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## Original Paper

# Identification of Lymph Node Metastases by Use of Polymerase Chain Reaction (PCR) in Melanoma Patients

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**Diagnosis of clinically suspected lymph node metastases in melanoma patients can be confirmed with high sensitivity and specificity by fine needle aspiration (FNA) cytology. However, small lymph nodes or haemorrhagic metastases may yield negative or unevaluable cytology. We tested whether the sensitivity and specificity of presurgical diagnosis could be improved by a polymerase chain reaction (PCR) method, identifying tyrosinase-mRNA in samples obtained by fine needle aspiration (FNA-PCR). PCR was positive in 17 of 18 histopathologically proven melanoma metastases, while conventional cytopathology detected 16 of 18. 4 of 9 disease-free melanoma patients with negative FNA cytology had positive PCR results, but controls gave negative results. FNA-PCR analysis cannot be recommended as superior to conventional FNA cytological examination. Whether the positive FNA-PCR in four of the nine clinically unsuspicious regional lymph nodes correlates with earlier disease progression or indicates lower specificity of the method will need further investigation.**

**Key words: tyrosinase, PCR, minimal residual disease, melanoma**
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## INTRODUCTION

EARLY DETECTION of lymph node metastases in melanoma patients at high risk of relapse of disease is one major aim of extensive follow-up care. Once the clinical possibility of lymph node metastasis arises, the most helpful diagnostic tools are ultrasound sonography (USS) and cytological examination of fine needle aspirates (FNA) in confirming metastatic events [1–6]. FNA cytology is reported to be highly sensitive, between 73% [4] and 98% [5], as is specificity, between 92% [4] and 100% [5]. Since lymphadenectomy or resection of metastases can be planned only if specific involvement has been proved, we addressed the question of whether currently available procedures could be improved by additional molecular biological investigation of the aspirates. Based on the description of the human tyrosinase gene [7–9], a method has been established of identifying single melanoma cells in peripheral blood [10] by detection of their tyrosinase transcripts with the polymerase chain reaction (PCR) technique. Using this technique, circulating melanoma cells have been demonstrated in the peripheral blood of patients with advanced metastatic melanoma [11, 12], and more recently even a correlation between the clinical stage of melanoma disease and the percentage of patients positive for circulating tumour cells has been shown [13].

The aim of our study was to investigate if sensitivity and specificity of FNA cytology could be improved by additional use of the tyrosinase-PCR technique.

## PATIENTS AND METHODS

*Patients*

FNA cytology samples were obtained from (i) patients with clincially suspected melanoma metastases ($n = 18$, 13 lymph nodes, five cutaneous); (ii) clinically unsuspected regional lymph nodes of melanoma patients in complete remission ($n = 9$); (iii) from patients with other malignant diseases ($n = 9$); or (iv) from lymph nodes clinically unrelated to neoplastic disease ($n = 6$) (Table 1).

*Fine needle aspiration cytology*

FNA was technically performed as previously described [4, 5]. The aspirates were divided and one half was placed on a glass microscope slide and air-dried before staining with the May–Grünwald–Giemsa technique or immunocytochemically for S100 and HMB45 antigens [31]. The other half was shock frozen in liquid nitrogen for subsequent molecular biological

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*Table 1. Clinical diagnoses and numbers of patients included in the study*

| Clinical diagnosis | FNA cytology target | Total number | PCR samples | FNAC samples | Histopathology samples | Number of USS |
|---|---|---|---|---|---|---|
| MM metastases | LN | 13 | 13 | 13 | 13 | 13 |
| MM metastases | CF | 5 | 5 | 5 | 5 | 5 |
| Regional LN of disease-free MM | LN | 9 | 9 | 9 | 1 | 9 |
| MFH | LN | 1 | 1 | 1 | 1 | 1 |
| Spinocellular carcinoma | LN | 1 | 1 | 1 | — | 1 |
| Immunocytoma | LN | 2 | 2 | 2 | — | 2 |
| Merkel cell carcinoma | CF | 1 | 1 | 1 | 1 | 1 |
| Oat cell carcinoma | LN | 1 | 1 | 1 | 1 | 1 |
| MF | LN | 3 | 3 | 3 | 1 | 3 |
| Control LN | LN | 6 | 6 | 6 | 0 | 6 |

In all patients, fine needle aspirates (FNA) were evaluated by PCR, conventional cytology (FNAC) and, if the targeted sample was surgically removed, by standard histopathology. MM, malignant melanoma; LN, lymph node; CF, cutaneous filia; USS, ultrasound sonography; MFH, malignant fibrous histiocytoma; MF, mycosis fungoides.

investigation. The following cytomorphological criteria [4] were analysed: presence of large disassociated and pleomorphic cells with large nuclei, high nucleus/plasma relation, atypical mitoses and megakaryotypic cells. In general, the morphological criteria led to the same results as the immunocytochemical analysis (see Results). Immunocytochemistry was never useful if cytological examination had been negative.

*Ultrasound sonography*

Clinically suspected lesions were investigated using a 7.5-MHz linear scanning device. If a balloon-shaped tumour with lack of central reflexes could be demonstrated, the lesion was considered to be malignant.

*Surgical intervention*

If clinical criteria, such as history of a fast growing tumour and palpation of a firm spherical nodule were present and USS or FNA cytology criteria suggested a malignant process, the lesion was removed surgically. Therefore, standard histopathological examination was possible in most cases. In cases where removal was refused, the lesion was followed up clinically for at least 6 months.

*RNA preparation and reverse transcription*

mRNA was extracted from the cell pellet obtained by FNA cytology as described above. The Quick-Prep Micro mRNA purification Kit (Pharmacia, Frieburg, Germany) combining guanidium thiocyanate [14] with oligo (dT)-cellulose chromatography was used. With the SuperScript preamplification system (Gibco), 1 μg of mRNA was reverse transcribed by the use of 50 ng of random hexamers, 10 mM dNTP mix (dATP, dCTP, dTTP and dGTP) and 200 units of SuperScript II RT. The mixture was incubated at 42°C for 50 min and another 15 min at 70°C to stop reverse transcription. After 10 min cooling on ice, 1 μl of RNAse was added for 20 min at 37°C. After another 5 min at 90°C, the sample was stored at 4°C until subsequent PCR procedure was started. Controls were performed using cellular pellets of the Sk-Mel-28 melanoma cell line [15].

*Synthetic oligonucleotides and PCR procedure*

Primers for tyrosinase-PCR designed for a nested PCR were used as previously described [10]. The outer primers HTYR1 (TTGGCAGATTGTCTGTAGCC) and HTYR2 (AGGCATTGTGCATGCTGCTT) amplify a product of 284 base pairs, while the inner (nested) primers HTYR3 (GTCTTTATGCAATGGAACGC) and HTYR4 (GCTAT CCCAGTAAGTGGACT) amplify a product of 207 base pairs. cDNA probes obtained from patients or from Sk-Mel-28 pellets were processed with PCR. Two microlitres of the cDNA solution were added to 50 μl PCR buffer, 0.3 μg of HTYR1 and HTYR2. The first, outer PCR run was initiated with a hot start, heating the samples to 90°C for 10 min. After cooling to 85°C, two units of TaqDNA polymerase (Gibco) were added and 30 cycles (94°C for 90 s, 60°C for 90 s and 72°C for 90 s) were carried out. Five microlitres of 1:100 dilution were further processed in a second, inner PCR cycle. HTYR3 and HTYR4 (0.3 μg each) were added and again 30 cycles were carried out as performed with the outer PCR. Seven microlitres of each PCR product were run on a polyacrylamide gel with the temperature gradient gel electrophoresis (TGGE) technique [16]. On each gel length, standards, positive and negative controls were run in addition to the patients' samples. Only gels with correct negative and positive controls were evaluated.

## RESULTS

*Metastases of malignant melanoma*

13 patients with malignant melanoma presented with lymph nodes clinically suspicious for metastases. 5 patients had melanoma metastases of the skin. In all cases, we were able to perform USS as well as FNA cytology for cytological and molecular biological investigations. All lesions were excised based on clinical criteria and their typical USS pattern. In all lesions, histopathological analysis confirmed the diagnosis of melanoma metastasis (Table 2).

*Table 2. Sensitivity of polymerase chain reaction (PCR) and fine needle aspirate cytology (FNAC) technique*

| Tumour type | *n* | Positive PCR/*n* | Positive FNAC/*n* | Lymph node size |
|---|---|---|---|---|
| Lymph node | 13 | 12/13 | 12/13 | 12 (5, 30) |
| Skin | 5 | 5/5 | 4/5 | 12 (12, 25) |

Positive results are numbers of malignant tumours. *n* = total number of samples. Lymph node size in mm is displayed as median (minimum, maximum).

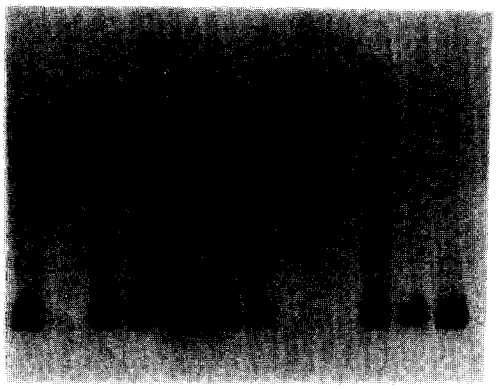


**Figure 1. TGGE-gel displaying the tyrosinase-PCR results for positive control (SK-mel, lane A), negative control (water, lane B) and fine needle aspirates obtained from suspected melanoma lymph node metastases (positive lanes C–G and J–L, negative lanes H and I).**

FNA-PCR was able to detect 17 of 18 melanoma metastases in skin or lymph nodes (Figure 1), while classic FNA cytology failed in 2 cases. Therefore, the sensitivity of the FNA-PCR method was 94% while the sensitivity of the FNAC was 89% in our study (statistically non-significant). The $10 \times 5$ mm lymph node which escaped detection of malignancy by FNA-PCR showed histologically the pattern of melanosis of the lymph node sinus (Figure 2). Both failures of the classical FNA cytology occurred in haemorrhagic necrotic tumours, where almost only red blood cells could be aspirated for cytological examination. The size of these two negative cases was 30 mm in lymph node and 12 mm in skin.

In parallel to conventional FNA cytology, the aspirates were investigated immunocytologically, using monoclonal antibodies against the HMB45 and the S100 antigen (Figure 3). In all cases, the immunocytological results paralleled the conventional FNA cytology results and therefore did not provide additional information.

*Regional lymph nodes of disease-free melanoma patients*

In 9 patients (Table 3), cytology and tyrosinase FNA-PCR were performed on regional lymph nodes clinically and sonographically judged as non-specific and not suspected for melanoma metastasis. All 9 patients had negative FNA cytology, but 4 had positive tyrosinase FNA-PCR results. All 9 patients were clinically in complete remission at the time of FNA cytology and FNA-PCR investigations and remained macroscopically disease-free for a follow-up time of at least 6 months. One of these which was negative clinically and by FNA cytology but positive by PCR lymph nodes was surgically removed by block resection together with the primary melanoma. The histopathological result did not reveal any clue of metastatic melanoma spread.

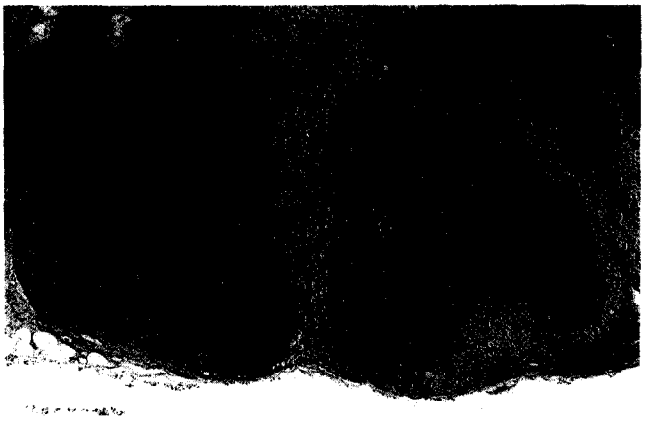

**Figure 2. Inguinal lymph node with sinus carcinosis. Haematoxylin-eosin staining. Magnification × 100.**

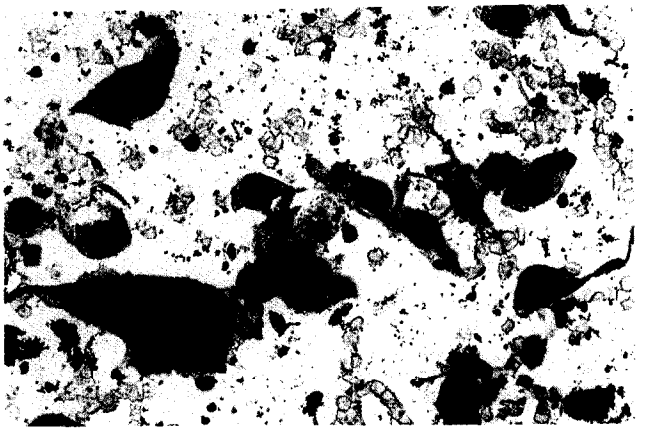

**Figure 3. Immunocytological investigation. Smear of a fine needle aspirate obtained from a melanotic lymph node metastasis. Staining against S100-protein. Magnification × 400.**

*Table 3. Tumour data of 9 patients in complete remission who had fine needle aspirate polymerase chain reaction (FNA-PCR) on non-specific regional lymph nodes, as judged clinically and by USS*

| Patient no. | FNA-PCR | Tumour type | Breslow thickness | Clark level | Time interval from pTX (months) |
|---|---|---|---|---|---|
| 1 | Pos | NMM | 4.0 | IV | 0 |
| 2 | Neg | SSM | 4.0 | IV | 21 |
| 3 | Neg | ALM | 2.8 | IV | 6 |
| 4 | Pos | ALM | 2.7 | IV | 23 |
| 5 | Pos | SSM | 2.5 | IV | 32 |
| 6 | Neg | SSM | 2.1 | IV | 34 |
| 7 | Neg | SSM | 1.0 | III | 21 |
| 8 | Pos | SSM | 0.4 | III | 6 |
| 9 | Neg | SSM | 0.3 | II | 14 |

pTX, primary tumour excision; NMM, nodular malignant melanoma; SSM, superficial spreading melanoma; ALM, acrolentiginous melanoma; Pos, positive; Neg, negative.

*Control lymph nodes*

6 patients gave informed consent to obtain aspirates from lymph nodes unrelated to neoplastic disease. FNA cytology and tyrosinase-PCR results of all lymph nodes investigated were negative (specificity 100%).

*Miscellaneous tumours*

In another series, lymph nodes of patients with proven non-melanoma malignancies were investigated (Table 4). In 6

*Table 4. Tyrosinase polymerase chain reaction (PCR) and fine needle aspirate cytology (FNAC) results of lymph node aspirates in non-melanoma tumours*

| Tumour type | Total no. | Positive PCR | Positive USS | Positive FNAC | Positive histology |
|---|---|---|---|---|---|
| Mycosis fungoides | 3 | 0/3 | 2/3 | 2/3 | Not performed |
| Immunocytoma | 2 | 2/2 | 2/2 | 2/2 | Not performed |
| Spinocellular carcinoma | 1 | 0/1 | 1/1 | 1/1 | Not performed |
| Merkel cell tumour | 1 | 0/1 | 0/1 | 1/1 | 1/1 |
| Malignant fibrous histiocytoma | 1 | 1/1 | 1/1 | 1/1 | 1/1 |
| Oat cell tumour | 1 | 1/1 | 1/1 | 0/1 | 1/1 |

USS, ultrasound sonography.

patients, lymph nodes could not be removed surgically, in 3 cases diagnosis could be confirmed by histopathological examination.

In aspirates of the two immunocytomas, the malignant fibrous histiocytoma and the oat cell tumour, tyrosinase transcripts were detected. The other tumours tested had negative tyrosinase-PCR.

## DISCUSSION

Tyrosinase is the key enzyme which catalyses the first step of melanin production and is therefore considered to be restricted to melanocytes, except some distinct *in vitro* conditions such as transfected mouse fibroblasts [17]. *In situ* hybridisation experiments in mice showed that expression of the tyrosinase gene is restricted to melanocytes in skin, hair follicles and the retina [18].

In Northern blot analysis of mouse RNA tyrosinase, transcription was only detected in melanoma cells and to a lesser extent in normal testes. Normal brain, lung, heart, kidney, liver and muscle were negative [19]. So far, only one study [11] has detected tyrosinase RNA in a variety of normal (skin, lymph node, antrum, colon, lung, testis, ovary, breast, peripheral nerve, spleen) and neoplastic (melanoma, lymphoma, ductal breast carcinoma, schwannoma, liposarcoma) human tissues. These experiments, which were mainly performed in very low numbers, are in contrast to the aforementioned papers which report strong tissue restriction of tyrosinase transcription.

PCR for detection of tyrosinase transcripts is sensitive enough to identify a single melanoma cell in 5 ml of heparinised blood [12]. Despite the high sensitivity of the method, no false positive results were observed in blood obtained from healthy volunteers. We, therefore, examined whether this method would add further relevant information to the also very sensitive and specific FNA cytology method, routinely used for investigation of suspicious lymph nodes in melanoma patients [3–5]. Since patients are currently monitored with USS and therefore small lymph nodes in high-risk patients are detected frequently, the need to optimise sensitivity and specificity of diagnostic procedures arises.

In our series of experiments on melanoma metastases, only one histopathologically proven lymph node metastasis escaped detection by FNA-PCR. This may reflect the problem of aspiration of at least one tumour cell. Alternatively, both melanoma metastases, which showed negative FNA cytology, were identified by FNA-PCR. Both were haemorrhagic necrotic metastases, which showed only masses of red blood cells instead of melanoma cells in the cytological preparation. Nevertheless, in our study, the sensitivity for both methods was very high with values of 89% for FNA cytology and 94% for FNA-PCR.

The four positive FNA-PCR results, obtained from analysis of nine regional lymph nodes of disease-free melanoma patients, need special consideration. Their clinical behaviour, with lack of growth for at least 8 months after the FNA-PCR procedure, excludes early detection of a fast growing metastasis. However, these positive FNA-PCR results could be due to single tumour cells that might be present in draining lymph nodes. The possibility of small numbers of tumour cells in lymph nodes, persisting for long periods in the state of immune surveillance, unable to cause overt tumour progression, should be considered. We cannot conclude whether these positive FNA-PCR results are indicative of very early micrometastasis or whether they will not be predictive of further development of systemic melanoma spread. Other possibilities which could explain the positive FNA-PCR results are the aspiration of circulating melanoma cells [12] from lymph node blood vessels, or the presence of melanocytic nevus cells in normal lymph nodes, but this was described as a very rare event occurring in only 3 of 17 000 investigated lymph nodes of non-melanoma patients [20]. Furthermore, contamination of specimens by cutaneous melanocytes consequent upon skin puncture could be considered as a source of false positives. However, negative tyrosinase-PCR results obtained from blood samples of healthy volunteers [12, 13] suggest that at least perforation of the skin for blood sampling is not a source of contamination. Since fine needles for collection of lymph node material are even thinner than those normally used for collecting blood samples, we feel that it is highly unlikely that skin melanocytes should be a source for false positives.

Tyrosinase transcripts are reported to be present in other neoplasia such as schwannoma, liposarcoma, ductal breast carcinoma or a neuroblastoma cell line [11, 21]. In our study, tyrosinase transcripts could be detected in immunocytoma, malignant fibrous histiocytoma and oat cell tumour, while mycosis fungoides, squamous cell carcinoma and Merkel cell tumours remained negative. Currently, the full spectrum of tumours with the capability of transcription of the tyrosinase gene is still undetermined, and it also remains uncertain if tyrosinase production could be paraneoplastic. In paraneoplastic endocrine syndromes, some tumours have a high probability, for example, oat cell carcinoma, to synthesise peptide hormones which may or may not be functionally active. Similarly, various tumours may sometimes have the capability of tyrosinase gene expression and other times not.

In conclusion, we can state that FNA-PCR analysis is at least as sensitive as conventional FNAC. However, special

attention should be given to the macroscopically disease-free melanoma patients with positive FNA-PCR results in regional lymph nodes. Whether the detection of tyrosinase mRNA in these clinically unsuspicious lymph nodes correlates with earlier disease progression or low specificity of the method, has to be clarified by further follow-up examination. So far, we discourage the use of this relatively costly and time-consuming technique and recommend performance of conventional FNAC.


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