastases but not in stage I melanoma patients is detectable. Even if the PCR products were blotted on a nylon membrane and hybridized with radiolabeled internal oligonucleotide, no increase of the sensitivity was reached (data not shown).

Foss et al[12] showed that only in a minority of melanoma patients could melanoma cells be detected in the peripheral blood. However, in this study we confirmed that circulation of melanoma cells is detectable in patients with lymph node metastasis. Other melanoma markers such as gp100 or MART-1[13,14] could be established to develop a multimarker test in case of negative tyrosinase RT-PCR due to mutations or deletions in the tyrosinase transcript during tumor cell transformation. As shown, RT-PCR is a highly sensitive and specific method,

which is technically simple and rapid. In the detection of occult metastasis, the tyrosinase RT-PCR proved its value by supporting the indication of ELND in the surgical management. A blood test for melanoma patients is now available and enables us to screen larger populations of melanoma patients. This could lead to the definition of melanoma patients with high and low risk of disease recurrence.

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