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F. Gudat · M. Zuber · U. Dürmüller · T. Kocher
C. Schaefer · C. Noppen · G. Spagnoli

The tumour-associated antigen MAGE-1 is detectable in formalin-fixed paraffin sections of malignant melanoma

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Abstract The *MAGE-1* gene encodes a protein encompassing a HLA-A1-restricted target epitope for cytolytic T lymphocytes. Monoclonal antibodies directed against the MAGE-1 protein were tested for usage in immunohistology of routine pathology material. Seven formalin-fixed, paraffin-embedded malignant melanomas were studied by the Avidin–Biotin complex (ABC) method with or without different antigen retrieval methods. Native, frozen tissues from the same tumours were used to validate the results by immunohistochemistry on frozen sections, by PCR for mRNA and by protein demonstration in tissue extracts using western blotting. Of 4 monoclonal antibodies tested, mAB 34B and mAB 77B were highly efficient in detecting MAGE-1 protein in deparaffinised sections with the regular ABC method after microwave pretreatment. In a series of an additional 28 patients 75% expressed MAGE-1, 50% in a substantial proportion. Follow-up studies in 6 patients indicate that the expression pattern remains stable but may change substantially within a short range. Immunohistology is thus a rapid and well-established method that might be used to select and monitor HLA-A1 positive patients with malignant melanoma and other candidate tumours for MAGE-1-directed immuno-therapy.

Key words Malignant melanoma · *MAGE-1* · Immunohistology · Tumour antigen · Tumour markers · Biological immunology

Introduction

The human *MAGE*-gene family (1–3) has been located to the X chromosome [9]. *MAGE-1* gene encodes an antigen originally detected on the melanoma cell line MZE-MEL and termed MZ2-E [19]. This HLA-A1-restricted antigen has been shown to serve as a target epitope for cytotoxic T lymphocytes [12, 18]. *MAGE-1* gene expression was detected by PCR in a fraction of malignant melanomas but also in neurogenic tumours [11], carcinomas of breast [2, 13], oesophagus [17], stomach [5], urinary bladder [10] and lung [15], and T- and B-cell leukaemia [16]. *MAGE-1* gene product has been identified as a cytoplasmic protein in melanoma cell lines [1, 14]. In HLA-A1-positive patients, the demonstration of MAGE-1 protein expression in tumour cells would provide the rationale for an active immunotherapy specifically directed against this rejection target. We report here the detection of the MAGE-1 protein by immunohistochemistry on native and formalin-fixed, paraffin-embedded melanoma biopsies, allowing rapid and easy identification of eligible patients.

F. Gudat (✉) · U. Dürmüller
Institute for Pathology, University of Basel,
Schönbeinstrasse 40, CH-4003 Basel, Switzerland
Fax: (41) 61–2 65 31 94

M. Zuber · T. Kocher · C. Schaefer · C. Noppen · G. Spagnoli
Departments of Surgery and Research, University of Basel,
Hebelstrasse 20, CH-4003 Basel, Switzerland

Material and methods

Detection of *MAGE-1* gene expression

Total cellular RNA was extracted from snap-frozen malignant melanoma samples and reverse transcribed as previously reported [4, 6]. The cDNA thus generated was tested in 25 and 35 cycles of polymerase chain reaction (PCR) assays in the presence of pairs of primers encompassing specific gene sequences encoding β-actin [6] or *MAGE-1* [2, 20]. Each cycle included 40 s denaturation at 94°C, 40 s annealing at 62°C and 1 min extension at 72°C. The amplification products were run on 1.5% agarose gels in the presence of ethidium bromide and photographed upon UV trans-illumination. The expected sizes of the amplified sequences are 661 bp for β-actin and 421 bp for *MAGE-1*. Specificities of PCR products were confirmed by restriction mapping [14].

Detection of MAGE-1 protein expression

Frozen melanoma samples were mechanically minced and lysed in a lysis buffer. Total protein content was then measured by spectrometry and 100 µg of protein from each sample were run on standard SDS-PAGE gels. Subsequently, gels were blotted on nitro-cellulose membranes, which after a 2-h incubation in TBE, 1%

casein were probed with undiluted supernatants of selected hybridomas. After an overnight incubation, specific binding was revealed by a chemiluminiscence based technique (ECL, Amersham, UK) as described [14].

Immunohistochemistry

The murine monoclonal antibodies used (27R, 77B, 14B, 34B) were produced after immunisation of mice with recombinant MAGE-1 protein as immunising antigen, as described elsewhere [14].

Frozen sections of tumour tissue stored at –70°C were thawed, fixed in acetone (10 min, room temperature) and incubated with undiluted supernatants overnight. This was followed by two washes in PBS and postfixation in paraformaldehyde-lysine-periodate [7]. The alkaline–anti-alkaline phosphatase (APAAP) method was used, with a commercial rabbit anti-mouse link antibody and APAAP complex for visualisation of specifically bound antibodies according to the recommendations of the supplier (DAKOPATTS A/S, Glostrup, Denmark).

Matched formalin-fixed tumour tissue embedded in paraffin was processed for immunohistochemistry according to our standard method [8] employing the avidin–biotin complex method (Vector, Burlingame, Calif.). Pretreatment of the sections after deparaffinisation included incubation with pronase (0.1%, 15 min) or heating in a microwave oven (60 min at 90°C). The final working conditions included microwaving and dilution of 1:16 of the primary antibody. This was applied to a series of 41 biopsy specimens taken from malignant melanomas in 28 patients. Three kidneys and two liver biopsies were used as normal tissue controls.

Specificity was monitored by replacement of the monoclonal antibody by phosphate-buffered saline and by an absorption control. For the latter, recombinant *MAGE-1* (r*MAGE-1*) antigen was prepared as previously described [14]. A semipurified product could be obtained by loading bacterial lysates on a nickel Sepharose column (Novagen, Madison, Wis.) and eluting the bound fusion protein at pH 4. The fusion protein was then immobilised on CNBr-activated Sepharose 4B beads at 1.5 mg of protein per ml of packed beads (Pharmacia Biotechnology, Uppsala, Sweden) according to the manufacturer's instructions. Equal volumes of beads and supernatant (77B) were incubated at room temperature. After 30 min the beads were centrifuged and the supernatant was used as absorbed control antibody.

Results

Gene expression

Expression of β-actin house-keeping gene was detectable in all samples following 25 cycles RT-PCR (Fig. 1A). In contrast, *MAGE-1* gene expression was only detectable with the same number of PCR cycles in sample 3 (Fig. 1B). Similar data were obtained following 30 PCR cycles. However, after 35 cycles of amplification a positive signal was observed in three more patients' samples, leaving only patients 1, 6 and 7 whose samples did not express *MAGE-1* (Fig. 1C, Table 1).

Protein expression

On immunohistochemistry, all four antibodies gave a positive cytoplasmic signal on frozen sections. On paraffin sections, this was seen only with antibodies 77B and 34B after microwave pretreatment, and not with enzymatic pretreatment (Fig. 2, Table 1). In formalin-fixed,

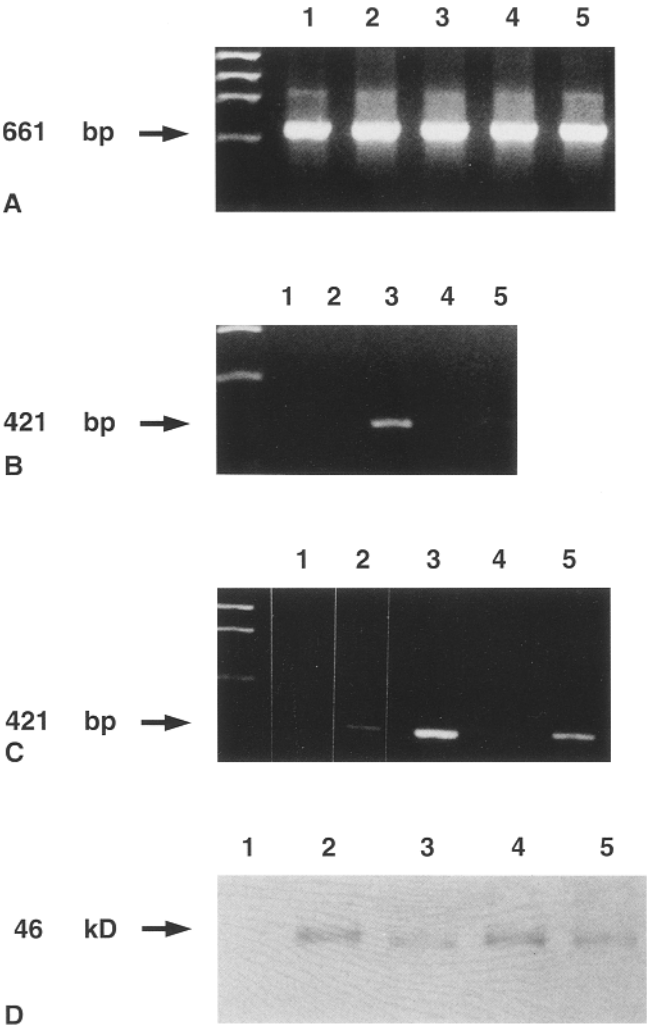

Fig. 1A–D *MAGE-1* gene expression and protein detection in clinical melanoma samples (patients 1–5 in Table 1). DNA obtained was amplified in the presence of beta-actin (**A**) on *MAGE-1*-specific primers (**B, C**). PCR was allowed for 25 (**A, B**) or 35 cycles (**C**). The Western blot shows the presence of 77B mAB-specific binding for patients 2–5 but not for patient 1 (**D**)

Fig. 2A–F Immunohistology of MAGE-1 in malignant melanoma. **A** Extensive cluster of positive cells showing a granular cytoplasmic staining with mAB 77B. Frozen section, APAAP technique, ×350. **B** Single positive cells with discrete red label (*arrows*) surrounded by pigmented melanocytes illustrating the lack of cross-reactivity between these proteins. Frozen section, APAAP technique, ×550. **C** Focal cytoplasmic expression showing heterogeneity of staining intensity. Note also cytoplasmic invaginations into nuclei which may be labelled (*arrow*). Paraffin section, ABC technique, ×350. **D** Control incubation for C (replacement of specific antibody by PBS; identical picture with MAGE-1 absorbed mAB 77B. Paraffin section, ABC technique, ×350. **E** Extensive expression showing the granular appearance of the antigen and only vague association with the inner aspect of the cell membrane. Paraffin section, ABC technique, ×350. **F** Lack of cross-reactivity with melanosomes in paraffin sections as demonstrated by two interspersed labelled cells (*arrows*). Paraffin section, ABC technique, ×550

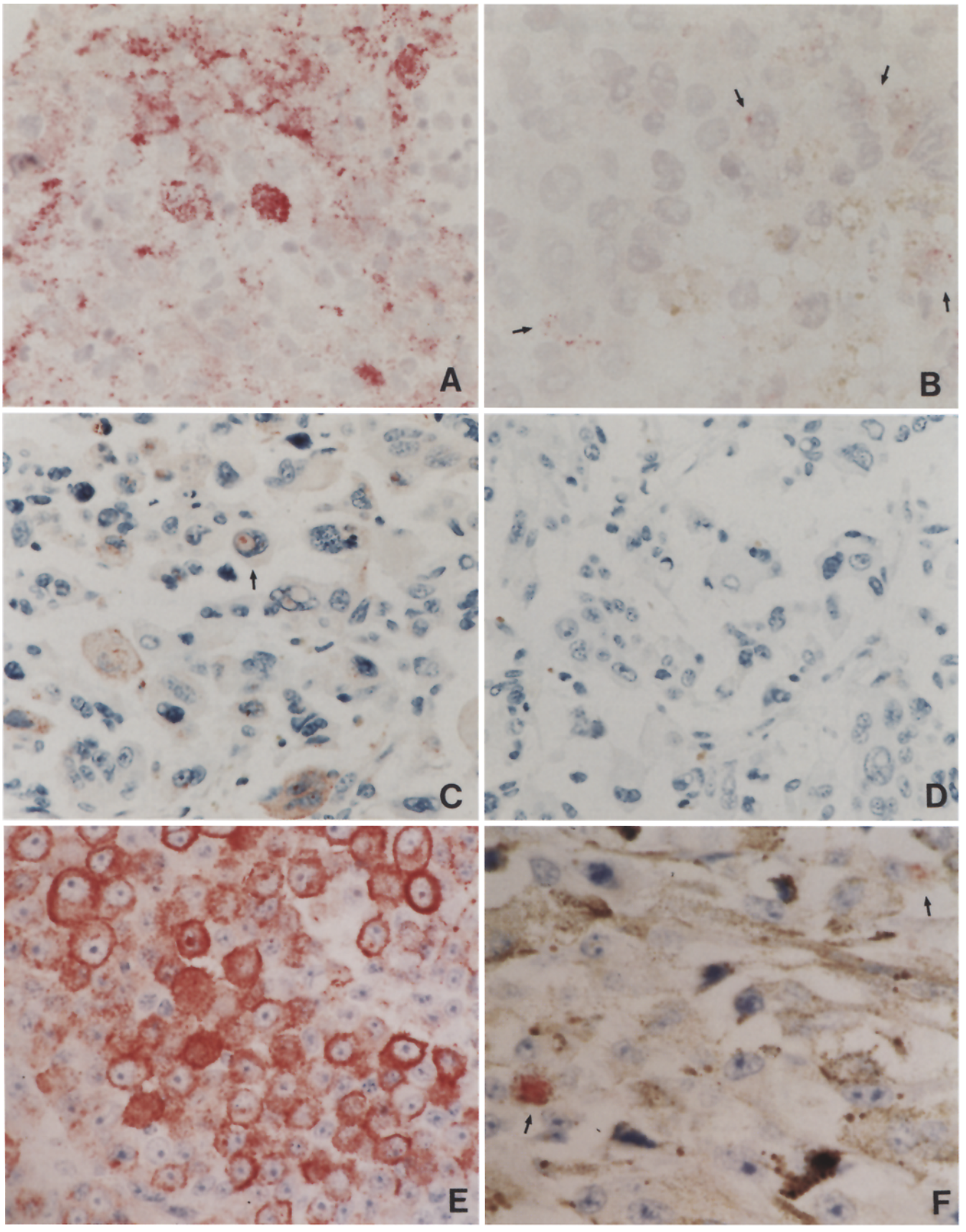
A
B
C
D
E
F

Table 1 MAGE-1 expression in frozen and formalin-fixed malignant melanomas on immunohistochemistry and PCR determination (*Neg.* negative, *focal* focally positive, *single cells* single cells positive, *pos (wk)* weakly positive)

	mAB 77B		mAB 34B		PCR	Western Blot
	Paraffin embedding Formalin	Frozen tissue Acetone-PLP fixation	Paraffin embedding Formalin	Frozen tissue Acetone-PLP fixation		
Malignant melanomas						
Patient 1	Neg.	Single cells	Neg.	Neg.	Neg.	Neg.
Patient 2	Focal	Neg.	Single cells	Single cells	Pos.**	Pos.
Patient 3	Focal	Focal	Focal	Focal	Pos.*	Pos.
Patient 4	Focal	Single cells	Focal	Focal	Pos.** (wk)	Pos.
Patient 5	Neg.	Neg.	Neg.	Neg.	Pos.**	Pos.
Patient 6	Focal	Single cells	Single cells	Neg.	Neg.	Pos. (wk)
Patient 7	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
Normal tissues						
Kidney (*n*=3)	Epithelia of distal tubules	Neg.	Epithelia of distal tubules	Neg.	Neg.	Neg.
Liver (*n*=2)	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.

[a] 25 and 30 cycles
[b] After 35 cycles only

paraffin-embedded material, the granular reaction product appeared slightly coarser and was detectable in more cells than in corresponding frozen tissue. MAB 77B was more effective than mAB 34B in this respect. The granules were either evenly distributed or, occasionally, oriented along the cell membrane. As a rule, there was a considerable tumour heterogeneity with respect to distribution of positive cells and staining intensity. Cytoplasmic invaginations into distorted nuclei were a constant finding, simulating intranuclear inclusions. Melanin granules, if present, were not stained (Fig. 2). Absorption of mAB 77B with the immobilised recombinant protein abolished the granular staining of the cytoplasm.

Two cases were negative for MAGE-1 expression, and one additional biopsy showed single cells only in the frozen section. Two of these cases were negative by PCR and Western blotting but one exceptional case (5) gave a discordant result in that PCR was weakly and Western blotting was strongly positive.

In a series of 28 consecutive primary biopsies embedded in paraffin, 7 (25%) were negative for *MAGE-1*, 8 (29%) contained single positive tumour cells, 7 (25%) showed focal and 6 (21%) extensive cellular expression. For six patients additional follow-up biopsies were available. Five of these were stable with respect to the range of labelled cells over observation times of 2, 6, 7, 9 and 16 months. One patient, however, had a shift from negativity to focal positivity after 3 months.

Discussion

The results show that *MAGE-1* antigen can be demonstrated efficiently on paraffin sections of tumour specimens by using mAB 77B and mAB 34B as a confirmatory antibody. Cross-linking by formalin fixation and subsequent antigen retrieval by microwave pretreatment enhances MAGE-1 protein detection over that possible with the frozen section technique.

Another important feature of the MAGE-1 protein is the heterogeneity of its expression within an individual tumour specimen. Basically, antigen expression was not homogeneous but focal, and the cellular antigen load varied considerably. The follow-up data indicate that the expression pattern remains stable within the observed range of 2–16 months. However, a significant change from no expression to focal change was observed in one case within 3 months.

An apparent discordance between gene expression and protein detection was observed, and this might be partly attributed to the heterogeneity of protein expression. However, considering that *MAGE-1* gene product was detected by immunohistochemistry in samples where no or little specific mRNA was amplified, the hypothesis could be set up that the protein expression is switched on and off at the transcriptional level and that intracellular protein accumulates. These data suggest that even in tumours where no *MAGE-1* gene expression is detectable following a standard 25 cycles of PCR, specific immunotherapy procedures could still be envisaged. Considering the so-called innocent bystander phenomenon, recognition of few targets displaying specific antigenic epitopes could lead to the cytokine-mediated killing of antigen devoid target cells. In addition, the cytokine environment generated by the specific antigen recognition might favour the induction of unselected tumour-specific cytotoxic T lymphocytes [3]. By the same token, in the one case with the reversed situation, in which protein was undetectable by immunohistochemistry despite evidence of active transcription and protein synthesis, an efficient immune elimination might be expected.

It is well established that the *MAGE-1* antigen is a T-lymphocyte-dependent, tumour-associated antigen that can be envisaged as a target for immunotherapy in patients co-expressing the *HLA-A1* allele [18]. In the series of 28 patients 75% expressed *MAGE-1*, and 50% in a substantial portion of the tumour. Such patients co-expressing the HLA-A1 haplotype could profit from immunohistology of pathology material prospectively and retrospectively: newly detected patients could be selected for specifically targeted immunotherapy. The effect of such therapy could be conveniently monitored and failures attributable to immunoselection could be recognised. These procedures could also be applied to archival material and to other tumours known to express the *MAGE-1* antigen system.

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