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Circulating melanoma exosomes as diagnostic and prognosis biomarkers

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ABSTRACT

Background: Malignant melanoma is an aggressive cancer with an increasing incidence. Exosomes are actively secreted microvesicles, whose characteristics reflect those of the cell they are originated in. The aim of this study was to identify and evaluate the presence of the melanoma biomarkers MIA, S100B and tyrosinase-related protein 2 (TYRP2) in exosomes and their potential clinical utility.
Methods: Serum samples were obtained from stage IV melanoma patients, melanoma-free patients and healthy controls. Exosomes were precipitated and TYRP2, MIA and S100B concentrations were quantified in serum, exosomes, and exosome-free serum.
Results: Both MIA and S100B were detected in exosomes and correlated significantly with serum concentrations (S100B: $r = 0.968$; MIA: $r = 0.799$; $p < 0.001$). MIA and S100B concentrations in exosomes were significantly higher in melanoma patients than in healthy controls and disease-free patients. However, TYRP2 concentrations in exosomes did not differ between these three groups. ROC curves analysis rendered AUCs for MIA of 0.883 ($p < 0.01$) and of 0.840 for S100B ($p < 0.01$). Patients with exosome MIA concentration higher than 2.5 μg/L showed shorter median survival related to those with lower level (4 versus 11 months; $p < 0.05$).
Conclusions: MIA and S100B can be detected in exosomes from melanoma patients and their quantification presents diagnostic and prognostic utility.

1. Introduction

Metastatic melanoma is a very aggressive cancer whose incidence is increasing worldwide. The prognosis is generally poor, although new treatments have improved recently overall survival. S100B, Melanoma Inhibitory Activity (MIA) and Lactate Dehydrogenase (LDH) are the most widely used tumor markers for prognosis and follow-up in advanced melanoma [1,2]. MIA is a small soluble protein of 11 kDa secreted by malignant melanoma cells. S100 is a 21 kDa dimeric protein composed of 2 subunits, α or β, being the αβ heterodimer expressed by melanoma cells [3]. LDH concentrations higher than reference range can classify patients with metastatic melanoma in a more advanced stage (M1c) [4]. Both MIA and S100B serum concentrations are elevated in advanced melanoma and their measurement can be useful as prognostic factors and to monitor the disease in stages III and IV [1,5]. However they show some limitations of specificity and sensitivity, especially LDH and therefore, they are not widely used [2,6,7].

Recently, other biomarkers have been investigated, such as cell-free nucleic acids and exosomes. BRAF mutations in cell-free DNA have also shown to be useful to monitor melanoma patients treated with BRAF inhibitors [8,9]. Exosomes are actively secreted microvesicles derived from the cellular endosomal membrane with sizes ranging from 30 to 200 nm [10]. Cancer cells, and particularly melanoma cells, can release large quantities of exosomes [11], in contrast to normal melanocytes [12]. These exosomes derived from cancer cells participate in tumor progression with immune-suppressive functions [13], contributing to angiogenesis [14], drug resistance [15] and cell migration [16]. As a result, exosomes are now considered to play a pivotal role in tumor development and progression [17].

The composition of nucleic acids and proteins cargo of exosomes reflects the cells they originate from [18]. In fact, Peinado et al. [17] found that exosomes isolated from stage IV melanoma patients characteristically contain the proteins tyrosinase-related protein 2 (dopachrome tautomerase, TYRP2), Very Late Antigen 4, Heat Shock Protein 70, HSP90 isoform, and MET oncoprotein [17]. In addition, melanoma exosomes seem to play a role in the metastasis to lymph nodes. Particularly, TYRP2 expression in exosomes has been associated with metastatic progression in advanced melanoma [17].

Abbreviations: AUC, Area Under Curve; LDH, Lactate Dehydrogenase; MIA, Melanoma Inhibitory Activity; ROC, Receiver Operating Characteristics; TYRP2, tyrosinase-related protein 2, Dopachrome tautomerase.

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Tumor-derived exosomes have been detected in several biological fluids and can carry tumor specific antigens, such as carcinoembryogenic antigen in ascitic exosomes from colon carcinoma [19], prostate specific antigen in urinary exosomes from prostate cancer [20], or CA125 in ascitic exosomes from ovarian carcinoma [21]. The expression of tumor-specific antigens by exosomes can render these particles useful for cancer diagnosis and monitoring. For this reason, the aim of the present work was to study the presence of melanoma biomarkers S100B and MIA in exosomes and to compare its utility with their determination in serum.

2. Material & methods

2.1. Sample collection

Peripheral blood samples were collected after obtaining informed consent from 53 advanced melanoma patients (mean age: 55 years; males: 54%), 18 melanoma disease-free patients (mean age: 54 years; males: 43%) and from 25 healthy volunteers (mean age: 41 years; males: 29%) used as age and sex matched control groups. Blood samples were centrifuged and serum was isolated and kept at −80 °C until analysis. The study was approved by our institution's Ethical Review Board.

M8 and UMBY melanoma cell lines were cultured in RPMI 1640 medium supplemented with 2 mmol/L L-glutamine, 10% exosome-free heat inactivated fetal bovine serum, penicillin G 100 U/mL, geniticin 1 mg/mL, streptomycin 100 μg/mL (Gibco, Grand Island, NY, USA), and were cultured in a 37 °C, 5% CO_2 humidified incubator.

2.2. Exosome isolation

Exosomes were obtained from serum with ExoQuick precipitation solution (System Biosciences, Mountain View, CA, USA) according to manufacturer's instructions. Briefly, 250 μL, of serum were mixed with 63 μL of ExoQuick™ solution and incubated for 1 h at 4 °C. After centrifugation at 1500 *g* for 30 min, pellets were suspended in 100–200 μL of PBS or in lysis buffer.

Exosomes from cell cultures were collected by ultracentrifugation at 110,000 *g* for 2 h at 4 °C after precleaning the media by two successive centrifugations and filtration through a 0.22 μm filter (EMD Millipore, Billerica, MA, USA). Pellets were resuspended in PBS or in lysis buffer.

2.3. Exosome size analysis

Isolated exosomes were diluted in PBS and particle size was analyzed using Malvern Zetasizer Nano-ZS (Malvern Instruments, UK) and its corresponding software (Zetasizer Ver. 7.03).

2.4. Protein concentration

Protein concentration in exosomes was estimated through absorbance at 280 nm with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). A correction factor was introduced to correct sample volume reduction from 250 to 200 μL.

2.5. Western blot

Proteins (20 μg) were denatured at 100 °C for 5 min in loading buffer containing 125 mM Tris (pH 6.8), 4% SDS, 30% glycerol, 5% β-mercaptoethanol and 0.4% bromophenol. Proteins were subjected to 10% polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE), with subsequent electroblotting transfer onto a nitrocellulose membrane. The membrane was blocked with 5% skimmed milk in PBS-Tween 0.1% during 30 min at room temperature, and then incubated during 1 h with anti-CD63 antibody (Santa Cruz Biotechnology, Texas, USA) diluted 1:1000 in PBS-Tween 0.1%. Immunoblot analysis was performed using an HRP-conjugated anti-mouse antibody (1:5000; Amersham Biosciences, Uppsala, Sweden), and developed using the ECL kit (Amersham Biosciences).

2.6. Immuno assays

MIA was determined by a manual commercial enzyme-linked immunosorbent assay (Roche, Mannheim, Germany) and S100B was analyzed by an electrochemiluminescence assay automatized in an e602 Module from a C8000 (Roche Diagnostics, Mannheim, Germany) [1]. Tyrosinase-related protein 2 concentration was measured using a commercial ELISA kit (Cloud-Clone Corp, Houston, USA). This is a sandwich enzyme immunoassay that uses two antibodies specific for TYRP2. A correction factor was introduced to correct sample volume reduction.

The concentrations of biomarker in exosome-free serum were calculated by subtracting the measured concentration in exosomes to the serum concentration.

2.7. Statistical analysis

Concentrations were expressed as median and 25th–75th percentile after determining their non-Gaussian distribution with the Kolmogorov–Smirnov and Shapiro–Wilk's tests. The non-parametric Kruskal–Wallis test and Dunn's multiple comparison test were applied to compare the levels of the tumor markers. Correlation analysis was performed using the Pearson correlation test. Overall survival was measured from the time of blood drawing to death or last follow-up and it was analyzed by the Kaplan–Meier method and compared by the Gehan–Breslow–Wicoxon test. Receiver operating characteristic (ROC) curves were constructed to determine the sensitivity and specificity of the assays. A two-tailed p-value <0.05 was considered to be statistically significant. Statistical analysis was performed with GraphPad Prism version 6.07 (La Jolla, CA, USA).

3. Results

3.1. Identification of serum exosomes

The mean size of the microvesicles obtained by precipitation with ExoQuick and by ultracentrifugation was lower than 200 nm, similar to that described for exosomes [10] (Fig. 1A). Exosomes isolated from plasma were characterized by Western blot using anti-CD63 and, as expected, we observed the predicted band at 53 kDa (Fig. 1B). In conclusion, we could identify these microvesicles obtained by precipitation as exosomes.

The concentration of exosomes isolated using the ExoQuick reagent was estimated by measuring the protein concentration. The median concentration of proteins in exosomes from melanoma patients was 8 μg/L (Q1–Q3: 6–10 μg/L), very similar to the control group (median: 9 μg/L; Q1–Q3: 8–10 μg/L). These results indicate that patients with advanced melanoma do not show significant differences in serum exosome levels, as compared to melanoma-free patients and healthy controls.

3.2. Biomarkers analysis in exosomes from melanoma patients

S100B and MIA were measured in serum (srm-S100B and srm-MIA respectively), exosomes (exo-S100B and exo-MIA respectively) and exosome-free serum (sn-S100B and sn-MIA respectively) (Table 1). There was a significant correlation between biomarker concentrations in exosomes and serum (S100B: $r = 0.968$; MIA: $r = 0.799$; $p < 0.001$ in both cases) (Fig. 2). However, while there was a good correlation of S100B concentration between exosomes and supernatant ($r = 0.943$; $p < 0.001$) this association was very poor in the case of MIA ($r = 0.189$; $p = 0.138$).

As expected, srm-S100B and srm-MIA concentrations were higher in stage IV melanoma patients compared to melanoma free patients and