Level of a Membrane-bound High-Molecular-Weight Melanoma-associated Antigen and a Cytoplasmic Melanoma-associated Antigen in Surgically Removed Tissues and in Sera from Patients with Melanoma

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ABSTRACT

Utilizing double-determinant immunoassays (DDIAs), the high-molecular-weight melanoma-associated antigen (HMW-MAA) was detected only in fetal skin and in one nipple of 54 normal tissues from adults tested, while the cytoplasmic MAA recognized by the monoclonal antibody 465.12S was found in most of the normal tissues tested. Among malignant lesions, the HMW-MAA was found in melanomas, astrocytomas, and skin carcinomas; the cytoplasmic MAA was found in all of the malignant lesions tested, even those which originated from normal tissues without detectable cytoplasmic MAA. The levels of the HMW-MAA and of the cytoplasmic MAA showed marked variations in malignant lesions removed from various patients, as well as in autologous metastatic lesions removed from four patients with melanoma. No relationship was found between the degree of expression of the two MAA analyzed and the clinical stage of the disease. Both types of MAA were found in sera from patients with melanoma or other types of cancers, as well as in sera from healthy donors. The level of the HMW-MAA tended to be higher in patients with Stage IV melanoma.

INTRODUCTION

The hybridoma technology has been successfully applied to develop monoclonal antibodies to human MAA, i.e., antigens expressed by melanoma cells but undetectable in resting melanocytes (1–3, 5, 9–11, 13–16, 18, 20, 22, 23). The high degree of specificity of these reagents has rekindled interest in the development of immunodiagnostic and immunotherapeutic approaches to melanoma. Essential to these reagents is the information about the expression of the corresponding antigens in normal and malignant tissues. Therefore, the analysis of the reactivity pattern of monoclonal antibodies has moved from the use of cell lines in long-term culture to that of surgically removed tissues (1, 6, 9–11, 19–22, 25, 27).

We have focused our studies on a membrane-bound HMW-MAA and on a cytoplasmic MAA; the latter is the only cytoplasmic MAA thus far identified with monoclonal antibodies, while the former is similar to the MAA identified with the monoclonal antibodies developed by Bumol and Reisfeld (2), Carrel et al. (3), and Hellstrom et al. (9). By IIF staining of a large number of surgically removed normal and malignant tissues, we have shown that the HMW-MAA is detectable only in melanomas, nevi, and a low percentage of skin carcinomas (20), while the cytoplasmic MAA has a much wider tissue distribution (22).

IIF relies on subjective evaluation of the results and yields qualitative data. To overcome these limitations, in this study, we have applied DDIAs to quantitate the HMW-MAA and the cytoplasmic MAA in normal and malignant surgically removed tissues, as well as in sera of healthy donors and patients with melanoma or other types of disorders.

MATERIALS AND METHODS

Cell Lines in Long-Term Culture and Surgically Removed Tissues.

The melanoma cell lines Colo 38, HO-1, SK-MEL 37, and SK-MEL 93 were perpetuated in medium RPMI 1640 supplemented with 2 mm L-glutamine, 10% calf serum, and gentamicin at a concentration of 10 μg/ml.

Normal tissues were obtained from a 20-week-old fetus and from patients undergoing plastic or ablative surgery. The latter, at the time of excision, were not treated with chemotherapy and/or radiotherapy. Monoclonal suspensions were prepared by mincing with scissors either the malignant tissue (following removal of normal surrounding tissue) or the normal tissue, tearing the tissues with forceps, and filtering them through a fine mesh metal net. Cells were then washed 5 times with PBS.

Extracts were prepared by incubating cells (2 x 10⁷) for 30 min at 4° in 1 ml of a 1% solution of Nonidet P-40 detergent in PBS containing 10 μm phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO). This extraction procedure was used because it had been shown to be the most efficient in solubilizing the HMW-MAA and cytoplasmic MAA (21). The extract was cleared by centrifugation at 7000 x g for 15 min and stored at -20° until use, unless otherwise stated.

Sera. Sera were obtained from healthy donors and from patients with melanoma or other types of malignant diseases. The latter were receiving various types of chemotherapy. Sera from 20 patients with melanoma were obtained within 48 hr after local hyperthermic perfusion. Clinical staging of melanoma patients was performed according to the method of Sugarbaker and McBride (24).

Monoclonal Antibodies to the HMW-MAA and Cytoplasmic MAA.

The monoclonal antibody 149.53, an IgG1, the monoclonal antibody 465.12S, an IgG2a, and the monoclonal antibody 763.74T, an IgG1, to distinct determinants of the HMW-MAA, and the monoclonal antibody 465.12S, an IgG2a, to distinct determinants of a cytoplasmic MAA, have been prepared and characterized as described elsewhere (7, 12, 22). Antigens to be used for coating of plates were purified from ascites fluid by sequential 33% ammonium sulphate precipitation and by ion-exchange chromatography on DEAE-cellulose. Antigens to be radiolabeled with 125I were further purified either by affinity chromatography on protein A-Sepharose, if belonging to the IgG2a subclass, or by gel exchange chromatography on DEAE-cellulose.
filtration on Sephadex G-200 (Pharmacia, Uppsala, Sweden), if belonging to the IgG1 subclass. The purified antibodies were radiolabeled with $^{125}$I using the chloramine T method (8).

**DDIAs to Quantitate the HMW-MAA and the Cytoplasmic MAA.** Unless otherwise stated, the combination insolubilized monoclonal antibody 763.74T:$^{125}$I-monoclonal antibody 225.28S was used to quantitate the HMW-MAA. The monoclonal antibody 465.12S was used both in the insolubilized form and in the radiolabeled form to quantitate the cytoplasmic MAA, since the corresponding determinant is expressed more than once on the cytoplasmic MAA. DDIAs were performed according to the procedure described elsewhere (7). Briefly, polyvinylchloride microtiter plates (Dynatech, Alexandria, VA) were coated with a monoclonal antibody solution in 0.1 M NaHCO$_3$ buffer, pH 9.5, and washed 3 times with 0.9% NaCl solution (saline) containing 0.05% T-20. Cell extracts or their dilutions in PBS:T20 supplemented with 5% calf serum were added to wells. After a 2-hr incubation, plates were washed 3 times with saline:T-20. Then, $^{125}$I-labeled monoclonal antibodies ($1 \times 10^6$ cpm) were added to single wells and incubated for an additional 2 hr. After 5 washings with saline:T-20, wells were cut, and the bound radioactivity was counted in a $\gamma$-counter. The radioactivity specifically bound by the antigen was determined by subtracting the non-specifically bound cpm from the total bound cpm. Results are expressed in ng of $^{125}$I-labeled monoclonal antibody 225.28S added to wells. After a 2-hr incubation, plates were washed 3 times with saline:T-20. Then, $^{125}$I-labeled monoclonal antibodies ($1 \times 10^6$ cpm) were added to single wells and incubated for an additional 2 hr. After 5 washings with saline:T-20, wells were cut, and the bound radioactivity was counted in a $\gamma$-counter. The radioactivity specifically bound by the antigen was determined by subtracting the non-specifically bound cpm from the total bound cpm. Results are expressed in ng of $^{125}$I-labeled monoclonal antibodies bound. Human sera were tested undiluted in the amount of 200 µl for the HMW-MAA and at a 1:50 dilution in the amount of 200 µl for the cytoplasmic antigen. The dilution was required, since the amount of the cytoplasmic antigen in undiluted sera is much higher than the upper level of sensitivity of the DDIA.

**Indirect Immunofluorescence.** IIF was performed as described previously (20). Briefly, 4-µm thick acetone-fixed cryostat sections were sequentially incubated with monoclonal antibody solutions (5 to 25 µg/ml) and with fluorescein-conjugated antimouse immunoglobulin xenonatiserum. Fluorescent patterns were observed in a Leitz Ortholux microscope equipped for phase-contrast microscopy and fluorescent epillumination. The immunofluorescence staining of lesions was scored as follows: negative, when no stain was detected, even with oil immersion objectives; bare trace, when the stain was detected only with oil immersion objectives; trace, when the stain was homogeneous and weak; 1+ when at least 50% of the lesion had trace stain; and 2+ when staining was homogeneously bright.

**RESULTS**

**Selection of Optimal Conditions for Storing Tissue Extracts without Affecting the Level of MAA.** Because of the high sensitivity of the HMW-MAA to proteolytic enzymes (26), preliminary experiments determined the optimal conditions for storing tissue extracts without affecting the level of the HMW-MAA and of the cytoplasmic MAA.

Overnight storage of cell extracts at 37° was associated with a 30% reduction in the level of the HMW-MAA. No reduction was detected when antiproteases had been added to tissue extracts. The level of the HMW-MAA did not significantly change in cell extracts and spent culture media stored at -20°, 4°, and 20° for up to 2 days, even when antiproteases had not been added. Furthermore, temperature of storage and addition of antiproteases did not affect the levels of HMW-MAA in spent culture media. The results of the DDIA were similar with all of the combinations of monoclonal antibodies 149.53, 225.28S, and 763.74T to distinct determinants of the HMW-MAA, indicating that the storage conditions investigated had no preferential effect on the subpopulations of the HMW-MAA measured with the monoclonal antibodies used.

The temperature of storage of cell extracts and spent culture media had no effect on the level of the cytoplasmic MAA. Therefore, tissue extracts as well as sera were stored at -20°.

**Level of the HMW-MAA and of the Cytoplasmic MAA in Normal Tissues and in Benign Lesions.** The HMW-MAA was detected in only 1 of 2 normal nipples of 54 normal tissues from adults tested. They include bladder (3 cases), brain cortex (2 cases), cartilage (2 cases), colon (2 cases), duodenum (1 case), endometrium (1 case), kidney (2 cases), liver (1 case), lung (4 cases), lymph node (3 cases), mammary gland (3 cases), ovary (1 case), pancreas (1 case), peripheral blood lymphocytes (4 cases), peripheral nerve (1 case), prostate (2 cases), salivary gland (3 cases), skeletal muscle (1 case), skin (5 cases), spleen (1 case), stomach (3 cases), thyroid (2 cases), tonsil (1 case), and testis (5 cases) (Charts 1 and 3).

On the other hand, all of these normal tissues, except for lymph nodes, mammary tissue, ovary, peripheral blood lymphocytes, and skin, contain the cytoplasmic MAA (Charts 2 and 3). Its level shows significant differences in the various tissues tested, as well as in the same type of tissue obtained from different donors. The level of the cytoplasmic MAA does not appear to correlate with the embryological origin of the tissues or with the age and sex of the donor. The only fetal tissue available was skin from a 20-week-old fetus. The HMW-MAA was found at a low level, while the cytoplasmic MAA was not detected.

Among the 11 dysplastic lesions and the 7 benign tumors tested, the HMW-MAA was detected only in a lymphangioma, while the cytoplasmic MAA was expressed by all of the lesions tested (Charts 1 and 2). Its level was within the ranges found in normal tissues.

**Level of the HMW-MAA and of the Cytoplasmic MAA in Melanoma Lesions and in Other Malignant Skin Tumors.** The HMW-MAA and the cytoplasmic MAA were found in 3 and 4 of 6 primary melanoma lesions and in 12 and 14 of 22 metastatic melanoma lesions, respectively (Chart 3). The level of both types of MAA was not different in primary and metastatic melanomas. In both types of lesions, there were marked variations in the levels of the 2 MAA analyzed. No relationship was found between...
MM in Tissues and Sera

Chart 2. Level of the cytoplasmic MAA in Nonidet P-40 extracts of the following: Lymphoid tissues, number of samples: peripheral blood lymphocytes, 3; lymph nodes, 3; spleen, 1; and tonsil, 1. Dysplastic lesions: breast dysplasia, 2; breast cyst, 1; and interstitial pneumonitis, 8; lymphoma, 1; neoplasia, 1; and intestinal polyp, 1. Brain tumors: astrocytoma, 4; glioblastoma, 3; meningioma, 1; and schwannoma, 1. Breast carcinomas: infiltrating duct carcinoma, 13. Gastrointestinal carcinomas: colorectal, 4; liver, 3; and stomach, 3. Genitourinary tract carcinomas: bladder, 2; kidney, 9; and ovary, 1. Lung carcinomas: adenocarcinoma, 3; mesothelioma, 1; and squamous cell carcinoma, 12. Sarcomas: botryoid sarcoma, 1; liposarcoma, 1; and rhabdomyosarcoma, 1. Open Symbols, level of HMW-MAA in Nonidet P-40 extracts of the normal tissue counterpart of malignant tumors. Bars, mean values; arrows, S.D. MoAb, monoclonal antibody. The cytoplasmic antigen was detected also in normal cartilage (2 samples) and normal thyroid (2 samples), which are not shown.

the degree of expression of the 2 MAA analyzed. The level of neither MAA appeared to correlate with the clinical stage of the lesion and with the site of origin of the metastasis. Metastases removed from 2 sites were available from 4 patients. In 2 patients, there were significant differences in the levels of the HMW-MAA and of the cytoplasmic MAA in autologous lesions (Table 1).

Among skin tumors of non-melanocyte origin, the HMW-MAA and the cytoplasmic MAA were found in 7 and 17, respectively, of 19 squamous cell carcinomas and in 1 of 3 basal cell carcinomas. The level of both types of MAA overlapped with that in melanoma (Chart 3).

Levels of the HMW-MAA and of the Cytoplasmic MAA in Malignant Tumors Other Than Melanoma and Skin Carcinomas. Among brain tumors, the HMW-MAA was found in all of the 4 astrocytomas and in 1 of 3 meningiomas tested, but it was not detected in the 3 glioblastomas tested. In 2 astrocytomas, the level of the HMW-MAA reached almost the highest level found in one primary melanoma. Among the remaining malignant tumors, the HMW-MAA was detected only in 2 squamous cell carcinomas and one adenocarcinoma of the lung, in one mesothelioma, in one liver carcinoma, in one botryoid sarcoma, and in one rhabdomyosarcoma. Except for the latter tumor, the level of the HMW-MAA in these lesions was low (Chart 1).

The cytoplasmic MAA was found in all of the types of solid malignant tumors tested, even those derived from normal tissues without detectable cytoplasmic MAA. The latter include mammary and ovary carcinomas. There was no significant difference in the mean value of the cytoplasmic MAA among various types of malignant tumors. The range of values within each group of tumors was broad (Chart 2).

Level of Subpopulations of HMW-MAA in Malignant Tumors. The DDIA performed with the monoclonal antibodies 149.53, 225.28S, and 763.74T in different combinations detects different levels of the HMW-MAA in Nonidet P-40 extracts and in spent media of melanoma cells in long-term culture. The amount of the HMW-MAA detected by the combinations of the 3 monoclonal antibodies does not correlate with the level of their binding to melanoma cell lines (Chart 4). All positive lesions were tested by the 3 possible combinations of monoclonal antibodies.

Table 1

<table>
<thead>
<tr>
<th>Patient</th>
<th>Metastasis</th>
<th>HMW-MAA</th>
<th>Cytoplasmic MAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. G.</td>
<td>M1</td>
<td>&lt;0.05&lt;</td>
<td>&lt;0.05&lt;</td>
</tr>
<tr>
<td>C. A.</td>
<td>M2</td>
<td>&lt;0.05&lt;</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>M1</td>
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<td>5.33</td>
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<tr>
<td></td>
<td>M2</td>
<td>0.85</td>
<td>&lt;0.05&lt;</td>
</tr>
<tr>
<td>B. C.</td>
<td>M1</td>
<td>&lt;0.05&lt;</td>
<td>13.60</td>
</tr>
<tr>
<td></td>
<td>M2</td>
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<td>13.10</td>
</tr>
<tr>
<td>F. P.</td>
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<td>6.21</td>
</tr>
<tr>
<td></td>
<td>M2</td>
<td>1.93</td>
<td>6.22</td>
</tr>
</tbody>
</table>

<0.05< represents the lowest amount of monoclonal antibody detected by the double determinant immunoassay.

Data about the level of the HMW-MAA in these samples have already been published in Ref. 7.
Ratios of subpopulations of the HMW-MAA exhibited a significant variation in about 50% of the lesions tested. The combination insolubilized monoclonal antibody 763.74T:[125I]monoclonal antibody 225.28S detected the highest levels of HMW-MAA in the majority of cases. Only in one case (Melanoma Case 84), one subpopulation of the HMW-MAA (the one recognized by the combination MoAb 225.28S and 763.74T) was not detected, while the other 2 subpopulations were found in significant amounts (Chart 5).

Agreement Between the Results of DDIA and of IIF. Eighty-eight lesions were tested with anti-HMW-MAA monoclonal antibodies in the 2 assays. The results were concordant in 86% of the cases. The discrepancies were caused by 7% of the extra-positive reactions in each of the 2 assays. The 2 assays agreed in the intensity of positivity in 64% of the positive reactions. Twenty-four % of the discordant results were caused by more intensely positive reactions in the DDIA than in IIF, while the remaining discrepancies were caused by a higher degree of positivity in the latter than in the former assays.

Testing of 85 lesions with the monoclonal antibody 465.12S utilizing the DDIA and IIF yielded concordant results in 75% of the cases. The discrepancies were caused by about 7 and about 18% of extrapositive reactions in IIF and in DDIA, respectively. Fifty-three % of the positive reactions had a similar degree of positivity in the 2 assays. Thirty and 17% of the discordant results were more intensely positive in the DDIA and in the IIF, respectively, than in the other assays.

Levels of HMW-MAA and of Cytoplasmic MAA in Sera From Patients with Melanoma, From Healthy Donors, and From Patients with Various Types of Disorders. The HMW-MAA was found in about 87% of sera from 134 patients with melanoma, in 94% of sera from 49 healthy donors, and in 88% of sera from 88 patients with various types of disorders. The latter include malignant diseases other than melanoma (Chart 6). The cytoplasmic MAA was found in the sera from all of the patients and controls tested (Chart 7). Levels as well as ranges of both types of MAA in patients with melanoma were not different from those in...
MAA in Tissues and Sera

Chart 8. Level of the HMW-MAA in sera from melanoma patients at different clinical stages; when blood was drawn, patients either were tumor free (O) or carried clinically detectable tumor masses (•). MoAb, monoclonal antibody. Bars, mean values; arrows, S.D.

Chart 9. Level of the cytoplasmic MAA in sera from melanoma patients at different clinical stages; when blood was drawn, patients either were tumor free (O) or carried clinically detectable tumor masses (•). MoAb, monoclonal antibody. Bars, mean values; arrows, S.D.

in controls and in patients with other types of tumors. Only in patients with breast carcinoma did the level of the cytoplasmic MAA tend to be higher than in other groups, but the difference was not statistically significant. The level of the HMW-MAA was higher in patients with stage IV melanoma than in those with melanoma in other stages. Furthermore, in Stage II and III melanoma, the level of the HMW-MAA was higher in patients with clinically detectable tumor masses than in patients who were tumor free at the moment of blood drawing (Chart 8). However, the differences in both groups did not reach the level of statistical significance. The level of the cytoplasmic MAA did not show any relationship with the stage of the disease in patients with melanoma (Chart 9).

Sera from 20 patients with melanoma were obtained before and within 48 hr after local hyperthermic perfusion (24). The level of both types of MAA increased in 6 patients and decreased in 3. In the remaining 11 patients, the change in the level of the 2 MAA was discordant; specifically, the increase in the level of the HMW-MAA was associated with lack of change and with a reduction in the level of the cytoplasmic MAA in 1 and 3 patients, respectively. The lack of change in the level of the HMW-MAA was associated with the increase of the cytoplasmic MAA in 2 patients and with its reduction in 2 patients. The reduction in the level of the HMW-MAA was associated with an increase of the cytoplasmic MAA in 2 patients and with lack of change of its level in 2 patients. The behavior of the 2 MAA did not appear to correlate with the clinical response to hyperthermic perfusion.

DISCUSSION

Analysis with highly sensitive DDIA's of a large number of surgically removed tissues has confirmed the results of IIF stainings about the tissue distribution of a HMW-MAA and a cytoplasmic MAA; the latter antigen is present in a large number of normal and malignant tissues and may be acquired by cells which undergo malignant transformation. On the other hand, the HMW-MAA was found in all of the astrocytoma lesions tested, about 50% of the melanomas and skin carcinomas tested, and rare cases of various types of malignant tumors. The HMW-MAA was found in fetal skin but was not detected, with one exception, in a large number of normal tissues from adults, suggesting that it may be an oncofetal antigen. Among MAA defined by monoclonal antibodies, the oncofetal nature is not unique to the HMW-MAA, since it has been described for a M, 95,000-97,000 MAA identified by Woodbury et al. (27) and Dippold et al. (5) and for a M, 100,000 MAA characterized in our laboratory (11). The restricted tissue distribution of the HMW-MAA is in agreement with radioimaging studies which have shown that injection of radiolabeled anti-HMW-MAA monoclonal antibodies in patients with melanoma and with other types of solid tumors results in the specific localization of radiolabeled anti-HMW-MAA monoclonal antibodies in melanoma lesions.

Both DDIA and IIF assays have shown that the cytoplasmic MAA and the HMW-MAA are heterogeneous in their expression in each type of tumor analyzed, as well as in autologous metastatic melanoma lesions removed from different sites. The mechanism(s) regulating the expression of the 2 types of MAA appear to be different, since no relationship was found between their levels in the lesions analyzed. Furthermore, recombinant DNA interferon has a differential effect on the expression and shedding of the 2 types of MAA.

6 P. Giacomini and S. Ferrone, unpublished results.
DDIs performed with various combinations of monoclonal antibodies to distinct determinants of the HMW-MAA have detected different amounts of the HMW-MAA. This finding is not likely to reflect differences in affinity of the 3 monoclonal antibodies utilized in the assay, since all of them have an equilibrium constant higher than $1 \times 10^4$ mol/liter. Furthermore, the ratio among the subpopulations of the HMW-MAA measured with the combinations of the 3 monoclonal antibodies varies in cell lines, as well as in surgically removed lesions. An alternative mechanism which may explain the different amounts of HMW-MAA measured by the different combinations of monoclonal antibodies used is differential binding of the 3 monoclonal antibodies to polyvinyl chloride microtiter plates. Although we cannot exclude this possibility, we favor the interpretation that the determinants recognized by the monoclonal antibodies 149.55, 225.28S, and 763.74T are recognized in various combinations on the pool of HMW-MAA solubilized from cell lines and from surgically removed lesions. This possibility is supported by results of immunodepletion experiments which have shown heterogeneous distribution of the determinants recognized by the monoclonal antibodies 149.53, 225.28S, and 763.74T on the pool of HMW-MAA-bearing molecules synthesized by melanoma cell lines (12). Whether the antigenic heterogeneity of the HMW-MAA is genotypic or phenotypic, whether it correlates with the degree of differentiation of tumor cells, and whether it reflects different ratios of the subpopulations of HMW-MAA synthesized by individual tumor cells or by the cell population as a whole is not known.

This study has shown, for the first time, that both the HMW-MAA and the cytoplasmic MAA are present in sera from healthy donors and from patients with melanoma. Their level appears to differ by about 3 orders of magnitude. While the cytoplasmic MAA is found also in urine and spinal fluids from healthy donors, the HMW-MAA is detectable in neither. The detection of HMW-MAA in normal sera has been a surprising finding, in view of the restricted tissue distribution of this antigen. Its most likely source may be represented by cells of the melanocyte lineage, since melanocytes in culture have been reported to express the HMW-MAA (17). The available information indicates that screening of sera for the HMW-MAA does not have diagnostic value. On the other hand, quantitation of the HMW-MAA may provide some information about the extent of the disease, since its level is increased in most of the patients with stage IV melanoma. The range of values of the HMW-MAA is broader in sera from patients with melanoma than in sera from healthy donors; the relationship between the level of HMW-MAA in serum and the stage of the disease suggests that the high values may reflect shedding from the tumor masses. The low values and the lack of detection may reflect complexing of HMW-MAA with autoantibodies which coat the determinants recognized by the monoclonal antibodies. The complexing of serum HMW-MAA with autoantibodies may also explain why free antibodies to the HMW-MAA have not been detected in patients with melanoma. If this antigen is indeed immunogenic in autologous combinations.

Melanoma cells have been described to be abnormally sensitive to hyperthermia (4). The present study has shown that hyperthermia treatment may be associated with changes in the level of the 2 MAA we have analyzed. Whether these changes may affect the interaction of tumor cells with the host’s immune system and/or provide any useful clinical information is not known.

From a technical viewpoint, the DDIs we have applied offer the advantages of being specific and highly sensitive, of relying on objective evaluation of data, of yielding quantitative results, and of being suitable for screening of a large number of samples. On the other hand, the DDIs require amounts of tissue which are not always available. Furthermore, the DDIs do not provide any information about the cellular distribution of MAA. The latter limitation can be overcome by combining the DDIA with binding assays, such as IIF.

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