Detection of neoplastic cells in blood of miniature pigs with hereditary melanoma

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ABSTRACT: Tyrosinase, a key enzyme of melanin biosynthesis, is widely used as a specific marker for the detection of disseminated or metastatic melanoma cells in peripheral blood and other tissues like lymph node or bone marrow, which are normally tyrosinase negative. The amplification of tyrosinase-specific mRNA by means of RT-PCR is a sensitive technique capable of detecting a single tumour cell in 5–10 ml of whole blood. We have utilised this method to analyse the peripheral blood of laboratory miniature pigs with advanced cutaneous melanoma for the presence of tumour cells. This highly invasive hereditary malignancy can serve as an experimental model for the study of melanoma development and dissemination. For amplification of porcine gene, oligonucleotide primers derived from the sequence of human tyrosinase were used. These primers amplified fragments of the predicted length and restriction enzyme digestion confirmed their homology with the sequences of human tyrosinase gene. After the second round of amplification, tyrosinase could be detected up to the amount of 1×10^{-5} µg of total RNA isolated from porcine melanoma per 1 µg of control RNA. Blood samples from eight animals with advanced melanoma and from five non-melanoma control animals were examined for tyrosinase expression. Tyrosinase mRNA was detected in five samples from animals with malignant melanoma. Non-melanoma control animals gave negative results.

Key words: MeLiM strain; melanoblastoma; circulating tumour cells; tyrosinase, tyrosinase-specific mRNA

INTRODUCTION

In humans, cutaneous melanoblastoma accounts for about 2% of all malignancies and belongs to those tumours whose incidence is worldwide rising steadily over the past few decades (Osterlind, 1992). The presence of disseminated tumour cells in peripheral blood indicates a high risk of developing metastases and has been associated with a poor prognosis of melanoma (Keilholz *et al*., 1997). Methods developed for the detection of metastatic cells in blood also enable to monitor the course of the disease during treatment.

Tyrosinase is a multi-functional enzyme involved in melanin biosynthesis, specifically expressed in melanocytes and most melanoma cells. This enzyme, which has not been found in normal blood cells, is a suitable marker for the detection of circulating neoplastic cells in melanoma patients. The detection of tyrosinase transcript by RT-PCR is the technique most widely used for monitoring melanoma cells in circulation. This highly specific assay can detect a single melanoma cell in 5 to 10 ml of peripheral blood (Hanekom *et al*., 1997).

Various research groups have examined melanoma patients by tyrosinase RT-PCR for the presence of circulating neoplastic cells in all clinical stages of the disease (Smith *et al*., 1991; Hanekom *et al*., 1997; Curry *et al*., 1998; Farthmann *et al*., 1998). In disease with distant metastases (stage IV), several authors have found circulating melanoma cells in a high proportion of individuals. However, discrepancies have also occurred between the published results. Other laboratories have reported much lower frequencies of positive results in advanced disease, or have found no positivity of the assay (Keilholz *et al*., 1997).

The MeLiM strain (Melanoblastoma-bearing Liběchov Minipigs) of laboratory miniature pigs with cutaneous hereditary melanoma has been established in the Institute of Animal Physiology and Genetics (Liběchov, Czech Republic). The tumours are often multiple and appear in about 57% of all animals. Numerous organ metastases

are regularly ascertained and 34% of animals die during the first two months of age. The invasiveness, metastatic capacity and morphological features of the melanoma in minipigs are similar to those of human melanoma (Fortýn *et al*., 1994a; Horák *et al*., 1999). The hereditary melanoma in minipigs can thus serve as a suitable model for studying dissemination of tumour cells in different clinical stages of the disease and for monitoring the response to experimental therapy by devitalization technique. This surgical method based on an ischemization of one of the cutaneous tumours was tested in more than 40 affected animals and caused the destruction of melanoma cells in all tumours and organ metastases during 4–6 months (Fortýn *et al*., 1994b; Horák *et al*., 1999).

The aim of our study was to examine the group of pigs with advanced melanoblastoma for the presence of circulating neoplastic cells and to assess the applicability of the nested RT-PCR assay for tyrosinase-specific mRNA for studying dissemination of melanoma cells.

MATERIAL AND METHODS

Animals

Miniature pigs of the MeLiM strain with hereditary melanoma were used for this study (Horák *et al*., 1999). Blood samples from the group of 8 pigs with the advanced disease (multiple cutaneous nodular melanomas and distant organ metastases) and additional blood samples of 5 healthy animals were tested.

RNA isolation

Total RNA was prepared from the fraction of leucocytes isolated from EDTA blood samples (5–10 ml) by gradient centrifugation on Histopaque 1119 (Sigma). The cells were rinsed in cold PBS and subjected to extraction with guanidinium thiocyanate/phenol-chloroform as de-
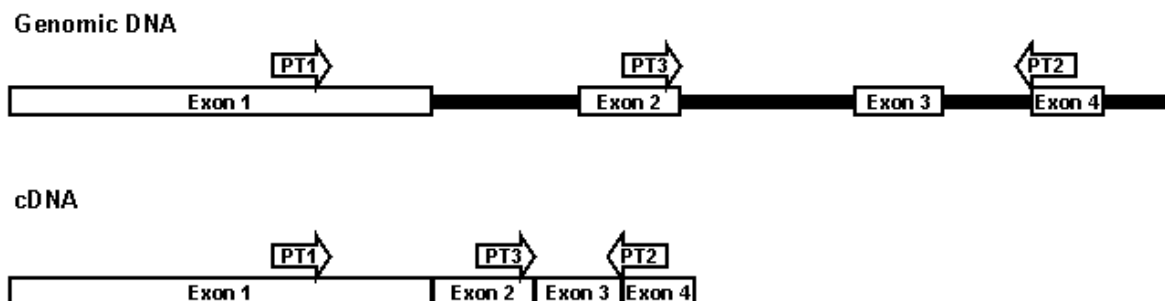
A

B

Figure 1A. Segments of human, mouse and chicken tyrosinase cDNA (accession numbers: M27160, X12782 and A023291) from which PCR-primers – PT1, PT3 and PT2 – were derived. Identical nucleotide sequences are indicated by shadow boxes; a = primer position in human tyrosinase gene, b = sense sequence of reverse primer

Figure 1B. Schematic diagram of exons 1–4 of human tyrosinase gene and cDNA showing the positions of PCR primers

scribed by Chomczynski and Sacchi (1987). Total RNA was further extracted from histologically confirmed human and porcine cutaneous melanomas. The tumour samples were homogenized in 1 ml of guanidinium thiocyanate solution per 100 mg of tissue in a glass/Teflon homogenizer and purified as described above. The examination of human RNA has been approved by the Ethical Committee at the Charles University in Prague. Informed consent was obtained from all patients prior to analysis of the samples from surgically removed melanomas.

Synthetic oligonucleotide primers

The primary purpose of our study was to develop the sensitive PCR-assay for the detection of tyrosinase-specific mRNA in peripheral blood of miniature pigs with hereditary melanoma. Samples of total RNA were tested for the presence of tyrosinase mRNA by the two step RT-PCR. Since the sequence of porcine tyrosinase is not known, PCR primers were derived from gene segments conserved between different animal species. Selected primers corresponded to segments of human gene (Takeda *et al*., 1989) with high degree of homology to known sequences of mouse (Yamamoto *et al*., 1987) and chicken (Tobita-Teramoto *et al*., 2000) tyrosinase. Conserved sequences of the tyrosinase gene, from which primers were derived, are shown in Figure 1A. The map of tyrosinase gene and cDNA showing the locations of primers is illustrated in Figure 1B. The outer primers PT1 (sense) and PT2 (antisense) amplified on the human tyrosinase cDNA a 662 base pair (bp) fragment. Primers used in the second round of PCR contained the inner sense primer PT3 and the antisense primer PT2 ("semi-nested" PCR). These primers amplified a fragment of 287 bp.

Detection of tyrosinase mRNA by RT-PCR

Reverse transcription was performed following the manufacturer's instructions (Roche Molecular Biochemicals). A two step PCR was used for amplification of selected fragments of tyrosinase transcript. Amplifications were carried out in 25 µl of PCR buffer (10 mmol/l Tris-HCl, pH 8.3, 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$) with 0.2 mmol/l dNTPs and 0.6 units of *Taq* DNA polymerase (Roche Molecular Biochemicals). The first round of PCR contained 10 pmol of each of the outer primers (PT1 and PT2) and a 2 µl aliquot of the reverse transcription reaction. PCR was carried out using a Peltier thermal cycler PTC-200 (MJ Research). Following denaturation at 94°C for 2 min, 32 cycles (94°C for 30 s, 60°C for 1 min, 72°C for 2 min) and final extension (72°C for 5 min) were performed. In the second round of PCR with 10 pmol forward (PT3) and reverse (PT2) primer, 1 µl of a 1 : 100

dilution of the first PCR product was amplified for additional 32 cycles. Cycling conditions were as above. PCR products were analysed on 1,5% ethidium bromide-stained agarose gels.

Restriction enzyme digestion

The specificity of amplified fragments and their homology with the human tyrosinase gene was demonstrated by restriction enzyme digestion. Products of the first and second round of PCR (10 µl of each product) were digested with 5 units of the restriction enzyme *MvaI* in a 20 µl reaction mixture overnight at 37°C. Resulting fragments were analysed on a 2% ethidium bromide-stained agarose gel.

PCR for β-actin

An aliquot of each transcribed cDNA sample was analysed by PCR with β-actin primers to check its integrity and acceptability for amplification. The following primers were used: sense primer 5´-TGACGGGGTCAC-CCACACTGTGCCCATCTA-3´ and antisense primer 5´-C T A G A A G C AT T T G C G G T G G A C G AT G-GAGGG-3´. Primers were derived from the sequence of human gene and amplified the fragment of 661-bp by one step PCR. Conditions of amplification were identical to those described above.

Testing of sensitivity

To establish the sensitivity of the assay, we prepared 10-fold serial dilutions of RNA extracted from porcine cutaneous melanoma in 1 µg aliquots of RNA isolated from peripheral blood leucocytes of healthy animals. Decreasing amounts of RNA (from 1 µg to 10^{-6} µg) were tested for tyrosinase-specific mRNA by nested RT-PCR as described above.

Positive and negative controls of PCR

Total RNA isolated from histologically confirmed porcine melanoma was used as a positive PCR control. Assay mixtures without template were used as negative controls of PCR.

RESULTS

Total RNA extracted from human cutaneous melanoma was initially used to test the specificity of the RT-PCR assay and applicability of designed primers. Specific
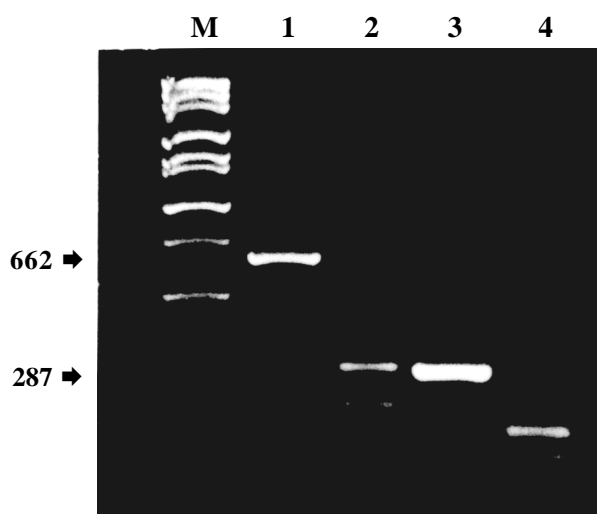
Figure 2. Digestion of PCR products with restriction enzyme *MvaI*. Lane M, wide range DNA marker (Sigma); lane 1 = first round PCR product; lane 2 = respective fragments obtained after digestion; lane 3 = second round PCR product; lane 4 = respective fragments obtained after digestion

PCR-products of predicted size (662 bp and 287 bp, respectively) appeared to be produced with both sets of primers. Fragments of the same length were also detected with RNA isolated from porcine melanoma (data not shown). The homology of sequences amplified on porcine cDNA with human tyrosinase was confirmed by restriction enzyme digestion. Restriction enzyme *MvaI* has two restriction sites within the first product amplified from human RNA and one site within the product obtained after the second round of amplification. Digestion of the first amplified product gives fragments of 314 bp, 224 bp and 124 bp, whereas the second PCR product is cut into 163 bp and 124 bp fragments. Fragments of the same sizes were detected from products amplified from porcine RNA after digestion with *MvaI* (Figure 2).

Total RNA isolated from porcine melanoma was used to determine the sensitivity of the PCR assay. Decreasing amounts of tested RNA were mixed with control RNA isolated from peripheral blood leucocytes of healthy animals and subjected to the nested RT-PCR for tyrosinase mRNA. After the amplification with outer primers alone,

Figure 4. Detection of tyrosinase mRNA and β-actin mRNA in blood samples from laboratory minipigs. A) Lane M, wide range DNA marker (Sigma); lanes 1, 3, 5, 7, and 8 = tyrosinase mRNA positive samples from animals with malignant melanoma; lanes 2, 4, and 6 = tyrosinase mRNA negative samples from animals with melanomas; lane 9 = positive control (the RNA sample from melanoma tissue); lane 0 = negative control (the assay mixture without template). B) Identical samples tested for the presence of β-actin mRNA
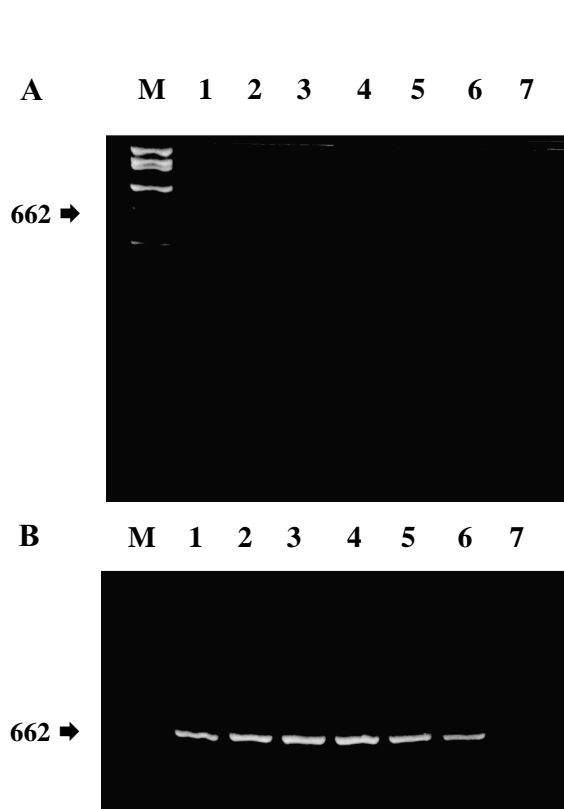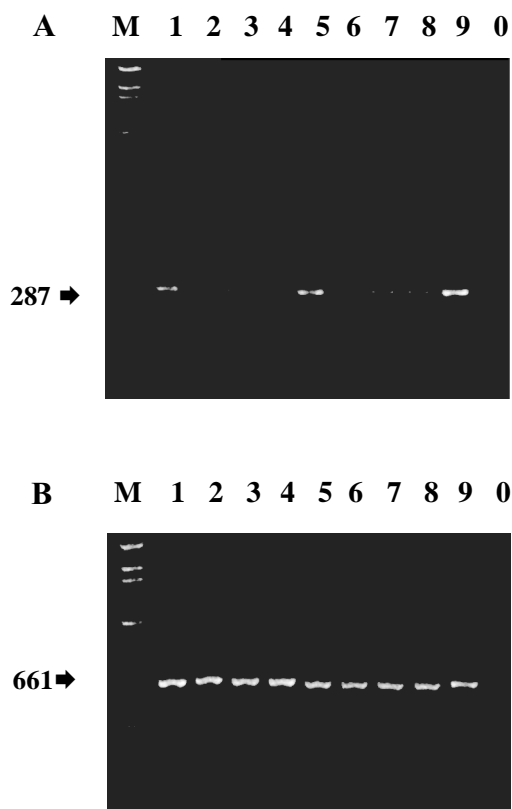
Figure 3. Assessment of sensitivity of the assay. Tyrosinase-specific mRNA amplified by the RT-PCR in samples with decreasing amounts of RNA from porcine melanoma. Sensitivity is indicated after the first (A) and second (B) PCR. Lane M = wide range DNA marker (Sigma); lanes 1–7 = samples containing from 1 μg to 1×10^{-6} μg RNA

a tyrosinase specific band was detected on an agarose gel up to the amount of 0.1 μg of the tested RNA per 1 μg of control RNA (Figure 3A). Slight visibility of this band (Figure 3A, lane 2) suggests that the limit of sensitivity was reached in the PCR reaction. The sensitivity of the assay was greatly increased by further amplification. After the second round of amplification with nested primers, tyrosinase could be detected up to the amount of 1×10^{-5} μg of tested RNA (Figure 3B).

Peripheral blood samples from a total of 8 animals with advanced malignant melanoma and from five non-melanoma control animals were examined for tyrosinase mRNA. After the first step of amplification, tyrosinase specific bands were not found on agarose gels. However, the semi-nested RT-PCR detected tyrosinase mRNA in five samples from animals with melanoblastoma (Figure 4A). Negative results were obtained with non-melanoma control animals. Aliquots of reverse transcription reactions were also assayed for the presence of β-actin cDNA. This housekeeping gene could be detected by the one step PCR in all analysed samples from melanoma (Figure 4B) and non-melanoma animals (not shown). The test confirmed the integrity of isolated RNA and suggested that the absence of tyrosinase mRNA in particular blood samples from melanoma animals was not the result of partial degradation of RNA, ineffective cDNA synthesis, or ineffective amplification.

DISCUSSION

Detection of tissue-specific expression of mRNA based on RT-PCR can be used to reveal the dissemination of tumour cells. It seems likely that the highly sensitive test could be useful in the assessment of prognosis of the malignant disease (Smith *et al.*, 1991). By this approach, disseminated tumour cells were demonstrated in numerous malignancies, for example in patients with prostate (Seiden *et al.*, 1994) and breast cancers (Datta *et al.*, 1994). In human metastatic melanoma, circulating tumour cells were frequently detected by several authors (Smith *et al.*, 1991; Keilholz *et al.*, 1997). Our findings in hereditary melanoma of laboratory miniature pigs indicate that the detection rates can be just as high.

Tyrosinase primers used in our experiments differed from those originally designed by Smith *et al.* (1991), which were most widely utilised by other groups. Primers corresponded to sequences conserved between different animal species and were represented on different exons. This location of primers excludes amplification of contaminating genomic DNA into products, which have the same length as fragments amplified from RNA. Considering the amount of leucocytes in peripheral blood, in our highly diluted samples the sensitivity of the assay can be similarly high to that reported by other laboratories. We did not find tyrosinase mRNA in peripheral blood

of control animals, which confirmed the specificity of the test.

Circulating tumour cells were detected in five out of eight animals with advanced disease. Histological examination of tumours did not show any differences between tyrosinase mRNA positive and negative animals. No differences were found in amount and vertical spreading of melanin-bearing cells and in metastatic capacity of tumours. Numerous distant metastases mainly into the spleen and lymph nodes were found in all animals. Disparate results of the RT-PCR assay in our sample of pigs with advanced melanoma may be due to the intermittent release of tumour cells into the circulation (Farthmann *et al.*, 1998). Another possible explanation is the difference in the expression of tyrosinase mRNA between tumour cells (Keilholz *et al.*, 1997). In patients with advanced melanomas, discrepant positivity rates were also reported for the PCR detection of tyrosinase mRNA. Some authors found the PCR positivity in the high percentage of patients, others obtained low positivity rates. These conflicting findings could also be explained by differences in processing blood samples and differences in reverse transcription and cycling conditions. Low frequency of melanoma cell detection can be found in patients with slow progression of the disease or in remission of the disease after treatment with interferon α (Keilholz *et al.*, 1997).

Our results confirm that the RT-PCR assay, performed with primers derived from the sequence of human gene, enables sensitive detection of tumour dissemination in advanced porcine melanoma. The applicability of the test for the evaluation of a course of melanoma destruction induced by devitalization is now under study.

Repeated examination of blood samples (e.g. every other day) or examination of multiple specific markers may increase the sensitivity of detection of circulating tumour cells. Our preliminary results indicate that the inclusion of the RT-PCR test for MART-1 (Melanoma Antigen Recognised by T cells-1) allows detection of circulating melanoma cells in a larger percentage of laboratory pigs with advanced melanoma.

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