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The detection of tyrosinase mRNA in peripheral blood samples is unlikely to aid in the management of patients with localised malignant melanoma

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Summary A number of authors have reported the detection of tyrosinase mRNA in the peripheral blood of patients with malignant melanoma using the reverse transcription polymerase chain reaction (RT-PCR). The precise value of this assay as a prognostic tool, however, remains in doubt. This is particularly so with relation to localised disease, where relatively little data has been accumulated. In this study we analysed the peripheral blood of 50 consecutive patients with primary malignant melanoma referred to a plastic surgical centre with the facility of a pigmented lesion clinic. Samples were analysed from an additional 35 patients with advanced melanoma disease and 35 patients with benign pigmented cutaneous lesions.

We were able to identify tyrosinase transcripts in the peripheral blood of only two of 50 patients with localised disease. Of those with more advanced disease, a positive finding was found in three with regional disease and four patients with metastatic spread. Stage of disease was found to correlate significantly with PCR status. No correlation was identified with other prognostic markers or with outcome over a three-year period.

This data would support the conclusion that the detection of tyrosinase mRNA in peripheral blood is likely to be of little value as an aid in the management of patients with early malignant melanoma.

It is well recognised that localised melanomas may behave unpredictably and aggressively.[1,2] In a publication from this centre, 3% of patients with disease (<1.5 mm) went on to develop metastases within a period of 5 years.[3] In order to identify those with recurrence as early as possible, it is a policy of many plastic surgical centres within the UK to follow up melanoma patients over many years. This practice, which places considerable demands upon resources, has lead to an increasing enthusiasm for a reliable serological marker to aid in patient management.

As long ago as 1869 Ashurst detected malignant cells in the peripheral circulation[4] and proposed that their detection might provide a means for

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determining prognosis. However, whilst it has been possible to detect tumour cells in the peripheral blood using conventional pathological techniques,[5] limitations in the sensitivity and specificity of these methods has prevented their adoption as routine assays.

Reverse transcription coupled to a polymerase chain reaction (RT-PCR) was first described as a method for the identification of tyrosinase mRNA in peripheral blood in 1991.[6] This enzyme, involved in melanin synthesis, provides an attractive target as it is highly specific to melanin producing cells and is not normally present in peripheral blood.

RT-PCR when used in this context is a technically demanding and capricious assay. False positives are readily generated by contamination either at the time of sampling (through extraneous inoculation of melanocytes) or at the time of processing through environmental contamination.[7–9] Equally, false negatives may arise for a number of reasons including loss of tumour cells during preparation, reduction of tyrosinase expression, loss of homogeneity and down regulation or incomplete expression.[10] Perhaps most importantly they may be caused as a result of the intermittent nature of cell shedding within the circulation.[11]

In addition to these technical issues, procedural issues may also impact on the outcome of RT-PCR analysis.[12] Data obtained from tertiary oncology units or where non-consecutive recruitment of patients has taken place may favour those with higher risk of relapse or preclude sampling preoperatively.

We report the findings of analysis of peripheral blood samples obtained on 50 patients with primary malignant melanoma recruited consecutively from a plastic surgical unit, using a pigmented lesion clinic as a primary source of patients. In addition, peripheral blood samples were obtained from a further 30 patients with benign pigmented lesions and from 35 patients with more advanced disease. Subjects were followed up over a three year period.

Materials and methods

Sample collection and RNA purification

Ethical approval for this study was obtained from the regional advisory body. Peripheral venous blood samples were obtained from consecutive patients with primary cutaneous malignant melanoma attending the Plastic Surgical Department at Frenchay Hospital, Bristol. A pigmented lesion clinic was used to source 60% of these and also obtain blood samples from further 35 patients with benign pigmented lesions. All samples were obtained prior to surgical intervention. Established prognostic features for melanoma were recorded including site, morphological type, Breslow thickness, Clarks level, mitotic activity, the presence of ulceration, host lymphocytic response and presence of regression. Melanoma was staged according to the American Joint Committee on Cancer staging system for cutaneous melanoma, 1997.[13]

A further 30 samples were obtained from patients with advanced disease (AJCC Stages III and IV). Blood samples were collected in ethylenediamine tetra-acetic (EDTA) vacutainor tubes. In order to minimise possibility of contamination with epithelial melanocytes a preliminary blood sample was discarded. Blood was stored at 4 °C for a maximum of 2 h before processing. Lymphocytes were extracted from whole blood by density gradient using Ficoll Paque™ and total RNA was extracted using the RNAzol B™ system.

Oligonucleotide primers

Primer sequences were as described by Smith.[6] HTYR1 and HTYR2 amplify a 284 base pair (bp) fragment of the tyrosinase cDNA. The oligonucleotide sequences HTYR3 and HTYR4 amplify an internal sequence (207 bp) within this PCR product. In order to test integrity of RNA transcription, parallel assays were performed using the housekeeping gene β-actin (sense, 5′-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3; antisense, 5′-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3′), amplifying a 660-bp fragment.

Reverse transcriptase polymerase chain reaction assay

For reverse transcription 5 μl of isolated total RNA was heated to 95 °C for 4 min and cooled rapidly (Perkin Elmer GeneAmp™ PCR System 2400). It was then incubated with a mixture containing 10 μl of ddw, 2 μl of 10× RT buffer (Promega), 1 μl of RNAsin (Promega), 2 μl of 10 mM dNTP's (Promega) and 1 μl of random hexanucleotide (Promega), primer in a final volume of 20 μl at 65 °C for 15 min. One microlitre of reverse transcriptase (Promega) was added, and the solution incubated for 1 h at 42 °C.

For the PCR, 20 μl of cDNA was incubated with 79.5 μl of PCR master mix (65.5 μl ddw, 10 μl of 10× Taq buffer (Promega), 2 μl × 10 mM dNTP's (Promega), 1 μl of each oligonucleotide primer, 3.5 mM $MgCl_2$). Following a 'hot start' procedure in which the mixture was heated to 95 °C for 5 min,

0.5 μl Taq DNA polymerase (Promega) was added and 25 cycles of PCR performed, each consisting of a denaturation step of 94 °C for 5 min, an annealing step for 70 s and an extension step of 72 °C for 70 s (final volume 100 μl). A final extension time of 10 min at 72 °C was added and the mixture was brought to 4 °C.

All RT-PCR assays were accompanied by positive and negative controls. Positive controls were obtained by spiking normal lymphocytes with the human melanoma-derived cell line SK-MEL-28. Negative controls comprised peripheral blood samples obtained from healthy donors. All RT-PCR methods were repeated to confirm results and considered positive only if reproducible in a second assay.

The products of the RT-PCR assay were separated by electrophoresis on a 2% agarose gel containing ethidium bromide and analysed by direct visualisation under ultraviolet light.

Results

The sensitivity and specificity of the RT-PCR assay were determined by analysing peripheral blood samples obtained from healthy donors and spiked with a serially reducing number of SK-mel-28 melanoma cells, grown in culture. With this method it was consistently possible to detect as few as five melanoma cells within a 10 ml of blood. Analysis of blood samples obtained from healthy individuals and patients with non-melanoma pathology were negative for tyrosinase (data not shown).

Of those with localised disease, only three of the patients have developed recurrence within the 3-year follow-up period. In each case recurrence occurred initially to regional lymphatics. An agarose gel showing the results of RT-PCR analysis for tyrosinase in patients with Stage II disease is shown in Fig. 1. Two patients have died as a result of further dissemination whilst another patient died of causes unrelated to the original melanoma. In one patient there was discordance between the two independent rounds of RT-PCR and as such was counted as negative. Of the two patients in whom tyrosinase transcripts were identified, one subsequently went on to develop regional metastases and one remains fit and well. In these samples transcripts were not identified without amplification using the nested primers. The relationship between prognostic markers and PCR result is shown in Tables 1 and 2.

Of those with Stage III disease, all had clinically apparent metastases and the lymphatic basin reflected the site of the original lesion in all cases but one, where spread occurred initially in the groin following a primary in the head and neck. Within the 3-year follow-up 13 of the 21 patients have died of the disease whilst all other patients remain well.

Table 1 Prognostic indicators and their relationship with PCR result

Prognostic marker	Patient numbers (*n*)	PCR results (%)
Tumour thickness		
Breslow <1.5 mm	30	0
Breslow 1.5–4 mm	16	1 (6.3%)
Breslow >4 mm	4	1 (25%)
Clark's level		
III	4	1 (25%)
IV	46	1 (2%)
Morphological type		
Superficial spreading	38	1 (2.6%)
Nodular	7	1 (14.2)
Lentigo maligna melanoma	1	0
Acral lentigenous	4	0
Mitotic activity		
< 5/h.p.f	34	1 (2.9%)
> 5/h.p.f	15	1 (6.7%)
NA	1	0
Ulceration		
Present	42	1 (2.4%)
None	8	1 (12.5)
Lymphocyte infiltration		
None	6	1 (17)
Minimum	15	0
Moderate	20	1 (5%)
Maximum	6	0
NA	3	0
Evidence of regression		
None	33	2 (6%)
Minimum	7	0
Moderate	1	0
Maximum	5	0
NA	4	0
Vascular invasion		
None	42	2 (4.8%)
Present	3	0
NA	5	0

NA, not available.

Table 2 Stage and PCR result

Stage (AJCC)	Patient numbers (*n*)	PCR positive (%)
I	30	0
II	20	2 (10%)
III	21	3 (14%)
IV	14	5 (36%)

AJCC, American Joint Committee on Cancer.

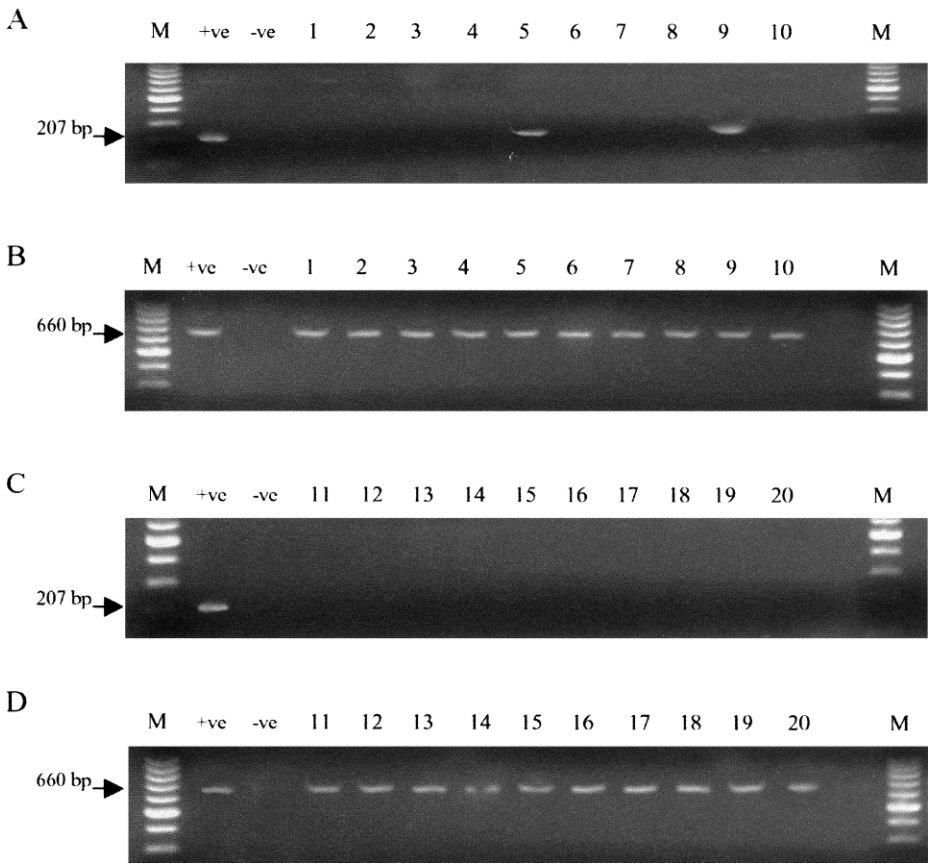

Fig. 1 Agarose gel (2%) analysis of amplified tyrosinase cDNA (10 μl) from patients 1–20 with Stage II (AJCC) malignant melanoma (A and C). Amplification of respective patients using β-actin primers are shown in the gels B and D. Positive controls (+ve) were obtained by spiking normal lymphocytes ($\sim 10^7$) with the human melanoma-derived cell line SK-MEL-28 (~100). Negative controls (−ve) were water controls. The molecular weight marker (M) corresponds to the 100 bp DNA ladder.

The number of histologically positive lymph nodes varied between one and 11 and was not found to correlate with the PCR result. Of those with Stage IV disease eight patients had visceral metastases, one cerebral metastases and in five cutaneous metastases.

Integrity of transcription was demonstrated in each case by analysis with 'house-keeping' gene primers. In no case were transcripts identified prior to nested amplification.

Statistical evaluation

The Pearson chi-squared test was used to examine the differences between stage of disease (Stages I and II analysed together) and the finding of a positive PCR result, the observed difference in rates across stages was found to be statistically significant ($p = 0.005$). Given the small numbers involved further analysis was undertaken using the Fisher's Exact test and again a significant correlation was drawn between stage and result ($p = 0.005$).

Given the small numbers of positive results it was not possible to draw statistical significance between recurrence or survival and the finding of tyrosinase transcripts within the blood samples. Furthermore no correlation could be established between the other parameters of prognosis and outcome.

Discussion

There is a clear need to develop prognostic indicators for patients with melanoma in order to better rationalise management, and to stratify for ongoing trials and for specific adjuvant therapies. The detection of occult circulating cells within the peripheral circulation provides a possible means of determining which tumours will ultimately metastasise. The extent to which such cells divide and consequently form distant metastases remains, however, to be fully established.[11,14,15]

The publication in 1991 using RT-PCR to detect tyrosinase mRNA[6] described detection of melanoma cells with exquisite sensitivity (one cell within a

single 10 ml blood sample). Although a number of other groups have since evaluated the method as a prognostic assay, the accumulated data remains inconclusive, particularly in relation to those with primary malignant melanoma.[12]

We were able to detect tyrosinase mRNA in the peripheral blood of only two of 50 patients with primary malignant melanoma; none from Stage I and two from Stage II (10%). This small number of positive findings prevented any valuable correlation with other markers of prognosis; as has been reported previously.[16,17] On the basis of these findings and in light of the fact that of the three recurrences within this cohort only one was found to be tyrosinase positive, we would support the conclusions drawn by previous studies that have concentrated primarily on RT-PCR analysis in early disease that, there is likely to be little clinical value for the assay in the management of these patients.[16,18]

The technique of RT-PCR for tyrosinase mRNA contains inherent difficulties when used in the context of an assay. These problems which relate to the intermittent nature of tumour shedding and to the transient nature of circulating metastatic cells have been discussed extensively elsewhere[19] and it is not an intention to reiterate these points in this discussion. Notwithstanding, in his analysis of 23 studies, Tsao[12] concluded that 'several procedural' problems (in addition to technical difficulties) might limit the value of the reported data. It was suggested, for example, that enrolment from oncology centres might favour patients with a higher risk of recurrence. By this, it is meant, that other 'risk factors', other than simple AJCC staging, might influence referral to a tertiary oncology. This would be particularly so where it is not a routine practice to refer all melanoma patients with localised or regional disease to oncology units, as in UK. We recruited consecutively from patients attending a plastic surgical centre that benefits from the presence of a pigmented lesion clinic. The specific purpose of this clinic is to facilitate and reduce the threshold for referral from primary care physicians. Recruitment from this population might therefore be seen to address, at least to some extent, these concerns.

Of further importance with relation to procedure is the technique of blood sampling itself and specifically the precise timing of the sampling. One almost inevitable consequence of sampling following referral to a tertiary oncology centre will be that sampling will follow excision. Whilst it remains uncertain how long melanoma cells will survive in the circulation, it is entirely possible that removing the source of malignant cells will substantially reduce the quantity of circulating cells. This may then impact upon RT-PCR result. In a study on breast cancer using RT-PCR in which sampling was undertaken before and after surgery it was found that the presence of circulating cells is transitory, presumably as a result of cells being 'mopped up' by other tissues such as the lung and reticulo-endothelial system. Indeed, in his study of melanoma patients, Curry found that 65% of patients with positive findings pre-operatively changed in the post-operative period.[20] In the majority of reports on RT-PCR for tyrosinase in patients with early disease and where details are given, it is a feature that samples were obtained post-operatively. Given that in our study all venepuncture was undertaken pre-operatively and in the presence of the tumour, it might have been expected that a higher positivity rate would be found but, this was not so.

In those with more advanced disease stage, we identified tyrosinase mRNA in three of 21 patients (14%) with Stage III and five of 14 (36%) patients with Stage IV disease. Overall, disease stage was found to correlate with PCR status. Whilst this lends some credibility to our findings, these rates remain below the 95% confidence intervals of 26–34% and 41–50%, respectively, reported in the meta-analysis.[12] Given that we found sensitivities for the RT-PCR assay to be comparable with those previously reported, the authors feel that the findings of this study serve only to substantiate the concerns highlighted in previous publications over the likely value of this assay when applied to localised melanoma.[19]

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