ORIGINAL ARTICLE

Serum levels of cytoplasmic melanoma-associated antigen at diagnosis may predict clinical relapse in neuroblastoma patients

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Abstract The high molecular weight melanoma-associated antigen (HMW-MAA) and the cytoplasmic melanoma-associated antigen (cyt-MAA/LGALS3BP) are expressed in melanoma. Their serum levels are increased in melanoma patients and correlate with clinical outcome. We investigated whether these molecules can serve as prognostic markers for neuroblastoma (NB) patients. Expression of cyt-MAA and HMW-MAA was evaluated by flow cytometry in NB cell lines, patients' neuroblasts ($_{FI}$-NB), and short-term cultures of these latter cells (cNB). *LGALS3BP* gene expression was evaluated by RT–qPCR on $_{FI}$-NB, cNB, and primary tumor specimens. Soluble HMW-MAA and cyt-MAA were tested by ELISA. Cyt-MAA and HMW-MAA were expressed in NB cell lines, cNB, and $_{FI}$-NB samples. *LGALS3BP* gene expression was higher in primary tumors and cNB than in $_{FI}$-NB samples. Soluble cyt-MAA, but not HMW-MAA, was detected in NB cell lines and cNBs supernatants. NB patients' serum levels of both antigens were higher than those of the healthy children. High cyt-MAA serum levels at diagnosis associated with higher incidence of relapse, independently from other known risk factors. In conclusion, both HMW-MAA and cyt-MAA antigens, and *LGALS3BP* gene, were expressed by NB cell lines and patients' neuroblasts, and both antigens' serum levels were increased in NB patients. Elevated serum levels of cyt-MAA at diagnosis correlated with relapse, supporting that cyt-MAA may serve as early serological biomarker to individuate patients at higher risk of relapse that may require a more careful follow-up, after being validated in a larger cohort of patients at different time-points during follow-up. Given its immunogenicity, cyt-MAA may also be a potential target for NB immunotherapy.

Keywords Cytoplasmic melanoma-associated antigen · Neuroblastoma · Clinical relapse · Serum biomarkers

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Introduction

The large majority of tumor markers are developmentally regulated molecules, expressed in specific tissues during fetal development and reexpressed in neoplastic cells after malignant transformation [1]. The number of potential markers is progressively increasing; however, for most malignancies, there are few surrogate serum markers able to predict at diagnosis outcome or tumor relapse.

Two melanoma-associated antigens have been well characterized in the last years by Ferrone et al. [2–9]. The high molecular weight melanoma-associated antigen (HMW-MAA) is a membrane proteoglycan consisting of two subunits of 280 and 440 kDa, expressed in a high proportion of melanomas [5, 9, 10]. Several immunotherapy schedules have been designed using anti-HMW-MAA monoclonal antibodies [7, 11] or vaccination with HMW-MAA whole protein or peptides [6, 12]. The cytoplasmic melanoma-associated antigen (cyt-MAA), also known as Mac-2-binding protein or galectin-3-binding protein (LGALS3BP) [13, 14], is an antigen composed of four polypeptides of 94, 75, 70, and 25 kDa expressed in the cytoplasm of normal cells [2, 4]. Its expression has been found increased in activated normal cells [4], as well as in metastatic melanoma and in other malignancies [8, 9]. It has previously been demonstrated that cyt-MAA is secreted by neuroblastoma cells and has a role in cancer progression, stimulating IL-6 release in bone marrow stromal cells [15].

Soluble HMW-MAA and cyt-MAA have been detected in sera from normal individuals [3, 16], and concentration of both antigens is higher in sera from metastatic melanoma patients than in healthy subjects [9]. High serum levels of cyt-MAA, but not of HMW-MAA, correlate with a higher incidence of relapse and a poorer clinical outcome in metastatic melanoma patients. Moreover, concentration of cyt-MAA in sera is a good marker of immunotherapy response, and patients whose cyt-MAA serum levels remained elevated during treatment were three times more likely to recur or progress [8]. IFN-α and IFN-β increased serum levels of cyt-MAA in melanoma patients [17] and augmented the release of cyt-MAA by melanoma cell lines in vitro [2].

Neuroblastoma (NB) shares a neuroectodermal origin with metastatic melanoma [18], as well as the expression of some tumor-associated antigens, such as MAGE [19], GAGE [20], PRAME [21] and NY-ESO-1 [22].

NB is a neuroectodermal tumor originating from the sympathetic nervous system, and it is the most common extracranial solid malignancy in childhood [23–25]. NB patients' clinical course is heterogeneous, ranging from localized and well-differentiated tumor with good prognosis (stage 1 and 2) or spontaneous regression (stage 4S), to fatal progression, characterized by metastasis and associated with tumor relapse (stages 3–4) [26].

Accurate risk estimation of the patients is mandatory to choose the most adequate therapy. The clinical variables currently employed are the following: (1) stage of disease, (2) age of patient at diagnosis, and (3) genetic alterations, such as amplification of *MYCN* oncogene and loss of the long arm of chromosome 11 [26, 27].

Since no surrogate serum biomarkers for risk estimation at diagnosis are available for NB, except lactate dehydrogenase (LDH), whose prognostic value is still controversial [28, 29], we evaluated (1) the expression, secretion, and shedding of cyt-MAA and HMW-MAA in NB cell lines, NB primary tumors, and metastatic neuroblasts, (2) serum levels of both antigens in NB patients compared with those in age-matched healthy subjects, and (3) correlations between altered serum levels of both antigens and clinical outcome of NB patients.

Materials and methods

Patients

The study was approved by the Ethical Committee of the G. Gaslini Institute, Genoa, Italy. Samples were collected at diagnosis from 47 patients with different stages of NB disease, namely 10 stage 1, 6 stage 2, 11 stage 3, 16 stage 4, and 4 stage 4S, according to the International Neuroblastoma Staging System [26]. All patients were untreated at study entry.

Twenty patients with localized NB (stages 1, 2 and 4s) received only surgery. Eight patients were enrolled in Localised Neuroblastoma European Study (LNESG1) [30], 6 patients were enrolled in multicenter study in Europe for infants (INES) [31], and 6 patients were enrolled in Italian Neuroblastoma protocol NB 92 [32]. Twenty-seven patients with metastatic NB (stages 3 and 4) were subjected to (1) only surgery (5 patients, LNESG1 or INES protocols) or (2) surgery, chemotherapy, and autologous stem cell transplantation (22 patients, European protocol NB-AR-01 and Italian protocol NB 85 and 97).

NB patients' characteristics and clinical features at diagnosis and at follow-up are summarized in Table 1. The median of follow-up length was 14.37 months (range, 1.87–88.3 months).

Cell lines and NB cell cultures

GI-LI-N, GI-ME-N, IMR-32, LAN-5, SH-SY-5Y NB cell lines and M14 melanoma cell line were purchased from American Type Culture Collection (ATCC, Rockville, MD). Tumor cell lines were maintained in DMEM (Sigma–Aldrich, St.Louis, MO, USA) supplemented with 10% fetal bovine serum (GIBCO, Invitrogen, Carlsbad, CA, USA), HEPES buffer, non-essential amino acids, and antibiotics (Cambrex Bio Science Verviers, Verviers, Belgium).

Fresh neuroblasts were obtained at diagnosis from bone marrow (BM) samples of five stage 4 NB patients. Erythrocytes were lysed using erythrocyte lysis buffer (Qiagen, Hilden, Germany) following manufacturer's protocol. Cells were then treated with Fc-blocking reagent (Miltenyi Biotech, Bergisch Gladbach, Germany) and stained with the

Table 1 Characteristics and clinical features of NB patients

Diagnosis	
Gender	
M	26/47
F	21/47
Age	
<18 months	19/47
>18 months	28/47
MYCN	
Normal	31/47
Amplified	16/47
Stage	
1	10/47
2	6/47
3	11/47
4	16/47
4s	4/47
Follow-up	
Overall survival	
CR	21/47
Alive with disease	14/47
Dead	12/47
Relapse-free survival	
Relapsed	17/47
Local	6/47
Disseminated	5/47
Both	6/47

murine anti-GD2 G2a mAb (IgG2a, obtained from 14.G2a hybridoma cell line, kindly donated by Dr. Mirco Ponzoni, Genoa, Italy) [33, 34] for 30 min at 4°C. Cells were then washed with washing buffer (PBS 0.5% BSA, 2 mM EDTA) and treated with Rat anti-mouse IgG2(a + b) microbeads (Miltenyi Biotech) following manufacturer's protocol and washed with washing buffer. GD2 $^+$ cells were then separated using magnetic columns (Miltenyi Biotech), counted, and plated in DMEM 20% FBS. In some experiments, fresh neuroblasts were cultured for few passages. Cells were detached using Trypsin–EDTA solution (Celbio, Milano, Italy) when culture reached the confluence and expanded in DMEM 20% FBS.

Serum samples

Samples were obtained from the serum bank of the Clinical Pathology Laboratory, G. Gaslini Children's Hospital. Together with samples from the 47 NB patients, 38 samples collected from age-matched healthy children, admitted at the Emergency Department of Gaslini Hospital for accidental traumatic injuries, were studied as controls. Samples were collected between 1997 and 2005, following written informed consent of the patients or legal guardians and stored at −80°C until use.

Monoclonal antibodies

The 465.125 monoclonal antibody (mAb), which recognizes a repeated epitope of cyt-MAA, and the TP41.2 and 763.74 mAbs, which recognize distinct and spatially distant determinants of HMW-MAA, were developed and characterized as previously described [3, 15]. The 465.125 mAb was used in assays as both capture and detection reagent. For detection, the 465.125 and 763.74 mAbs were biotinylated using AnaTag protein biotinylation kit (Ana-Spec Inc., San Jose, CA, USA), following manufacturer's protocol.

Flow cytometry

Intracellular and surface staining of NB cell lines was performed as previously described [35]. Briefly, cells were detached using Trypsin–EDTA solution (Celbio) and washed in PBS 1% FBS. For HMW-MAA detection, cells were incubated for 30 min at 4°C with 1 μg/10^6 cells of TP41.2 mAb, washed with PBS 1% FBS, and then incubated with goat F(ab)$_2$ anti-mouse IgG1 PE-conjugated secondary antibody (Southern Biotech, Birmingham, AL, USA) for 30 min at 4°C.

For cyt-MAA detection, cells were fixed and permeabilized using BD Perm/Wash (BD Biosciences, Franklin Lake, NJ, USA) following manufacturer's protocol and then incubated for 30 min at 4°C with 1 μg/10^6 cells of 465.125 mAb. Cells were then washed with Perm/wash 1× buffer and incubated with goat F(ab)$_2$ anti-mouse IgG2a PE-conjugated secondary antibody (Southern Biotech) for 30 min at 4°C.

In both cases, cells were subsequently washed with PBS 1% FBS and then subjected to flow cytometry using FACScalibur (BD Biosciences). Cell Quest software (BD Biosciences) was used for data analysis. Results are expressed as percentage of positive cells or as mean relative fluorescence intensity (MRFI) obtained as a ratio between mean fluorescence intensity (MFI) of cells stained with specific mAb and MFI obtained with isotype control.

Soluble cyt-MAA and HWM-MAA ELISA

ELISA for soluble cyt-MAA and HMW-MAA was performed using MaxiSorp Nunc-Immuno 96-microwell plates (Nunc A/S, Roskilde, Denmark) coated overnight at 4°C with 465.125 or TP41.2 mAbs, respectively (0.1 μg/ml in carbonate/bicarbonate buffer, pH 9.6, 100 μl/well). After three washes with PBS 0.05% Tween 20 (washing buffer),

plates were blocked with 200 μl/w of PBS 0.3% Tween 20 (blocking buffer) for 1 h at room temperature (RT).

Serum levels of each antigen were calculated from the standard curve. For cyt-MAA, standard curve was performed using purified antigen and expressed in ng/ml. For HMW-MAA, standard curve is expressed in units (U), where 1U equaled the amount HMW-MAA in 1 μg per ml of the standard cell lysate. Standard melanoma cell lysate was prepared from 1×10^7 M14 melanoma cells resuspended in lysis buffer (PBS 0.5% NP-40, with protease inhibitors). The preparation was sonicated and then centrifuged for 15 min at $10,000 \times g$. The supernatant was collected, and the protein concentration was determined by the BCA assay (Pierce, Rockford, Illinois). Serum samples were diluted in blocking buffer 1:1,600 for cyt-MAA and 1:50 for HMW-MAA. One hundred microliters of samples or standards was added to each well and incubated at 4°C overnight. Plates were then washed three times with washing buffer and then incubated with 100 μl/well of biotin-conjugated 465.125 or 763.74 mAbs (0.1 μg/ml in blocking buffer) at 37°C for 2 h. After three washes, plates were incubated with streptavidin–horse radish peroxidase (GE Healthcare, Chalfont St. Giles, UK) 1:4,000 in PBS 0.1% Tween 20, 0.1% BSA, for 1 h at RT. After additional three washes, plates were incubated with the substrate (3′-3′-5′-5′ Tetramethylbenzidine, Sigma) for 5 min at RT. Finally, H_2SO_4 5 M (100 μl/well) was added, and optical densities were measured at 450 nm.

The assay's lowest threshold was 4.33 ng/ml for cyt-MAA and 2.5 U/ml for HMW-MAA. Each sample was tested in duplicate.

RNA extraction, reverse transcription, and quantitative real-time PCR (qPCR)

Total RNA was extracted from (1) freshly isolated GD2+ cells obtained following magnetic separation from BM samples of five NB patients with stage 4 disease (FI-NB) and (2) the same cells cultured for a few passages in vitro (cNB), using the RNeasy Micro kit (Qiagen), according to the manufacturer's procedures. A DNASe I digestion was performed to eliminate contaminating DNA.

Total RNA was extracted from 9 primary NB tumors samples, 5 Schwannian stroma-poor NBs (3 stage 4 and 2 stage 3), and 4 Schwannian stroma-rich neuroblastomas (2 stage 1, 1 stage 3, and 1 stage 4) using PerfectPureTM RNA Tissue Kit (5Prime, Hamburg, Germany), including RNase-free DNase I treatment.

RNA (1 μg) was reverse transcribed using 20 pmol of random hexamers (Eppendorf, Hamburg, Germany) and 200 U of SuperScript II enzyme (Invitrogen Life Technologies, Carlsbad, CA) in a total reaction volume of 20 μl. The mixture was incubated in a Mastercycler epGradient S (Eppendorf) at 25°C for 10 min, 42°C for 60 min, and 85°C for 5 min to stop the reaction. cDNA was diluted 10-fold in molecular biology grade water to a final concentration of 5 ng/μl, assuming 100% reverse transcription efficiency. Reverse transcription–quantitative PCR (RT–qPCR) assays for cyt-MAA (TaqMan Gene Expression Assay (ID: Hs00174774_m1), Applied Biosystems, Foster City, CA, USA) and β2-microglobulin (β2-M) (Applied Biosystems) were set up in 96-white-well Twin.tec real-time plates (Eppendorf) by EpMotion 5070 Liquid Handling Workstation (Eppendorf) and performed in duplicate on Mastercycler RealPlex4 S system (Eppendorf) using 2 μl of cDNA, 2.5× RealMaster Mix Probe (5Prime) and 20× primer/probe mix in a final volume of 10 μl. Negative controls (water as template and RNA reverse transcribed without enzyme) and positive controls (cDNA of GI-LI-N and GI-ME-N cell lines) were run simultaneously. Cycling conditions were as follows: 95°C for 2 min, 40 cycles at 95°C for 15 s and at 60°C for 1 min. RealPlex software v. 2.0 (Eppendorf) was used to determine quantification cycle (Cq). Results of qPCR analysis were expressed as relative values (ΔΔCq method [36]) using β2-M as reference gene and GI-ME-N cell line as calibrator sample.

Statistical analysis

For each NB patient, demographic, clinical, biological, and follow-up data were retrieved from the Italian NB Registry that collects patients' information at diagnosis, during treatment and yearly after treatment discontinuation.

The median serum levels measured in patients and controls were compared by Student's t test, using Prism software (GraphPad Software Inc., La Jolla, CA).

To determine the cutoff level of each antigen to be considered elevated, ROC curves [37] were constructed using MedCalc software (Mariakerke, Belgium), using as read-out: (1) NB patients' sera versus control sera, (2) relapsed versus not-relapsed NB patients, and (3) alive versus dead NB patients.

Relationship between patients' clinical outcome and MAA serum levels was determined according to the Kaplan–Meier method. Relapse-free and overall survival curves were compared by the log-rank test using MedCalc software. A P value <0.05 was considered as statistically significant.

Multivariate analysis of survival in relationship with serum cyt-MAA levels, age, and MYCN amplification was performed by Cox multiple regression model, using Stat-Plus Professional software (AnalystSoft Inc., Vancouver, Canada).

Results

NB cell lines and neuroblasts from NB patients express HMW-MAA and cyt-MAA

First, surface expression of HMW-MAA and intracellular expression of cyt-MAA were evaluated by flow cytometric analysis of five NB cell lines (GI-ME-N, GI-LI-N; SH-SY-5Y, IMR-32, and LAN-5). As shown in Fig. 1a, cyt-MAA expression was detected in all five NB cell lines tested (black bars), whereas HMW-MAA (grey bars) was expressed on the surface of three out of five NB cell lines (GI-ME-N, GI-LI-N, and IMR-32). The expression of both melanoma-associated antigens in NB samples was much lower than that detected on the M14 melanoma cell line, tested as positive control. Mean of three different experiment carried out ±SD is shown.

Next, the expression of cyt-MAA and HMW-MAA was assessed on metastatic $GD2^+$ neuroblasts isolated from the bone marrow (BM) of five stage 4 NB patients, either freshly isolated ($_{FI}$-NB) or cultured in vitro for few passages (cNB). As shown in Fig. 1b, both cyt-MAA (black bars) and HMW-MAA (grey bars) were expressed in 3 out of 5 $_{FI}$-NB and in all 5 cNB samples. A statistical analysis was performed to compare the expression of both antigens in $_{FI}$-NB and cNB. As shown in Fig. 1c, the expression of cyt-MAA was significantly higher in cNB samples than in $_{FI}$-NB samples (median MRFI: 14.8 vs. 1.1, $P = 0.035$). Conversely, the expression of HMW-MAA was similar between $_{FI}$-NB and cNB samples.

Expression of cyt-MAA/LGALS3BP gene in neuroblastoma samples

The expression of cyt-MAA encoding gene, LGALS3BP, was then evaluated by RT–qPCR. HMW-MAA gene expression could not be evaluated since specific RT–qPCR probes are not available so far.

LGALS3BP gene expression was evaluated on (1) nine primary NB tumor samples (five stroma-poor and four stroma-rich NB frozen tissue samples), (2) five $_{FI}$-NB samples, and (3) five cNB samples.

As shown in Fig. 2a, LGALS3BP gene was highly expressed in primary NB tumors, regardless of tumor histology. Moreover, LGALS3BP gene was expressed in metastatic neuroblasts, and its expression was significantly higher in cNB samples than in $_{FI}$-NB samples (mean of $-2^{-\Delta\text{CCt}}$ values: 0.13 ± 0.03 vs. 0.04 ± 0.03, $P = 0.002$). These results were in accordance with the flow cytometry data obtained in the same cell preparations.
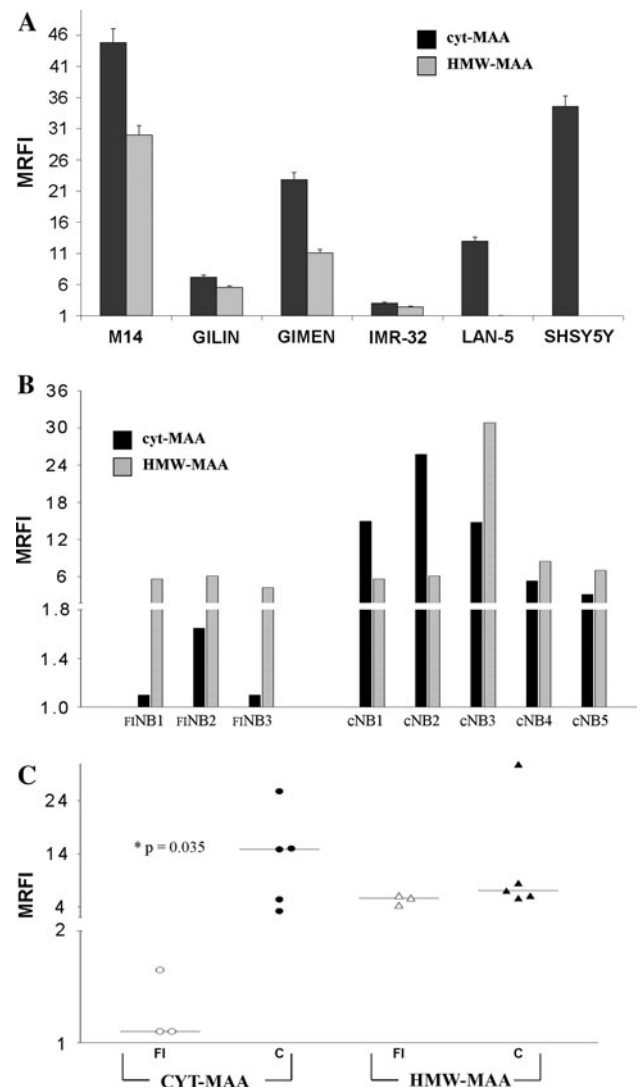

Fig. 1 Expression of cyt-MAA and HMW-MAA. **a** FACS analysis of cyt-MAA (black bars) and HMW-MAA (grey bars) expression in 5 NB cell lines (GI-LI-N, GI-ME-N, IMR-32, LAN-5, SH-SY-5Y). M14 melanoma cell line was tested as positive control. Results are expressed as MRFI (mean relative fluorescence intensity = mean fluorescence intensity obtained with specific mAb/mean fluorescence intensity obtained with an irrelevant isotype-matched antibody). Mean of three different experiments carried out ± SD is shown. **b** FACS analysis of cyt-MAA (black bars) and HMW-MAA (grey bars) expression in three freshly isolated neuroblasts from patients' BM ($_{FI}$-NB) and in five short-term culture of freshly isolated neuroblasts from five patient's BM (cNB). Results are expressed as MRFI (mean relative fluorescence intensity = mean fluorescence intensity obtained with specific mAb/mean fluorescence intensity obtained with an irrelevant isotype-matched antibody). **c** Statistical analysis of cyt-MAA and HMW-MAA expression in freshly isolated ($_{FI}$-NB) and short-term cultured neuroblasts (cNB) from patients' BM. Scatter plots indicate MRFI values obtained by flow cytometric analysis of MAAs expression on $_{FI}$-NB ($N = 3$) and cNB ($N = 5$). Horizontal bars indicate medians. P value is indicated where the difference is statistically significant
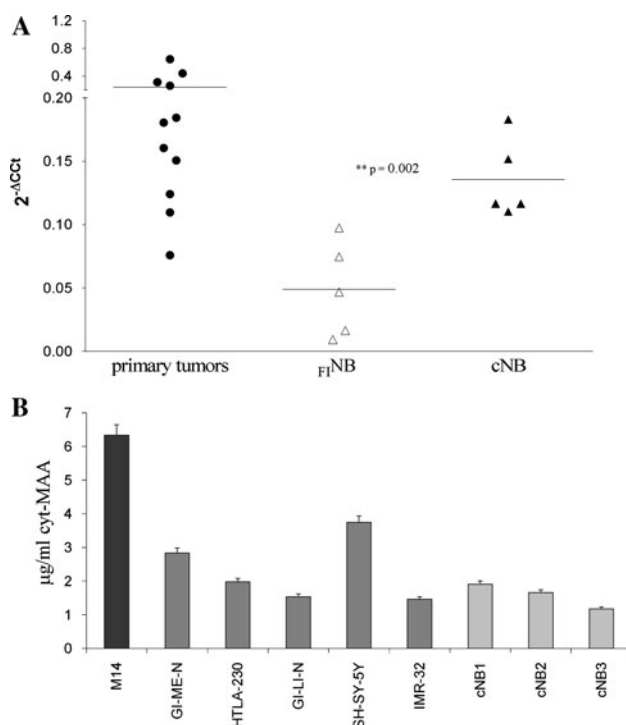
Fig. 2 Expression of *LGALS3BP* gene and release of soluble cyt-MMA. **a** *LGALS3BP* gene expression was evaluated by RT–qPCR analysis using mRNA extracted from (1) nine NB tumor samples, (2) five $_{FI}$-NB samples, and (3) five cNB samples. *Scatter plot* indicated $2^{-\Delta\Delta Ct}$ values from each sample. *Horizontal bars* indicated medians. *P* values are indicated where the difference is statistically significant. **b** Quantification of soluble cyt-MAA in supernatants from 5 NB cell lines (GI-LI-N, GI-ME-N, IMR-32, LAN-5, and SH-SY-5Y) and 3 cNB samples. M14 melanoma cell line supernatant was tested as positive control. Results are expressed as μg/ml. Mean of three different experiments carried out ± SD is shown

Investigation of soluble cyt-MAA and HMW-MAA in supernatants of NB cell lines

The release of the soluble form of both cyt-MAA and HMW-MAA was then evaluated by specific ELISA on 5 NB cell lines supernatants collected after 48 h culture. Supernatant from M14 melanoma cell line was tested as positive control. As shown in Fig. 2b, soluble cyt-MAA was detected in supernatants from all five NB cell lines (range 0.06–2.75 μg/ml) and in supernatants from 3 out of 5 cNB samples (range 1.18–1.91 μg/ml). Conversely, soluble HMW-MAA was undetectable in all samples (data not shown).

Serum levels of soluble cyt-MAA and HMW-MAA are increased in NB patients

Serum levels of soluble cyt-MAA and HMW-MAA were then tested in sera from 47 NB patients with different stages

of disease (10 stage 1, 6 stage 2, 11 stage 3, 16 stage 4, and 4 stage 4s) and from 38 age-matched healthy children.

As shown in Fig. 3a, the concentration of soluble cyt-MAA was significantly higher in sera from NB patients (median 99.2 ± 64.9 ng/ml) than in sera from healthy children (median 39.78 ± 47.19 ng/ml, P = 0.0005). The concentration of soluble HMW-MAA (Fig. 3b) was also significantly higher in sera from NB patients (median 2.65 ± 1.2 U/ml) than in sera from healthy children (median 0.86 ± 0.15 U/ml, P = 0.035). However, serum levels of both cyt-MAA and HMW-MAA were similar among patients with different NB stages (Fig. 3c, d, respectively).

Relationships between serum levels of cyt-MAA and clinical outcome of NB patients

To evaluate whether serum cutoff levels could discriminate between NB patients and healthy children, ROC curves were obtained. As summarized in Table 2A, the number of NB patients with serum levels above the cutoff levels was significantly higher than that of healthy children, for both cyt-MAA and HMW-MAA. Next, ROC cutoff levels for both antigens were calculated by considering as read-out the NB patients' outcome (relapse-free vs. relapsed and alive vs. dead). As shown in Table 2B, cyt-MAA serum levels above the cutoff level (91.47 ng/ml) significantly correlated with a higher incidence of relapse (9/30 vs. 11/17, P = 0.032), whereas no correlation was found between HMW-MAA serum levels and incidence of relapse. Neither cyt-MAA nor HMW-MAA serum levels correlated with overall survival (Table 2C). Accordingly, as shown in Fig. 4a, Kaplan–Meier analysis confirmed that soluble cyt-MAA serum levels higher than 91.47 ng/ml correlated with a higher incidence of relapse (P = 0.0028), whereas no impact of cyt-MAA serum levels was found on overall survival (Fig. 4b). Kaplan–Meier analysis confirmed that HMW-MAA serum levels had no correlation with either relapse-free or overall survival (data not shown).
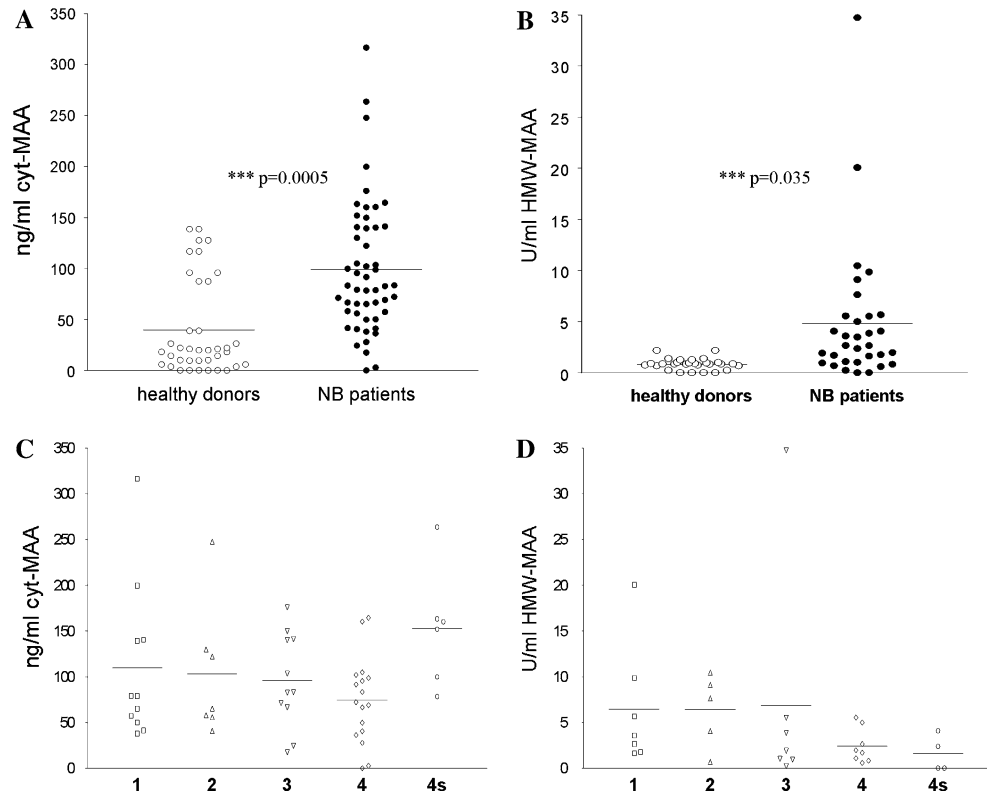
Next, correlation between cyt-MAA levels below or over the estimated ROC cutoff level (91.47 ng/ml) and different clinical parameters, i.e., gender (M vs. F), *MYCN* amplification (Yes or No), age at diagnosis (>18 months vs. <18 months), and disease stage (stages 1, 2, 3, and 4s vs. stage 4), was analyzed by Fisher exact test. As shown in Table 2D, cyt-MAA serum levels were independent from all parameters (P > 0.05). To confirm these data, a multivariate analysis using Cox regression model was performed on cyt-MAA serum levels below or above cutoff level and other known prognostic factors. As summarized in Table 2E, cyt-MAA serum levels displayed a significant

Discussion

Serological markers for human tumors may help predicting clinical outcome. Serological markers currently used for human NB are neuron-specific enolase, ferritin, and LDH. These markers, however, with the possible exception of LDH [28, 29] have limited prognostic role.

In the present study, we show that cyt-MAA, as well as surface HMW-MAA, was expressed by NB cell lines and, more importantly, by metastatic neuroblasts isolated from BM samples. These latter cells showed a low expression of cyt-MAA immediately after isolation, which increased upon short-term in vitro culture. In contrast, HMW-MAA expression levels were similar in freshly isolated and cultured metastatic neuroblasts. Cyt-MAA encoding gene LGALS3BP was expressed by metastatic neuroblasts and primary NB tumors, regardless of tumor histology. Soluble cyt-MAA was released in culture supernatants by NB cell lines and freshly isolated metastatic neuroblasts after short-term culture. Indeed, soluble cyt-MAA and soluble HMW-MAA, which are generated through proteolytic cleavage by plasmin and other endogenous protease [15], were detected in sera from NB patients. Concentration of both antigens was higher than in healthy children, regardless of tumor

stage. While serum HMW-MAA levels did not correlate with outcome, soluble cyt-MAA serum levels above the cutoff level of 91.47 ng/ml (calculated with ROC curves) significantly correlated with a higher incidence of relapse, independently from other known risk factors, with a high sensitivity and specificity. Conversely, serum cyt-MAA levels did not predict a lower overall survival of NB patients, accordingly the low specificity obtained with ROC curves calculations.

Taken together, these data support that high cyt-MAA/LGALS3BP serum levels at diagnosis may serve as early serological biomarker to identify patients at higher risk of relapse that may require a more careful follow-up. The lack of correlation between cyt-MAA serum levels and tumor stage was in line with other reports concerning both serological and histological markers of NB relapse [38–40].

The role of cyt-MAA/LGALS3BP in cancer is not entirely understood, and LGALS3BP has been shown to have multiple functions. In addition to metastatic melanoma [8, 9], cyt-MAA/LGALS3BP is expressed in numerous cancer cell types, including non-small cell lung cancer, astrocytoma, and colon cancer, where it is a predictor of poor survival [41–43]. Cyt-MAA/LGALS3BP stimulates the secretion of many cytokines in peripheral blood mononuclear cells, including tumor necrosis factor-α (TNF-α), IL-1, and IL-6 [44], which contribute to inflammation and tumor progression. Moreover, cyt-MAA/LGALS3BP binds to multiple proteins of the extracellular

association with relapse ($P = 0.01$, HR $= 4.11$), independently from the other parameters.

Table 2 Statistical analysis of cyt-MAA and HMW-MAA serum levels

(A)

Antigen	Patients n % (n = 47)	Controls n % (n = 38)	P value	Sensitivity	Specificity
CYT-MAA				92.8%	64.7%
Median serum level	99.2 ng/ml	39.78 ng/ml	0.0005[§,***]		
% elevated (>26 ng/ml)	43 (92%)	8 (41%)	<0.0001[†,***]		
HMW-MAA				71.8%	92.8%
Median serum level	2.65 U/ml	0.86 U/ml	0.035[§,*]		
% elevated (>1.38 U/ml)	34 (72%)	3 (14%)	0.0004[†,***]		

(B)

Antigen	Relapse-free n % (n = 30)	Relapsed n % (n = 17)	P value[†]	Sensitivity	Specificity
CYT-MAA				64.7%	70%
% elevated (>91.47 ng/ml)	9 (30%)	11 (65%)	0.032*		
HMW-MAA				66.7%	56.5%
% elevated (>2.65 U/ml)	13 (44%)	10 (56%)	0.69		

(C)

Antigen	Alive n % (n = 35)	Dead n % (n = 12)	P value[†]	Sensitivity	Specificity
CYT-MAA				36.3%	88.9%
% elevated (>40.42 ng/ml)	31 (89%)	8 (72%)	0.33		
HMW-MAA				80%	59%
% elevated (>2.65 U/ml)	14 (40%)	7 (59%)	0.62		

(D)

CYT-MAA	Gender		MYCN ampl.		Age		Stage	
	M	F	Yes	No	>18 m	<18 m	1, 2, 3, 4s	4
Above cutoff (>91.47 ng/ml) n = 21	12	9	7	14	11	10	14	7
Below cutoff (<91.47 ng/ml) n = 26	12	14	8	18	18	8	17	9
P value[†]	0.56		1		0.36		1	

(E)

	HR	95% Cl	P value°
MYCN (amplified vs. single copy)	2.08	0.73–5.88	0.17
Age (>18 months vs. <18 months)	1.68	0.54–5.23	0.37
Serum cyt-MAA (cutoff 91.47 ng/ml)	4.11	1.36–12.43	0.01**

CYT-MAA cytoplasmic melanoma-associated antigen, *HMW-MAA* high molecular weight melanoma-associated antigen

[§] Student's *t* test

[†] Fisher exact test

° Cox multiple regression analysis (HR = hazard ratio)

* Significance: * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$

matrix [45], which are critical during tumor cell invasion and migration. Finally, the engagement of galectin-3 receptor, which binds to cyt-MAA/LGALS3BP, leads to the release of elastin-derived peptides VGVAPG and VAPG, which are chemotactic for tumor cells and stimulate the expression of CXCR4, CXCL12 and metalloproteases MMP-2 and MMP-3, thus facilitating the spreading of tumor cells [46].
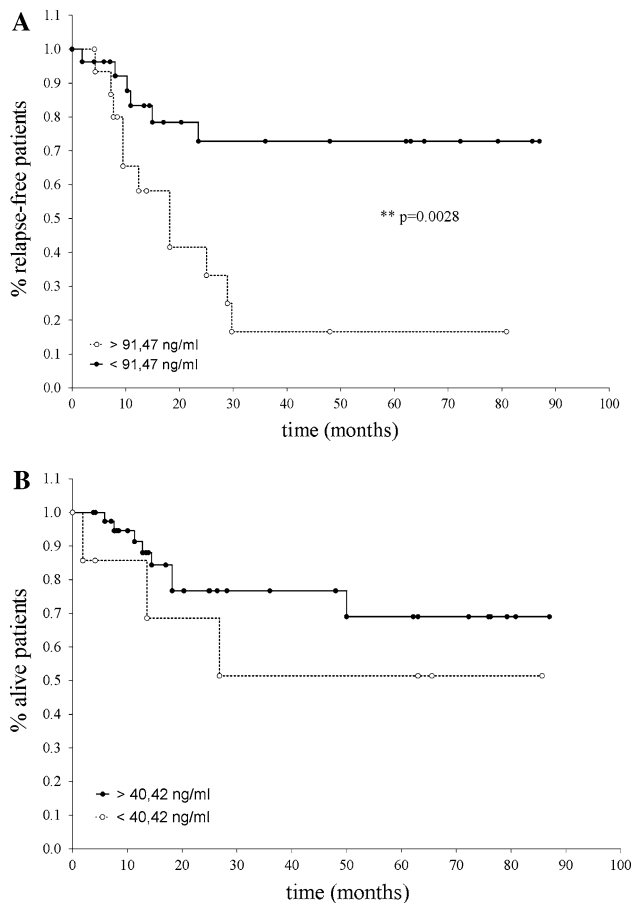
Fig. 4 Kaplan–Meier analysis of relapse-free survival and overall survival of NB patients. **a** Relapse-free survival in NB patients with cyt-MAA serum levels above (*dashed line*) or below (*black line*) the ROC cutoff level (91.47 ng/ml). *Y*-axes indicate proportion of relapse-free patients, whereas *X*-axes indicate time to relapse (months). **b** Overall survival in NB patients with cyt-MAA serum levels above (*dashed line*) or below (*black line*) the ROC cutoff level (40.42 ng/ml). *Y*-axes indicate proportion of alive patients, whereas *X*-axes indicate survival time (months)

Expression of cyt-MAA antigen was low to absent in freshly isolated metastatic NB and similar to primary tumor cells after short-term in vitro culture, suggesting that factors present in the BM microenvironment may suppress cyt-MAA expression. Fukaya et al. have previously reported that cyt-MAA was detectable in conditioned medium of NB cell lines. The soluble cyt-MAA, following interaction with the Galectin-3 receptor on stromal cells was shown to stimulate the release of IL-6 [15], which in turn induced proliferation of NB cells, creating a favorable microenvironment for progression of metastatic NB [47]. Elevated soluble cyt-MAA levels were found in NB patients, regardless of tumor stage, suggesting that soluble cyt-MAA was secreted either by the primary NB tumor cells or by normal cells [2, 4], stimulated by factors released by NB cells.

Ozaki et al. have previously demonstrated that cyt-MAA/LGALS3BP is highly immunogenic, since HLA-A2 restricted epitopes of this antigen are recognized by specific T lymphocytes [48] and anti-cyt-MAA/LGALS3BP-specific antibodies are elevated in cancer patients [49]. Our finding that NB cells express high levels of cyt-MAA/LGALS3BP may be very interesting in the view of a possible use of this antigen as target for T-cell-mediated immunotherapy of NB. Moreover, since cyt-MAA/LGALS3BP is also recognized by B lymphocytes, it would be interesting to evaluate in further studies whether serum levels of anti-cyt-MAA/LGALS3BP antibodies are elevated in NB patients, as previously demonstrated in patients with lung cancer [49]. In addition, a possible inverse correlation between serum levels of these antibodies and incidence of relapse may be analyzed.

In conclusion, the quantification of soluble cyt-MAA in serum samples at diagnosis may help NB patients' risk stratification. Validation in a larger cohort of NB patients and at different time-points during follow-up is required in a translational perspective, also in view of its possible use as surrogate marker of treatment response, as observed in metastatic melanoma patients [8]. Moreover, our study paves the way to further investigations on cyt-MAA as target for NB immunotherapy.

Conflict of interest The authors disclose any conflict of interest.

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