Clinica Chimica Acta 442 (2015) 119–124

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Versican and its associated molecules: Potential diagnostic markers for multiple myeloma

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ARTICLE INFO

Article history:
Received 18 November 2014
Received in revised form 16 January 2015
Accepted 16 January 2015
Available online 23 January 2015

Keywords:
Multiple myeloma
Versican
β-Catenin
β1 Integrin
FAK
Diagnostic marker

ABSTRACT

Background: Multiple myeloma (MM) represents a malignancy of B-cells characterized by proliferation of malignant plasma cells in the bone marrow (BM). Versican (VCAN), an extracellular matrix (ECM) protein, appears to be involved in multiple processes in several cancers. Identifying optimum diagnostic markers and delineating its association with disease severity might be important for controlling MM.
Methods: Expression of VCAN and its associated molecules (β-catenin, β1 integrin and FAK) were investigated in 60 subjects to evaluate their usefulness as diagnostic marker. Circulatory and molecular levels of above molecules were analyzed in their BM and Blood using ELISA, Q-PCR and western blotting along with their ROC curve analysis.
Results: Circulatory levels of VCAN, β-catenin and FAK were significantly higher in patients with varying significance in each stage. β-Catenin and FAK intracellular levels were significantly elevated in patients. mRNA levels of all molecules were significantly higher in BMMNCs while VCAN and β-catenin also showed increase in PBMCs. Upregulation of these molecules was also observed at protein level. ROC curve analysis for VCAN showed absolute combination of sensitivity and specificity for diagnosis in serum.
Conclusions: Significant elevation of VCAN and its associated molecules imply their role in MM. Optimal sensitivity and specificity of VCAN might utilize its importance as potential marker for active disease.

1. Introduction

Multiple myeloma (MM) is a malignant neoplasm of plasma cells in the BM accounting for 13% of all hematological malignancy and nearly 1% of all malignancies. Multistep genetic and microenvironmental changes lead to the transformation of these cells into a malignant neoplasm and produce large quantities of an abnormal immunoglobulin [1]. BM microenvironment plays an important role in survival, growth and differentiation of diverse lineages of blood cells but also provides optimal growth environment for multiple hematological malignancies including MM. This also confers drug resistance to malignant plasma cells providing the preclinical evidences for targeting MM cells and BM stromal cells (BMSCs) as an anticancer strategy in this disease [2,3]. Components of the ECM as well as their proteolytic digestion products have been shown to stimulate the in vitro migration of a variety of normal and malignant cell types [4].

Versican (VCAN), a large ECM chondroitin sulfate proteoglycan, provides a loose and hydrated matrix during key events in development and disease. It participates in cell adhesion, proliferation, migration and angiogenesis via interaction with its domains and hence, plays a role in tissue morphogenesis and maintenance [5,6]. VCAN was also found to play a key role in both malignant transformation and tumor progression. Increased VCAN expression has been observed in a wide range of malignant tumors and has been associated with both cancer relapse and poor patient outcomes [6–8]. Recently, Corral et al. [9] showed the role of the VCAN transcript deregulation in the transition from a normal plasma cell to a clonal plasma cell and from an indolent clonal plasma cell to a malignant plasma cell. There are further studies exploring VCAN in solid tumors and other hematological malignancies [10–12]. These reports emphasize the importance of VCAN in malignant transformation and differentiation. The transcription of VCAN is mediated by β-catenin (a central molecule in wnt pathway) [13]. β-Catenin is a multifunctional structural protein that acts as a transcription co-activator. The activation of wnt pathway leads to translocation of β-catenin into the nucleus to form a transcription factor complex that activates various target genes [14] and VCAN is one of its target gene [15,16]. Wnt pathway and subsequently β-catenin were found to be upregulated in MM [17–19], and their inhibition was found to suppress growth and progression of myeloma cells in vitro and in vivo [20–22].

VCAN interacts with a cell surface receptor (β1 integrin) via its G3 domain [23]. β1 Integrin promotes myeloma cell adhesion to the BM that mediates MM cell trafficking and drug resistance [24,25]. The binding of VCAN to β1 integrin activates the focal adhesion kinase (FAK) [5], which is an important regulator of cell migration and proliferation

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http://dx.doi.org/10.1016/j.cca.2015.01.012
0009-8981/

required for invasion and metastasis. FAK was found to be overexpressed in MM and shows its involvement with disease progression and poor patient outcome [26]. The inhibition of FAK resulted in the inhibition of proliferative and invasive ability of myeloma cells [27]. These studies emphasize the role of FAK as a potentially important therapeutic target. It is evident from the previous reports that VCAN, which is produced predominantly as a result of wnt pathway, interacts with β1 integrin and activates FAK, which plays a role in cell proliferation and migration. Therefore, in this maiden attempt, we have studied the circulatory and molecular expression of VCAN along with its associated molecules to explore its potential involvement in MM.

2. Materials and methods

2.1. Study samples

Twenty-five newly diagnosed MM patients registered at Department of Medical Oncology, BRA-IRCH, AIIMS, New Delhi, were included in the study (Table 1). BM aspirate and blood were obtained from these patients. The controls recruited for the study were divided in two groups: the first group included 10 patients with idiopathic thrombocytopenic perpurae (ITP) registered at Department of Hematology, AIIMS, and the second group included 25 healthy volunteers. BM aspirate was taken from the first group and blood sample from the second group for accurate comparison of expression changes of molecules with patients in two compartments. The study was approved by the ethical committee at AIIMS, and written informed consent was taken. Five milliliters of BM aspirate from patients and controls (first group) was collected in heparinized vials at the time of their diagnostic procedure and centrifuged at 3,000 rpm for 10 min for BM supernatant fluid (BMSF) isolation (stored at −80 °C), and the pelleted cells were used for BM mononuclear cells (BMMNCs) isolation. These obtained BMMNCs were then used for RNA isolation and cell lysate preparation. Similarly, 10 ml of blood was withdrawn from patients and controls (second group). Plain sterile tubes free of endotoxins were used to withdraw blood (4 ml) and serum was isolated, whereas EDTA vials were used to collect blood (6 ml) for peripheral blood mononuclear cells (PBMCs) isolation. For serum isolation, blood was kept at room temperature for 10 min, and then centrifugation was done for 10 min at 3,000 rpm. Isolated serum was stored at −80 °C for further use, while the Ficoll gradient was used for PBMCs isolation which was used for RNA isolation and cell lysate preparation.

Table 1
Demographic and clinical data for MM patients and control subjects. Values are represented as mean ± SD.

Patients	
Total no. (*n*)	25
Male/female	16/9
Age (years) (range)	59.96 ± 9.39 (42–79)
Anemia (Hb <10 g/dl)	56% (14/25)
% Plasma cells	51.9 ± 24.6
	≥50% (15)
	<50%(10)
M-band (g/dl)	3.2 ± 1.2
β2 microglobulin (mg/L)	<3.0 (3)
	>3.5 (22)
Globulin (g/dl)	5.3 ± 1.8
Albumin (g/dl)	3.6 ± 0.7
Urea (mg/dl)	46.8 ± 33.3
Creatinine (mg/dl)	1.6 ± 1.2
Clinical staging	
Stage I	03
Stage II	10
Stage III	12
Controls	
Total no. (*n*) (bone marrow/blood)	35 (10/25)
Male/female	24/11
Age (years) (Range)	38.2 ± 14.1 (21–75)

2.2. ELISA for determination of circulatory levels of VCAN, β-catenin, β1 integrin and FAK and intracellular levels of β-catenin, β1 integrin and FAK

High-sensitivity commercially available enzyme-linked immunosorbent assay (ELISA) kits were used for determining the circulatory levels of VCAN, β-catenin, β1 integrin and FAK in BMSF and blood serum. Intracellular levels of cytosolic proteins (β-catenin and FAK) and cell surface receptor (β1 integrin) were assayed in 15 patients and 20 controls (5 BM controls and 15 blood controls) using cell lysates normalized by their total protein concentration. VCAN, β-catenin, β1 integrin and FAK ELISA kits were supplied by USCN Life Science Inc., USA.

2.3. Quantitative mRNA expression by real-time PCR

The relative mRNA expression of VCAN, β-catenin, β1 integrin and FAK were analyzed by relative quantitation using Biorad CFX96™ real-time system. Total RNA was isolated by ethanol-chloroform precipitation from BMMNCs and PBMCs using sigma TRI Reagent®. One microgram of the DNase-treated RNA was used for single strand cDNA synthesis using random hexamers (Thermo Scientific) that was used as template in Quantitative PCR (Q-PCR). GAPDH was used as an endogenous control for relative quantitation. The forward and reverse primers used for each molecule were as follows:

VCAN	Forward-5′ GTGAGCAAGATACCGAGAC 3′
	Reverse-5′ GCCCACACGATTAACAAAC 3′
β-Catenin	Forward-5′ TGCTTGGTTCACCAGTGGATT 3′
	Reverse-5′ GATGAGCTTGCTTTCTTGGTTG 3′
β1 Integrin	Forward-5′ TAAGATCAGGGGAGCCACAG 3′
	Reverse-5′ GCCAAATCCAATTCTGAAGTCC 3′
FAK	Forward-5′ CCTCAACCAGGGATTATGAG 3′
	Reverse-5′ CGCATTGTTAAGGCTTCTTG 3′
GAPDH	Forward-5′ AGAAGGCTGGGGCTCATTTG 3′
	Reverse-5′ AGGGGCCATCCACAGTCTTC 3′

Relative mRNA expression was calculated for each molecule using $2^{-\delta Ct}$ method, where Ct values of the molecules were normalized to that of GAPDH and compared with their respective controls.

2.4. Western blotting

BMMNCs were lysed in RIPA lysis buffer (150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0) containing protease inhibitor cocktail and EDTA. Protein quantification was done by Bradford assay. Thirty micrograms of total protein was then resolved in SDS–PAGE and transferred from gel onto a nitrocellulose membrane. Membrane was blocked in 5% BSA–TBST (Tris-buffered saline, 0.1% Tween 20, pH 7.2) for 2 h. VCAN polyclonal antibody, β-catenin, β1 integrin and FAK monoclonal antibodies were incubated for overnight at 4 °C. Horseradish peroxidase (HRP)-conjugated secondary antibody was then added for 2 h at room temperature. Chemiluminescence detection reagent (Thermo Scientific) was used to develop blots, and band images were acquired with FluorChem E (Cell Biosciences) followed by quantification using Image J analyzer software.

2.5. Statistical analysis

Stata 1.0 was used for statistical assessment. Data were presented as mean ± SD. Student's *t*-test and Mann–Whitney test were used to statistically analyze the differences in mean values of the parameters between MM patients and control subjects. Pearson correlation was done to correlate the different molecules with each other and with clinical parameters. BM and blood concentration of each molecule were analyzed for determining the receiver operating characteristic (ROC)