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Meeting Highlight

Diagnostic PCR in Melanoma, Methods and Quality Assurance. Epalinges, Switzerland, 26/27 January 1996

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INTRODUCTION

REVERSE TRANSCRIPTION of RNA and subsequent polymerase chain reaction (RT-PCR) facilitates assessment of gene expression under many circumstances. Tyrosinase PCR is emerging as a powerful diagnostic procedure to detect circulating tumour cells [1–8] or histologically undetectable lymph node involvement [9], and PCR detection of the MAGE family [10], MelanA/MART1 [11, 12] and gp 100 [13] are increasingly being used to determine the expression of these potential tumour rejection antigens. Since PCR methods are very sensitive for artifacts and the methods used for sample preparation, RNA extraction and cDNA synthesis differ among laboratories, standardised methods and standard quality control measures are necessary in order to facilitate comparison of results obtained by different research groups. This task is important for studying melanoma, but also for other solid tumours and haematological malignancies, where occult haematogenous spread and residual disease are monitored. Based on previous excellent experience with small expert meetings, a two day workshop organised by the immunotherapy subgroup of the EORTC Melanoma Cooperative Group on this topic was held at the Ludwig Institute in Epalinges, Switzerland.

Diagnostic PCR is mainly employed to address two questions, and the requirements for the PCR-based methods differ accordingly: (1) To detect circulating tumor cells. (2) To determine the expression of putative tumour rejection antigens.

Localised disease

For detection of circulating melanoma cells in patients with localised disease according to clinical staging, maximal sensitivity is desired. All workshop participants use the tyrosinase gene as the 'reporter gene' and the primers HTYR1–HTYR4 initially described by Smith and associates [1]. Tyrosinase is usually amplified by the highly sensitive nested PCR with two times 30 cycles (denaturation at 94°C for 90 sec, annealing at 60°C for the outer primers and 55°C for the inner primers, respectively, for 90 sec, and extension at 72°C for 90 sec). The times may be considerably shortened for several PCR machines without losing sensitivity. Most groups employ a 'hot start' technique to avoid non-specific bands. Nested PCR facilitates the detection of a single melanoma cell expressing the tyrosinase RNA in 10 ml of blood. Sue Burchill (Leeds) reported a similar sensitivity for a single round PCR with 40 cycles using the inner primers HTYR3 and HTYR4, if the PCR product is visualised by liquid hybridisation.

Advanced disease

In patients with haematogenous spread of melanoma, qualitative and semiquantitative assessment of circulating melanoma cells is of interest for studying tumour biology and for follow-up of minimal residual disease after treatment. In untreated stage IV patients, the percentage of blood samples tested positive for tyrosinase mRNA expression varies greatly, ranging from 35% to over 90% in different studies. It is currently unclear whether this is due to different patient groups, differences in sample preparation, or different PCR protocols. This topic was discussed in great detail, covering sample storage, RNA extraction procedures and protocols for cDNA synthesis.

Sample preparation

All laboratories require blood samples to be stored at −70°C within 2 h of being obtained from the patient. If longer intervals cannot be avoided, the samples should be kept at room temperature, but even then the sensitivity of the analysis may decrease. There was consensus that whole blood methods are superior to gradient separation techniques. Ulrich Keilholz (Heidelberg) reported a split sample analysis, where 5 out of 5 patient samples were tested positive for tyrosinase mRNA expression if a whole blood sample was analysed, but only one out of these five samples gave a positive result if the mononuclear cell fraction, obtained by density gradient separation over a specific gravity of 1.077 g/l, was tested. If the specific density of the separation medium was increased to 1.09 g/l, which yields separation of all peripheral blood leucocytes from erythrocytes, all five samples again gave positive results. In contrast to most melanoma cell lines, circulating

melanoma cells apparently possess sedimentation characteristics similar to granulocytes rather than mononuclear cells, and a proportion may be lost in the erythrocyte pellet.

Generally, the serum is decanted from the pellet of whole blood samples prior to storage, and the RNA extraction buffer is added prior to storage to inactivate RNAses. The impact of these measures on RNA quality is uncertain. Sue Burchill (Leeds) added that lysis of erythrocytes using lysis buffers should be avoided, since this leads to significant release of RNAses and frequent RNA degradation.

RNA extraction

A guanidinium-isothiocyanate-phenol-chloroform based method is employed for RNA extraction in all laboratories for detection of circulating tumour cells, since CsCl gradient methods, which yield RNA of higher purity, are too time consuming for routine measurements and require ultracentrifugation. Various modifications of the method first described by Chomczynski and Sacchi [14] have been introduced in most laboratories, but their impact on RNA yield and purity is only partially known. The RNA quantity is determined photometrically at wavelengths of 260 nm and 280 nm, but contaminating DNA and proteins can lead to false high RNA quantities. The RNA purity can be assessed using an ethidium bromide stained MOPS agarose gel, which also allows the assessment of relative quality and the detection of significant degradation.

cDNA synthesis

The protocols and enzymes vary among laboratories. Various reverse transcriptases are used. Random hexamer priming as well as oligo dT priming are employed with or without a prior incubation step for 5 min at 65°C to linearise secondary structures. The integrity of the cDNA can be verified by PCR amplification of a housekeeping gene. The value of this assessment for efficiency of reverse transcription should not be overestimated, since the housekeeping genes are so abundantly expressed that they can be detected even in cDNAs of rather poor quality, not suited for reliable detection of the tyrosinase gene, with a several orders of magnitude lower template number in peripheral blood. This problem can be reduced by limiting the amplification of the housekeeping gene to a number of cycles where the PCR is still in the exponential phase (approximately 20 cycles for tumour tissue and cell lines and up to 26 cycles for peripheral blood).

Internal control

It would be desirable to implement a constant internal control for each sample. Martina Willhauck (Heidelberg) presented a system where 1000 cells of the T-cell line Jurkat are added to the blood sample prior to processing. The Jurkat-specific T-cell receptor α chain is amplified with primers spanning parts of the Jurkat-specific variable region and the joining region. Tyrosinase is co-amplified in the same tube, and primer competition was not observed. Compared to control amplification of housekeeping genes, this system has the advantage that the control gene is expressed in the sample at a level comparable to the tyrosinase gene, allowing amplification of both genes for the same number of cycles. This can be equally useful for quantitative as well as qualitative assays.

Tumour antigens

Biopsies from tumour lesions and, to a lesser extent, peripheral blood samples are tested for expression of putative melanoma rejection antigens in the context of developing novel antitumour vaccines. For this purpose, maximal sensitivity of the PCR is not desired, since expression of antigens at a very low level or by a tiny subset of tumour cells would probably be irrelevant, i.e. too low to elicit a T-lymphocyte response [15].

The *MAGE* family was the first set of genes to be described as melanoma rejection antigens for T-lymphocytes. The family comprises 12 genes with a remarkable homology (69–98%) between coding sequences. For this reason, it has been difficult to develop specific primers for each gene, but the primer panel developed at the Brussels branch of the Ludwig Institute [10] discriminates between all 12 members of this family. Francis Brasseur (Brussels) stated that *MAGE* gene expression has been detected in more than 80% of skin, lymph node or visceral metastases of cutaneous melanoma in stage III and stage IV patients, and co-expression of two or more of *MAGE-1*, *-2* *-3* and *-4* genes has been observed in 70% of these lesions [16]. Bernard Lethé (Brussels) demonstrated that under stringent conditions and with reduced numbers of cycles, the PCR amplification of *MAGE-1*, *β-actin* and *GAPDH* is highly efficient and quantitative, facilitating quantitative assessment of MAGE expression in tumour tissue.

MelanA/MARTI and gp100 denominate gene products frequently serve as target antigens of tumour infiltrating lymphocytes. Various primers are being used for PCR amplification yielding good results for MelanA (Donata Rimoldi, Epalinges; Martina Willhauck, Heidelberg; Francis Brasseur, Brussels), but unsatisfactory results for gp100. The genomic sequence of gp100 is not fully known. Teun de Vries (Nijmegen) reported that several primers for gp100 co-amplify genomic DNA. Optimal primer pairs for gp100 remain to be developed, possibly employing the advantage of the only known 102 nucleotides long intron, thus enabling discrimination between genomic DNA and mRNA amplification. Donata Rimoldi and Stefan Carrel (Epalinges) found MelanA and tyrosinase to be either co-expressed (90%) or both absent (10%) in the majority of melanoma specimens. The pattern of expression is usually conserved in multiple metastases from an individual patient.

Carmen Scheibenbogen (Heidelberg) presented data concerning the heterogeneity of the gp100 protein expression in melanoma metastases. In a considerable number of patients with advanced disease, the tumour cells do not stain with the antibody HMB45, but the mRNA for gp100 can be detected with nested PCR in all samples. In acccordance, several melanoma cell lines negative for gp100 protein by flow cytometry and immunocytology express gp100 mRNA. Apparently, positive signals obtained with the highly sensitive nested PCR are not sufficient proof of significant gp100 protein expression. Bernard Lethé and Francis Brasseur reported that strong gp100 signals can be obtained with tumour samples after only 22 cycles of single round PCR.

Future prospects

A series of blinded samples for analysis of tyrosinase mRNA has been distributed to prospectively compare sensitivity and reliability of the assays among the participating laboratories. Furthermore, standard cell lines and cDNAs as well as detailed laboratory protocols have been exchanged to optimise and standardise the PCR methods for tumour antigens.

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