Detection of Melanoma Micrometastases in the Sentinel Lymph Node and in Nonsentinel Nodes by Tyrosinase Polymerase Chain Reaction

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The aim of our study was to investigate the metastatic pathways of melanoma cells in sentinel and other regional lymph nodes. The term "sentinel lymph node" means that the first lymph node of the draining site of a primary tumor is never bypassed in malignant melanoma. In this case lymph node dissection would be necessary only when melanoma cells are detected in the sentinel node. Tyrosinase reverse transcriptase–polymerase chain reaction was applied to search for metastatic melanoma in the sentinel lymph node and in further lymph nodes of a complete lymph node basin in patients who underwent lymph node dissection. In 24 patients with malignant melanoma the draining site of the tumor was marked by lymphoscintigraphy and by intraoperative injection of patent blue V in the area around the primary tumor. The lymph nodes of the affected basin were excised and prepared for histopathologic, immunohistochemical, and molecular biologic examinations. Regarding the sentinel lymph node, 10 of 24 patients showed morphologic evidence for metastases, three additional patients showed only tyrosinase transcripts. In 11 of these 13 cases we found one or more nonsentinel lymph nodes with morphologically detectable melanoma cells and/or tyrosinase mRNA. Interestingly, in seven of 24 patients a positive tyrosinase reverse transcriptase–polymerase chain reaction was received in nonsentinel lymph nodes, whereas the sentinel lymph node was negative, not only for all histologic examinations but also by tyrosinase reverse transcriptase–polymerase chain reaction. In five of seven patients of the latter group, gp100 reverse transcriptase–polymerase chain reaction was carried out, showing also gp100 mRNA in nonsentinel lymph nodes only. Our data indicate that the concept of the sentinel lymph node may miss micrometastases. Whether such micrometastases cause a recurrence or a metastasis of malignant melanoma, or can be destroyed by the immune system, remains to be clarified. ***Key words: lymph node dissection/lymph node mapping/melanoma cells/reverse transcriptase-PCR. J Invest Dermatol 113:554–559, 1999***

Melanoma accounts for 1–3% of all malignant tumors (Brossart *et al*, 1993). The dramatic increase of the incidence of malignant melanoma in Europe, Australia, and the U.S.A. during the last decades has been mainly attributed to exposure to high ultraviolet doses (Sober *et al*, 1991). Although today melanoma is diagnosed more frequently in early stages of disease and therefore shows a better overall survival (Sober *et al*, 1991), once tumor cells can be detected in the regional lymph node (LN), the patients have a poorer prognosis. For example, in patients with clinical and pathologic stage I disease (Breslow thickness below 1.50 mm, Clark level III and without involvement of regional LN) a 5 y survival rate between 80% and 100% was reported, depending on tumor thickness (Slominski *et al*, 1995). Prognosis rapidly decreases to a 5 y survival rate of 20–30% in patients with LN metastases (Koh *et al*, 1986). Therefore, the excision of the tumor and LN dissection represent an effective therapy in malignant melanoma, despite an ongoing discussion about the value of elective LN dissection in patients with clinically unsuspicious LN and medium- or high-risk melanoma.

The technique of sentinel LN (SLN) mapping and selective lymphadenectomy has been introduced to optimize melanoma treatment (Wong *et al*, 1991; Morton *et al*, 1992; Lingam *et al*, 1994). It is based on the concept that melanoma cells via the lymphatics regularly enter the first regional LN (SLN). If the SLN would reflect the metastatic status of the entire LN area, only patients with melanoma cell positive SLN should be subjected to elective LN dissection. In patients with negative SLN local excision of the primary tumor would be an appropriate therapy (Ross *et al*, 1993; Reintgen *et al*, 1994; Glass *et al*, 1995; Godellas *et al*, 1995).

Recently, there have been attempts to detect small numbers of melanoma cells by the polymerase chain reaction (PCR) technique. Smith *et al* (1991) were the first to describe a reverse transcription (RT–PCR) for tyrosinase mRNA to detect melanoma cells in peripheral blood. Others modified this technique to detect micro-

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Abbreviations: ALM, acrolentiginous malignant melanoma; LN, lymph node; NM, nodular malignant melanoma; RT–PCR, reverse transcriptase–polymerase chain reaction; SLN, sentinel lymph node; SSM, superficial spreading malignant melanoma.

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Table I. Survey on the patients investigated

Pat. no.	Age	Sex	Melanoma type	Thickness	Clark level	Site affected	LN basin affected	Dissected LN n	Clinical stage	TNM
1	67	m	SSM	3,06	IV	trunk	axilla	7	III	pT4,N0,Mx
2	52	f	SSM	1,4	IV	trunk	axilla	7	II	pT3,N0,Mx
3	60	f	ALM	2,34	IV	leg	groin	10	III	pT2,N1,Mx
4	55	f	NM	1,59	IV	leg	groin	12	III	pT3,N1,Mx
5	62	f	SSM	1,45	IV	trunk	axilla	6	I	pT3,N0,Mx
6	68	m	NM	1,62	III	arm	axilla	8	I	pT2,N0,Mx
7	60	m	SSM	1,94	III	trunk	axilla	8	I	pT2,N0,Mx
8	61	m	SSM	4,2	IV	trunk	axilla	12	II	pT3,N0,Mx
9	44	f	NM	1,85	IV	trunk	axilla	10	II	pT3,N0,Mx
10	34	m	SSM	1,27	IV	arm	axilla	7	III	pT3,N1,Mx
11	46	f	SSM	2,37	IV	trunk	axilla	12	II	pT3,N0,Mx
12	60	m	NM	3,0	IV	trunk	axilla	8	II	pT3,N0,Mx
13	46	m	SSM	2,31	IV	trunk	axilla	9	III	pT3,N1,Mx
14	58	m	#[a]	9	IV	hand	axilla	8	III	pT4a,N1,Mx
15	61	f	SSM	1,9	IV	leg	axilla	6	III	pT3,N1,Mx
16	54	f	NM	2,7	IV	leg	groin	19	III	pT3,N1,Mx
17	64	f	#[a]	1,19	IV	leg	groin	20	III	pT3,N1,Mx
18	45	m	NM	10	IV	leg	groin	18	III	pT4a,N1,Mx
19	68	m	SSM	1,53	III	leg	groin	14	III	pT3,N1,Mx
20	65	m	NM	2,07	IV	leg	groin	7	III	pT3,N1,Mx
21	63	f	NM	4,0	IV	buttock	groin	22	II	pT3,N0,Mx
22	45	m	SSM	0,72	III	chest	axilla	10	I	pT2,N0,Mx
23	63	m	NM	4,3	IV	chest	axilla	12	II	pT3,N0,Mx
24	71	f	SSM	2,8	IV	arm	axilla	12	II	pT3,N0,Mx

[a]No classification possible.

metastases also in LN (Wang *et al*, 1994; Schwürzer-Voit *et al*, 1996; Blaheta *et al*, 1998). It was demonstrated, that LN from patients with tumors other than melanoma do not express RT–PCR detectable tyrosinase mRNA (Blaheta *et al*, 1998). As tyrosinase expression is restricted to melanocytes, melanocytic nevus cells (nevocytes), and in melanoma cells, the detection of tyrosinase mRNA indicates the presence of melanoma cells in the LN investigated (Foss *et al*, 1995). Nodal nevocytes causing a positive tyrosinase RT–PCR, however, were found in dissected LN from melanoma patients (Carson *et al*, 1996). Thus, a careful interpretation of positive tyrosinase RT–PCR results in patients with a melanoma-negative histopathology is strongly recommended. In our study, the SLN as well as the other LN of a given LN basin from 24 melanoma patients were investigated following mapping by histopathology, immunohistochemistry, and tyrosinase RT–PCR in order to detect the presence of LN metastasis or micrometastasis.

MATERIALS AND METHODS

Biopsy material The entire regional LN from 24 patients with malignant melanoma in clinical stages I–III (classification of the American Joint Committee on Cancer) were examined. Informed consent was obtained. The patients were recruited from the Department of Dermatology, Charité University Hospital and had complete physical and medical histories. Tumor thickness and level of invasion were documented as well as tumor type and stage of disease according to the TNM classification of the International Union Against Cancer (UICC, Hermanek *et al*, 1989). In eight patients with malignant melanoma of the leg, LN were excised from the inguinal and iliacal region. In the other cases the axillary LN basin was affected: 12 patients had malignant melanoma of the trunk or shoulder and four patients had malignant melanoma of the arm or thumb. All clinical and histologic characteristics are summarized in **Table I**.

Lymphography and LN mapping By combination of lymphoscintigraphy and patent blue injection the SLN was detected in all patients. Lymphoscintigraphy was performed 24 h before elective LN dissection by intracutaneous injection of $^{Tc}99^{m}$-Nano colloid in four to six aliquots around the primary tumor or the site of previous excision. Imaging was performed using a gamma camera, and the lymphatic drainage was marked on the skin. Similarly, patent blue V was injected directly before surgery to visualize the afferent lymphatics and the SLN. First, the SLN was excised. All following non-SLN of the draining basin were sectioned *en bloc* together with the surrounding fatty tissue. LN mapping was performed systematically node by node, beginning at the site of the tumor and SLN. The LN were numbered in sequence as found. Between six and 22 LN per basin (mean value 11) were excised. In total, 264 LN were examined. After being sliced the nodes were inspected macroscopically to detect structures suspicious of metastatic malignant melanoma, e.g., discolored or hemorrhagic tissue. One part was snap-frozen in liquid nitrogen for molecular biologic analyses. The other parts were prepared for hematoxylin and eosin staining and immunohistochemical staining for HMB-45 protein.

Six LN of five patients with a cutaneous T cell lymphoma, without any signs or history of melanoma, were included as negative controls for the tyrosinase and gp100 RT–PCR (see below).

RNA extraction Fifty to 250 mg of the frozen LN specimens were lyzed with heated lysis buffer (QIAGEN, Hilolen, Germany) and immediately homogenized with a rotor-stator homogenizer. The subsequent extraction of the total RNA by the Oligotex Direct mRNA Kit (QIAGEN) was performed as described by the manufacturer.

cDNA synthesis Reverse transcription was carried out with SuperScript Preamplification System (Gibco BRL, Eggenstein, Germany) directly following RNA extraction as recommended by the manufacturer. All RNA extraction and reverse transcription procedures were performed under sterile conditions with autoclaved tubes and pipettes.

PCR To avoid contamination components of the PCR were handled under a laminar air flow hood in a separate room. The PCR assays were performed in a programmable thermocycler (Landgraf, Langenhagen, Germany) as previously described with some modifications immediately following the cDNA synthesis. Primers for human tyrosinase were used as published (Smith *et al*, 1991; Ponnazhagan *et al*, 1994). The outer primers HTYR 1 and 2 produce a PCR fragment of 284 bp. The inner primers HTYR 3 and 4 produce a PCR fragment of 207 bp. The reaction mix consisted of 51.5 μl sterile water, 7.5 μl 10 × PCR buffer (Perkin Elmer, Weiterstadt, Germany), 7.5 μl $MgCl_2$ (25 mM, Perkin Elmer), 3.0 μl dNTP (10 mM each, Pharmacia Biotech, Freiburg, Germany), 0.4 μl AmpliTaq Polymerase (5 U per μl, Perkin Elmer), 1.5 μl (20 pmol) of each primer, and 3 μl of cDNA. PCR conditions were set up as follows: 95°C for 5 min, 30 cycles of 60°C for 90 s, 72°C for 90 s, and 94°C for 90 s. For re-amplification with inner primers, 3 μl of a 1 in 50 dilution of the PCR products were processed as described above. PCR products, i.e., 8 μl of each reaction mixture were separated by electrophoresis on ethidium bromide containing 2% agarose gels with *Hinc*II digests of ϕX 174 (USB) as DNA molecular weight marker (**Fig 1**). To control the integrity of each mRNA sample a PCR with primers for the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was set up as published (Platzer *et al*, 1994). Samples without DNA were subjected to

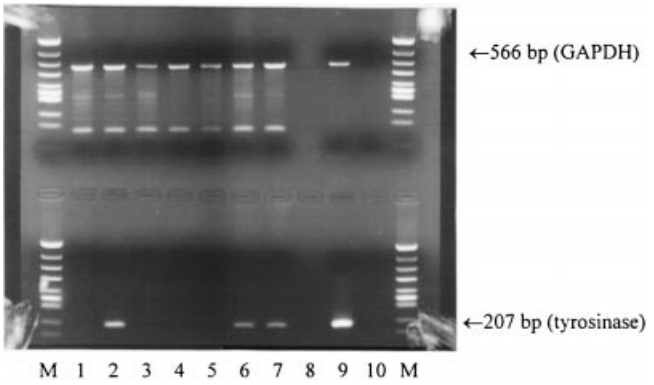

Figure 1. Tyrosinase and GAPDH RT–PCR products separated on an ethidium bromide stained 2% agarose gel. cDNAs of patient 8 (see **Table V**), *lane 1*, SLN; *lanes 2–7*, LN 2–7; *lane 8*, empty; *lane 9*, positive control (melanoma cell line); *lane 10*, negative control (no DNA); M, size standard *Hinc*II digest of phi X174 DNA.

PCR as negative control. In order to exclude artifacts caused by contaminating genomic DNA, PCR was performed without prior reverse transcriptase reaction. cDNA produced from the melanoma cell line MeWo was used as positive control for the GAPDH and tyrosinase RT–PCR. For some melanoma patients and the control LN, a RT–PCR for gp100 mRNA was performed according to Spagnoli *et al* (1995). The conditions were as follows: 94°C for 5 min, 40 cycles of 56°C for 60 s, 72°C for 120 s, and 94°C for 60 s. The PCR was regarded as positive if a 334 bp fragment was generated. The melanoma cell line SK-Mel 29 served for positive control.

Sequencing of tyrosinase RT–PCR products The correctness of the base sequence of the PCR-generated tyrosinase DNA fragments was confirmed by direct DNA sequencing. The amplification products of three different templates were purified from agarose gels (NuSieve, 3%, FMC Bioproducts, Rockland, U.S.A.). The band of interest (207 bp DNA fragment) obtained with primers HTYR 3 and 4 was cut out and the DNA was recovered. The sequencing reaction was performed as dye terminator cycle sequencing by means of the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer). For each reaction 50 ng DNA were used as template with 5 ng of the primers HTYR 3 or 4, respectively. The sequencing reaction, the purification of its products and the preparing of the samples were carried out following the kit protocol. Finally, the samples were analyzed on the automated ABI 373A sequencer. For each sample the nucleotide residues 822–1019, i.e., a 196 bp segment of the corresponding 207 bp DNA fragment was found correctly (not shown here).

RESULTS

Overall findings in 264 LN from 24 patients In 223 of 264 LN samples of the 24 melanoma patients (stages pT 2–4) mRNA extraction and cDNA were successful as demonstrated by the GAPDH RT–PCR. These 223 samples were subjected to tyrosinase RT–PCR, and in selected samples also to gp100 RT–PCR. All cDNA derived from the control specimen provided positive GAPDH RT–PCR but negative tyrosinase and gp100 RT–PCR. From all patients, the entire LN of the respective regional LN area were investigated. Regarding all 24 patients, 10 cases (42%) had histologic and/or immunohistologic evidence for melanoma metastasis in the SLN, three patients (13%) showed mRNA transcripts for tyrosinase without morphologic evidence for melanoma metastasis in the SLN, whereas four patients (17%) were completely negative using these techniques. Seven patients (29%) had tyrosinase mRNA in the non-SLN whereas the SLN was negative by all methods applied.

A survey on the results of those 223 LN samples providing a functional cDNA (22 SLN, 201 non-SLN) is shown in **Table II**: The frequency of morphologic demonstration of melanoma metastasis as well as tyrosinase mRNA was clearly higher in the SLN as compared with the non-SLN, indicating the functional power of SLN to capture and retain metastatic melanoma cells for some time. From the 201 non-SLN with an intact RNA/cDNA, 15 (7.5%) showed morphologic evidence for metastatic melanoma, whereas 41 (20,5%) contained only tyrosinase transcripts. Regarding the non-SLN, 145 of 201 specimen (72%) were completely negative. It is important to realize that these figures for non-SLN reflect the overall number of non-SLN but not percentages of the patients investigated.

Table II. Results of histologic, immunohistologic, and tyrosinase RT–PCR in 22 SLN and 201 non-SLN samples with a positive GAPDH signal

Positive samples by	SLN		Non-SLN	
	n	%	n	%
Histology + immunohistology + RT−PCR	5	23	14	7
Histology + immunohistology	2	9	1	0.5
Immunohistology + RT−PCR	−	−	−	−
RT−PCR	3	14	41	20.5
Immunohistology	1	4	−	−
None of the applied methods	11	50	145	72

To assess these overall findings on a patient-based analysis, we formed four groups: first, patients with morphologically affected SLN; secondly, those with no morphologic evidence for melanoma in the SLN but demonstration of tyrosinase transcripts; a third group with completely negative findings in SLN but positive in at least one non-SLN; and lastly, patients without any positive findings.

Patients with morphologically affected SLN As can be seen from **Table III**, in this group of 10 patients six had further morphologically affected non-SLN. The number of morphologically affected non-SLN varied from one (patients 4, 13, 16, 19) to five (patients 3 and 15, both with Clark level IV melanomas). Tyrosinase mRNA was detected in seven patients in this group. Whereas in patient 19 the histologically positive non-SLN was negative for tyrosinase mRNA despite positive GAPDH control, patients 10 and 14 showed tyrosinase transcripts in three morphologically negative non-SLN. From the SLN of patients 14 and 17 no functional cDNA was received. Most morphologically positive LN with an intact cDNA showed also tyrosinase transcripts (19 of 23, 83%).The number of reverse transcriptase-positive non-SLN (27) exceeded that of morphologically positive non-SLN (14) substantially.

Patients with morphologically negative but tyrosinase RT–PCR positive SLN Patients 5, 9, and 11 expressed tyrosinase mRNA in their SLN without morphologic evidence for melanoma (**Table IV**). In all three cases, also non-SLN (one, eight, or seven, respectively) contained tyrosinase transcripts.

Patients with morphologically and tyrosinase RT–PCR negative SLN and positive non-SLN Seven of 24 patients (29%) showed a positive tyrosinase RT–PCR in one or more non-SLN, whereas the SLN was negative by all methods applied (**Table V**). In this group, two tumors were low-risk melanomas (Breslow thickness below 1.50 mm), the remaining five cases were intermediate- and high-risk tumors. In four cases, only one of the examined non-SLN was found to be positive for tyrosinase transcripts. In patient 8, tyrosinase RT–PCR was positive in four non-SLN (shown in **Fig 1**). In one case (patient 20) the tyrosinase mRNA expression corresponded to morphologic detection of metastatic melanoma. In all cases, where a RT–PCR for gp100 was performed, gp100 transcripts were also not found in the SLN (**Table V**). We detected gp100 mRNA in tyrosinase mRNA-positive non-SLN, however, with only one exception (patient 20). In this patient the detection of gp100 mRNA failed, although the tyrosinase mRNA containing non-SLN was also morphologically positive. Particular clinical features distinguishing patient 20 from the four patients which showed gp100 transcripts were not found (see also **Table I**).

Table III. Histologic, immunohistologic, and molecular analysis of regional LN in patients with morphologically positive SLN[a]

Patient 3, LN	1	2		3	4	5		6		7	8			9	10					
Histopathology	+	+		+	+	−		−		+	−			−	+					
Immunohistology	+	+		+	+	−		−		+	−			−	+					
mRNA tyrosinase	+	+		+	+	#		#		+	#			+	+					
Patient 4, LN	1	2		3	4	5		6		7	8			9	10	11				12
Histopathology	+	−		−	−	+		−		−	−			−	−	−				−
Immunohistology	+	−		−	−	+		−		−	−			−	−	−				−
mRNA tyrosinase	+	−		+	−	+		−		−	−			+	−	−				−
Patient 10, LN	1	2		3	4	5		6		7										
Histopathology	−	−		−	−	−		−		−										
Immunohistology	+	−		−	−	−		−		−										
mRNA tyrosinase	−	−		+	+	+		−		−										
Patient 13, LN	1	2		3	4	5		6		7	8			9						
Histopathology	+	−		−	−	+		−		−	−			−						
Immunohistology	+	−		−	−	+		−		−	−			−						
mRNA tyrosinase	+	+		−	+	+		+		+	−			+						
Patient 14, LN	1	2		3	4	5		6		7	8									
Histopathology	+	−		−	−	−		−		−	−									
Immunohistology	+	−		−	−	−		−		−	−									
mRNA tyrosinase	#	#		#	#	#		+		+	+									
Patient 15, LN	1	2		3	4	5		6												
Histopathology	+	+		+	+	+		+												
Immunohistology	+	+		+	+	+		+												
mRNA tyrosinase	+	+		+	+	+		+												
Patient 16, LN	1	2		3	4	5		6–18		19										
Histopathology	+	−		−	−	−		−		+										
Immunohistology	+	−		−	−	−		−		+										
mRNA tyrosinase	+	−		−	−	#		−		+										
Patient 17, LN	1	2		3	4	5–7	8		9		10–13		14	15	16–19		20			
Histopathology	+	−		−	−	−	−		−		−		−	−	−		−			
Immunohistology	+	−		−	−	−	−		−		−		−	−	−		−			
mRNA tyrosinase	#	#		−	−	#	−		#		−		#	−	#		−			
Patient 18, LN	1	2−7	8	9	10	11		12		13–16		17	18							
Histopathology	+	−		−	−	−		−		−		−	−							
Immunohistology	+	−		−	−	−		−		−		−	−							
mRNA tyrosinase	−	−		#	−	#		−		#		−	#							
Patient 19, LN	1	2		3	4	5		6		7	8			9	10	11–14				
Histopathology	+	−		−	−	−		−		−	−			−	+	−				
Immunohistology	+	−		−	−	−		−		−	−			−	+	−				
mRNA tyrosinase	−	−		−	−	−		−		−	−			−	−	−				

[a]LN1 = SLN; #, not tested: negative GAPDH RT−PCR.

Table IV. Histologic, immunohistologic, and molecular analysis of regional LN in patients with morphologically negative and tyrosinase RT–PCR positive SLN

Patient 5, LN	1	2	3	4	5	6						
Histopathology	−	−	−	−	−	−						
Immunohistology	−	−	−	−	−	−						
mRNA tyrosinase	+	−	+	−	−	−						
Patient 9, LN	1	2	3	4	5	6	7	8	9	10		
Histopathology	−	−	−	−	−	−	−	−	−	−		
Immunohistology	−	−	−	−	−	−	−	−	−	−		
mRNA tyrosinase	+	+	+	+	+	+	+	+	+	−		
Patient 11, LN	1	2	3	4	5	6	7	8	9	10	11	12
Histopathology	−	−	−	−	−	−	−	−	−	−	−	−
Immunohistology	−	−	−	−	−	−	−	−	−	−	−	−
mRNA tyrosinase	+	+	−	+	+	−	+	+	+	#	+	#

LN1 = SLN; #, not tested: negative GAPDH RT−PCR.

Additionally, also some of the tyrosinase RT–PCR negative non-SLN contained gp100 mRNA. There was no association between morphologic changes which usually can be found in regional LN of malignant tumors, i.e., hyperplasie of lymph follicles, histiocytosis of the marginal sinus or infiltration of the fibrotic capsule with the presence of tyrosinase or gp100 mRNA in the RT–PCR positive non-SLN.

DISCUSSION

The aim of our study was to investigate the metastatic pathways of melanoma cells in regional LN. Detailed understanding of these processes would be important in new surgical concepts such as that of the SLN (Morton *et al*, 1992; Ross *et al*, 1993; Reintgen *et al*, 1994; Godellas *et al*, 1995). Because of the mainly lymphatic spread of malignant melanoma, the SLN ideally would provide information concerning the probability of metastatic involvement of other LN in the LN basin and further systemic dissemination of melanoma cells, respectively.

The first question to be discussed is that of specificity of the techniques used. It is generally accepted, that in most instances histopathologic and immunohistochemical assessment distinguish between melanoma cells and other atypical cells in the LN, particularly in patients with high-risk melanoma. In our study, the melanoma cell detection in LN was performed using three techniques of different sensitivities and specificities, i.e., histology, immunohistochemistry, and RT–PCR for tyrosinase and gp100. Concerning tyrosinase RT–PCR, some authors reported on false positive results as a major technical problem either attributed to carryover contamination (Foss *et al*, 1995) or to too high numbers of amplification cycles in the PCR set up (Smith *et al*, 1991). With appropriate controls we made sure to avoid these problems.

There are also doubts about the specificity of melanoma cell detection in LN by tyrosinase RT–PCR. In melanoma patients, a nodal frequency of nevus cell aggregates from 0.54 to 1.2% was described (Bautista *et al*, 1994; Carson *et al*, 1996; Blaheta *et al*, 1998). These nodal nevi may imitate melanoma metastasis providing also a positive outcome of tyrosinase RT–PCR. Although as much as 3.9% of the investigated SLN showed nodal nevi, however, by far fewer non-SLN, i.e., only 0.01% did so (Carson *et al*, 1996). In all LN investigated in our study an experienced pathologist did not find any melanocytic nevus cells by histology or immunohistochemistry. Generally, we see such nevus cell aggregates in LN of melanoma patients very rarely. Five of seven cases of our patient group, assumed to have so-called "skip" metastases, were also tested for gp100 mRNA presence. Overall, the data of the tyrosinase RT–PCR were confirmed by the gp100 assay. In conclusion, we assume that in the vast majority of LN samples melanoma micrometastasis were indeed responsible for the positive tyrosinase RT–PCR, also in the morphologically negative specimen. This is in accordance with recent findings of Blaheta *et al* (1998), who applied the same PCR technique. In repeated histopathologic examinations they found in none of 87, morphologically negative but tyrosinase mRNA positive LN nodal nevocytes. The authors demonstrated a correlation between positivity in the tyrosinase RT–PCR on one hand and Breslow's tumor thickness on the other. Thus, the probability that the positive tyrosinase RT–PCR is caused by nodal nevi is low, particularly in morphologically negative non-SLN.

In a few of LN we received a negative tyrosinase RT–PCR despite of histopathologic and immunohistochemical evidence for melanoma cells. Obviously, in these samples the negative tyrosinase RT–PCR was a consequence of the LN preparation procedure: immediately after excision, every LN was cut into two sections, one used for RNA isolation, the other one for histologic staining. In LN carrying low numbers of melanoma cells, these cells might be restricted to one of the sections. This would explain the finding of LN specimens with negative tyrosinase RT–PCR and positive histopathology/immunohistochemistry as well as those with positive RT–PCR but morphologically negative. The latter combination occurred much more frequently, however, most likely due to the substantially higher sensitivity of the RT–PCR.

Because the problem of investigating different LN sections for histopathologic examinations and tyrosinase RT–PCR remains, we would recommend the investigation of each SLN with serial sections to improve the sensitivity of morphologic detection. Both, serial sections and the technique of RT–PCR, however, are hardly possible in the routine diagnostic procedures because of an enormous expenditure and time demand.

Table V. Histologic, immunohistologic, and molecular analysis of regional LN in patients with morphologically and tyrosinase RT–PCR negative SLN but positive non-SLN

Patient 1, LN	1	2	3	4	5	6	7													
Histology	−	−	−	−	−	−	−													
Immunohistology	−	−	−	−	−	−	−													
mRNA tyrosinase	−	−	−	−	+	−	−													
Patient 2, LN	1	2	3	4	5	6	7													
Histology	−	−	−	−	−	−	−													
Immunohistology	−	−	−	−	−	−	−													
mRNA tyrosinase	−	−	−	+	−	−	−													
Patient 8, LN	1	2	3	4	5	6	7	8	9	10	11	12								
Histology	−	−	−	−	−	−	−	−	−	−	−	−								
Immunohistology	−	−	−	−	−	−	−	−	−	−	−	−								
mRNA tyrosinase	−	+	−	−	−	+	+	−	+	#	#	#								
gp100	−	+	+	−	−	*	+	−	+	#	#	#								
Patient 12, LN	1	2	3	4	5	6	7	8												
Histology	−	−	−	−	−	−	−	−												
Immunohistology	−	−	−	−	−	−	−	−												
mRNA tyrosinase	−	−	+	−	−	−	#	−												
gp100	−	−	*	−	−	+	#	+												
Patient 20, LN	1	2	3	4	5	6	7													
Histology	−	−	+	−	−	−	−													
Immunohistology	−	−	+	−	−	−	−													
mRNA tyrosinase	−	−	+	−	−	−	−													
gp100	−	−	−	−	−	−	−													
Patient 21, LN	1−2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17−19	20	21	22	
Histology	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
Immunohistology	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
mRNA tyrosinase	−	#	−	#	−	#	−	−	−	+	−	−	−	−	#	−	−	+	−	
gp100	−	#	−	#	−	#	−	+	−	+	(+)	+	−	+	#	−	*	−	*	
Patient 22, LN	1	2	3	4	5	6	7	8	9	10										
Histology	−	−	−	−	−	−	−	−	−	−										
Immunohistology	−	−	−	−	−	−	−	−	−	−										
mRNA tyrosinase	−	−	−	−	−	+	−	−	#	+										
gp100	−	−	−	−	+	+	+	−	#	+										

LN1 = SLN; #, not tested: negative GAPDH RT−PCR; *, not tested.

To our knowledge, there are no other studies investigating all LN of a given LN basin step by step morphologically as well as by tyrosinase RT–PCR. Regarding all methods applied, in 13 of 24 patients (54%) with pT2–pT4 malignant melanoma the SLN indicated metastatic involvement.

In three cases the SLN as well as one or more non-SLN were positive only by tyrosinase RT–PCR. Obviously, these LN contained only small, morphologically undetectable numbers of melanoma cells. The frequency of SLN and non-SLN, positive only for tyrosinase RT–PCR, was found to be 13% and 20%, respectively. Therefore, up to one-fifth of local LN in pT2–pT4 melanomas may harbor micrometastatic melanoma cells, undetectable by conventional microscopy. Occasionally, a positive tyrosinase RT–PCR, however, might be also due to the presence of nodal nevocytes. Moreover, a clustering of melanoma cells in the part used for RT–PCR can not be excluded.

Currently it is not clear, whether melanoma cells demonstrated only by RT–PCR give rise to clinically overt LN metastasis or systemic disease. In fact, we have observed patients with fatal outcome following dissection of morphologically negative SLN, supporting the clinical impact of undetected micrometastasis. Patients with morphologically positive SLN showed frequent involvement of non-SLN (eight of 10 cases), seen by morphology and/or indicated by tyrosinase RT–PCR.

Interestingly, in seven of 24 cases (29%) we found the SLN to be negative by all techniques applied but the non-SLN were positive for tyrosinase and/or gp100 transcripts. One of these cases additionally showed morphologic evidence for melanoma cells. As discussed, we believe that nevocytes instead of melanoma cells were only rarely indicated in the non-SLN by the lineage-specific mRNA. Thus, up to 30% of patients may harbor metastasis despite the nondetection of melanoma cells in the SLN. The finding of negative SLN and positive non-SLN in a given LN dissection specimen should influence surgical treatment protocols in melanoma. In this context, the SLN concept (Glass *et al*, 1995) depending on a probability of only 1–2% for the so-called "skip" melanoma metastases (Morton *et al*, 1992; Thompson *et al*, 1995) would have to be questioned. Although the number of melanoma patients investigated in our study is too small yet for a clinically relevant conclusion, a further evaluation of the SLN concept is recommended by our findings.

In comparison with light microscopy, the tyrosinase RT–PCR has the potential to detect considerable lower numbers of tumor cells. The question remains, which number of melanoma cells might cause a recurrence or a metastasis by escaping the immune surveillance. Nevertheless, the estimation of tyrosinase or other lineage-specific mRNA in LN of melanoma patients can contribute to a better understanding of the biologic significance of micrometastasis.

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