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Extracellular vesicles as a source for non-invasive biomarkers in bladder cancer progression

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ABSTRACT

Bladder cancer is the second most frequent malignancy of the urinary tract after prostate cancer. Current diagnostic techniques, such as cystoscopy and biopsies are highly invasive and accompanied of undesirable side effects. Moreover, there are no suitable biomarkers for relapse or progression prognosis. We analysed whether the specific composition of microRNAs (miRNAs) and proteins of extracellular vesicles (EVs) that urothelial tumour cells of bladder mucosa release into the urine, could reflect their pathologic condition. For this purpose, urinary EVs were isolated and their protein and miRNA composition evaluated in healthy donors and low or high-grade bladder cancer patients. Using a microarray platform containing probes for 851 human miRNAs we found 26 deregulated miRNAs in high-grade bladder cancer urine EVs, from which 23 were downregulated and 3 upregulated. Real-time PCR analysis pointed to miR-375 as a biomarker for high-grade bladder cancer while miR-146a could identify low-grade patients. Finally, several protein markers were also deregulated in EVs from tumour patients. Our data suggest that the presence of ApoB in the 100,000 pellet is a clear marker for malignancy.

1. Introduction

Bladder cancer is a public health problem, both because of its high incidence and the high rate of relapses in all phases of the disease. It is ranked the 4th most common cancer in men and the 8th most common cancer in women worldwide *(http://globocan.iarc.fr, accessed on 09/01/2013)* and the second most common urologic tumour after prostate. Although it is more common in men, its incidence in women is also relevant, and habit changes (mostly tobacco abuse) are balancing both sexes in the last years. Tobacco abuse and industrial carcinogens such as aromatic amines and polycyclic aromatic hydrocarbons (contained for example in dyes, solvents, paints, combustion products, rubber, and textiles) are the most common risk factors. Chronic urinary tract infections, schistosomiasis, cyclophosphamide use and exposure to radiotherapy have also been associated with the development of bladder cancer (Burger et al., 2013; Kantor et al., 1984). Moreover, in the last decades, the incidence rate of bladder cancer is also increasing because of the overall aging of the population (Barocas et al., 2012).

Urothelial cell carcinoma (UCC) is the predominant histologic type of bladder cancer. It has been classified by the World Health Organization (2004 WHO) in low and high-grade based on cellular and structural atypia and non-infiltrating and infiltrating subtypes according to the degree of tissue invasion. Low-grade urothelial carcinoma, which has an incidence of 70% to 80%, is usually non-infiltrating and has an excellent prognosis but shows frequent relapse. About 30% of these recurrent tumours may progress to higher grade with stromal invasion. High-grade non-infiltrating urothelial carcinoma, including papillary and flat types, namely carcinoma in situ, often progresses to infiltrating carcinoma. High-grade infiltrating carcinoma usually has a poor prognosis (Eggener et al., 2004; Messing and Catalona, 1998).

Treatment of high grade UCC has not varied in the last decades. It consists of an endoscopic trans-urethral resection and intracavitary immune-prophylaxis with BCG to delay tumour progression in order to try

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to keep the bladder of the patient. However, even though BGC immune-prophylaxis has been shown to reduce relapse in 27%, up to 40% of patients will still present tumour relapses, 20% in the first two years. From those tumours that present relapses in the first year after BCG treatment, 44% will progress and 35% of those will cause the death of the patient (Gunlusoy et al., 2005; Witjes, 2006). Because of this perspective, many groups opt for a radical cystectomy from the beginning (Thalmann et al., 2004). Although radical cystectomy is currently the gold standard treatment for invasive localized disease or tumours with high risk of dissemination, it is a very aggressive surgery, with a marked impact on the patient quality of life. With this radical approach oncological control is achieved but it would mean an overtreatment of a significant proportion of patients (Turner, 2004).

Diagnosis and treatment is often difficult to establish, especially in high-grade non-muscle invasive bladder cancer, which constitute a very heterogeneous type of tumour, whose evolution and response to medical treatment is difficult to predict. Current diagnostic methods used in bladder cancer are invasive, with low sensitivity and costly. Moreover, urothelial bladder cancer diagnosis lacks markers for long-term prognosis. In the recent years, a series of immunological (Thalmann et al., 2000), genetic (Konety and Lotan, 2008) or proteomic (Lin et al., 2006; Pisitkun et al., 2006) screens have been conducted but results have not been standardized to the clinical practice. Thus, the identification of novel non-invasive biomarkers for diagnosis and prognosis of this kind of urologic cancer is an important challenge. Our results suggest that protein or microRNA (miRNA) analysis from urine extracellular vesicles could represent an important clinical tool.

Extracellular vesicles (EVs) are released by all kind of cells and they are present in all biological fluids, including urine (Yanez-Mo et al., 2015). They represent a potent mechanism of intercellular communication since they contain membrane and cytosolic proteins, bioactive lipids, mRNA and miRNA. Urine samples are easy to obtain from patients and contain EVs secreted by the cells lining the urinary tract, including glomerular podocytes, renal tubule cells, and urinary bladder to urine (Gamez-Valero et al., 2015). Due to the intimate relationship between EVs and the extracellular milieu, EVs may be considered a non-invasive "liquid biopsy" of the physiological state of the urinary system (Fais et al., 2016). As in other pathological disorders, EV levels in urine may be significantly altered by the tumour, and concentrate potential biomarkers, which may remain otherwise undetectable by dilution in whole urine. Recent studies evidence the potential or urine EV as biomarker source. Urinary EVs from renal cell carcinoma patients have a particular protein profile (Raimondo et al., 2013). Patients of prostate cancer present prostate markers in their urinary EVs (Gamez-Valero et al., 2015) so that their protein composition signature may allow to distinguish among metastatic prostate cancer patients, prostate hyperplasia patients and patients without metastasis (Bijnsdorp et al., 2013; Mitchell et al., 2009). Moreover, a panel of miRNAs recently described could be used as biomarker for metastatic prostatic cancer (Corcoran et al., 2014). Regarding bladder cancer, urinary EVs play a role in cancer progression and previous studies suggest that they contain an altered repertoire of mRNA (Beckham et al., 2014; Chen et al., 2012).

The miRNAs, small non-coding RNAs, have been described as key regulators of tumour initiation and progression (Croce, 2009; Garzon et al., 2009) and extensively studied for their diagnostic and prognostic implications in a variety of human tumours, including urologic cancers (Catto et al., 2011; Friel et al., 2010; Kosaka et al., 2010; Schaefer et al., 2010). Remarkably, miRNAs may have a dual role as tumour suppressors or oncogenes depending on the functions of their target genes. Since some circulating miRNAs are contained inside EVs to be protected from degradation, the evaluation of EV miRNA profile represents a promising diagnostic and prognostic non-invasive tool in bladder cancer. In this study, we provide proof of concept for this perspective in a pilot study of bladder cancer patients.

2. Materials and methods

2.1. Donor cohort

A total of 34 urine samples from patients with suspected bladder cancer based on previous cystoscopy from the Urologic Unit of La Princesa Hospital were collected. In addition, samples from 9 non-smokers healthy volunteer donors were used as controls (Table 1). All patients signed an informed consent form. All cases were thereafter histologically confirmed and classified: 18 high-grade and 16 low-grade bladder cancer donors.

2.2. Sample collection

First morning urine was collected in one litre collection containers immediately prior to transurethral resection of bladder tumour from fasted eligible patients with suspected bladder cancer based on previous cystoscopy. 200 ml of the total collected urine were processed immediately to collection. Urine samples were first centrifuged at 3500 *g* for 25 min at 4 °C and the supernatant filtered through a 220 nm filter (Millipore) to remove cells and cellular debris. Then urine samples were used to isolate EVs by serial ultracentrifugation.

2.3. Bladder cell lines

UMUC-3, J82 and SW780 bladder cancer cell lines were grown in DMEM supplemented with 10% EV-depleted FCS.

2.4. EVs isolation

EVs were concentrated by serial ultracentrifugation. Urine samples or cell lines culture supernatant were first centrifuged at 3500 *g* for 25 min at 4 °C and the supernatant filtered through a 220 nm filter (Millipore) to remove cells and cellular debris. 200 ml of sample were transferred to 35 ml open top Ultra-Clear™ centrifuge tubes (Beckman Coulter, Brea, CA) and ultracentrifuged for 1 h at 100,000 *g* at 4 °C in a Beckman Coulter Avanti J-30 i centrifuge (Js-24,38 rotor, Beckman Coulter). The pellet was resuspended in 33 ml of filtered PBS for a washing step and centrifuged again under the same conditions. The final pellet containing EVs was resuspended in 200 μl of filtered PBS. One aliquot was used to check the presence of EVs in samples by electronic microscopy and their quantification by NTA. The pellet of two tubes were gathered and mixed with 3 volumes of Trizol to microRNA analysis. The pellet from three remaining tubes was pooled for proteomic analysis.

2.5. Nanoparticle tracking analysis (NTA)

Determination of particle concentration and size of EV samples was performed by Nanoparticle Tracking Analysis (NTA) using a Nanosight LM10 and NTA 2.3 Software (Nanosight, Wilt- shire, UK). EV samples were diluted in filtered PBS. Three 60-second videos were recorded for each sample with camera level and detection threshold set at 10. Temperature was monitored throughout the measurements.

2.6. Transmission electron microscopy

EV samples were adsorbed on carbon-coated nickel grids by floating an ionized grid onto a drop of the sample. The grids were contrasted with 2% uranyl acetate. Preparations were examined in a Jeol JEM-1010 electron microscope.

2.7. RNA isolation and microarray detection

Trizol/chloroform extraction was performed on EV aliquots intended for RNA analyses. EV-miRNA was isolated with a miRNeasy Mini Kit (Quiagen) following the manufacturer's instructions. Prior to