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Original Paper

Detection of Early Micrometastases in Subcutaneous Fat of Primary Malignant Melanoma Patients by Identification of Tyrosinase-mRNA

T.M. Proebstle,[1] R. Huber[1] and W. Sterry[2]

[1]Department of Dermatology, University of Ulm, Oberer Eselsberg 40, D-89081 Ulm; and [2]Department of Dermatology of the Charité, Humboldt University of Berlin, Germany

To improve survival following melanoma excision, wide margin resection including subcutaneous fat, is indicated. Subcutaneous fat, resected with primary melanomas, was investigated using the tyrosinase PCR method originally described for detection of melanoma cells in peripheral blood. Identification of tyrosinase transcripts was possible in 4 melanoma patients (3 Clark level IV, 1 level III), while negative results were obtained from 6 other patients (2 level IV, 3 level III and 1 level I). In control experiments, after infiltration of the reference cell line SkMel into normal fat tissue, the PCR was positive in 33% (3/9) with 10^2 infiltrated cells, 69% (9/13) with 10^4 cells and in 100% (7/7) with 10^6 cells. We conclude that detection of melanoma cells in subcutaneous fat tissue is possible by tyrosinase PCR. Follow-up studies should show whether such positive tyrosinase PCR can define patients at very high risk of tumour relapse.

Key words: melanoma, micrometastasis, subcutis, tyrosinase, PCR
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INTRODUCTION

EPIDEMIOLOGICAL DATA allow the calculation of risk of melanoma relapse with respect to various risk factors at the point of primary tumour excision. The parameter most useful for the estimation of survival rate is maximal vertical tumour thickness [1]. For instance, a tumour thickness of 4.0 mm is linked to an overall survival rate of around 50 per cent 5 years after primary melanoma excision. Since most patients present with primary tumour thicknesses below 4.0 mm, the identification of patients at very high risk for relapse remains difficult. Other additional risk factors, such as trunk location, male sex or histological melanoma types, are rather weak multipliers. However, identification of "very high risk patients" is important for the selection of participants for future adjuvant treatment trials, when antitumour-drugs with relatively strong side-effects, such as high-dose cytokines or cisplatin, will be administered.

Based on the description of the human tyrosinase gene [2–4], circulating melanoma cells have been detected by use of the tyrosinase PCR method in peripheral blood [5–7] of patients with metastatic melanoma, and, in correlation with the primary tumour's Breslow thickness, also in the peripheral blood of disease-free follow-up patients [8]. The aim of our study was to detect early spreading melanoma cells in the subcutaneous fat below the primary melanoma. Patients with such early metastasis may form a group at very high risk of tumour relapse.

Correspondence to T.M. Proebstle.
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PATIENTS AND METHODS

Patients

To determine the sensitivity of the method, normal subcutaneous fat tissue was obtained from patients receiving dermatosurgery for excision of benign lesions such as tattoos or congenital naevi. Subcutaneous fat from melanoma patients was obtained simultaneously with removal of primary melanoma, or when additional resection with wide margins between 1 and 3 cm was performed.

Fat dissociation in melanoma patients

Samples were obtained from 10 melanoma patients. After deep excision of the skin and subcutis in one block, the subcutaneous fat was removed throughout the wide resection sample with scissors. A margin of subcutaneous fat was left

connected to the dermis to avoid contamination of the resected fat with tyrosinase-containing structures of the corium. Standard histopathological investigation (H&E staining) of serial slices was performed on the dermal preparation in each case. The obtained fat was divided into parts of 3–5 g. Each of these smaller parts was dissected into pieces of 10–100 mm^3 and put into a 50 ml Falcon tube together with 25 mg of collagenase (activity 500 U/mg) and 10 ml Ringer's solution. The mixture was incubated for 2 h at 37°C in a shaker ($f = 1$ Hz) and then washed twice with PBS. After centrifugation for 5 min at 400*g* the pellet was dissolved in GTC buffer and either immediately proceeded to RNA preparation or stored at −85°C until then.

Fat dissociation for sensitivity experiments

After removal of the subcutaneous fat from the dermis of non-melanoma patients, either 10^6, 10^4, 10^2 or 0 cells of the tyrosinase-positive reference cell-line SkMel-28 [9], dissolved in 0.5 ml Ringer's solution, were randomly infiltrated into the whole specimen of fat. Random inoculation of the resected fat block was performed with a 1 ml syringe and a 20G × 1.5″ needle. The cell suspension was infiltrated in a "fan-like" manner leaving depots of 0.05–0.1 ml volume in each arm of the fan. After incubation for 1 h, further fat dissociation proceeded as described above.

RNA preparation and reverse transcription

Total RNA was extracted from the cell pellet which was obtained by fat dissociation as described above. The acid guanidinium thiocyanate-phenol-chloroform extraction method was applied as previously described [10, 11]. Reverse transcription was performed with 1–5 μg of total cellular RNA as previously described [6] by use of 1 μl of random hexamers. After incubation at 70°C for 10 min, 1 μl of 10 mmol/l dNTP-Mix (dATP, dCTP, dTTP and dGTP), 200 U Superscript II RT and 2 μl 10-fold synthesis buffer were added. After 10 min at room temperature, the mixture was incubated at 42°C for 50 min, and another 5 min at 90°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 the PCR procedure was started. For control purposes, the procedure was performed in parallel on cellular pellets of SkMel-28 cultures.

Synthetic oligonucleotides and PCR procedure

Primers for tyrosinase PCR and the procedure itself, designed as a nested PCR, were used as previously published [7]. The outer primers HTYR1 (TTGGCAGATTGTC TGTAGCC) and HTYR2 (AGGCATTGTGCATGCTG CTT) amplify a product of 284 base pairs, while the inner (nested) primers HTYR3 (GTCTTTATGCAATGGAA CGC) and HTYR4 (GCTATCCCAGTAAGTGGACT) amplify a product of 207 base pairs. PCR was performed on a thermocycler quattro TC-40 (Biozym Hameln, Germany). cDNA probes obtained from either total cellular RNA from fat of melanoma patients, from SkMel-28-injected fat of non-melanoma patients for sensitivity testing or from pure SkMel-28 pellets, were processed with PCR.

Two microlitres of the cDNA solution were added to 50 μl PCR buffer with nucleotide mix, 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, 2 units of Taq DNA Polymerase (Promega) 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.

Four microlitres of each PCR product were run on a temperature gradient gel electrophoresis (TGGE) as described [12]. On each gel, length standards, two positive and two negative controls, were run in addition to the fat derived samples. Only gels with correct negative and positive controls were evaluated.

RESULTS

Standard histopathology

Histopathological investigation of the excised dermal tissue—with subcutaneous fat already withdrawn—was performed to determine the Clark level and Breslow's tumour thickness (see Table 1), or to confirm that the corial part of the dermis was not injured by the fat dissection procedure. No melanoma specimen revealed extension of the tumour into the subcutaneous fat (Clark level V) and, moreover, no melanoma cells were detected in the subcutaneous fat still adherent to the corium after fat preparation.

Table 1. *Tyrosinase-PCR results of subcutaneous fat samples obtained from melanoma patients. Ranking according to Breslow's tumour thickness.*

Rank	Breslow index [mm]	PCR-result [−/+]	Clark level	Tumour type	Localisation	pTNM stage	AJC stage
1	3.7	+	IV	SSM	trunk	pT3b	II
2	3.0	+	IV	NM	trunk	pT3a	II
3	2.1	−	IV	SSM	arm	pT3a	II
3	2.1	+	IV	NM	arm	pT3a	II
5	2.0	−	IV	SSM	arm	pT3a	II
6	0.8	+	III	SSM	trunk	pT2	I
6	0.8	−	III	SSM	leg	pT2	I
8	0.7	−	III	SSM	trunk	pT2	I
9	0.4	−	III	SSM	trunk	pT2	I
10	0.3	−	II	SSM	trunk	pT1	I

SSM, superficial spreading melanoma; NM, nodular melanoma; −, negative; +, positive.

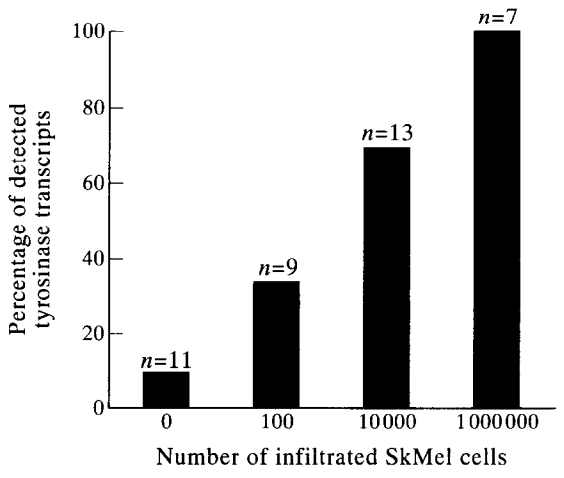

Figure 1. Percentage of positive tyrosinase PCR results drawn against the number of Sk-Mel cells infiltrated into subcutaneous fat obtained from non-melanoma patients. *n* denotes the number of experiments performed.

Sensitivity of the method

In total, 40 experiments were carried out to determine the sensitivity of the method. Ringer's solution (0.5 ml) containing zero ($n = 11$), 10^2 ($n = 9$), 10^4 ($n = 13$) or 10^6 ($n = 7$) SkMel-28 cells was infiltrated into fat obtained from non-melanoma patients. One false-positive result was obtained in 11 control experiments without SkMel cells. Figure 1 shows the sensitivity of the experimental procedure for the various amounts of Sk-Mel tested. Sensitivity of 50% was estimated to be between 10^2 and 10^4 infiltrated SkMel cells.

Tyrosinase PCR in melanoma patient derived subcutaneous fat

In four of ten investigated fat samples, tyrosinase PCR was positive. Figure 2 shows a representative gel of 10 PCR samples derived from fat tissue, a positive control (SkMel) and a negative control. In this, all fat infiltrated with SkMel cells were considered to be positive after the final (inner) PCR cycle (lanes A, C, D, E, H, I) as was one specimen from below a primary melanoma (lane K). Weaker positive results were also achieved after the first PCR cycle but only in specimens infiltrated with SkMel cells (lanes a, c, i) and the positive control (lane l). Table 1 shows ranking of tested patients, according to Breslow's tumour thickness. Detection of tyrosinase mRNA in subcutaneous fat below primary melanomas was more frequent with higher tumour thickness, but was positive in 4 of the 10 patient samples.

DISCUSSION

Tyrosinase is the key enzyme catalysing the first step of melanin production and is therefore considered to be restricted to melanocytes except for some distinct *in vitro* conditions [13] or neuroblastoma cells [14]. *In situ* hybridisation experiments in mice have shown that expression of the tyrosinase gene is restricted to melanocytes in skin, hair follicles and the retina [15]. When the RNA of a variety of murine tissues was investigated using Northern blot analysis, tyrosinase transcription was only detected in melanoma cells and, to a lesser extent, in the testes, but not in brain, lung, heart, kidney, liver or muscle [16]. However, in contrast, another study [5] described the presence of tyrosinase RNA in various normal and neoplastic human tissues.

The present study indicates the possibility of detecting melanoma cells in subcutaneous fat, since a few hundred melanoma cells, experimentally infiltrated into normal fat tissue, could be detected by use of the tyrosinase PCR method. Unfortunately, the method is currently not as sensitive as that using peripheral blood, where one single melanoma cell in 5 ml blood can be detected [6]. It is likely that many melanoma cells were destroyed by the described enzymatic fat dissociation process. However, in subcutaneous fat located below a primary melanoma, detection of tyrosinase transcripts was possible in some cases, suggesting the presence of early spreading melanoma cells. This raises the question of why local recurrence of melanomas is so rare. The reason might be that the presence of tumour cells in the subcutaneous fat or its lymphatic vessels does not fulfill all the conditions for the development of local or in-transit metastases.

Our findings also support a more dynamic model of the metastatic process in malignant melanoma. In some patients, it seems possible that the tumour sheds large numbers of tumour cells at very early clinical stages, and includes early spread of melanoma cells via the blood as reported by Vormwald-Dogan and colleagues [8]. However, although the detection of melanoma cells in peripheral blood has been correlated to Breslow's tumour thickness, including around 10 per cent of pT1 patients, the prognostic value of these findings has

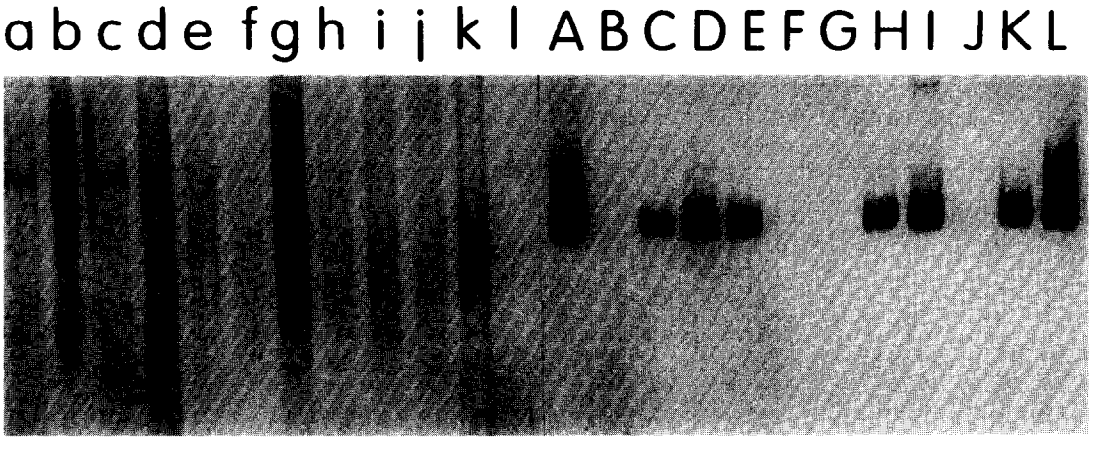

Figure 2. A representative gel showing PCR products from the first (outer) cycle (lanes a–l) and the second (inner) cycle (lanes A–L).

not been shown [5–8]. Similarly, the prognostic value of melanoma cells in the subcutaneous fat tissue beneath a primary melanoma is still unclear. Nevertheless, it seems of interest to evaluate whether the detection of tumour cells in subcutaneous fat is associated with reduced survival or reduced disease-free survival.

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