

# A Polymerase Chain Reaction-based Semiquantitative Assessment of Malignant Melanoma Cells in Peripheral Blood<sup>1</sup>

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## ABSTRACT

Malignant melanoma cells can be detected with high sensitivity in peripheral blood of patients using reverse transcription-PCR. The detection of tyrosinase mRNA that is actively expressed only in melanocytes and melanoma cells indicates the presence of melanoma cells in peripheral blood. As shown previously, tyrosinase transcripts can be found in a variety of patients with metastatic malignant melanoma. For semiquantitative analysis of these cells in peripheral blood and evaluation of possible influence of immunotherapy on the amount of circulating cells, we describe an assay combining reverse transcription-PCR and Southern blotting. In this system, the amount of circulating tumor cells was determined by interpolating the amplified tyrosinase signal strength of patient samples to an equivalent tyrosinase signal of diluted SK-mel 28 cells. We found that the amount of circulating tumor cells correlates with the tumor burden. Furthermore, in patients with regression of melanoma metastases after immunotherapy, a decrease of the amount of tumor cells in the peripheral blood was observed. Quantitative estimates of residual disease may be an accurate and sensitive predictor for the clinical course.

## INTRODUCTION

A variety of methods has been used to detect malignant cells in the peripheral blood of patients, including immunological detection by mAbs (1, 2). It is by PCR that the sensitivity of this approach is dramatically increased (3-10). Tyrosinase, a key enzyme in melanin biosynthesis, has been proposed for detection of malignant melanoma cells (8, 11). The tyrosinase gene is actively expressed only in melanocytes and melanoma cells. Because melanocytes do not usually circulate in peripheral blood, the detection of tyrosinase RNA indicates the presence of melanoma cells.

Using a nested PCR method, we have shown previously that malignant melanoma cells can be determined with high sensitivity in blood samples of patients with malignant melanoma (12, 13). Circulating melanoma cells were detected in all patients with metastatic malignant melanoma. Examination of patients responding to immunotherapy revealed that residual melanoma cells can be detected in peripheral blood and bone marrow of all patients in prolonged complete remission after successful treatment. This analysis was done by a nested PCR with two pairs of specific primers. This approach is highly sensitive but not applicable for a quantitative estimate.

For quantitation of these cells in peripheral blood and evaluation of possible influence of immunotherapy on the amount of circulating cells, we describe a semiquantitative assay combining RT-PCR<sup>3</sup> and Southern blotting.

## MATERIALS AND METHODS

**Cell Lines.** The lines MKr and MKrZ had been grown in our own laboratories (14). The remaining human melanoma cell lines had all been established and were provided by different laboratories (SK-mel 28 and MeWo, A. Houghton, Memorial Sloan Kettering Cancer Center; Colo 38, S. Ferrone, Valhalla, NY). By light microscopy all cell lines are amelanotic. All lines were cultured in tissue culture flasks in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% FCS (GIBCO), 1 mM L-glutamine, and 1% penicillin/streptomycin (GIBCO). For RNA extraction, 10<sup>7</sup> cells were used.

**Synthetic Oligonucleotides.** Tyrosinase primers were devised from published sequences (8, 11). HTYR 1 (TTGGCAGATTGTCTGTAGCC) and HTYR 2 (AGGCATTGTGCATGCTGCTT) amplify a PCR product of 284 bp. We chose this outer primer pair because the product is of similar length as the GAPDH PCR product. GAPDH primers were devised from published gene sequence (15). GA 1 (GGTCGGAGTCAACGGATTG; sense) and GA 2 (ATGAGCCCCAGCCTTCTCCAT; antisense) produce a fragment of 320 bp. For hybridization of the GAPDH PCR product, we used an oligonucleotide probe derived from the internal part of the amplified fragment (GTAAAC-CATGTAGTTGAGGTCAATGAAGGG).

**RT-PCR Method.** Total RNA was prepared from the cell lines and blood samples by acid guanidinium thiocyanate/phenol-chloroform extraction according to the method of Chomczynsky and Sacchi (16). For RT, 1 µg of total cellular RNA was added to 25 µl of RT buffer containing 0.4 µmol of random hexamers, 1 mmol each dNTP (dATP, dCTP, dTTP, dGTP), 24 units of avian myeloblastosis virus reverse transcriptase (Promega), and 40 units of RNasin (Promega). After incubation at 42°C for 1 h, 2 µl of the sample were suspended in 50-µl PCR buffer containing 0.3 µg HTYR 1 and HTYR 2 primer and 2 units Taq DNA polymerase (Promega). PCR was carried out with a Thermal cycler (Perkin Elmer Cetus): denaturation at 94°C for 90 s, annealing at 60°C for 90 s, and extension at 72°C for 90 s. Primer and RNA concentrations, as well as PCR cycles, were titrated to establish standard curves for tyrosinase and GAPDH amplification and to permit quantitative analysis of signal strength. Eight µl of reaction product were run on 2% agarose gel and stained with ethidium bromid. Hybridization and chemiluminescent detection were performed using a commercially available kit (Lumigen PPD, Boehringer Mannheim). After hybridization, the band signal was determined using Image 1.41 software (National Cancer Institute, Bethesda, MD) for integration and normalized to GAPDH.

To generate linear dilution curves for quantitation of melanoma cells in peripheral blood, SK-mel 28 cells were serially diluted into 10 ml of peripheral blood from a healthy donor. RNA isolation and RT were performed as described above. Samples of the cDNA of patients were run along with diluted samples. Quantitation of circulating tumor cells was determined by interpolating the signal strength of the patient samples to an equivalent signal of the diluted SK-mel 28 cells.

**Patients and Treatment Regimen.** Fifty patients with metastatic malignant melanoma (14 patients with low tumor burden, 21 with medium, and 15 with high tumor burden) were examined. Tumor volume was calculated according to standard WHO criteria as the sum of the products of the largest diameter and two perpendicular diameters of all detectable lesions by imaging procedures or clinical examination. Tumor volume was categorized into low (<50 cm<sup>3</sup>), medium (50-250 cm<sup>3</sup>), and high (>250 cm<sup>3</sup>) tumor burden. A more discrete distinction, especially in small volumes, is unreliable because of the limited precision of the staging methods (e.g., the ultrasonic scan). Furthermore, samples of 14 melanoma patients who underwent immunotherapy with high dose interleukin 2 and IFN-α as described elsewhere were analyzed (17). In no patient without a malignant melanoma or with another cancer were

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<sup>3</sup>The abbreviations used are: RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HTYR, human tyrosinase.

tyrosinase transcripts detectable; their signal always showed background activity.

**Statistical Analysis.** Wilcoxon signed rank test was used to test for statistically significant differences between the three patient groups. A paired Wilcoxon test was performed to compare pre- and posttreatment data.

**RESULTS**

**Expression of Tyrosinase mRNA in Melanoma Cell Lines.** A semiquantitative RT-PCR technique was used to investigate tyrosinase mRNA expression in 5 human-derived melanoma cell lines (SK-mel 28, Colo 38, Mevo, MKr, and MKrZ). Primer concentrations, amounts of RNA, and PCR cycles were titrated to establish standard conditions, to document linearity, and to permit quantitative analyses of signal strength. PCR comprises 23 cycles for GAPDH and 26 cycles for tyrosinase amplification. After hybridization with a digoxigenin-labeled PCR-product (284 bp) as a probe, the band signal was determined using Image 1.41 software (National Cancer Institute) for integration and normalized to GAPDH. The signal strength of the amplified tyrosinase product of tested cell lines varied in a range of less than 10% in all 5 cell lines (Table 1). The SK-mel 28 cell line was used as a standard in further studies.

**Detection and Quantitation of Melanoma Cells in Peripheral Blood of Melanoma Patients.** We used an approach combining RT-PCR and Southern blotting. We first performed 10-fold serial dilution experiments. Decreasing numbers of cells of human melanoma-derived cell line SK-mel 28 were mixed with 10 ml of heparinized blood, and total RNA was extracted from the cell mixture. After 26–30 rounds of PCR with specific primers for tyrosinase and Southern blot hybridization with a digoxigenin-labeled PCR-product (284 bp) as a probe for hybridization, we were able to reliably detect 10 tumor cells in 10 ml of blood (Fig. 1). To verify the detection sensitivity, we performed 7 replicates of the experiment. The PCR product was detected in 7 of 7 assays using 10 SK-mel 28 cells in 10 ml of blood.

After 26–28 cycles, the relation between absorbance and log of SK-mel 28 melanoma cells was reproducibly almost linear in the range of 10–10<sup>4</sup> melanoma cells/10 ml blood (Fig. 1 and Fig. 2). Examination of patient samples revealed a higher sensitivity after 28 PCR cycles (samples of 5 patients that were negative for tyrosinase signal after 26 cycles of amplification and Southern blotting became positive after 28 cycles or using nested PCR with two specific primer pairs for tyrosinase as described previously).

We next performed a study in which artificial mixes were gener-

Table 1 Expression of tyrosinase in human melanoma cell lines using RT-PCR and Southern blotting

Cell lines	Signal strength (absorbance) <sup>a</sup>
SK-mel 28	96 ± 9
MeVo	92 ± 8
Colo 38	89 ± 10
MKr	86 ± 9
MKrZ	94 ± 6

<sup>a</sup> The signal strength (absorbance) was determined using Image 1.41 software (National Cancer Institute) for integration. Mean values (n = 3) and SEM are shown.

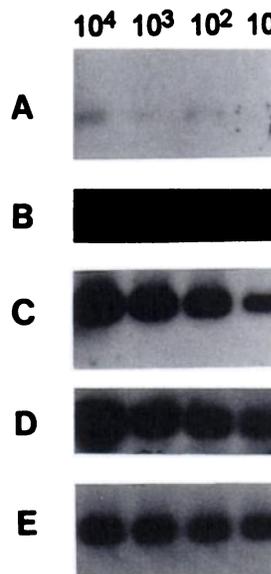


Fig. 1. Detection of tyrosinase mRNA in heparinized blood samples from a healthy subject mixed with a 10-fold serial dilution of SK-mel 28 cells (10–10<sup>4</sup>) after 22–30 cycles of PCR amplification with primers HTYR 1 and HTYR 2 and Southern blotting. A, after 22 cycles; B, 26 cycles; C, 28 cycles; D, 30 cycles; E, GAPDH signal after 23 cycles of PCR amplification with primers GA1 and GA2 and Southern blot hybridization.

ated. Within the linear range of titration, unknown concentrations of SK-mel 28 cells could be determined correctly within a range of 10% error. Moreover, when multiple assays were performed on the same sample, the intensity of the PCR products typically was within 10% variation (5 replicates of each experiment were performed).

Blood samples from 50 patients with metastatic malignant melanoma were examined. The strength of the amplified tyrosinase signal in the blood samples correlates with the tumor burden (P < 0.001) and

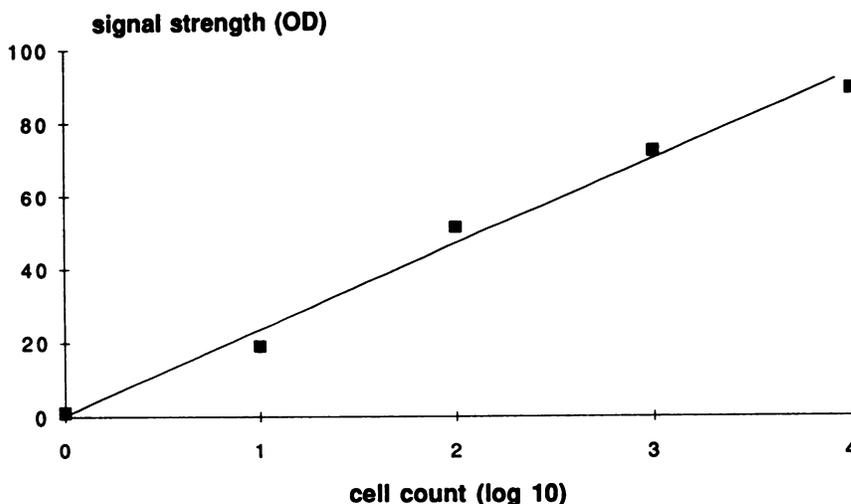


Fig. 2. Generation of a standard curve from serial diluted SK-mel 28 cells (10–10<sup>4</sup>) after 28 cycles of PCR amplification with specific primers for tyrosinase and Southern blotting. The signal strength (OD) was determined using Image 1.41 software (National Cancer Institute) for integration and normalized to GAPDH.

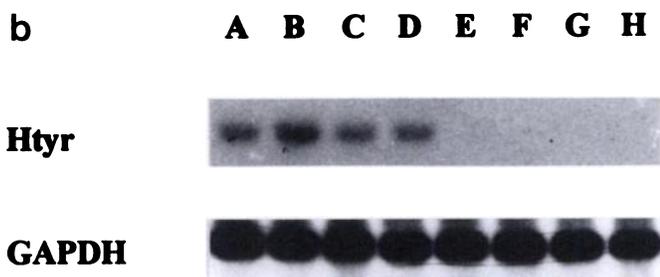
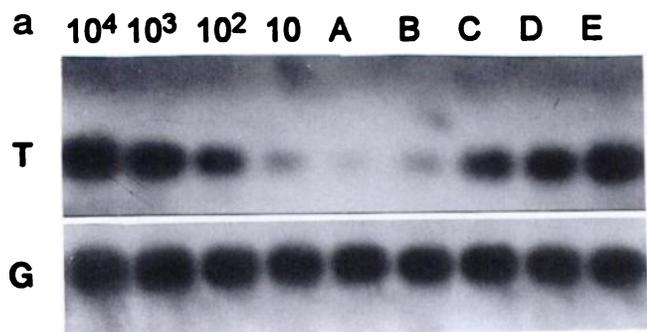


Fig. 3. *a*, Southern blot analysis of PCR-amplified tyrosinase (*T*) and GAPDH (*G*) expression in serial diluted SK-mel 28 cells (10<sup>4</sup>–10<sup>1</sup>) and patient samples. RNA was extracted from 10 ml of heparinized peripheral blood and analyzed by PCR using specific primers as described. PCR comprises 23 cycles for GAPDH and 28 cycles for tyrosinase amplification. Lanes 1–4, number of added SK-mel 28 cells, Lanes A–E, blood samples from patients with metastatic melanoma (Lanes A and B, tumor burden <50 cm<sup>3</sup>; Lane C, 50–250 cm<sup>3</sup>; Lanes D and E, >250 cm<sup>3</sup>). *b*, Southern blot analysis of PCR-amplified Human tyrosinase (*Htyr*) and GAPDH expression in patients and probands. A and B, patients in complete remission after immunotherapy. C and D, patients with no evidence of disease after immunotherapy and surgical removal of rest tumor. E–H, healthy controls and patients with colon respiratory mammary carcinoma.

varies in a range of less than 10–10<sup>4</sup> compared to an equivalent signal of SK-mel 28 cells (Figs. 3 and 4).

To evaluate the possible influence of immunotherapy on the amount of circulating tumor cells, we further tested blood samples from 14 patients with metastatic melanoma before and after treatment with IFN- $\alpha$  and interleukin 2. Tyrosinase mRNA was detected in blood samples from all patients before and after immunotherapy. In

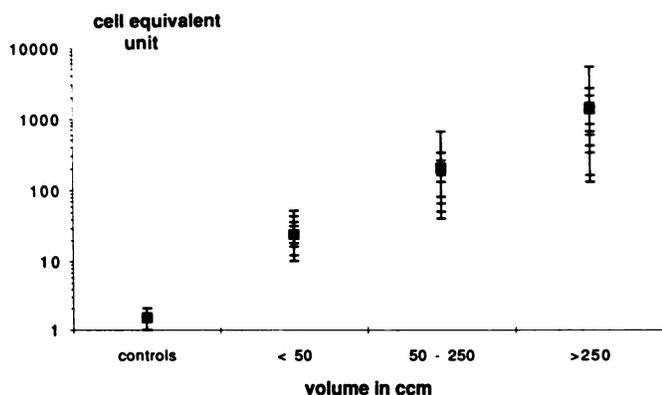


Fig. 4. Quantitative assessment of tumor cells in blood samples from melanoma patients with different tumor burden. RNA was extracted from 10 ml of heparinized peripheral blood and analyzed by PCR using specific primers as described. PCR comprises 23 cycles for GAPDH and 28 cycles for tyrosinase amplification. After Southern blot hybridization, the signal strength of the amplified tyrosinase product was determined using Image 1.41 software (National Cancer Institute) for integration and normalized to GAPDH. Quantitation of melanoma cells in blood samples was performed by comparison of the amplified tyrosinase signal with a corresponding tyrosinase signal of diluted SK-mel 28 cells. Points, median. The difference between the three patient groups is statistically significant (Wilcoxon signed rank test;  $P < 0.001$ ).

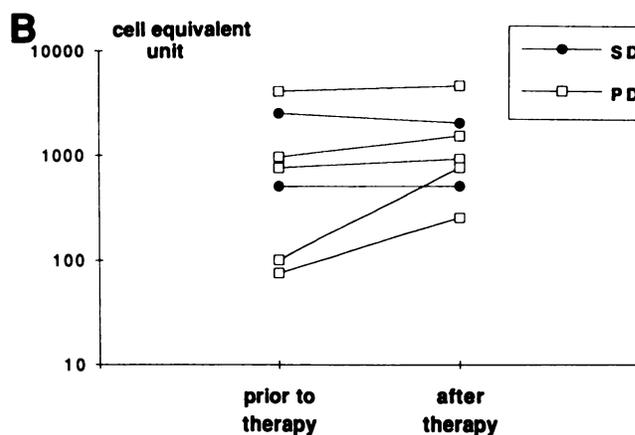
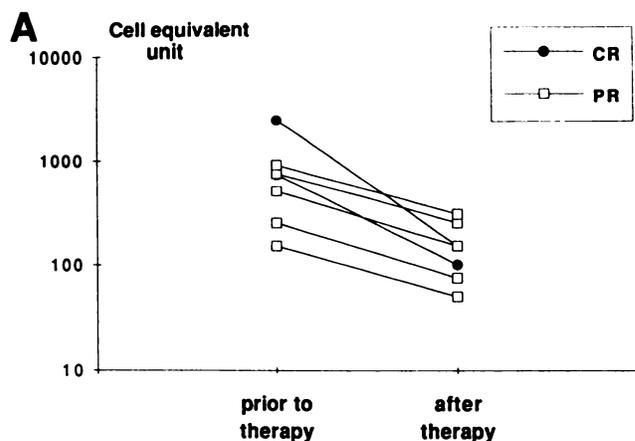


Fig. 5. Quantitative assessment of tumor cells in blood samples from patients with malignant melanoma before and after immunotherapy with interleukin 2 and IFN- $\alpha$  using RT-PCR and Southern blotting. The signal strength of the amplified tyrosinase product was determined using Image 1.41 software (National Cancer Institute) for integration and normalized to GAPDH. Quantitation of melanoma cells in blood samples was performed by comparison of the amplified tyrosinase signal with a corresponding signal of diluted SK-mel 28 cells. CR, complete remission; PR, partial remission; SD, stable disease; PD, progressive disease. The difference between CR and PR is statistically significant (paired Wilcoxon test;  $P < 0.01$ ). In SD and PD, results are not significantly different.

patients responding to immunotherapy (2 patients in complete remission, 5 in partial remission), we observe a statistically significant decrease of the tyrosinase signal strength from a median of 725 to a median of 150 ( $P < 0.01$ ), whereas in patients with stable (2 patients) or progressive (5 patients) disease, the signal strength was unchanged or even increased (Fig. 5).

## DISCUSSION

In this study, we applied RT-PCR and Southern blotting to detect and quantitate circulating tumor cells in peripheral blood of patients with malignant melanoma. Tyrosinase expression was used as target for determination. Quantitation of melanoma cells in blood samples was performed by comparison of the amplified tyrosinase signal with a corresponding signal of diluted SK-mel 28 cells. The assumption of this procedure is an equivalent tyrosinase expression in tumor cells *in vivo* and tested SK-mel 28 cells. Supporting this premise, the analysis of human melanoma cell lines for expression of tyrosinase mRNA by semiquantitative RT-PCR revealed a variation of tyrosinase expression of less than 10%. However, it can not be excluded that the expression of tyrosinase in tumor cells *in vivo* varies in an extended range. Therefore, the results obtained with this approach represent the amount of RNA equivalent to tyrosinase mRNA content in a defined

number of SK-mel 28 cells rather than the precise number of an individual patient's tumor cells.

In a cohort of melanoma patients, the amount of circulating tumor cells correlates with tumor burden of the patient. Our data also indicate that successful treatment reduces the amount of circulating tumor cells in peripheral blood; this, however, is preliminary because of the limited number of patients.

The quantitative estimates of residual disease could provide an important measure of response to therapy and could be a sensitive predictor of remission. In addition, PCR analyses can provide rapid screening of sequential samples that can be useful in evaluating therapeutic responses. Future applications may include the monitoring of patients to determine the ability of the assay to predict relapse at earlier timepoints. However, although RT-PCR appears to be a more sensitive technique than immunochemistry for analyzing peripheral blood specimens, the interpretation of such data must be verified through prospective analysis of more patients and longer follow-up.

## REFERENCES

- Moss, T. J., and Sanders, D. G. Detection of neuroblastoma cells in blood. *J. Clin. Oncol.*, **8**: 736-740, 1990.
- Schlimok, G., and Riethmüller, G. Detection, characterization and tumorigenicity of disseminated tumor cells in human bone marrow. *Semin. Cancer Biol.*, **1**: 207-215, 1990.
- Lee, M-S., Chang, K-S., Cabanillas, F., Freireich, E. J., Trujillo, J. M., and Stass, S. A. Detection of minimal residual cells carrying the t(14;18) by DNA sequence amplification. *Science (Washington DC)*, **237**: 175-178, 1987.
- Billadeau, D., Quam, L., Thomas, W., Kay, N., Greipp, P., Kyle, R., Oken, M. M., and Van Ness, B. Detection and quantitation of malignant cells in the peripheral blood of multiple myeloma patients. *Blood*, **80**: 1818-1824, 1992.
- Naito, H., Kuzumaki, N., Uchino, J., Kobayashi, R., Shikano, T., Ishikawa, Y., and Mastumoto, S. Detection of tyrosinase hydroxylase mRNA and minimal neuroblastoma cells by the reverse transcription-polymerase chain reaction. *Eur. J. Cancer*, **27**: 762-765, 1991.
- Deguchi, T., Doi, T., Ehara, H., Ito, S., Takahashi, Y., Nishino, Y., Fujihira, S., Kawamura, T., Komeda, H., Horie, M., Kaji, H., Shimokawa, K., Tanaka, T., and Kawada, Y. Detection of micrometastatic prostate cancer cells in lymph nodes by reverse transcriptase-polymerase chain reaction. *Cancer Res.*, **53**: 5350-5354, 1993.
- Mattano, L. A., Moss, T. J., and Emerson, S. G. Sensitive detection of rare circulating neuroblastoma cells by the reverse transcriptase-polymerase chain reaction. *Cancer Res.*, **52**: 4701-4705, 1992.
- Smith, B., Selby, P., Southgate, J., Pittman, K., Bradley, C., and Blair, G. E. Detection of melanoma cells in peripheral blood by means of reverse transcriptase and polymerase chain reaction. *Lancet*, **338**: 1227-1229, 1991.
- Moreno, J. G., Croce, C. M., Fischer, R., Monne, M., Vikho, P., Mulholland, S. G., and Gomella, L. Detection of hematogenous micrometastasis in patients with prostate cancer. *Cancer Res.*, **52**: 6110-6112, 1992.
- Katz, A. E., Olsson, C. A., Raffo, A. J., Cama, C., Perlman, H., Seaman, E., O'Toole, K. M., McMahon, D., Benson, M. C., and Buttyan, R. Molecular staging of prostate cancer with the use of an enhanced reverse transcriptase-PCR assay. *Urology*, **43**: 765-775, 1994.
- Kwon, B. S., Haq, A. K., Pomerantz, S. H., and Halaban, R. Isolation and sequence of a cDNA clone for human tyrosinase that maps at the mouse *c-albino* locus. *Proc. Natl. Acad. Sci. USA*, **84**: 7473-7477, 1987.
- Brossart, P., Keilholz, U., Willhauck, M., Scheibenbogen, C., Möhler, T., and Hunstein, W. Hematogenous spread of malignant melanoma cells in different stages of disease. *J. Invest. Dermatol.*, **101**: 887-889, 1993.
- Brossart, P., Keilholz, U., Scheibenbogen, C., Möhler, T., Willhauck, M., Hunstein, W. Detection of residual tumor cells in patients with malignant melanoma responding to immunotherapy. *J. Immunother.*, **15**: 38-41, 1994.
- Scheibenbogen, C., Keilholz, U., Meuer, S., Dengler, T., Tilgen, W., and Hunstein, W. Differential expression and release of LFA-3 and ICAM-1 in human melanoma cell lines. *Int. J. Cancer*, **54**: 494-498, 1993.
- Ercolani, L., Florence, B., Denaro, M., and Alexander, M. Isolation and complete sequence of a functional human glyceraldehyde-3-phosphate dehydrogenase gene. *J. Biol. Chem.*, **30**: 15335-15341, 1988.
- Chomczynski, P., and Sacchi, N. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162**: 156-159, 1987.
- Keilholz, U., Scheibenbogen, C., Tilgen, W., Bergmann, L., Weidmann, E., Seither, E., Richter, M., Brado, B., Mitrou, P. S., and Hunstein, W. Interferon- $\alpha$  and interleukin-2 in the treatment of malignant melanoma: a comparison of two Phase II trials. *Cancer (Phila.)*, **72**: 607-614, 1993.

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