

The product of the human AHI-1 (Abelson helper integration site) gene: experimental *in vitro* data point to its involvement in tumor cell invasion

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Abstract

Introduction. Each of the steps involved in invasion of tumors requires specific molecular program in which the modulation of adhesive and migratory properties of disseminating cells plays an essential role. The improvement in the knowledge of these mechanisms can lead to discovery of new target candidates in drug development. In this study we focused attention on the product of the human AHI-1 (Abelson helper integration site) gene Jouberein (Jbn).

Methods. In particular, we explore by *in vitro* invasion assay, AHI-1 knockdown and electron microscopy, if Jbn is involved in the signaling machinery that regulates tumor invasion. To this purpose tumor cells of different histological derivation (brain, breast, skin) were employed.

Results. We found that Jbn expression correlates with the proliferation, invasive potential and invasion strategy of the tested tumor cells, and that its downregulation reduces their capability of migrating and invading the extracellular matrix.

Conclusions. The results obtained in this study for the first time point to Jbn as a new candidate involved in the invasion process of tumor cells, and as potential molecular target in anticancer therapy.

Key words

- tumor cells
- invasion
- Jouberein
- electron microscopy
- cell signalling

INTRODUCTION

The migratory and invasive properties of cancer cells is one of the features that most contributes to the cancer progression. However, the molecular mechanisms of metastasis are poorly understood. Given its importance, it is of interest to better understand the molecular mechanisms that regulate this complex and dynamic process.

Each of the steps involved in this process requires specific molecular program in which the modulation of the adhesive and migratory properties of the disseminating tumor cells plays an essential role. Adhesive and migratory processes involve adhesion receptor families, receptor tyrosine kinases (such as ERK, FAK and AKT), cytoskeleton proteins, adapter and signalling proteins that interplay in a complex scenario, not yet fully understood [1]. The improvement in the knowledge of mechanisms behind cell migration/invasion can lead to discovery of new target candidates in drug development.

In this work, we focused attention on the product of the human AHI-1 (Abelson helper integration site)

gene Jouberein (Jbn), also identified as “Abelson helper integration site 1 protein homolog”. The Ahi-1 locus was initially identified as a common helper provirus integration site in 16% of Abelson murine leukemia virus (A-MuLV)-induced pre-B lymphomas [2].

The Ahi-1/AHI-1 gene is highly conserved in mammals and encodes a unique protein with one Src homology 3 (SH3) domain, multiple proline-rich motifs (PXXP) SH3-binding sites, and seven WD40-repeats, all known mediators of protein-protein interaction. PEST sequences and tyrosine kinase phosphorylation sites are also contained [3]. Both murine and human Ahi-1/AHI-1 genes encode at least three isoforms and thus this gene is also subject to alternative splicing in normal cells. Human AHI-1 isoform II, the shortest isoform, lacks the SH3 domain and isoform III, which is still shorter than isoform I, contains additional coding sequences in its C-terminus which are absent from both isoform I and II [4]. Interestingly, Jbn is the only protein thus far identified to contain both WD40 repeats and a SH3 domain. Moreover, the human AHI-1

protein contains a coiled-coil domain in its N-terminal region, also involved in protein-protein interactions [5], which is entirely absent in the mouse and rat Ahi-1 proteins [3]. Thus AHI-1 has multiple features of a unique adaptor protein regulating specific signaling pathways [4]. Normally Ahi-1/AHI-1 transcript levels are down-regulated during both early murine and human hematopoietic cell differentiation [6].

Mutations in human AHI-1 gene encoding Jbn have been associated with pathological conditions such as Joubert syndrome, an autosomal recessive brain disorder with cerebellar ataxia, oculomotor apraxia, hypotonia, neonatal breathing abnormalities and psychomotor delay [7-9]. In individuals with point mutations in human AHI-1, particularly within the WD40-repeat and SH3 domains, an abnormal development and axonal decussation is found [9]. In mouse, when Ahi-1 protein interacts with Huntingtin-associated protein 1 it forms a stable complex that is critical for neonatal development [10]. AHI-1 isoforms and its mutations also underlie other diseases, including Joubert syndrome-associated nephronophthisis and autism, and metabolic syndromes, such as type 2 diabetes [9, 11-15].

Evidence for an oncogenic role of AHI-1 has been reported. Ahi-1/AHI-1 transcript levels are highly up-regulated in certain human leukemic cells, including leukemic stem cells from patients with chronic myeloid leukemia (CML) and leukemic Sezary cells in cutaneous T-cell lymphoma (CTCL) [6, 13]. Moreover, over-expression of Ahi-1 alone in primitive hematopoietic cells confers a proliferative advantage *in vitro* and induces a lethal leukemia *in vivo* [15].

Strikingly, Jbn is a positive modulator of the canonical Wnt pathway through facilitation of β -catenin nuclear translocation [16, 17], event associated with epithelial-to-mesenchymal transition (EMT) process [18].

Thus, in this work, we explore if AHI-1 transcript Jbn is involved in the signaling machinery that regulates tumor invasion. To this purpose tumor cells of different histological origin were employed. The results obtained in this study by *in vitro* invasion assay, AHI-1 knock-down and electron microscopy for the first time point to Jbn as a new protein involved in the invasion process of tumor cells.

MATERIALS AND METHODS

Cell cultures

Established tumor cell lines from different histological origin were used: human glioblastoma cells (brain/right frontal parieto-occipital cortex: LN229 cells from ATCC), drug-resistant variant human melanoma cells (M14 ADR cells, selected from human cutaneous metastatic melanoma M14 cell line, in the laboratories of the Department of Technology and Health, Istituto Superiore di Sanità, Rome, Italy) and human breast adenocarcinoma cells (mammary gland/breast; derived from metastatic site: pleural effusion: MDA-MB-231 and MCF-7 WT cells from ATCC).

LN229, MDA and MCF-7 cell lines were grown in DMEM (Euroclone, Pero, Italy) with high glucose; M14 ADR cell line was grown in RPMI 1640 (Euroclone). The media were supplemented with 1% non es-

sential amino acids, 1% L-glutamine, 100 IU/ml penicillin, 100 IU/ml streptomycin, 10% fetal bovine serum (Euroclone) and 1% vitamins (HAM'S F-12 only) (Euroclone). Cell lines were cultured at 37 °C in a 5% CO₂ humidified atmosphere in air.

Growth curves

For growth curves, LN229, MDA and MCF-7 cells were detached and counted in a Neubauer camera at 1:2 dilution with trypan blue dye exclusion. Viable cells were plated at a density of 2.5 x10³ cells per well in 24 well plates. Two wells for each cell line were seeded in each plate. After 24, 48, 72 and 96 h cells were detached and counted in a Neubauer camera.

Invasion assays

To analyze invasive potential of tumor cell lines, the transwell chamber invasion assay was employed. Briefly, inserts (8.0 μ m pore) (BD Biosciences, Erembodegem, Belgium) which stood in 6-well plates (Corning Inc., Corning, NY) were employed. Matrigel™ was diluted to 1 mg/ml in serum-free RPMI medium. 200 μ l of 5 mg/ml Matrigel™ were placed on the lower side of each insert. The insert and the plate were incubated overnight at 4 °C. The following day, cells were harvested and suspended in RPMI. The inserts were washed with serum-free RPMI, then 1x10⁶ cells were added to each insert and 3 ml of RPMI containing 10% FCS were added to the well underneath the insert. Cells were incubated at 37 °C up to 24 hours. After this time, the inner side of the insert was wiped with a wet swab to remove the cells while the outer side of the insert was gently rinsed with PBS and stained with 0.25% crystal violet (Sigma) for 10 minutes, rinsed again and then allowed to dry. The inserts were then viewed under a CCD camera equipped Nikon (Tokyo, Japan). To calculate the number of cells, membranes were analyzed by Image J program. The image processing techniques that were employed include thresholding and analysis of particles.

Scanning electron microscopy

For SEM analysis, the membranes containing cells were removed from the inserts. At the indicated times, membranes were fixed with 2% glutaraldehyde (Merck, Darmstadt, Germany) in 0.1 M cacodylate buffer (pH 7.4) (Sigma-Aldrich Corporation, MO, USA) at room temperature for 30 minutes, post-fixed with 1% OsO₄ (Merck) in the same buffer, dehydrated through a graded ethanol series, critical point dried with CO₂, and gold coated by sputtering. Samples were examined with a Cambridge Stereoscan 360 scanning electron microscope (Cambridge Instruments Ltd, Cambridge, UK).

Knockdown of AHI-1

For the inhibition of AHI-1 protein expression in M14 ADR, LN229, MDA and MCF-7 cell lines, RNA interference experiments were carried out according to the manufacturer's protocol (Santa Cruz Biotechnology, Inc. CA, USA). In particular, AHI1 siRNA (h) (sc-72465) and control siRNA-A (sc-37007) were employed.

Western blotting

For the analysis of protein expression, western blotting experiments were performed on cell lysates both in the absence and in the presence of migratory stimulus, performed as described in invasion assay, but in the absence of Matrigel™. After 24 h in transwell chambers, cells were harvested from the insert and subsequently washed twice in ice-cold Tris-buffered saline (TBS; 20mM Tris-HCl, pH 7.6, 140 mM NaCl) and lysed at 4 °C in 200 µl of lysis buffer (10 mM Tris-HCl, pH 7.6, 50mM NaCl, 30 mM sodium pyrophosphate, 5 mM EDTA, 0.5% Nonidet P40, 1% Triton X-100, 50 mM NaF, 0.1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, and complete mini proteinase inhibitors). Cell lysates were obtained by centrifugation at 17000 g for 30 minutes at 4 °C; protein concentration in the supernatant was determined by DC Protein Assay (Bio-Rad Laboratories), and lysates were adjusted to equivalent concentrations with lysis buffer. Total cell lysate (10-40 µg) was then separated on SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes that were blocked for 1 hour at room temperature with 5% BSA in TTBS. Incubations with primary antibodies (anti AHI-1; Santa Cruz Biotechnology) (anti p-AKT, p-ERK, p-FAK; Cell Signaling Technology, Danvers, MA, USA) and with horseradish peroxidase-conjugated secondary antibody were performed in blocking solution overnight at 4 °C and for 1 hour at room temperature, respectively. Immunoreactive bands were visualized by the ECL kit (GE Healthcare Biosciences, Pittsburgh, PA, USA). For loading control, membranes were incubated with monoclonal anti- α -tubulin (Sigma Aldrich). The western blot densitometry values were normalized with those of the loading control.

Statistical analysis

Reported values are the means \pm SD from three or six independent experiments. Statistical analyses were performed using Wilcoxon-Mann-Whitney test. P-values lower than 0.05 were considered significant.

RESULTS AND DISCUSSION

During invasion process tumor cells activate highly regulated intracellular network of pathways that are necessary for cell growth, replication, death, and survival. This complex intracellular signalling involves ligands, receptors, kinases, adapter molecules, transcription factors, and other molecules, many with multiple roles and functions (Reviewed in [1, 19]) and other not yet known. Evidence for an oncogenic role of the product of AHI-1 gene, the Jbn protein (AHI-1) has been reported. Overexpression of AHI-1 alone in primitive hematopoietic cells confers a proliferative advantage *in vitro* and induces a lethal leukemia *in vivo* [15]. Of interest, it has been recently demonstrated that AHI-1 interact with ABL protein kinases in CML cells [20].

Thus, because of its putative oncogenic role [15] and link with ABL family of protein kinases [20], we was interested in investigating whether AHI-1 had a role in the invasive potential of tumor cells. Cell lines from human breast (MDA-MB-231, MCF-7 WT), brain (LN229) and skin (M14 ADR) cancers were used. The

invasive potential (IP) of cancer cells was evaluated by using the “transwell chamber invasion assay”, since the invasive abilities of tumor cells in this assay are usually related to their *in vivo* invasion behavior [21, 22]. Tumor cell lines tested in this study featured different IP (Figure 1, panels A and B). The IP was positively related to cell growth rate (Supplementary data 1 available online). In particular, human breast cancer estrogen triple negative MDA-MB-231 cells showed the highest capacity of invading the Matrigel™ (i.e. they occupied 40% of the lower side-membrane area) especially when compared with estrogen-receptor positive MCF-7 WT cells (7.5%). The LN229 cells derived from patient with right frontal parieto-occipital glioblastoma were more efficient (26.7%) than the M14 ADR cells (22.6%), resistant variants derived from human cutaneous metastatic melanoma M14 cell line [23].

The combination of 2D and 3D *in vitro* assays, biochemical tests and ultrastructural investigations by scanning electron microscopy (SEM) proved to be particularly suitable for the investigation of tumour cell migration and invasion [24]. Thus, we performed SEM observations to analyse the behaviour of tested tumour cells on membranes employed in “transwell chamber invasion assay” (Figures 2 and 3). The experiments were carried out either in the absence of Matrigel™ film (migration) (Figure 2), in order to analyse the behaviour of tumour cells without the stimulus of ECM proteins, or in the presence of a Matrigel™ film, gelled on the lower side of the porous membranes (invasion) (Figure 3). 3D imaging offered by SEM [24] can provide evidence of “individual” or “collective” behaviour adopted by tumour cells to migrate through membranes or to invade Matrigel™. In the first one, cells detach from the cell population and pass through the pores separately. In the “collective behaviour” cells move on the upper side of the porous membrane in clusters closely linked each other. As showed in Figure 2A, MDA-MB-231 cells with the highest IP adhered to the membrane as separated cells, except for dividing ones. They also preferentially move through the membrane pores as single cells, thus adopting an “individual behaviour”. Accordingly to quantitative data by transwell chamber invasion assay, SEM observations revealed a high number of MDA-MB-231 cells that migrated to the lower side of the porous membranes (Figure 2B). Similarly, a high number of LN229 cells was seen on the lower side of the migration membrane (Figure 2D), but differently from MDA-MB-231 cells they tended to form little chains on the upper side (Figure 2C). On the contrary, M14 ADR cells (Figures 2E and 2F) and MCF-7 WT cells (Figures 2G and 2H), that featured a low IP when compared to LN229 and MDA-MB-231 cells, appeared to be tightly clustered on the upper side of the porous membranes. Thus, they adopted a “collective behaviour”. Also in this case, SEM observations confirmed quantitative data, since very few M14 ADR and MCF-7 WT cells were observed on the lower side of the filters (Figures 2F and 2H, respectively).

SEM allowed us to look in the details of the ECM-cell interaction during the invasion process. On the lower side of the porous membranes covered with gelled

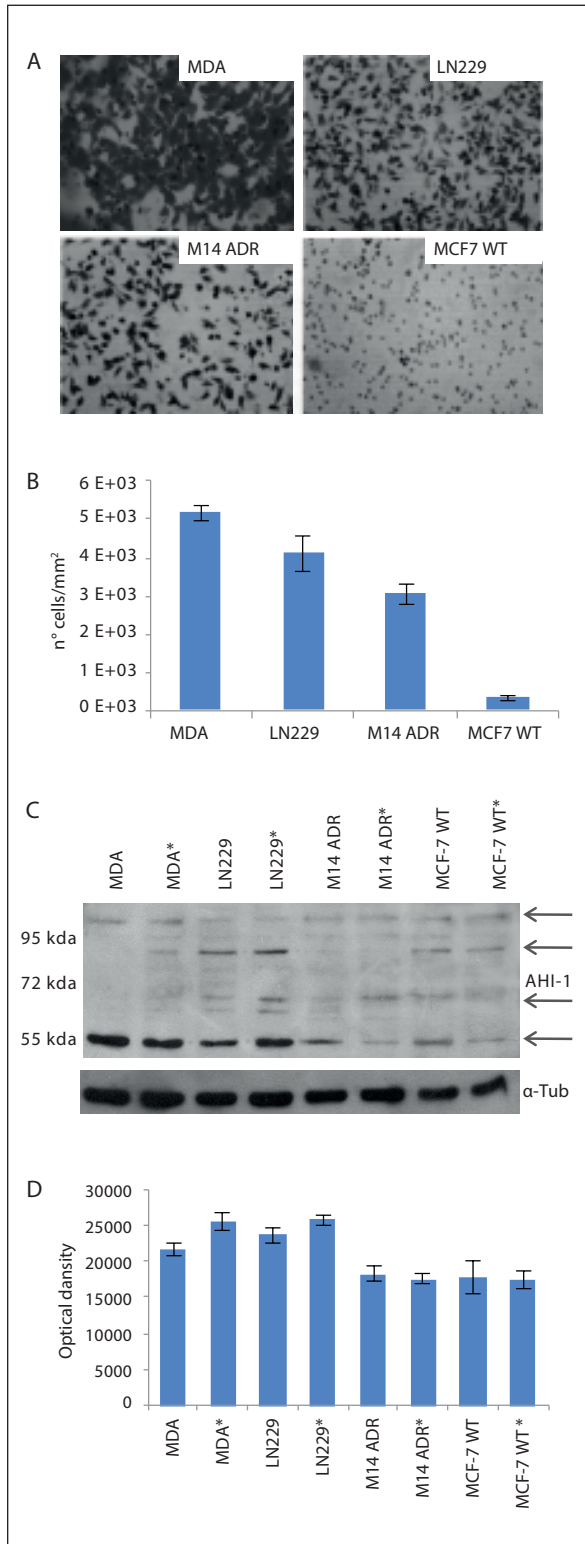


Figure 1
Invasion potential and expression of Jouberin (Jbn) in tumor cells. (A) Images of tumor cells migrated on the lower side of porous membranes covered by Matrigel™. (B) Invasion potential expressed as number of cells migrated on the lower side of porous membrane. Average + SD from six independent experiments. (C) Western blot of tumor cells expressing Jbn in the absence or in the presence (asterisk) of migratory stimulus. (D) Densitometric analysis of Jbn expression reported in C. Average + SD from three independent experiments.

Matrigel™ cell protrusions interacting with extracellular matrix are showed (Figures 3B-H). Literature data report two types of behaviour adopted by cancer cells to invade the extracellular matrix, “mesenchymal” and “ameboid” type, depending on the presence or the absence of focused proteolysis, respectively [25]. An area of Matrigel™ degradation was seen around the invadopode when SEM observations were performed on the lower side of MDA-MB-231 and LN229 cell samples (Figure 3B and D). The invadopode of M14 ADR cells (Figure 3F) was able to penetrate the extracellular matrix, apparently without degrading it, suggesting that an “ameboid behaviour” was adopted. The MCF-7 WT cells with the lowest IP scarcely invaded the extracellular matrix on the lower side of porous membranes (Figure 3H).

Subsequently, we evaluated the AHI-1 expression in MDA-MB-231, LN229, M14 ADR, and MCF-7 cells either in absence or in presence of the migratory stimu-

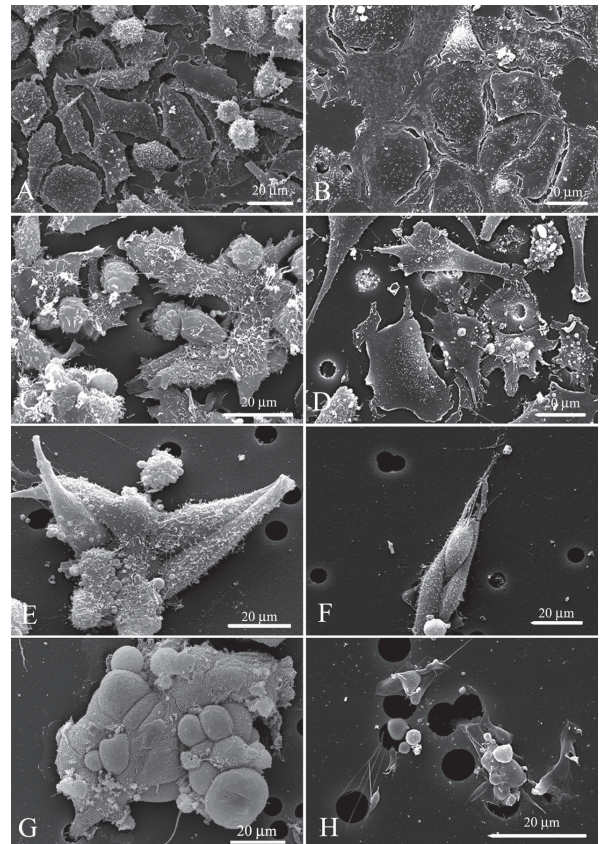


Figure 2
Scanning Electron Microscopy (SEM) observations of tumor cell migration. (A, C, E, G) Upper side of porous membrane. (B, D, F, H) Lower side of porous membrane. (A, B) MDA-MB-231, (C, D) LN229, (E, F) M14 ADR (G, H) and MCF-7 WT cells. 3D imaging by SEM provided evidence of “individual” (A, B) or “collective” (E, G) behaviour adopted by tumour cells to migrate through membranes. Accordingly to quantitative data, SEM observations revealed a high number of MDA-MB-231 (B) and LN229 (D) cells migrated to the lower side of the porous membranes. Conversely, few M14 ADR (F) and MCF-7 WT (H) cells were detected.

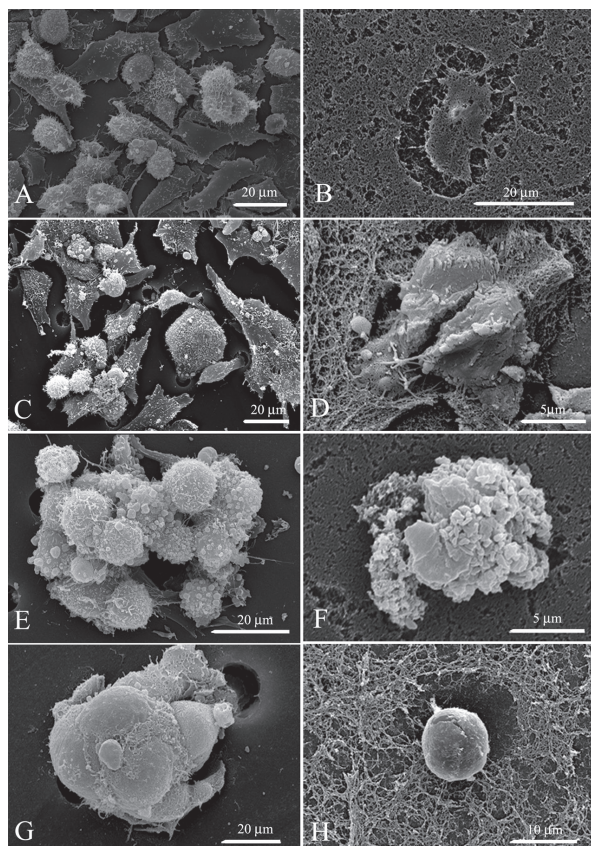


Figure 3 Scanning Electron Microscopy (SEM) observations of tumor cells invasion. (A, C, E, G) Upperside of porous membrane. (B, D, F, H) Lower side of porous membrane. (A, B) MDA-MB-231 (C, D), LN229 (E, F), M14 ADR and (G, H) MCF-7 WT cells. An area of Matrigel™ degradation was seen around the invadopode of MDA-MB-231 (B) and LN229 (D) cells. The invadopode of M14 ADR cells (F) was able to penetrate the extracellular matrix, apparently without degrading it, suggesting that an “amoeboid behaviour” was adopted. The MCF-7 WT cells scarcely invaded the extracellular matrix (3 h).

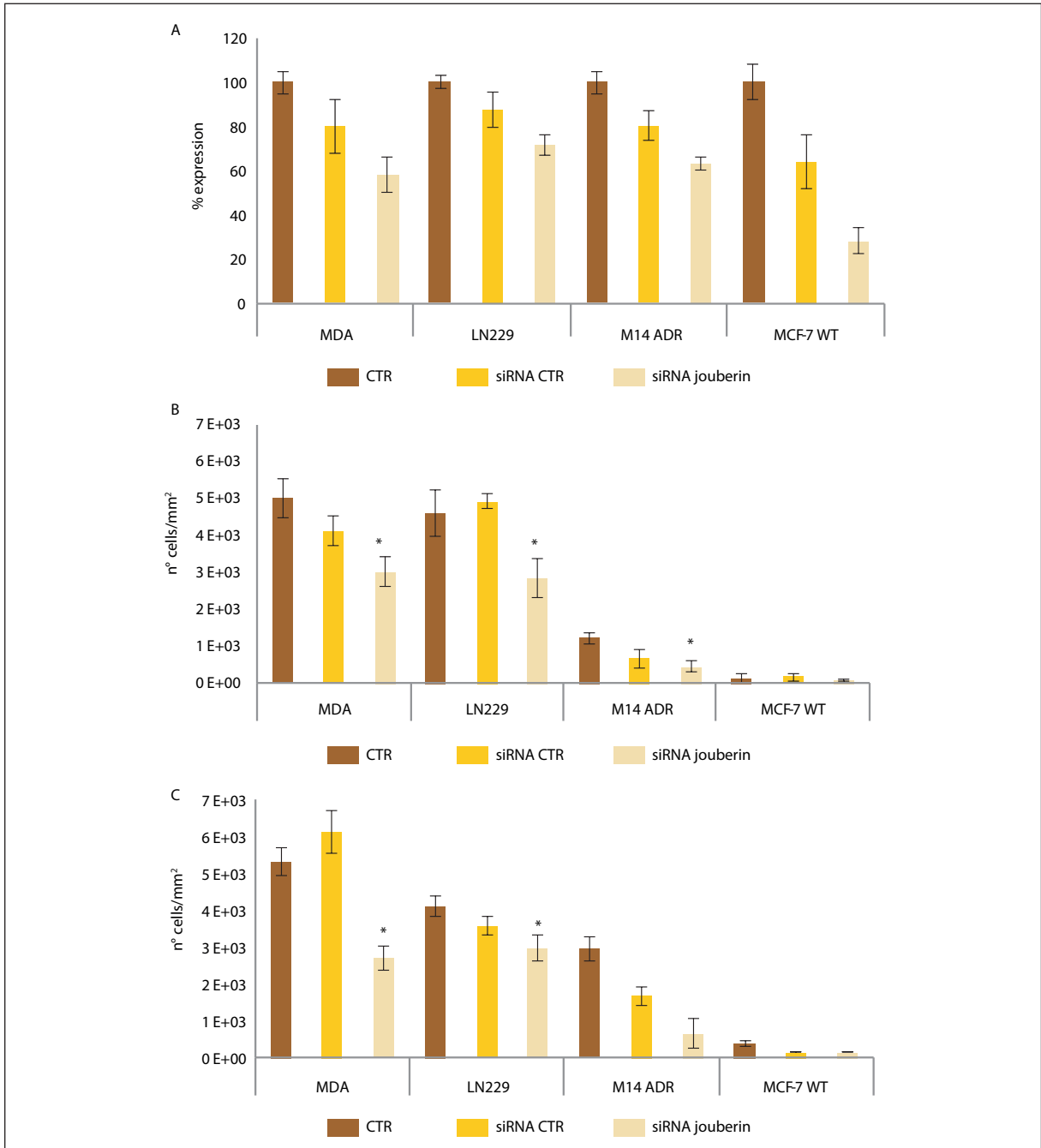
lus (chemotactic gradient). The results showed in *Figure 1*, panels C and D, demonstrated that the expression of AHI-1 was positively related to the IP, as showed by the densitometric quantification in Western blotting (WB) experiments. Thus, MDA-MB-231 and LN229 cells, which showed the highest IP in invasion assay, expressed Jbn levels higher than displayed by M14 ADR and MCF7 cells. Under migratory stimulus (*Figure 1*, panels C and D, samples with asterisk) an increase of O.D. was observed in MDA-MB-231 and LN229 cell samples, but it was not detectable in M14 and MCF-7 cell ones. Thus, the highest expression of AHI-1 gene product in tumour cells featuring the most aggressive phenotype with highest IP and an invasive individual behaviour.

In order to look insight the functional role of the AHI-1 in the migration and invasion processes, RNA interference (siRNA) experiments were performed. MDA-MB-231, LN229, M14 ADR, and MCF-7 cell lines were silenced for AHI-1 expression according to the manufacturer’s protocol. The gene-silencing efficiency

of siRNA experiments was evaluated by WB analysis (*Figure 4A*). Afterwards, the IP of tumour cells silenced for AHI-1 was evaluated by the “transwell chamber invasion assay”. The results of these experiments demonstrated that the siRNA-mediated suppression of AHI-1 mRNA significantly reduced the capability of migrating (*Figure 4B*) and invading (*Figure 4C*) of all tested cell lines.

Migration and invasion processes of tumour cells involve a protein complex scenario among which the signalling cascade of MAPKs. MAP kinases play an important role in various physiological processes such as cell proliferation, differentiation, as well as cell invasion [1]. In addition, in our previous study it was demonstrated that the highest invasive potential was associated with the activation of several signalling proteins such as AKT, ERK, and FAK [24]. ERK is activated during invasion process by some growth factors and ECM components through signalling pathways involving Ras, Raf and MEK1/2. Once activated, ERK phosphorylates different substrates including MLCK, calpain, paxillin and FAK (focal adhesion kinase) [26-28]. Activated ERK regulates membrane protrusions and focal adhesion turnover via phosphorylating MLCK and promotes focal adhesion disassembly via phosphorylating and activating calpain. Phosphorylation of FAK and paxillin by ERK may regulate focal adhesion dynamics, probably by influencing the paxillin-FAK interaction [29, 30]. FAK is involved in signal transduction from integrin-enriched focal adhesion sites that mediate cell interaction with the extracellular matrix [31, 32]. FAK-enhanced signals have been shown to mediate the survival of anchorage-dependent cells and are critical for efficient cell migration in response to growth factor receptor and integrin stimulation. Moreover FAK contributes to the secretion of matrix-metalloproteinases [31]. Akt promotes cancer cell invasion via increased motility and metalloproteinase production [33]. In particular, it has been demonstrated that PI3K/Akt signaling contributes to translocation of MT1-MMP that can influence MMP-2 proteolytic activity and facilitate formation of invadopodia [32].

In order to investigate the possible role of Jbn in these pathways, we performed the analysis of the activation of ERK, FAK, and Akt on the tested tumour cell lines, before and after AHI-1 silencing by RNA interference experiments. The results obtained by western blotting experiments demonstrated that the suppression of AHI-1 m-RNA reduced the expression of phosphorylated proteins in all tested cell lines with the exception of p-ERK in M14 ADR cells (*Figure 5*). Overexpression of P-gp in tumour cells induces multidrug-resistance and more aggressive phenotype [23, 34]. As we reported elsewhere [23], the M14 ADR cell lines overexpress the ATP-binding cassette (ABC) family member ABCB1 (P-glycoprotein, P-gp). In particular, our previous studies demonstrated that P-gp cooperates with the adhesion molecule CD44 in the activation of ERK1/2 and p38 mitogen-activated protein kinase (MAPK) protein. Thus, the effect of AHI-1 m-RNA downregulation on ERK phosphorylation in M14 ADR cells was very likely counterbalanced by P-gp/CD44

**Figure 4**

(A) Densitometric analysis of Jbn expression after m-RNA AHI-1 silencing. (B, C) Effect of Jbn downregulation on (B) migration and (C) invasion potential of tumor cells. Experiments were performed in triplicate. SD are reported. (*) P-values lower than 0.05 were considered significant (siRNA jouberin vs siRNA CTR).

activation. Strikingly, a new AHI-1-BCR-ABL-JAK2 interaction complex has been identified. This complex regulates transforming activities and drug resistance in CML stem/progenitor cells [20]. The WD40-repeat domain of AHI-1 interacts with BCR-ABL, whereas the N-terminal region interacts with JAK2 [20]. The ABL family of protein kinases links diverse extracellular stimuli to intracellular signalling pathways that control cell growth, survival, invasion, adhesion and migration [35-38]. Its role in solid tumors has not been compre-

hended until recently. However, the activation of ABL family proteins has been revealed in tumour cells derived from breast, colon, lung and kidney carcinomas, as well as melanoma (reviewed in [39]). In particular, it has been demonstrated a key role of ABL signalling in the regulation of cell adhesion, motility, and invadopodia through the modulation of the actin cytoskeleton [19, 40, 41] and the expression of several MMPs and other genes involved in epidermal growth factor receptor (EGFR)-mediated invasion [42, 43]. It could be

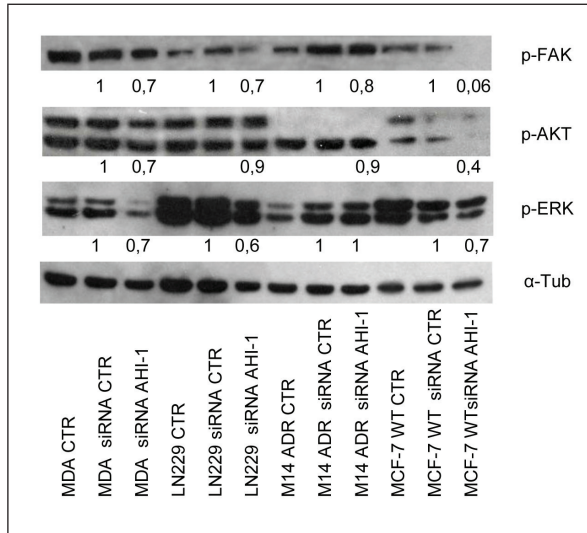


Figure 5
Western blot of p-FAK, p-AKT and p-ERK after m-RNA AHI-1 silencing. Suppression of AHI-1 m-RNA reduced the expression of phosphorylated proteins in all tested cell lines with the exception of p-ERK expressed by M14 ADR cells. Changes in the levels of p-FAK, p-AKT and p-ERK after being normalized to the levels of α -tubulin are shown below each blot. Control samples are considered as the unit. The results shown are representative of three independent experiments.

speculated that the interaction of AHI-1/Jbn with BCR/ABL/JAK2 complex lead to the activation of different pathways that via intermediate signalling proteins, such as AKT, ERK, and FAK upregulate cell proliferation, adhesion, migration and invasion.

CONCLUSIONS

In conclusion, our *in vitro* study for the first time demonstrate the involvement of AHI-1 transcript Jbn in the migration and invasion of tumor cells of different histological origin. In particular, the expression of Jbn is positively related to the IP of the tested tumor cells. In addition, it is more expressed in cells adopting an “individual/mesenchimal” behaviour. Noteworthy the AHI-1 m-RNA downregulation reduce the capability of migrating and invading the ECM in all tested cell lines. These results should candidate the product of the human AHI-1 gene Jbn as a new potential molecular target in anticancer therapy. Further studies are now in progress in order to analyse other signalling proteins involved downstream the AHI-1/ABL/JAK2.

Conflict of interest statement

The authors have no competing interests.

Received on 9 March 2016.

Accepted on 27 October 2016.

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