Runx2 expression: A mesenchymal stem marker for cancer

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Abstract. The transcription factor runt-related transcription factor 2 (Runx2) is a master gene implicated in the osteogenic differentiation of mesenchymal stem cells, and thus serves a determinant function in bone remodelling and skeletal integrity. Various signalling pathways regulate Runx2 abundance, which requires a number of molecules to finely modulate its expression. Furthermore, this gene may be ectopically-expressed in cancer cells. Recent studies have reported the involvement of Runx2 in cell proliferation, epithelial-mesenchymal transition, apoptosis and metastatic processes, suggesting it may represent a useful therapeutic target in cancer treatment. However, studies evaluating this gene as a cancer marker are lacking. In the present study, Runx2 expression was analysed in 11 different cancer cell lines not derived from bone tumour. In addition, the presence of Runx2-related cell-free RNA was examined in the peripheral blood of 41 patients affected by different forms of tumours. The results demonstrated high expression levels of Runx2 in the cancer cell lines and identified the presence of Runx2-related cell-free RNA in the peripheral blood of patients with cancer. As compared with normal individuals, the expression level was increased by 14.2-fold in patients with bone metastases and by 4.01-fold in patients without metastases. The results of the present study therefore opens up the possibility to exploit Runx2 expression as a cancer biomarker allowing the use of minimally invasive approaches for diagnosis and follow-up.

Introduction

The osteogenic differentiation process of mesenchymal stem cells involves either systemic hormones or specific local molecules, including transforming growth factor-β 1/2 (TGF-β), fibroblast growth factor-2 (FGF-2), bone morphogenic proteins (BMPs), insulin-like growth factor (IGF), prostaglandins, vascular endothelial growth factors (VEGFs) and the Wnt/β-catenin pathway (1). As a result, intracellular signalling promotes the expression of transcription factors. Among these, runt-related transcription factor 2 (Runx2) serves a pivotal role and it is considered a master gene for osteogenic differentiation (1). Runx2 induces the expression of specific downstream genes, including collagen type I, bone alkaline phosphatase, osteopontin and osteocalcin (2), and it is essential for terminal chondrocyte differentiation (3). Runx2 knock-out mice are affected by cleidocranial dysplasia syndrome (3), while Runx2 overexpression in mice impairs mineralization, suggesting that this gene affects bone formation in different ways (4). A previous study demonstrated that the expression of Runx2 in circulating mesenchymal stem cells was lower in patients with osteoporosis when compared with normal donors (5). Runx2 expression is modulated by several regulatory pathways. Important negative regulators include histone deacetylases (HDACs), in particular HDAC3, HDAC4, HDAC5, HDAC6 and HDAC7 (6). Twist proteins (7), activator protein 1, transcription factor 4 and osterix are additional regulators of Runx2 expression (2). Furthermore, it has been demonstrated that Runx2 function may be downmodulated by microRNA (miR) action, in particular miR-3960 (8), and phosphorylation induced by the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway results in Runx2 activation (9). The involvement of Runx2 in the oncogenic process has been recently suggested to occur in human osteosarcoma (10), in addition to other forms of malignancy such as pancreatic and thyroid cancer, and increased expression correlates with a poor prognosis (11,12).

Epithelial-mesenchymal transition (EMT) is involved in carcinogenesis and promotes metastatic spreading (13-15). Following its recognition as a regulator gene in transformed epithelial cells in breast, lung and thyroid carcinoma (13-15), it has been suggested that Runx2 may promote breast cancer metastasis by EMT (13). The cancer caused by EMT is a consequence of complicated reprogramming process involving differentiation, epigenetics and metabolic balance disruption (16). In this scenario, Runx2 has been identified as a regulator gene of transformed epithelial cells in breast, lung and thyroid carcinoma (13-15), and it has been suggested that this gene promotes breast cancer metastasis via EMT (13).

A number of researchers have focused on identifying cancer markers that may provide clinical information a less

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invasive way. A previous study reported that Runx2 expression was elevated in the tissue, serum and circulating cells of patients with thyroid cancer suggesting that Runx2 may serve as a useful biomarker for thyroid malignancies (17).

On the basis of these findings, the present study speculated that Runx2 may be expressed in cells derived from malignancies other than bone tumours. Therefore, the expression of this gene was analysed in pancreatic, melanoma, breast and prostate cancer cell lines. In addition, in order to evaluate potential applications in oncological malignancies, Runx2 cell-free RNA was examined in sera obtained from patients affected by various forms of cancer.

Materials and methods

Cell culture. A total of 4 pancreatic, 2 breast, 3 prostate and 2 bone human cancer cell lines, purchased by American Type Culture Collection (Rockville, MD, USA), were used in the present study (Table I). Table I specified the previous applications of these cell lines studies (18-28). The pancreatic cancer cell lines were cultured in RPMI 1640 (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) with 10% foetal bovine serum (FBS) (Sigma-Aldrich; Merck Millipore), whereas the breast, prostate and bone cell lines were cultured as previously described (29-33). For cell synchronization, cell cycles were arrested at G_1 phase by adding 400 mM mimosine (Sigma-Aldrich; Merck Millipore) for 24 h as previously described (34). Cells subsequently underwent three washes with PBS (Sigma-Aldrich; Merck Millipore) and were cultured in serum-free RPMI 1640 medium for 3 days. Finally, cells were cultured in fresh RPMI 1640 medium with 10% FBS (plus 2 mM L-glutamine and penicillin/streptomycin) until they reached 70% confluence. Adherent cells and supernatants for each cell line were harvested to perform expression analyses. For each cell line, three different cultures were tested.

Patients. Characteristics of the population analysed are presented in Table II. A total of 41 patients with cancer were positively diagnosed from 2010 to 2013 by pathologists (Pancreas Institute; Integrated University Hospital of Verona, Verona, Italy) prior to providing blood samples, and 41 age-matched donors, who were hospitalized in Clinic of Internal Medicine, Integrated University Hospital of Verona for cardiovascular or metabolic diseases, were recruited as controls. Bone metastases were present in 17 patients. All subjects had provided written informed consent and the study was approved by the local Institutional Ethics Committee of the Integrated University Hospital of Verona.

Serum preparation. Serum samples were obtained following three rounds of centrifugation (800 x *g*, 1,000 x *g* and 1,500 x *g* at 4˚C) of collected blood to keep lymphocyte contamination to a minimum as previously described (35).

RNA extraction and reverse transcription. RNA from cancer cell lines was extracted using the RNeasy® Mini kit (Qiagen, Hilden, Germany), and RNA extraction from sera and culture supernatants was performed using the QIAamp® UltraSens® Virus kit (Qiagen) with DNAse I treatment according to the manufacturer's protocol. First-strand cDNA was generated using the High-Capacity cDNA Archive kit with random hexamers (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. cDNA products were stored at -80˚C until use.

Quantitative polymerase chain reaction (qPCR). PCR was performed in a total volume of 50 μl containing 1X Taqman Universal PCR Master mix, No AmpErase® UNG and 5 μl cDNA. The real time amplifications included 10 min at 95˚C, followed by 40 cycles at 95˚C for 15 sec and at 60˚C for 1 min. Predesigned, gene-specific primers and a probe set for Runx2 were obtained from Assay-on-Demand™ Gene Expression products (Applied Biosystems; Thermo Fisher Scientific, Inc.). In order to normalize the results, the following three housekeeping genes were used: β-actin (structural gene), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; metabolism-related gene) and β-2 microglobulin (component of major histocompatibility complex class I gene). The primer sequences were pre-designed by the supplier (Applied Biosystems; Thermo Fisher Scientific, Inc.). The relative expression levels of the Runx2 gene were calculated for each sample following normalization using the $2^{-\Delta\Delta Ct}$ method for comparing differences in relative fold expression (36). The data are reported as mRNA fold expression.

Western blot analysis. Cells were lysed on ice for 45 min in a buffer containing protease inhibitor cocktail [1% IGEPAL®, 1% sodium dodecyl sulfate (SDS), 10% glycerol, 1 mM ethylenediaminetetraacetic acid, 5% b-mercaptoethanol, 1.5% Triton X-100 and 4% Protease Inhibitor Cocktail (Sigma-Aldrich; Merck Millipore)]. Cell lysates were then centrifuged (10,000 x *g*) for 15 min at 4˚C to remove insoluble materials. Protein concentration in the supernatants was measured using the Coomassie Protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Proteins (70 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis and electrotransferred onto a polyvinylidene fluoride membrane. The membrane was subsequently blocked for 30 min with 3% bovine serum albumin (Sigma-Aldrich; Merck Millipore) in 0.05% Tween 20 with Tris-buffered saline (t-TBS) at room temperature. For immunodetection, blots were incubated for 2 h at room temperature on titer plate agitator with anti-Runx2 antibodies (cat no. 05-1478; dilution 1:500; clone AS110; EMD Millipore, Billerica, MA, USA). The membranes were washed three times in t-TBS, incubated at room temperature with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (dilution, 1:2,500) in TBS for 1 h and washed in fresh t-TBS three times for a total of 20 min. Bands were detected using Luminata™ Forte Western HRP Substrate (Merck Millipore) and a G:BOX Chemi XX6 (Syngene, Frederick, MD, USA).

Statistical analysis. Results are expressed as the mean ± standard error. The Wilcoxon signed-ranked test was used for non-parametric data. Analysis of variance followed by Bonferroni correction was performed as a *post-hoc* analysis and the results are expressed as the mean ± standard error of the mean. $P<0.05$ was considered to indicate a statistically significant difference. Analyses were applied to experiments

Table I. Cancer cell lines.

Author, year	Cell line	Source	Tumour	Refs.
Morgan *et al*, 1980	Colo357	Metastatic	Pancreatic	(18)
Kim *et al*, 1989	HPAF	Metastatic	Pancreatic	(19)
Lieber *et al*, 1975	Panc1	Primary	Pancreatic	(20)
Parekh *et al*, 1994	BON	Metastatic	Pancreatic	(21)
Soule *et al*, 1973	T47D	Metastatic	Breast	(22)
Keydar *et al*, 1979	MCF7	Metastatic	Breast	(23)
Stone *et al*, 1978	DU145	Metastatic	Prostatic	(24)
Tai *et al*, 2011	PC3	Primary	Prostatic	(25)
Horoszewicz *et al*, 1983	LNCaP	Metastatic	Prostatic	(26)
Niforou *et al*, 2008	U2OS	Primary	Osteosarcoma	(27)
Billiau *et al*, 1977	MG63	Primary	Osteosarcoma	(28)

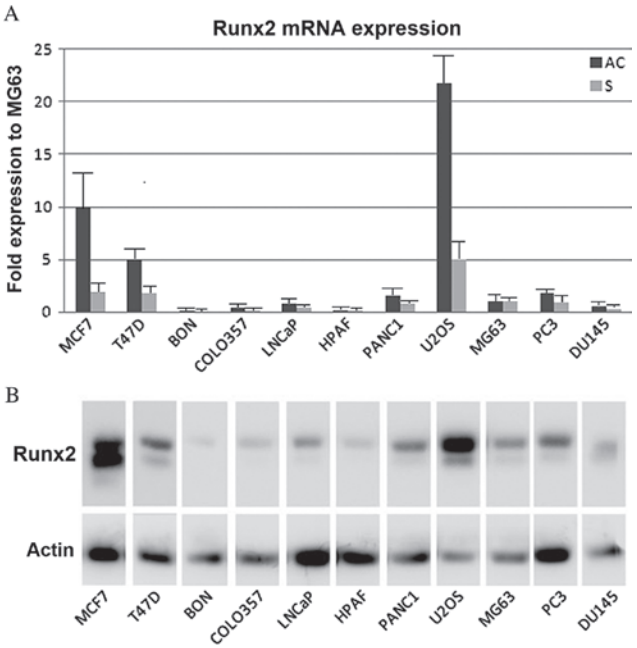

Figure 1. Runx2 mRNA fold expression in ACs and Ss of cancer cell lines. (A) All cell lines expressed Runx2 mRNA and (B) immunoblotting demonstrated that the ACs also expressed Runx2 protein. Runx2, runt-related transcription factor 2; ACs, adherent cells; Ss, supernatants.

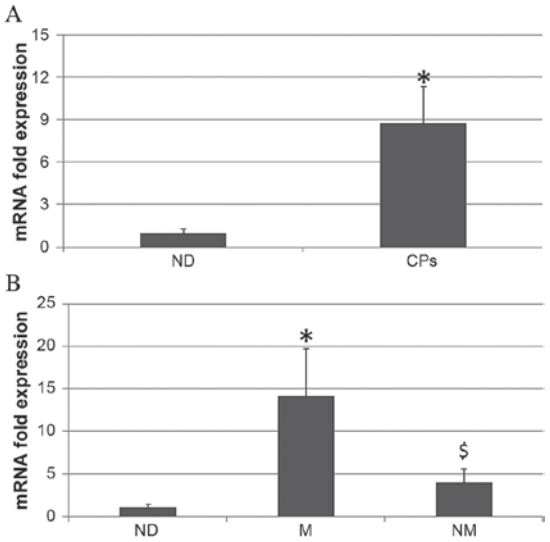

Figure 2. Runx2 expression in patients with cancer. (A) Circulating Runx2 mRNA in CPs was higher than in NDs (*P<0.01), and (B) patients with M expressed higher levels of Runx2 (*P<0.01) than NM patients with respect to NDs ($P<0.05). Runx2, runt-related transcription factor 2; CP, cancer patients; NDs, normal donors; M, bone metastases; NM, non-metastatic.

carried out at least three times, and statistical analyses were performed using SPSS v16.0 (SPSS, Inc., Chicago, IL, USA).

Results

Runx2 expression in cancer cell lines. Runx2 gene expression was analysed in adherent cells and in culture supernatants, and the MG63 cell line was used as a calibrator (fold of expression). It was observed that Runx2 mRNA was expressed in adherent cells and supernatants of the cancer cell lines, although expression was largely varied across the different cell types (Fig. 1A). In order to analyse the expression of Runx2 protein in adherent cells, immunoblotting using anti-Runx2 antibodies was performed. The results demonstrated that the protein was also expressed in all cell lines (Fig. 1B).

Runx2 gene expression in patients with cancer. The expression data of patients with cancer was reported as fold of expression in respect to a calibrator (40 normal donors). Patients with cancer and normal donors each expressed Runx2 mRNA; however, their expression levels were different. Notably, the expression of Runx2 in the patients with cancer was 8.74 (±3.5)-fold higher than the normal donors (P<0.01; Fig. 2A). In addition, Runx2 mRNA expression in patients with bone metastases was higher than in patients without metastases. Runx2 expression in patients with metastases was 14.12 (±4.2)-fold higher than the normal donors (P<0.01), whereas in patients without metastases Runx2 expression was 4.01 (±2.01)-fold higher than the normal donors (P<0.05) (Fig. 2B).

Discussion

In order to establish less invasive methods for the diagnosis and follow-up of patients with cancer, research has aimed

Table II. Characteristics of the study population.

N	Gender	Age, years	Diagnosis	TNM
1	M	65	Neuroendocrine adenocarcinoma	TxN1M1
2	M	80	Intestinal adenocarcinoma	T1-2N0M0
3	M	71	Hepatocarcinoma	T3N1M0
4	M	55	Prostatic adenocarcinoma	T2N0M0
5	M	83	Prostatic adenocarcinoma	T3N0M1
6	M	87	Prostatic adenocarcinoma	T4N1M0
7	M	66	Prostatic adenocarcinoma	T2N0M0
8	M	67	Kidney adenocarcinoma	T4N0M0
9	M	73	Intestinal adenocarcinoma	TxN0M0
10	M	94	Gastric adenocarcinoma	T3N0M0
11	M	81	Gastric adenocarcinoma	T3N2M0
12	M	70	Lung carcinoma	T1N1M1
13	M	70	Mesenchymal cancer	T4NxM1
14	M	81	Prostatic adenocarcinoma	T1N1M1
15	M	92	Breast carcinoma	T2N1M1
16	M	60	Intestinal adenocarcinoma	T1-2N0M0
17	M	75	Pancreatic adenocarcinoma	T3N1M1
18	M	60	Pancreatic adenocarcinoma	T3N1M1
19	M	64	Pancreatic adenocarcinoma	T3N0M0
20	M	67	Pancreatic adenocarcinoma	T1N0M0
21	M	78	Bladder carcinoma	T1N0M0
22	F	87	Hepatocarcinoma	T3N0M0
23	F	72	Intestinal adenocarcinoma	T1N0M0
24	F	27	Adrenal carcinoma	T3-4N1M1
25	F	82	Intestinal adenocarcinoma	TxN0M0
26	F	82	Lung carcinoma	T2N0M0
27	F	52	Esophageal carcinoma	T4N1M1
28	F	68	Ovarian carcinoma	T3N2M1
29	F	80	Breast carcinoma	T2N1M1
30	F	86	Breast carcinoma	T0N1M1
31	F	75	Lung carcinoma	T1NxM0
32	F	81	Pancreatic adenocarcinoma	T3N0M0
33	F	71	Bladder carcinoma	T3aN1M0
34	F	62	Pancreatic adenocarcinoma	T3N1M1
35	F	71	Pancreatic adenocarcinoma	T4N0M0
36	F	75	Pancreatic adenocarcinoma	T3N0M0
37	F	70	Pancreatic adenocarcinoma	T1N0M0
38	F	49	Pancreatic adenocarcinoma	T1N0M0
39	M	78	Prostatic adenocarcinoma	T2N0M1
40	M	80	Prostatic adenocarcinoma	T2N0M1
41	M	75	Prostatic adenocarcinoma	T3N1M1

TNM, tumour-node-metastasis.

to identify cell-free RNA encoding for genes upregulated in cancer malignancies (17,35,37). Previous studies primarily focused on osteosarcoma and metastatic breast and prostate cancer have linked Runx2 to neoplastic transformation (38-41). The present study enrolled patients affected by various types of tumours, including pancreatic, prostatic, intestinal, lung, breast, gastric, liver, neuroendocrine, kidney, mesenchymal, adrenal gland, oesophageal and ovarian cancer. Notably, the results of the current study demonstrated an increase in Runx2 circulating mRNA in multiple forms of cancer, thus opening the possibility to investigate it as a relatively comprehensive biomarker.

The Runx gene family is comprised of three related transcription factors, which are involved in the differentiation

of multiple haematopoietic lineages (Runx1), cartilage and bone (Runx2) and epithelial tissues (Runx3). However, all three genes are implicated in cancer by promoting (Runx1 and Runx2) or suppressing (Runx3) neoplastic transformation (42). Multiple mechanisms contribute to Runx2 functional modulation, including post-translational modification, in addition to protein-protein interaction and direct stimulation (11). Several hypotheses, such as the involvement of integrin alpha5 (39), p53 (43) or microRNA-205 (40) have been put forward to describe the molecular process of Runx2 in carcinogenesis. In osteosarcoma, loss of p53 upregulates Runx2 expression (43); this cause-effect relationship may explain Runx2 ectopic expression in various forms of cancer.

P53 and Runx2 have been demonstrated to be part of the regulatory network controlling EMT (44). P53 controls miRNAs, major EMT-related signalling pathways (TGF-β, Wnt, IGF, and signal transducer and activator of transcription), and EMT-associated transcription factors that promote a chemoresistant phenotype, invasion and loss of cell polarity (44). The direct involvement of Runx2 in cancer was demonstrated by downmodulation experiments in thyroid carcinoma cells (15) and upregulation experiments in breast cancer (45). EMT represents an early event of tumour progression and is mediated by well-characterized transcription factors (e.g. Snail and Twist family and helix-loop-helix factors) (46). The present study speculates that Runx2 participates in these events to promote invasion and metastasis in a larger number of cancer forms than previously anticipated. The data from the current study demonstrated an increase in the concentration of circulating cell-free Runx2 cell-free mRNA in patients with metastasis. In agreement with these results, Runx2 has been repeatedly identified as a regulator of bone metastases in breast and prostate cancer in previous studies (47-49). Bone is particularly recurrent as a target of metastasizing cells, thus a master skeletal transcription factor like Runx2 may be extremely relevant in potentiating tumour cell invasiveness of bone marrow, among others, contributing directly to the osteolysis process (38). Further studies with a larger number of patients should be performed in order to validate the predictive value of minimally invasive tests based on Runx2 cell-free mRNA.

In conclusion, the present study demonstrated that Runx2 is expressed at high levels in osteosarcoma and expanded this finding to non-osseous cells, thus supporting the possible use of Runx2-related cell-free RNA as a cancer marker for screening purposes. In addition, this useful, less invasive method may allow clinicians to monitor the development of metastases in patients with cancer.

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