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Migratory behaviour of tumour cells: a scanning electron microscopy study

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Abstract

Background. Tumour cells utilize different migration strategies to invade surrounding tissues and elude anticancer treatments. It is therefore important to understand the mechanisms underlying migration process, in order to aid the development of therapies aimed at blocking the dissemination of cancer cells.

Aims. In this study tumour cell lines of different histological origin were analysed by combining 2D and 3D *in vitro* assays, biochemical tests and high resolution imaging by scanning electron microscopy (SEM) in order to look insight strategies adopted by tumour cells to invade extracellular matrix.

Results. Quantitative (computer-assisted colour camera equipped-light microscopy) and qualitative analysis (SEM) indicated that the most aggressive tumour cells adopt an "individual" behaviour. The analysis of intracellular signalling demonstrated that the highest invasive potential was associated with the activation of AKT, ERK, FAK and ERM proteins. The "individual" behaviour was positively related to the expression of VLA-2 and inversely related with the E-cadherin expression.

Conclusions. The combination of 2D and 3D *in vitro* assays, biochemical tests and ultrastructural investigations proved to be a suitable test for the investigation of tumour cell migration and invasion. The high resolution imaging by SEM highlighted the interrelationships between cells in different migratory behaviours of tumour cells.

Key words

- migration
- invasion
- tumour cells
- scanning electron microscopy

INTRODUCTION

Metastasis is the most frequent cause of death for patients with cancer. The ability of a malignant tumour to become metastatic begins with the hallmarks of motility and invasiveness [1]. Cell movement (migration) is controlled by internal and external signals, which activate complex signal transduction cascades resulting in highly dynamic and localised remodelling of the cytoskeleton, cell-cell and cell-substrate interactions [2].

Cancer cells exhibit various types of migration, such as "mesenchymal" or "amoeboid" migration and individual or collective migration [3, 4]. In particular, depending on the cell type and tissue environment, cells migrate individually, when cell-cell junctions are absent, or collectively as multicellular groups, when cell-cell adhesions are retained [5-8]. The process that underlies both types of migration is the remodelling of the cell cytoskeleton that couples with cell surface receptors interacting with surrounding tissue structures; thus, the cytoskeleton serves as the motor that drives the cell, and the cell surface receptors act as its transmission [9].

Moreover, mesenchymal migration requires extracellular matrix (ECM) proteolysis through production of matrix metalloproteinases (MMPs). Conversely, amoeboid motility is characterised by squeezing movements that allow cancer cells to move through the matrix without the use of MMPs and integrin engagement [5].

By shifting between different migration strategies, migrating cells can adapt to environmental changes and matrix stiffness to elude anticancer treatments. It is therefore important to understand the molecular mechanisms underlying migration process, which is the pernicious step in most solid tumour diseases, in order to develop strategies aimed at blocking the dissemination of cancer cells [5, 10, 11].

A lot of methods have been invented to investigate tumour cell migration, but not all are equally suited and no method alone is able to deliver a complete picture of tumour cell migration. Most studies on cell motility have been performed in two-dimensional (2D) culture systems, which limits our understanding of mechanisms of cell migration, as cells use different cell migration strategies in physiological three-dimensional (3D) culture systems [12, 13].

One of the earliest 2D culture systems employed to investigate cell migration is the "monolayer wound-healing" assay. In this method cancer cells are seeded

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on a substrate and cultured until they form a confluent monolayer. Thereafter a scratch is made in the monolayer, and the time required for cells to fill the voided gap is measured. In the microliter-scale migration assays, selected molecules are deposited on a substrate (*e.g.* 10-well Teflon printed microscopic slides), the cell-containing solution is placed at the centre of these wells and the radial migration of cells is then monitored [14]. The "Boyden chamber" assay (another 2D assay) examines migration of cells through a porous insert (Transwell™) in response to specific attractant. Cells are seeded on the top of the insert and the cells crossing through the membrane pores are analysed [15]. However, *in vivo* tumour cells must attach and degrade a basement membrane matrix in order to metastasize. The migration of cells from one tissue compartment to another is closely associated with extracellular matrix (ECM) remodelling and represents a fundamental step both in physiological and pathological cell movement. More than 25 years ago a 3D *in vitro* invasion assay was developed by using Matrigel™ as a ECM model in a modified two well Boyden migration chamber. This assay is in wide use today and has been very important for the study of cell migration and invasion [16, 17].

We herein suggest a combination of methods for the investigation of tumour cell migration and invasion. This combination comprises 2D (Boyden chamber assay) and 3D (Modified Boyden chamber assay) *in vitro* assays, biochemical tests and ultrastructural investigations by scanning electron microscopy (SEM). The analysis performed by SEM allowed us to visualize at high resolution the behaviour adopted by tumour cells to migrate and invade the ECM model. Cell behaviours (collective/individual, amoeboid/mesenchymal) were compared with the aggressiveness of tumour cells (migration and invasion potential), and with the signalling pathways involved.

MATERIALS AND METHODS

Cells cultures

Established tumour cell lines from different histological origin were used: murine (C6) and human (LN229) glioblastoma, human melanoma (M14 WT and M14 ADR), human colon adenocarcinoma (LoVo WT and LoVo ADR), and human breast cancer (MDA-MB-231, MCF-7 WT and MCF-7 ADR) cells.

C6, LN229, MDA, MCF-7 WT and MCF-7 ADR cells were grown in DMEM with high glucose; M14 WT and M14 ADR cells were grown in RPMI 1640; LoVo WT and LoVo ADR cells were grown in Ham's nutrient mixture (F-12). The media were supplemented with 1% non essential amino acids, 1% L-glutamine, 100 IU/ml penicillin, 100 IU/ml streptomycin, 10% fetal calf serum (Flow Laboratories) and 1% vitamins (F-12). Cell lines were cultured at 37 °C in a 5% CO_2 humidified atmosphere in air.

Invasion and motility assays

To analyse the migration and invasion potential of tumour cells, 2D (Boyden chamber assay) and 3D (Modified Boyden chamber assay) assays were employed. Briefly, inserts (8.0 μm pore) (Falcon) which stood in 6-well plates (Costar) were used. For 3D assay Matrigel™ was placed on the lower side of each insert. Cells were incubated at 37 °C up to 24 h. 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. For quantitative analysis inserts were stained with 0.25% crystal violet for 10 min, rinsed again and then allowed to dry. The detection of cells that passed through the membrane pores (migration) and invaded Matrigel™ (invasion) was obtained by a computer-assisted colour camera equipped Nikon Optiphot microscope and the percentage of area occupied by migrated cells was analysed by dedicated software (Optilab Graftek). The image processing techniques employed included thresholding and morphological filtering. For qualitative analysis (ultrastructural observations by SEM) inserts were processed as below described.

Scanning electron microscopy

For SEM studies, inserts were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3), added with 2% sucrose. After post-fixation with 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.3), cells were dehydrated through graded ethanol concentrations, critical point-dried in CO_2 (CPD 030 Balzers device, Bal-Tec, Balzers) and gold coated by sputtering (SCD 040 Balzers device, Bal-Tec). The samples were then examined with a Cambridge Stereoscan 360 scanning electron microscope (Cambridge Instruments, Cambridge, United Kingdom).

Western blotting

The analysis of MAPK phosphorylation was performed in tumour cells under migration assay. After 24h cells were harvested from the insert, washed twice in ice-cold Tris-buffered saline (TBS; 20 mM Tris–HCl, pH 7.6, 140 mM NaCl), and lysed at 4 °C in 200 μl lysis buffer (10 mM Tris–HCl, pH 7.6, 50 mM NaCl, 30 mM sodium pyrophosphate, 5 mM EDTA, 0.5% Nonidet P40, 1% Triton X-100, 50 mM NaF, 0.1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and complete mini proteinase inhibitors). Cell lysates were obtained by centrifugation at 17 000 *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 with 5% BSA in TTBS, for 1 hour at room temperature. Incubations with primary antibodies and with horseradish peroxidase-conjugated secondary antibodies were performed in blocking solution overnight at 4 °C and for 1 hour at room temperature, respectively. Immunoreactive bands were visualised by the ECL kit. For loading control, membranes were incubated with monoclonal anti-alpha-tubulin.

Flow cytometry

For flow cytometry analysis of the surface adhesion molecules (E-Cadherin, VLA2, VLA5, CD44) cells

were detached and incubated in phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA). Then, cells were incubated for 30 minutes at 4 °C with specific monoclonal antibody directed against surface antigens. After incubating, the mixtures were centrifuged twice using cold PBS solution containing 0.5% BSA and incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies for 30 minutes at 4 °C. Finally, cells washed twice using PBS containing 0.5% BSA were immediately analysed. Propidium iodide (PI) was used to recognize dead cells. Negative controls were obtained by incubating cells with isotypic primary antibodies, and then with FITC-conjugated secondary antibodies.

Samples were analysed with a FACScan flow cytometer (Becton Dickinson, CA) equipped with a 15 mV argon ion laser, 488 nm. The fluorescence signal of fluorescein (FITC) was collected with a 530 nm band-pass filter while propidium iodide signal with a band-pass filter 575 nm.

Table 1
Percentage of area occupied by cells migrated on the lower side of the filter in the absence (migration) and in the presence (invasion) of Matrigel™

Migration (%)		Invasion (%)	
MDA	50.0	MDA	58.0
C6	22.0	C6	29.2
M14 ADR	17.8	LN229	26.7
LN229	15.0	M14 ADR	22.7
M14 WT	11.3	M14 WT	10.9
MCF-7 ADR	2.8	MCF-7 ADR	4.2
LoVo ADR	2.0	LoVo ADR	3.7
LoVo WT	1.7	LoVo WT	3.3
MCF-7 WT	1.2	MCF-7 WT	0

RESULTS AND DISCUSSION

Study of the migration and invasion potential

In this study several tumour cell lines of different histotypes were employed. In particular, three human breast carcinoma cell lines (MDA-MB-231, MCF-7 WT and their resistant variants MCF-7 ADR), two glioblastoma cell lines (LN229, human; C6, murine), two human melanoma cell lines (M14 WT and their resistant variants M14 ADR), and two human colon adenocarcinoma cell lines (LoVo WT and their resistant variants LoVo ADR) were employed. Drug-resistant variants overexpress the multidrug transmembrane P-glycoprotein, capable of transporting structurally and functionally not related xenobiotics, thus responsible for the pleiotropic resistance of tumour cells [18]. The migration and invasion potential of the tumour cell lines were analysed by the *in vitro* 2D (Boyden chamber assay) and 3D (modified Boyden chamber assay) assays, respectively. These assays can provide a rapid quantification of the invasive and metastatic potential of cell lines, corresponding to that determined by *in vivo* tests on the same experimental models [15, 19]. In the migration assay cells were placed on a membrane with 8 µm pores and subjected to a chemotactic stimulus (10% FCS) for 20 hours. For the invasion assay a Matrigel™ film was deposited on the lower side of the porous membranes. The percentage of area occupied by cells, migrated through the membrane pores in the absence (migration) or in the presence (invasion) of Matrigel™ film, was then evaluated (Supplementary data 1 and 2 available online at www.iss.it/anna).

In our experimental conditions different migratory and invasive potentials were found and the percentage of area occupied by the tested tumour cell lines that have been listed in a descending order in *Table 1*.

Human breast cancer MDA-MB-231 cells displayed the highest capacity of migrating, as showed by the percentage of occupied area on the lower side of the porous membrane (50.0%). This value appeared to be exceptionally high when compared with both the breast carcinoma cell lines MCF-7 WT and MCF-7 ADR cells and the other tumour cells of different histotypes. However, MCF-7 ADR cells showed to be faster (2.8%) than their sensitive counterparts MCF-7 WT cells (1.2%). Also in the presence of Matrigel™ MDA-MB-231 cells displayed the highest potential of invasion (58.0%) in comparison with the other tumour cells. Differently from their sensitive counterparts, MCF-7 ADR resistant variants appeared to be stimulated by the Matrigel™ as showed by the duplicated percentage of area occupied (4.2%). As elsewhere reported, the proteins of the extracellular matrix through the binding with adhesion molecules expressed by the plasma membrane trigger molecular mechanisms underlying actin assembly and invadopodia formation [20].

Glioblastoma and melanoma cells followed in the list order MDA-MB 231 cells. Murine C6 cells showed a migratory (22.0%) and invasive (29.2%) potential higher than human LN229 cells (15.0% and 26.7%, respectively). Drug resistant human melanoma cells (M14 ADR) proved to be more efficient than drug-sensitive M14 WT cells in both migration (17.8% *vs* 11.3%) and invasion assays (22.7% *vs* 10.9%). Invasion assay highlighted that the overexpression of the drug-transporter P-glycoprotein conferred a higher migration and invasion potential of human melanoma and human cancer breast cells. M14 ADR and MCF-7 ADR cells, in fact, displayed a higher capability of invading the Matrigel™ when compared to their drug sensitive counterparts. The overexpression of P-glycoprotein seems to be directly involved in increasing cell motility, and confers to tumour cells a more aggressive phenotype through the phosphorylation ERM proteins and MAPK signalling (ERK 1/2 and p38/ MAPK) [21, 22]. It is interesting to note that under migratory stimulus phosphorylation of ERM proteins was found in both M14 ADR cells, but not in MCF7-ADR and LoVo ADR cells (data not shown). Accordingly, the difference WT *vs* ADR appeared to be less significant in human colon adenocarcinoma cells both in migration (1.7% *vs* 2%) and invasion assays (3.7% *vs* 3.3%).

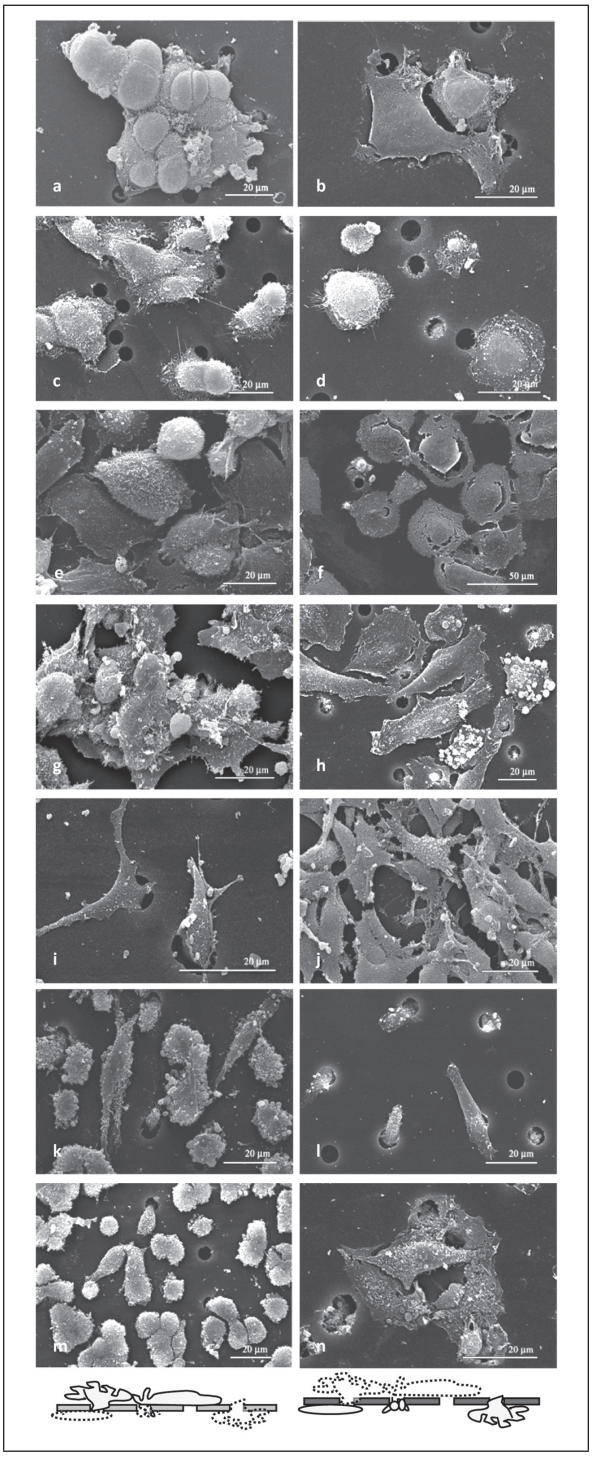

Figure 1
Scanning electron microscopy observations performed on the upper side (**a, c, e, g, i, k, m**) and on the lower side (**b, d, f, h, j, l, n**) of the filter during the migration process. The observations performed on the upper side provide evidence that MCF-7 WT (**a**), LN229 (**g**) and LoVo ADR (**m**) cells adopted a "collective" behaviour, whereas MDA-MB-231(**e**), C6 (**i**) and LoVo WT (**k**) cells adopted a "individual" behaviour. MCF-7 ADR (**c**) cells showed a "mixed" behaviour. Cells that adopt an individual behaviour tend to separate from the rest of the cell population and to pass through the pores individually. In the "collective" behaviour clusters of cells move closely linked each other. In these groups of cells it can be identified a leader (arrows) dragging the other cells. These leader cells generate the traction force necessary for the migration of the group, through the activity of pseudopodia, pulling behind resting cells. Observations performed on the lower side of porous membranes, confirmed data obtained by the quantitative analysis. MCF-7 WT cells samples (**b**) showed the smallest number of migrated cells on the lower side of the filter. In MCF-7 ADR cell samples (**d**) the number of migrated cells increased, while migrated MDA-MB-231 cells covered almost the whole area of the lower side of the filter (**f**). In C6 cell sample (**j**) numerous cells completed the migration process, in contrast with LN229 cells that moved more slowly (**h**). Finally, LoVo WT samples (**l**) displayed a number of cells lower than their resistant counterpart LoVo ADR cells (**n**).
Enlarged version of the figure 1 is available in Supplementary data 3 at www.iss.it/anna

Study of the migratory and invasive behaviour by SEM

The study of migratory and invasive behaviour adopted by the various tumour cells was analysed by SEM. 3D imaging offered by SEM can provide evidence of "individual" or "collective" behaviour adopted by tumour cells to migrate through membranes or to invade Matrigel™. Cells that adopt an "individual" behaviour detach from the cell population and pass through the pores separately. In a "collective" behaviour cells move in clusters closely linked each other. In these groups of cells it can be identified a leader, called by some authors "Guerilla cell" [23]. These leader cells generate the traction force necessary for the migration of the group through the activity of pseudopodia, pulling behind resting cells.

Among human breast carcinoma MDA-MB-231, MCF-7 WT and MCF-7 ADR cells different migratory behaviours were identified *(Figure 1 a-f)*. The greatest migration potential of this tumour appeared to be associated with an individual behaviour. In fact, MCF-7 WT cells less active in the migration assay tended to form large clusters on the upper side of the porous membrane *(Figure 1 a)*. MCF-7 ADR cells displayed an increase of migratory potential when compared to the parental cells and showed a "mixed" behaviour. In fact, they appeared to be organized in small groups of two or three cells overcrowding the same membrane pore during migration *(Figure 1 c)*. MDA-MB-231 cells, which in the quantitative analysis occupied the highest percentage of filter area (50%), adopted a distinct "individual" behaviour. Indeed, they were not arranged in clusters but moved separately towards the lower side of the membrane *(Figure 1 e)*. SEM observations performed on the lower side of porous membranes, confirmed data obtained by the quantitative analysis carried out by image analysis. In fact, in MCF-7 WT cell samples, a small number of migrated cells was observed on the lower side of the filter *(Figure 1 b)*. Accordingly, the number of migrated cells increased in MCF-7 ADR cell samples *(Figure 1 d)*, whereas migrated MDA-MB-231 cells covered almost the whole area of the lower side of the filter *(Figure 1 f)*.

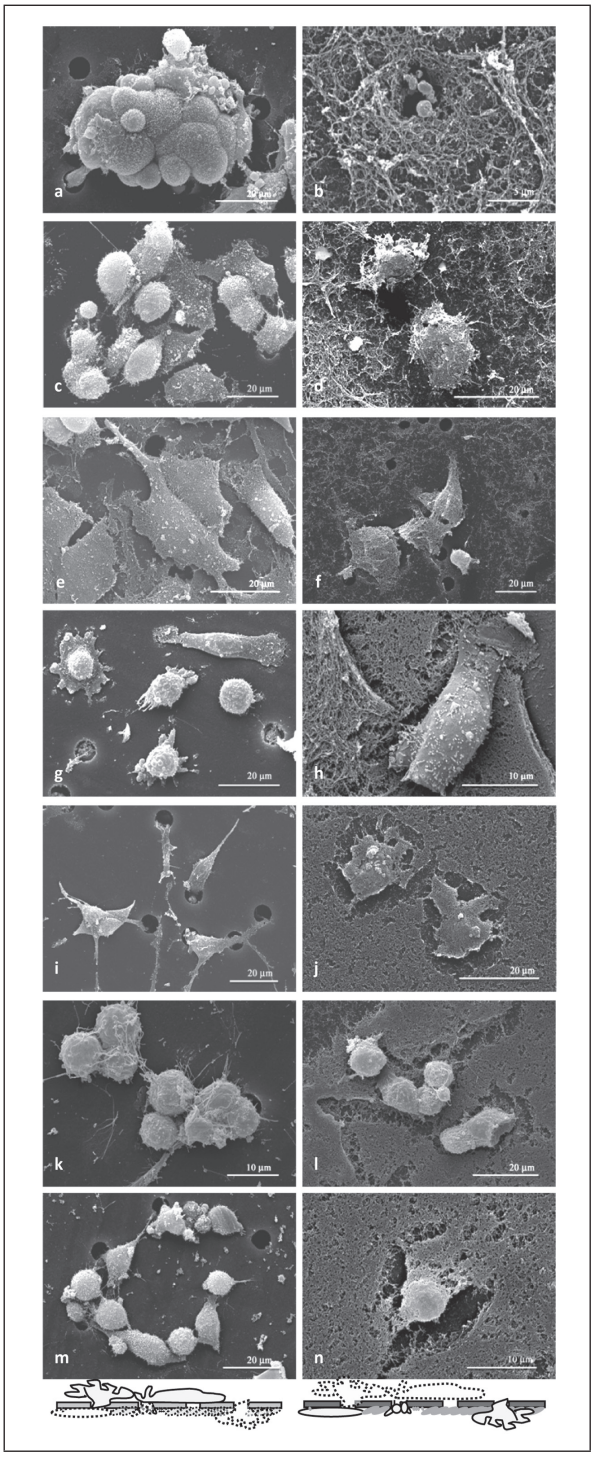

Figure 2
Scanning electron microscopy observations performed on the upper side (**a, c, e, g, i, k, m**) and on the lower side (**b, d, f, h, j, l, n**) of the filter during the invasion process in presence of Matrigel™. The observations performed on the upper side provide evidence of "individual" or "collective" behaviour adopted by tumour cells. In the presence of a film of Matrigel™, MCF-7 WT (**a**), LoVo WT (**k**) and LoVo ADR (**m**) cells adopted a "collective" behaviour, whereas MDA-MB-231 (**e**), LN229 (**g**), and C6 (**i**) cells adopted an "individual" behaviour. MCF-7 ADR (**c**) cells showed a "mixed" behaviour. The observations on the lower side provide information on the type of tumour cell-extracellular matrix interactions during the invasion process. MCF-7 WT (**b**), MCF-7 ADR (**d**), MDA-MB-231 (**f**) cells adopted a "mesenchymal" behaviour whereas, C6) (**j**), LoVo WT (**l**) and LoVo ADR (**n**) adopted an "amoeboid-like" behaviour. Cells that adopt a "mesenchymal" behaviour showed an intense proteolytic activity focused around invadopodia. Focused proteolysis was due to proteases strongly concentrated near the binding sites between integrins and extracellular matrix. Cells that adopt an "amoeboid" behaviour penetrated the fibers of Matrigel™ without degrading it, but infiltrating and invading the extracellular matrix. However, there are cells that show a "mixed" behaviour (LN229, **h**): while in some areas matrix degradation was observed, suggesting that cells recurred to an invasion of mesenchymal type, in other areas cells appear to infiltrate through the mesh of the matrix in "ameboid-like" manner.
Enlarged version of the figure 2 is available in Supplementary data 4 at www.iss.it/anna

Even the glioblastoma cell lines showed differences in migratory behaviour and also in the case the greatest migratory potential appeared to be associated with an individual behaviour. Human glioblastoma LN229 cells organized in clusters whence some cells spread out and tried to pass through pores, meanwhile other cells, rounded in shape, took advance of leading migratory forces, without actively participating in the process (*Figure 1 g*). The murine glioblastoma C6 cells migrated individually: on the top of the membrane all cells appeared to be equally stretched, trying to actively pass through the pores *(Figure 1 i)*. In the lower side of the filter, numerous C6 cells showed to have completed the migration process and covered completely the substrate *(Figure 1 j)*. Accordingly with quantitative data, human LN229 cells moved more slowly and a number of them were rich in blebs *(Figure 1 h)*, morphological markers suggestive of an active reorganization of the actin cytoskeleton [24].

SEM observations on human colon adenocarcinoma LoVo WT and LoVo ADR cells substantially confirmed the quantitative analysis. Human adenocarcinoma cells migrated with difficulty through the filter. On the upper side, cells passed through the pores alone or linked each other in small groups *(Figures 1 k and m)*. On the lower side of the filter very few if not at all LoVo cells were observed *(Figure 1 l and n)*.

The experiments of invasion were carried out in the presence of a Matrigel™ film gelified on the lower side of the membrane. In literature data two types of behaviour adopted by cancer cells to invade the extracellular matrix are described: the "mesenchymal" and the "ameboid" type, depending on the presence or the absence of focused proteolysis, respectively [5]. SEM observations performed on the upper side of the porous membrane confirmed the strong tendency of MCF-7 WT cells to organize in clusters within which it was difficult to distinguish individual cells *(Figure 2 a)*. On the contrary, the resistant cells tended to migrate alone or organized in small groups of two or three cells trying to pass through the same pore *(Figure 2 c)*. Both MCF-7 WT and MCF-7 ADR cells seemed to adopt a "mesenchymal" behaviour with matrix proteolysis as suggested by the structure of Matrigel™ film which appeared thin

and loose (*Figure 2 b and d*, respectively). However, in agreement with the data obtained by quantitative analysis MCF-7 WT cells showed a scarce ability to invade the Matrigel™ and only the ends of invadopodia peeking out from the pores were visible *(Figure 2 b)*. By the contrast, MCF-7 ADR cells proved to be more able to invade the extracellular matrix and they appeared covered by film residues, surrounded by an area of proteolysis *(Figure 2 b)*. Differently from MCF-7 cells, highly invasive breast cancer MDA-MB-231 cells showed an individual organization on the upper side of the filter *(Figure 2 e)*. The observations performed on the lower side of the filter revealed large areas of matrix degradation, suggesting that also MDA-MB-231 cells recurred to an invasion of mesenchymal type *(Figure 2 f)*.

Human LN229 glioblastoma cells *(Figure 2 g and h)* where strongly stimulated by the presence of the extracellular matrix and changed the "collective" in "individual" behaviour in the invasion process, similar as murine C6 cells *(Figures 2 i and j)*. Both human *(Figure 2 h)* and murine cells *(Figure 2 j)* penetrated the fibers of Matrigel™ apparently without degrading it. In fact, the invadopodia seemed to infiltrate the extracellular matrix principally by mechanical forces, as demonstrated by the numerous cracks crossing the compact film all around the invading cells.

The presence of Matrigel™ also stimulated the migration and invasion processes of adenocarcinoma LoVo WT and LoVo ADR cells, even if in a lesser extent. Both the sensitive *(Figure 2 k)* and drug-resistant cells *(Figure 2 m)* adopted a "collective" behaviour and organized in small clusters and chains on the upper side of the membrane. Adenocarcinoma cells penetrated the fibers of Matrigel™ seemingly in the absence of matrix proteolysis.

Intracellular signalling underlying migration

Functional and ultrastructural observations were associated to the analysis of intracellular signalling triggered under migratory stimulus. The main aim of these experiments was to assess intracellular signalling engaged by tumour cells of different histotype during cell migration. The analysis was performed on samples obtained both from cells cultured in flask and cells under migration in the invasion chambers. The kinases examined were p-ERK 1/2 (extracellular-regulated kinase 1/2), p-PKB (protein kinase B, also named AKT), p-FAK (focal adhesion kinase), and p-ERM (ezrina, radixina, myosin). MAP kinases play an important role in various physiological processes such as cell proliferation, differentiation, inflammation and response to stress [25]. Evidence showed that MAPKs are essential for cell migration and their role relates to the actin assembling resulting in formation of cell protrusions on leading edge [26, 27], stress fibers structures, and membership that stimulate directional migration of the cells [28].

An increased expression of p-ERK was observed in most of the cell lines tested under migration stimulus, except for MCF-7ADR and LoVo WT cells *(Figure 3 a)*. The MAPK ERK 1/2 is a kinase regulated by extracellular signals. Some growth factors and components of the matrix are able to activate ERK1/2 and several observations showed that it is directly involved in cell motility [29-31]. ERK activation can also modulate invasion and migration through several cellular pathways: influencing cell survival, gene transcription factor AP-1 or directly regulating the enzymes (MLCK) necessary for locomotion [32]. p-FAK, is one of the substrates of ERK 1/2 and its activation by phosphorylation can regulate focal contact dynamics. Accordingly with the activation of ERK, an increased activation of p-FAK was revealed in the cell lines most invasive MDA-MB-231, C6 and M14 ADR cells *(Figure 3 a)*.

A similar study was performed on both cultured and stimulated cells to evaluate the AKT/PKB activation. Literature data showed the involvement AKT pathway, in cell proliferation and metastasis [33]. Arboleda and colleagues [34] demonstrated that breast cancer cells transfected with AKTβ cDNA displayed increased cellular invasiveness *in vivo* and *in vitro*. There are three isoforms of PKB protein, the antibody used in this study recognizes and binds the β and γ isoforms. An increase of p-AKT expression was revealed in the most invasive MDA-MB-231, C6, M14 WT and M14 ADR cells *(Figure 3 a)*.

By analysing the results in their complexity a positive correlation of protein kinases activation with the migration and invasion potential was established. Under migratory stimulus, the largest number of activated molecular pathways was revealed in the most aggressive cell lines confirming the important role of these kinases in the invasive phenotype of tumour cells.

Expression of adhesion molecules

Flow cytometry analysis allowed us to evaluate the expression of adhesion molecules involved in the metastatic process such as E-cadherin, VLA2, VLA5 and CD44 [35-40] in the different tumour cell lines.

E-cadherin is an adhesion molecule belonging to the superfamily of cadherins. In epithelial tumors the progressive loss of E-cadherin expression is correlated with an increased aggressiveness [41]. The expression of this trasmembrane receptor, in fact, provides to both the stability of cell-cell junctions and the recruitment of β-catenin. However, gene coding for E-cadherin may undergone to promoter ipermetilation and switch off. This involves loss of cell-cell adhesions, thus promoting cell motility essential for the metastatic process. Also the β-catenin, no longer confined into the cytosol, migrates into the nucleus and activates genes involved in cell proliferation, including c-myc and cyclin D1. The flow cytometry analysis was performed only on human cell lines *(Figure 3 b,* Supplementary data 5 and 6 available online at www.iss.it/anna) and showed low levels or loss of E-cadherin expression in the cell lines with the highest migratory and invasive potential, MDA-MB-231, LN229, M14 WT, and M14 ADR cells. On the other hand, the cell lines that displayed a low potential in migration and invasion assays, expressed high levels of E-cadherin: MCF-7 WT, MCF-7 ADR, LoVo WT and LoVo ADR cells. These results confirmed the relationship between loss of expression of E-cadherin and tumour aggressiveness widely reported in the literature

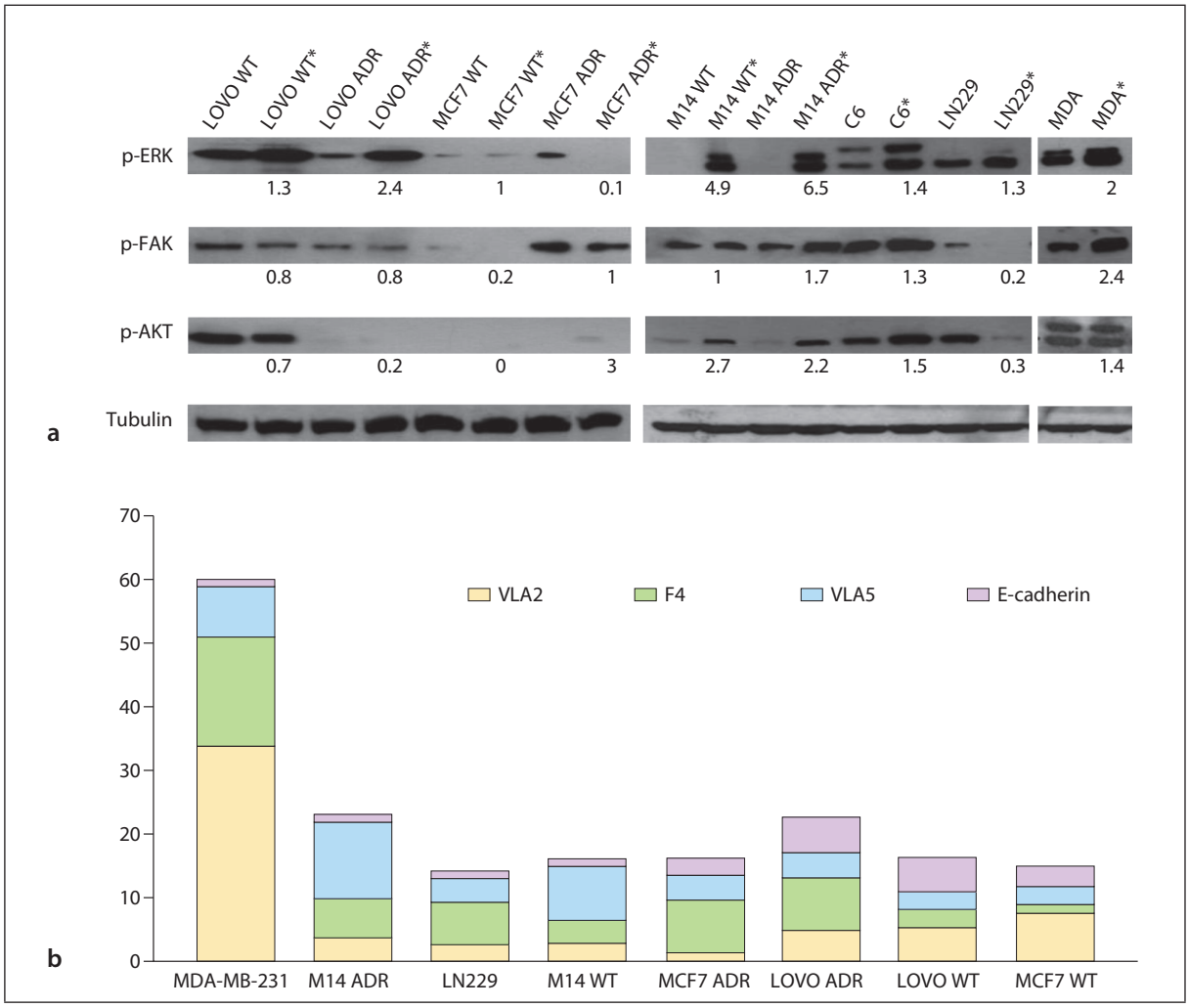

Figure 3
(**a**) Western blotting analysis of MAP kinase (p-ERK 1/2, p-AKT, p-FAK) activation under the migratory stimulus*. Cells with highest migration and invasion potential generally displayed, under migratory stimulus, the highest number of activated molecular pathways. (**b**) Flow cytometry analysis of the adhesion molecule expression. The analysed molecules were E-cadherin, VLA2, VLA5 and CD44. Values in ordinate are expressed in arbitrary units and represents positive sample/negative control ratio.

[35, 42] and validates migration and invasion assays used in this study. In fact, the loss of E-cadherin well correlates with the acquisition of the "individual" behaviour by aggressive cells analysed in the present study.

The acquisition of aggressive phenotype by tumour cells has been associated with the expression of both VLA5 and VLA2 integrins, and CD44 molecule. Literature data correlate the expression of these molecules with a more invasive and metastatic potential [40]. The results obtained showed that MDA-MB-231 cells express the highest level of the tested proteins (*Figure 3 b*, Supplementary data 5 and 6 available online at www.iss.it/anna). VLA2 and VLA5 belong to the superfamily of integrins. These two molecules are heterodimers consisting of $\alpha 2\beta 1$ and $\alpha 5\beta 1$ subunits respectively. They mediate tumour cell interaction with the extracellular matrix [43]. Analysing the expression of VLA2 in the various tumour cell lines, we observed that MDA-MB-231 cells were characterized by the highest invasive potential and highest expression of this integrin among the cell lines tested. Also the other tumour cells expressed on their membrane VLA2 integrin but at a lower level then MDA-MB-231 cells. When the VLA5 expression was examined the human melanoma resistant M14 ADR cells, which belong to the more invasive cell lines, showed the highest level of protein expression.

The CD44 adhesion molecule is a monomeric transmembrane protein that can bind to various components of the extracellular matrix, such as hyaluronic acid, laminin, fibronectin and collagen. CD44 is expressed on various types of neoplastic cells and on their metastases [38, 39]. Among the cell lines tested the MDA-MB-231 cells were those expressing the highest level of protein in the membrane. The CD44 was also the protein most expressed in the resistant human adenocarcinoma cells and human glioblastoma. It is interesting to note that the CD44 expression in drug resistant cell lines (MCF-7 ADR, LoVo ADR and M14 ADR cells) was higher than in the relative sensitive counterparts. The

expression of CD44 well correlated with drug-resistant phenotype and the increased invasion potential can be explained by the active cooperation between CD44 and P-glycoprotein previously demonstrated in human melanoma and breast carcinoma cells [21, 44]. However, the highest expression of CD44 can account for the highest invasive potential of resistant melanoma cells where it is associated to both P-glycoprotein and ERM proteins [22]. These proteins under migratory stimulus are phosphorylated only in human resistant melanoma cells, but not in the resistant breast cancer and adenocarcinoma cells (data not shown). This could account for a low number of intracellular pathways activated by the interaction with the extracellular matrix.

CONCLUSIONS

Cancer invasion is a multi-step process determined by both molecular properties of the tumor cells and mechanical and signaling input from the tumor microenvironment. Defined by context, tumors develop either single-cell or collective invasion modes. *In vivo* tumour cells invade tissue either individually as single cells in elongated, mesenchymal or rounded, amoeboid-like behaviour after cell-cell junctions were abandoned. Alternatively, cancer cells retain cell-cell junctions and migrate collectively as cohesive multicellular units into the peritumoural stroma however, the mechanical and molecular programs underlying such plasticity of invasion programs remain unclear [45, 46].

The combination of 2D and 3D *in vitro* assays, biochemical tests and ultrastructural investigations by SEM proved to be particularly suitable for the investigation of tumour cell migration and invasion. The high resolution imaging by SEM highlighted the interrelationships between cells in different migratory behaviours of tumour cells. The results obtained by quantitative (computer-assisted colour camera equipped-light microscopy) and qualitative analysis (scanning electron microscopy) indicated that the most aggressive tumour cells adopt an "individual" behaviour. The analysis of the intracellular signalling demonstrated that the highest invasive potential was associated with the activation of ERM, AKT, ERK, and FAK proteins. The "individual" behaviour was positively related to the expression of VLA-2 and inversely related with the E-cadherin expression. However, the results obtained in our experimental conditions indicated that under the stimulus of ECM proteins, tumour cells can adopt a more advantageous behaviour (collective *vs* individual) as showed by human glioblastoma cells. In addition, the results obtained in this study confirm that the acquisition of the multidrugresistant (MDR) phenotype increases the invasive potential of tumour cells by the involvement of MDR markers (P-glycoprotein) and ERM proteins in the MAPK intracellular signalling.

Conflict of interest statement

There are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

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Supplementary Materials for

Migratory behaviour of tumor cells: a scanning electron microscopy study

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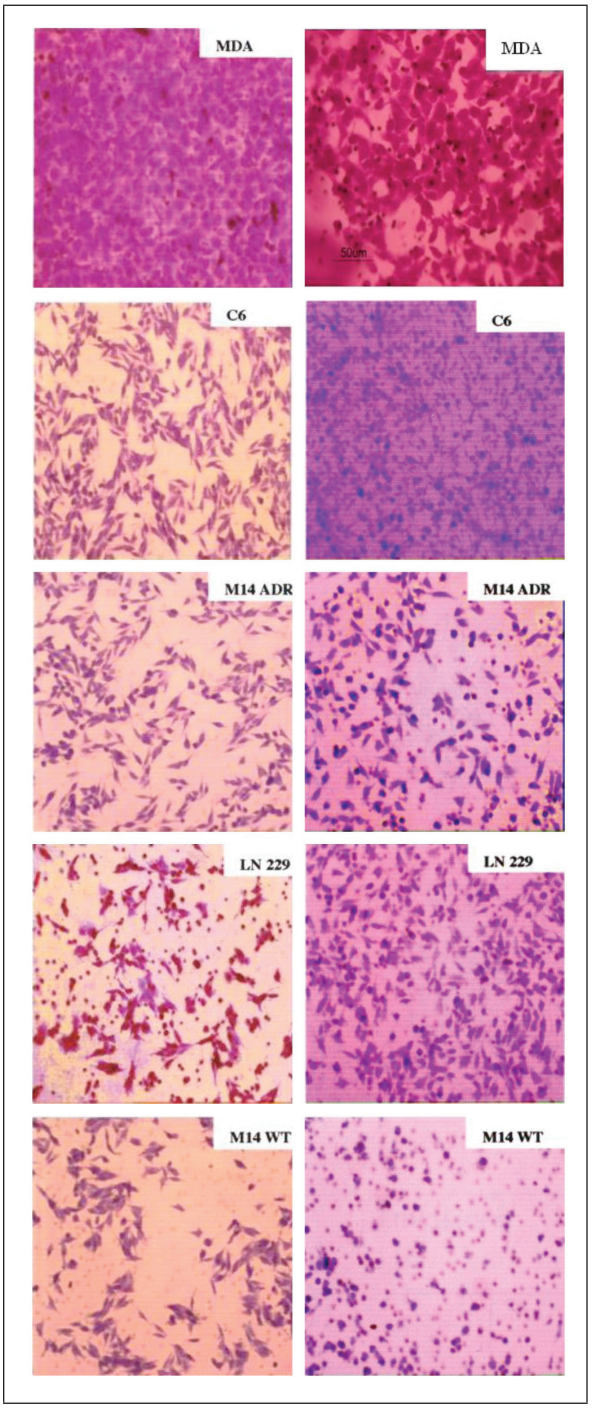
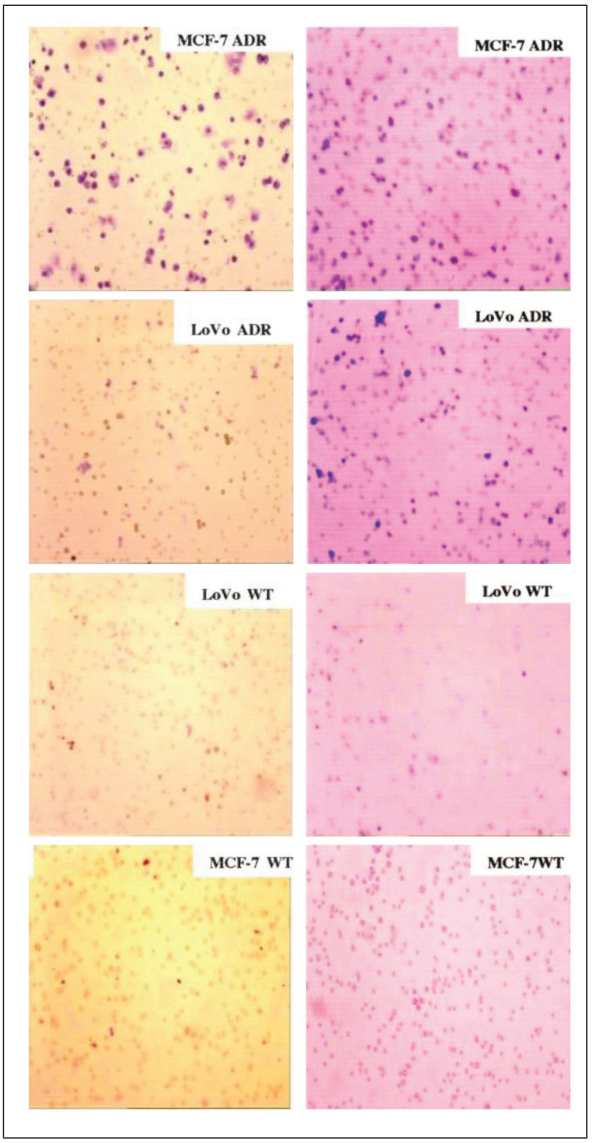
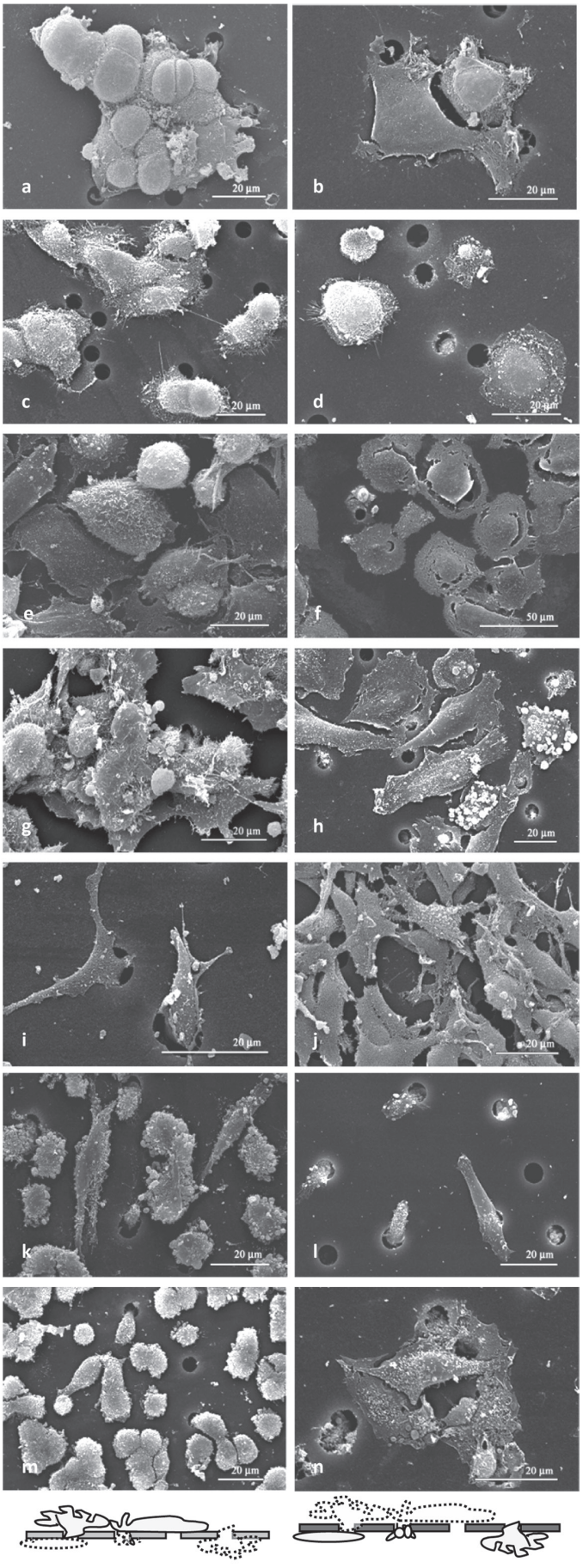
This PDF file includes:
Supplementary data 1-6



Supplementary data 1
Membranes of modified Boyden chamber assay stained with crystal violet. Left panels migration assay. Right panels invasion assay.

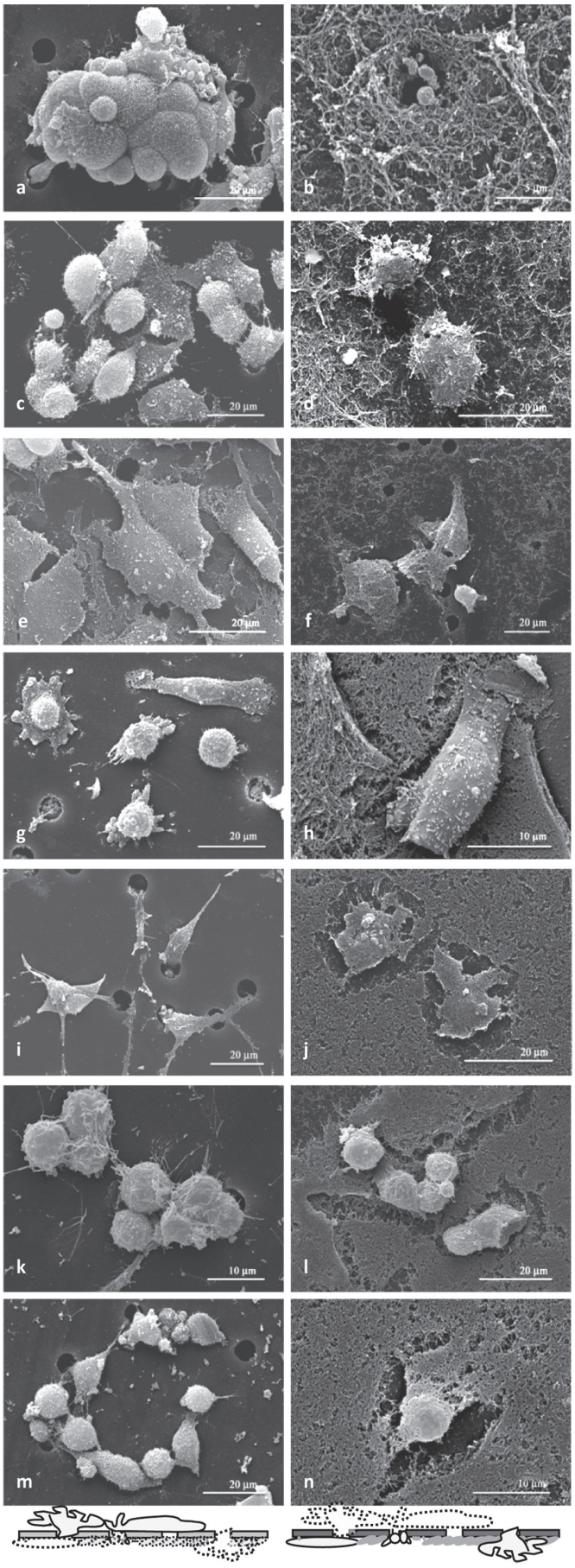


Supplementary data 2
Membranes of modified Boyden chamber assay stained with crystal violet. Left panels migration assay. Right panels invasion assay.



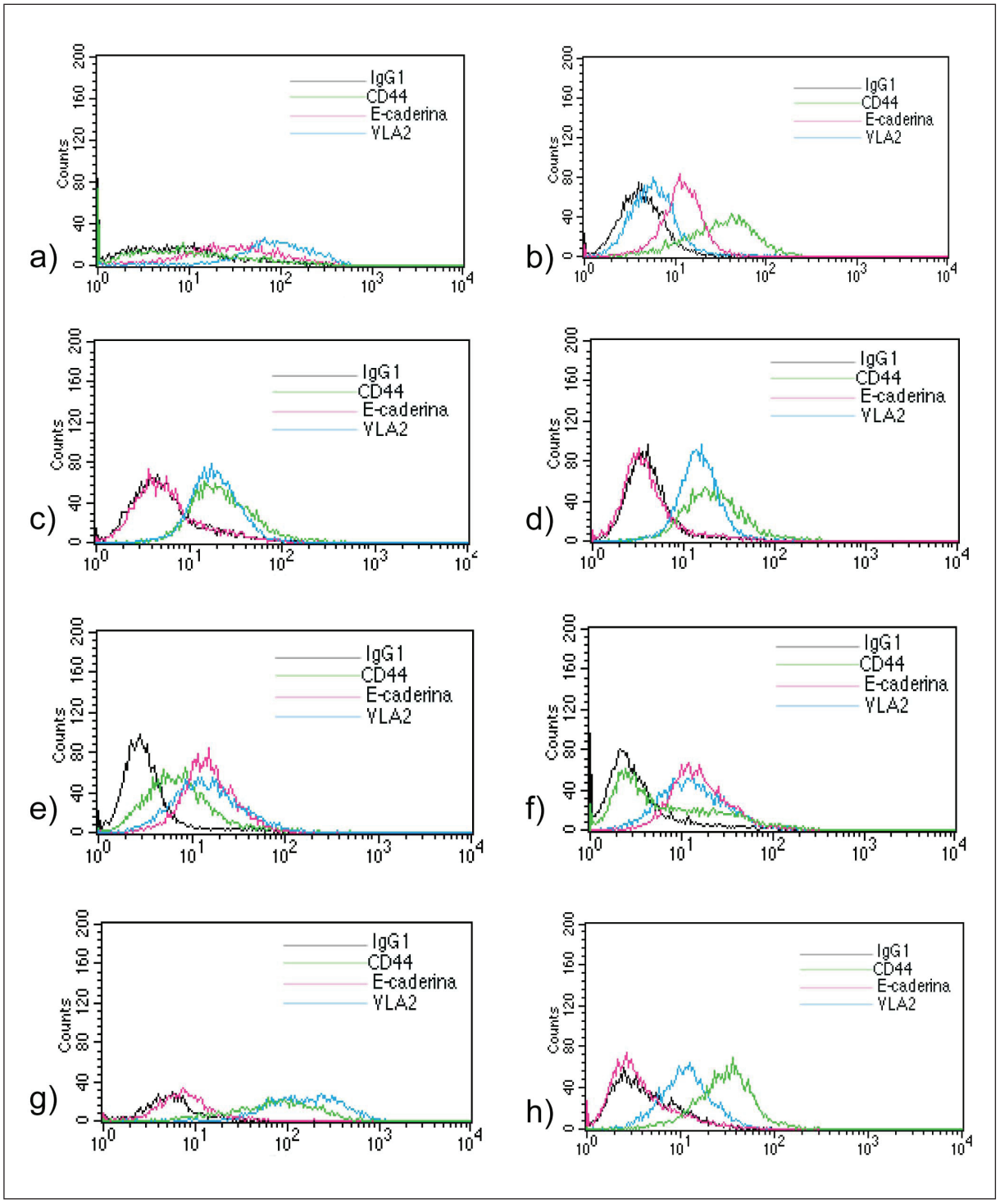
Supplementary data 3

Scanning electron microscopy observations performed on the upper side (**a, c, e, g, i, k, m**) and on the lower side (**b, d, f, h, j, l, n**) of the filter during the migration process. The observations performed on the upper side provide evidence that MCF-7 WT (**a**), LN229 (**g**) and LoVo ADR (**m**) cells adopted a "collective" behaviour, whereas MDA-MB-231(**e**), C6 (**i**) and LoVo WT (**k**) cells adopted a "individual" behaviour. MCF-7 ADR (**c**) cells showed a "mixed" behaviour. Cells that adopt an individual behaviour tend to separate from the rest of the cell population and to pass through the pores individually. In the "collective" behaviour clusters of cells move closely linked each other. In these groups of cells it can be identified a leader (arrows) dragging the other cells. These leader cells generate the traction force necessary for the migration of the group, through the activity of pseudopodia, pulling behind resting cells. Observations performed on the lower side of porous membranes, confirmed data obtained by the quantitative analysis. MCF-7 WT cells samples (**b**) showed the smallest number of migrated cells on the lower side of the filter. In MCF-7 ADR cell samples (**d**) the number of migrated cells increased, while migrated MDA-MB-231 cells covered almost the whole area of the lower side of the filter (**f**). In C6 cell sample (**j**) numerous cells completed the migration process, in contrast with LN229 cells that moved more slowly (**h**). Finally, LoVo WT samples (**l**) displayed a number of cells lower than their resistant counterpart LoVo ADR cells (**n**).

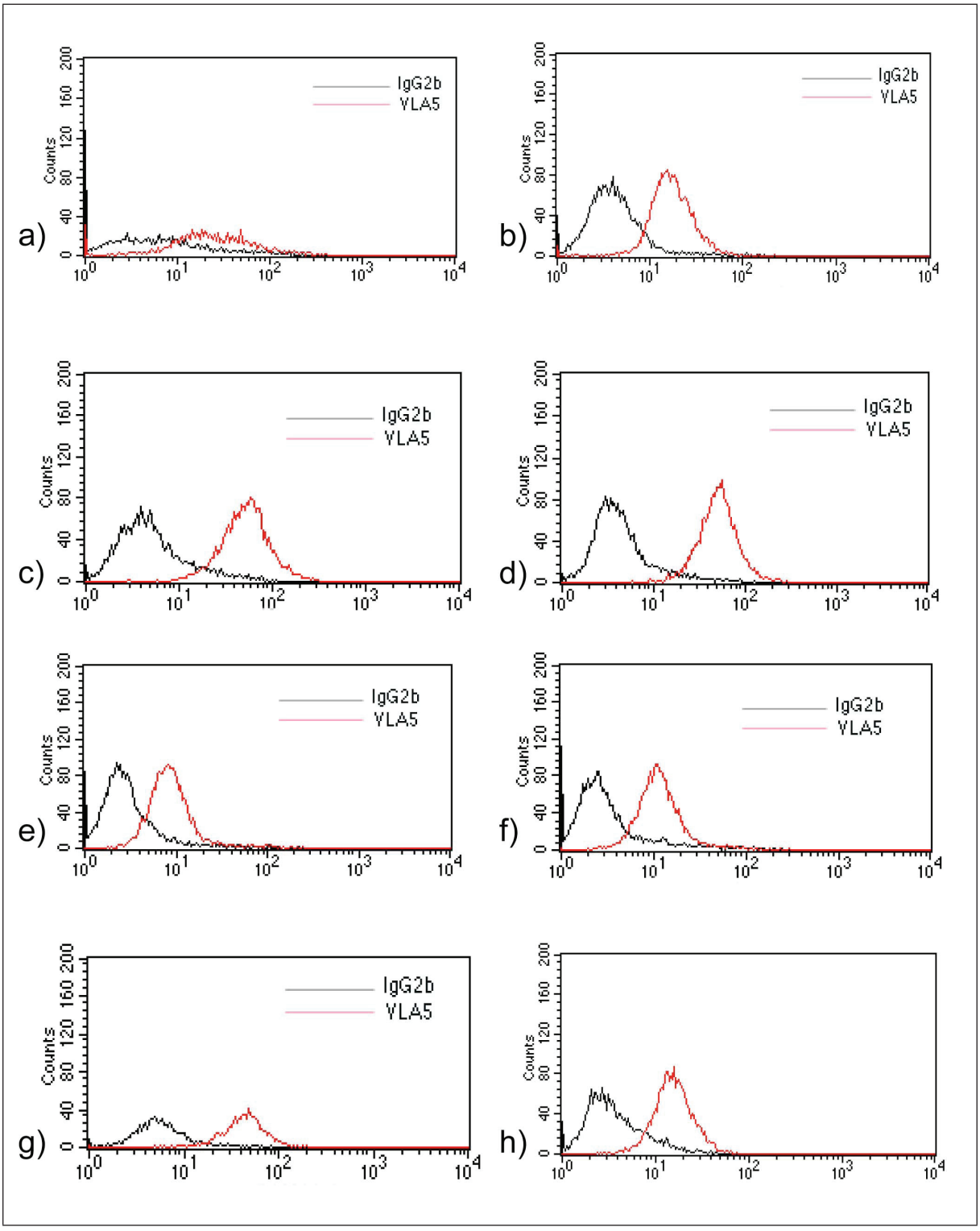


Supplementary data 4

Scanning electron microscopy observations performed on the upper side (**a, c, e, g, i, k, m**) and on the lower side (**b, d, f, h, j, l, n**) of the filter during the invasion process in presence of Matrigel™. The observations performed on the upper side provide evidence of "individual" or "collective" behaviour adopted by tumour cells. In the presence of a film of Matrigel™, MCF-7 WT (**a**), LoVo WT (**k**) and LoVo ADR (**m**) cells adopted a "collective" behaviour, whereas MDA-MB-231 (**e**), LN229 (**g**), and C6 (**i**) cells adopted an "individual" behaviour. MCF-7 ADR (**c**) cells showed a "mixed" behaviour. The observations on the lower side provide information on the type of tumour cell-extracellular matrix interactions during the invasion process. MCF-7 WT (**b**), MCF-7 ADR (**d**), MDA-MB-231 (**f**) cells adopted a "mesenchymal" behaviour whereas, C6) (**j**), LoVo WT (**l**) and LoVo ADR (**n**) adopted an "amoeboid-like" behaviour. Cells that adopt a "mesenchymal" behaviour showed an intense proteolytic activity focused around invadopodia. Focused proteolysis was due to proteases strongly concentrated near the binding sites between integrins and extracellular matrix. Cells that adopt an "amoeboid" behaviour penetrated the fibers of Matrigel™ without degrading it, but infiltrating and invading the extracellular matrix. However, there are cells that show a "mixed" behaviour (LN229, **h**): while in some areas matrix degradation was observed, suggesting that cells recurred to an invasion of mesenchymal type, in other areas cells appear to infiltrate through the mesh of the matrix in "ameboid-like" manner.



Supplementary data 5
Flow cytometry analysis of CD44, E-cadherin and VLA2 proteins on: (a) MCF7 WT, (b) MCF7 ADR, (c) M14 WT, (d) M14 ADR, (e) LoVo WT, (f) LoVo ADR, (g) MDA-MB-231, (h) LN229 cells.



Supplementary data 6
Flow cytometry analysis of VLA5 protein on: (a) MCF7 WT, (b) MCF7 ADR, (c) M14 WT, (d) M14 ADR, (e) LoVo WT, (f) LoVo ADR, (g) MDA-MB-231, (h) LN229 cells.