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Galectin-3 Expression Alters Adhesion, Motility and Invasion in a Lung Cell Line (DLKP), *In Vitro*

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Abstract. *Background: Galectin-3, a ß-galactosidase-binding protein, is involved in regulating many physiological and pathological cellular processes. The significance of galectin-3 in human lung and nasal carcinoma cells has not yet been elucidated. Materials and Methods: Using RT-PCR and Western blotting techniques, the constitutive level of galectin-3 in the human non-small cell lung carcinoma cell line, DLKP, was investigated. Following galectin-3 cDNA transfection into these cells, growth, toxicity, adhesion, motility and invasion assays were used to investigate the relevance of galectin-3 over-expression. Results: Galectin-3 over-expression did not induce a multi-drug resistance phenotype or significantly affect cell growth rate, but it did result in enhanced (i) adhesion to extracellular matrix components; (ii) cell motility; and (iii) in vitro invasiveness. Furthermore, studies of RPMI-2650 variants suggest that galectin-3 expression correlates with nasal carcinoma cell invasiveness. Conclusion: Our results suggest that galectin-3 expression levels in both lung and nasal tumour cells may play a role in cell motility, invasion, and metastasis.*

Galectin-3, a member of the ß-galactosidase-binding protein family, has been associated with a variety of physiological and pathological conditions and has been found to be widely distributed in many tissue types. Galectin-3 has been implicated in cell growth, differentiation, inflammation (1), malignant transformation (2), RNA processing (including pre-mRNA splicing), apoptosis (3, 4), angiogenesis (5), adhesion and invasion (6). The precise role of galectin-3 in many of these events, however, has not been conclusively defined, as conflicting observations have been reported which may, at least in part, be due to cell-type or tissue-type specificity. Galectin-3, for example, has been associated with changes in growth rates in certain cell types. However, whereas Yang *et al.* (3) reported higher growth rates in human leukemia T (Jurkat) cells transfected with galectin-3 cDNA compared to, untransfected cells, no differences in proliferation rates were detected in stable transfected breast carcinoma cell lines when compared to the non-transfected population (7).

Conflicting data has been reported from studies focussed on studying galectin-3 expression in a range of malignancies. Galectin-3 expression has been associated with neoplastic progression and metastatic potential in stomach (8), thyroid (9), central nervous system (10), liver (11) and colon (12) cancers. Galectin-3 translocation from nuclear to cytoplasmic expression has been associated with cancer progression and reduced disease-free survival in tongue carcinoma (13). In contrast, galectin-3 expression is down-regulated in ovary (14), uterus (15) and breast (16) cancers. Galectin-3 serum levels in patients with lung, breast, gastrointestinal, melanoma, non-Hodgkin's lymphoma and ovarian cancers are significantly elevated compared to those detected in healthy individuals (17). Expression patterns of galectin-3 and its effects in human lung cancer cells and nasal cancer cells have not yet been reported.

Galectin-3 is apparently involved in regulating cell death by apoptosis, having a functional BH1 (NWGR) domain of the Bcl-2 family (4). This observation was reported by Yang *et al.* (3) where galectin-3 over-expressing T cells were rendered resistant to apoptosis induced by anti-Fas and staurosporine. Akahini *et al.* (4) reported that galectin-3 transfection into human breast carcinoma cells (BT549) inhibited cisplatin-induced apoptosis. Similarly, Moon *et al.* (18) showed galectin-3 transfection to protect human breast carcinoma cells (BT549) from nitric oxide-induced apoptosis. Furthermore, inflammatory cells from galectin-3 deficient mice are more susceptible to undergoing apoptosis than those from normal mice (1). Whether or not galectin-3 can protect lung cells from drug-induced death remains to be determined.

The significance of galectin-3 in tumour cell metastasis is not yet conclusively defined. Galectin-3 expression levels have been reported to be increased (19, 20), decreased (21), or apparently not significantly modified either way (22), in

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tumour metastasis. To further elucidate the role of galectin-3 in the invasive and metastatic process, Matarrese *et al.* (7) over-expressed galectin-3 in a human breast carcinoma cell line, Esva-T. In agreement with results from studies by Choi Kim *et al.* (23) involving galectin-3 over-expressing BT549 breast epithelial cells, Matarrese *et al.* (7) concluded that galectin-3 may confer selective survival advantage and resistance to anoikia, by specifically influencing cell adhesion to the extracellular matrix and so may play a significant role in tumour cell invasion and metastasis.

As previously indicated, however, the status of galectin-3 expression in lung and nasal cancer cells has not yet been defined. This study addresses the significance of galectin-3 expression in a novel poorly-differentiated lung squamous cell carcinoma cell line, DLKP (24), and in variants of a nasal carcinoma cell line, RPMI-2650, with different patterns of multiple-drug resistance (MDR), integrin expression and *in vitro* invasiveness (25), which have been established in our laboratory.

Materials and Methods

Cell line culture. DLKP cells and transfected variants were grown in DMEM/ Hams F12 with 5 % FCS and 2mM L-glutamine at 37°C. RPMI-2650 (RPMI) and its selected variants, RPMI-Melphalan (*i.e.* RPMI-2650Ml) and RPMI-Taxol (*i.e.* RPMI-2650Tx), as well as HT1080 (obtained from the American Type Culture Collection (ATCC) were cultured in MEM medium containing 5 % FCS, 2mM L-glutamine, 1% sodium pyruvate and 1% non-essential amino acids, at 37°C and 5 % CO_2. Routine sterility checks, including screening for *Mycoplasma*, indicated that all cells were clear of contamination.

Stable transfection of DLKP. DLKP cells were seeded at 2×10^5 cells/25-cm^2 flask 24 hours prior to transfection. Human galectin-3 cDNA in pREB9 plasmid (a gift from Fu-Tong Liu, The Scripps Research Institute, CA, USA) was transfected into the DLKP cells, using Lipofectin reagent (GibcoBRL, Life Technologies; Paisley, Scotland). A mixed population and subsequent clonal populations (resistant to 1 mg/ml of geneticin) resulted. The empty pREB9 plasmid vector was also transfected into DLKP cells, as a control.

RT-PCR analysis. For RNA analyses, cells were grown in 25-or 75-cm^2 tissue culture flasks until approximately 80 % confluency was reached. Total RNA was isolated from pelleted cells by extracting with TriReagent (Sigma; Poole, England) according to the manufacturer's instructions. First-strand cDNA was synthesised from 1 μg RNA using oligo dT primers (Oswel; Southampton, England). Five μl cDNA was then amplified in a 50 μl PCR reaction solution containing 1.5 mmol/l $MgCl_2$, 0.2 mmol/l deoxynucleotide triphosphates, 20 μmol/l oligonucleotide primers and 2.5 U *Taq* polymerase enzyme (Sigma). Forward and reverse primers for galectin-3 cDNA amplification, spanning exons 3-4 and 5-6 respectively, were selected: (forward):
5' GCTGGGCCACTGATTGTGCCTTAT 3' and (reverse)
5' ACCAGTACTTGTATTTTGAATGGT 3'
amplifying a 281 bp product. ß-actin (forward):
5' GAAATCGTGCGTGACATTAAGGAGAAGCT 3' and
(reverse) 5' TCAGGAGGAGCAATGATCTTGA 3'primers were used, together with galectin-3 primers, to co-amplify a 383 bp ß-actin product (26). The semi-quantitative PCR cycle used was as follows: 95°C for 2 minutes; 20 cycles of 95°C for 30 seconds, 54°C for 30 seconds, 72°C for 30 seconds; completion step of 72°C for 5 minutes;.

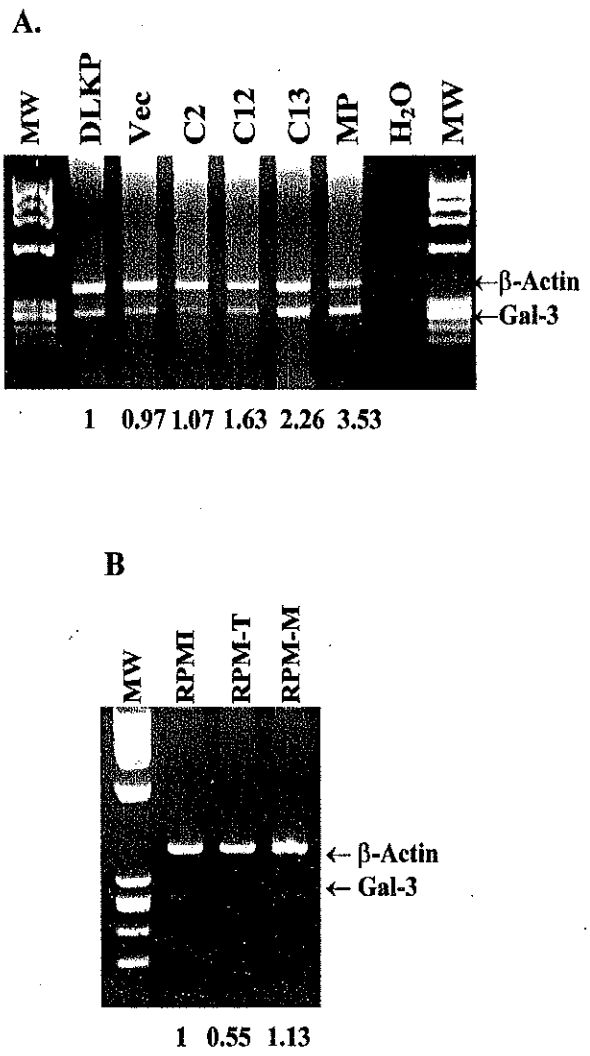


Figure 1. *Semi-quantitative reverse transcriptase-polymerase chain reaction analysis of galectin-3 (Gal-3; 281 bp) in (A) DLKP and it's transfected variants, Vec, C2, C12, C13 and MP, and (B) RPMI, RPMI-Taxol (RPMI-T) and RPMI-Melphalan (RPMI-M). β-actin (383 bp) was included as an endogenous control and H_2O as a negative control. Numerical values under each lane represent relative galectin-3 band intensities after normalising galectin-3 intensity with the corresponding β-actin band intensity (assessed by densitometry) and correcting the value for (A) DLKP and (B) RPMI to a value of 1. MW indicates molecular weight markers.*

Western blot analysis. For Western blots, cells were trypsined, rinsed with PBS and they were then lysed by re-suspending in protease inhibitor (Roche) and sonicating, on ice. Twenty μg aliquots of protein samples were separated by running at 250 V and 45 mA on a 15 % polyacrylamide gel, proteins were transferred onto PVDF membranes and were, subsequently, blocked for 2 hours at room temperature with 5 % semi-skimmed dried milk in Tris-buffered saline (TBS: 0.05 mol/l Tris-HCl, 0.15 mmol/l NaCl, pH 7.4). The membranes were probed over-night, at 4 °C, with a galectin-3 primary antibody (1/1000 dilution in TBS; a gift from Fu-Tong Liu, The Scripps Research Institute, CA, USA). Following a series of washes, the membranes were probed with a peroxidase-labelled secondary antibody for 1 hour and visualised by chemiluminescence using ECL reagents (Amersham Pharmacia Biotech.; Buckinghamshire, England).

Table I. *Proliferation rates of cell lines.*

Cell Line	Doubling Time (Hr.)
DLKP	28.99 ± 1.62
Vec	31.85 ± 2.23
C2	30.30 ± 1.03
C12	30.77 ± 1.85
C13	24.24 ± 2.18
MP	32.36 ± 3.04
RPMI	31.25 ± 2.13
RPMI-Taxol	30.78 ± 2.35
RPMI-Melphalan	30.77 ± 2.52

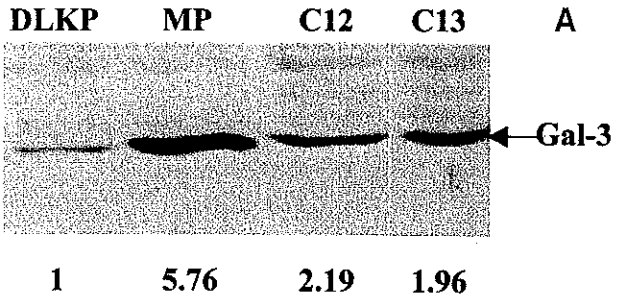
Doubling-times (proliferation rates) of all cell lines were determined from 3 repeat experiments and the data above represents means ± SD.

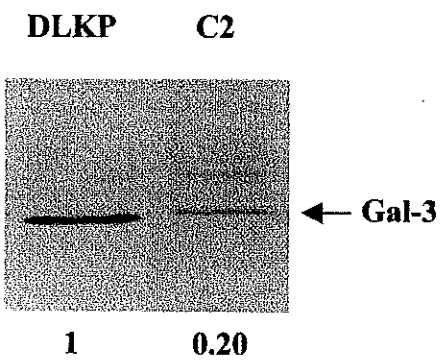
Table II. *The relative resistance of DLKP variants to a range of chemotherapeutic drugs, with respect to the drug sensitivity of DLKP.*

Cell Line	Adriamycin	Carboplatin	Taxol
DLKP	1	1	1
Vec	1.05	0.93	0.56
C2	1.44	0.60	0.46
C12	0.74	1.27	0.50
C13	0.65	1.52	0.51
MP	0.98	2.37	1.19

The fold-resistance was determined by dividing the average 50% inhibitory concentration (IC_{50}) value for the DLKP variant of interest by the corresponding average IC_{50} value for DLKP parent cell line. The IC_{50} values are the means of a minimum of 3 repeat experiments, with 8 intra-assay repeats in each experiment. The IC_{50} values of the drugs to DLKP cells are: adriamycin 14.2 ng/ml; carboplatin 1.86 μg/ml; taxol 2.11 ng/ml.

Proliferation rate assays. The proliferation rate (doubling times) of the cells was determined by monitoring their growth over consecutive 24 hour time periods. For this, the cell-were seeded at 2.5×10^5 cells in each of 7 × 25cm^2 flasks. The cells were incubated overnight at 37 °C and 1 flask of cells was tryspinised and the cells were counted on each of the 7 days following seeding. Proliferation rate assays were carried out on all cell lines a minimum of 3 times. Cell doubling times were calculated from a graph of cell numbers against time.





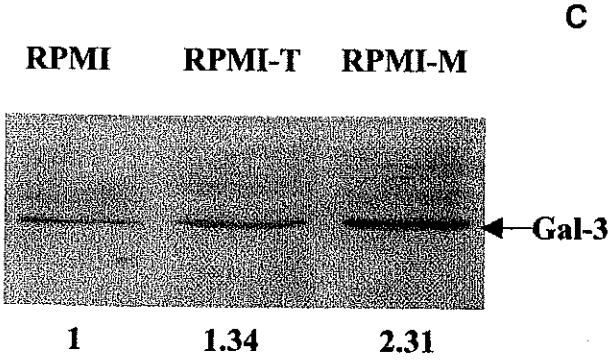


Figure 2. *Western blot analysis of galectin-3 (Gal-3) protein, indicating over-expression of Gal-3 in (A) MP, C12 and C13 and under-expression in (B) C2, compared to the DLKP parental cell line. Similarly, galectin-3 is apparently over-expressed in (C) RPMI-Melphalan (RPMI-M) compared to RPMI-Taxol (RPMI-T) and RPMI parent cell lines. Numerical values under each lane represent relative galectin-3 band intensities (analysed by densitometry), after correcting the (A & B) DLKP and (C) RPMI intensities to a value of 1. Results are representative of Western blot analyses performed at least 3 times.*

Cytotoxicity assays. For cytotoxicity analyses, the cells were seeded at 1×10^3 cells/well in a 96-well plate and were incubated overnight at 37 °C and 5% CO_2. Following this, the cells were continuously exposed to a range of concentrations of adriamycin, carboplatin and taxol for 5-7 days, as previously described (27). Cell viability was assessed using the

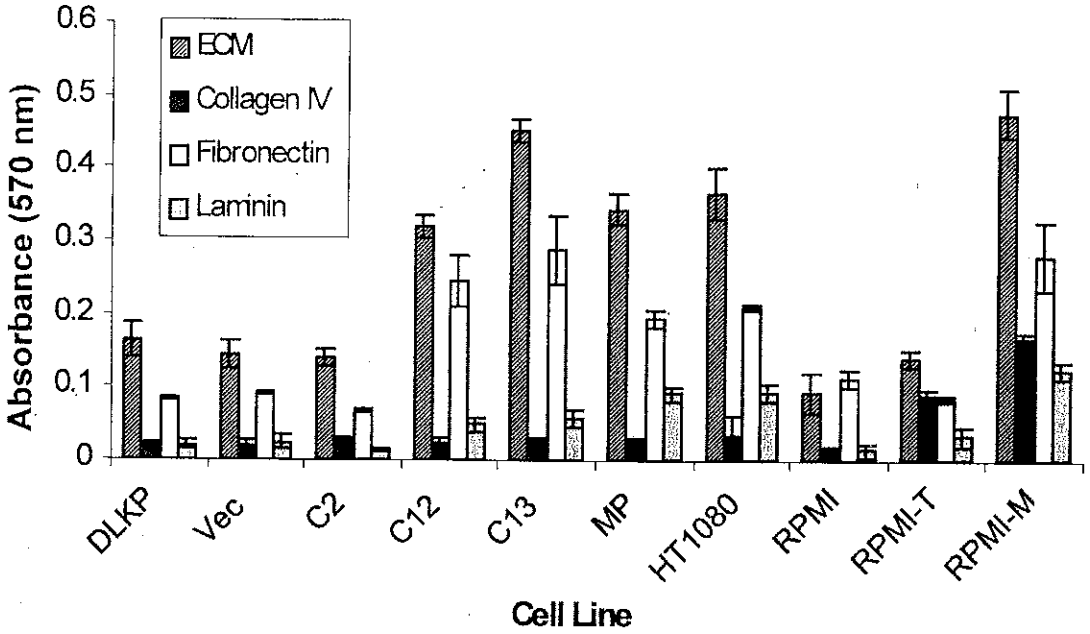


Figure 3 *Adhesion of DLKP and RPMI cell lines to ECM, collagen IV, fibronectin and laminin. Levels of galectin-3 expression in DLKP cell types apparently correlate with cell adhesion to ECM fibronectin and laminin, but not to collagen IV. RPMI-Melphalan (RPMI-M), which over-expresses galectin-3 protein, was more adhesive to all substrates than RPMI and RPMI-Taxol (RPMI-T). Results are expressed as absorbance (at 570 nm) of crystal violet-stained attached cells. Results represent means +/-SD for at least 3 experiments.*

acid phosphatase method (28). Toxicity assays were carried out on each cell line a minimum of 3 times, with each drug. All assays contained eight replicas of each drug concentration.

Adhesion assay. Adhesion assays were performed in accordance with the protocol described by Liang *et al.* (25). Collagen type IV (Sigma), fibronectin (Sigma) and laminin (Sigma) were diluted to 25 μg/ml with PBS. ECM (Sigma), a reconstituted basement membrane, was diluted to 1 mg/ml. Two hundred and fifty μl aliquots were placed into each well of a 24-well plate (Costar) and the plates were incubated at 4 °C overnight. The following day, the supernatants were removed and the wells were rinsed twice with PBS. To inhibit non-specific binding, 0.5 ml aliquots of 1 % BSA/PBS (w/v) were added to each well and the plates were incubated, for 20 minutes at 37°C / 5 % CO_2. The plates were then rinsed twice with PBS. Cells were trypsinised, resuspended in serum-free medium and plated, in triplicate, at a density of 2.5×10^4 cells per well. These were subsequently incubated at 37 °C / 5 % CO_2 for 60 minutes, after which medium and unattached cells were removed and the plates were rinsed gently with PBS. Attached cells were then stained with 0.25 % crystal violet dye (0.5 ml per well) for 10 minutes. Excess crystal violet dye was then removed, the plates were rinsed with H_2O and allowed to air-dry. The dye was eluted with 200 μl of 33 % glacial acetic acid/well. One hundred μl aliquots were transferred from each well to corresponding wells of a 96 -well plate and the absorbance was read at 570 nm.

Invasion assays and motility assays. Invasion assays were performed in accordance with the protocol described by Liang *et al.* (25). ECM (Sigma) was diluted to 1 mg/ml in serum-free medium. One hundred μl aliquots of 1 mg/ml ECM were placed into each insert (8.0 μm pore size; Falcon) which had been placed in wells of a 24-well plate (Costar). The plates (with inserts) were incubated at 4 °C overnight. The following day the inserts were washed 3 times with 100 μl aliquots of serum-free medium. The cells were harvested and resuspended, at a density of 1×10^6/ml, in medium containing 5 % FCS and 100 μl of cell suspension was added to each insert, as appropriate. A 250 μl aliquot of medium containing 5 % FCS was added to each well, beneath the insert. The cells were incubated at 37 °C for 48 hours. After this time-period, the inner side of the insert was wiped (3 times) with a swab which had been soaked in PBS to remove cells which had not migrated through the membrane. The outer side of the membrane was gently rinsed (3 times) with PBS and was subsequently stained, for 10 minutes, with 0.25 % crystal violet dye, rinsed again and allowed to air-dry. Control inserts were not wiped to remove cells and were stained with 0.25 % crystal violet dye to indicate that, in the test cases, only cells which had migrated through the membrane remained in place and so were stained with crystal violet. Inserts were then examined by light microscopy and were photographed. As some of the cells which had passed through the membrane had attached to the surface of the plate, following examination by light microscopy, crystal violet was eluted, with 200 μl of 33 % glacial acetic acid/well, from the insert and the corresponding well, simultaneously. One hundred μl aliquots were transferred from each well to corresponding wells of a 96-well plate and the absorbance was read at 570 nm.

The procedure for motility assays was identical to that for invasion assays with the exception that the inserts were not coated with ECM.

Results

Cell lines. Transfection of DLKP cells with human galectin-3 cDNA in pREB9 plasmid resulted in a geneticin-resistant (1 mg/ml geneticin) mixed population (MP) of cells and several resistant clones, including C2, C12 and C13. A mixed population resulting from transfection of the empty

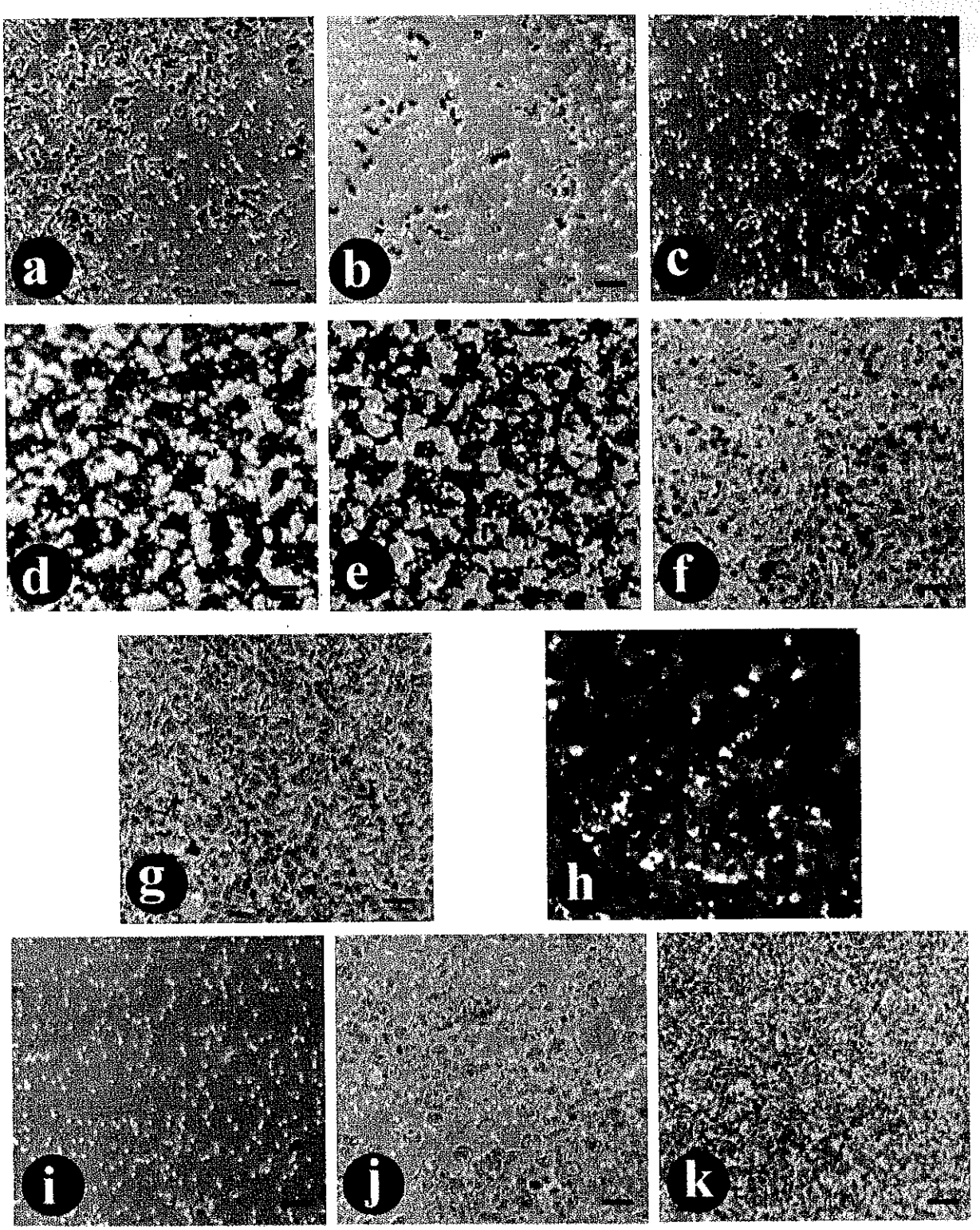


Figure 4(A) *Motility assay of (a) DLKP, (b) Vec, (c) C2, (d) C12, (e) C13, (f) MP, (g) HT1080, (i) RPMI, (j) RPMI-Taxol and (k) RPMI-Melphalan cells. (h) DLKP "unwiped" insert shown as a control. Scale bar: 200 μm.*

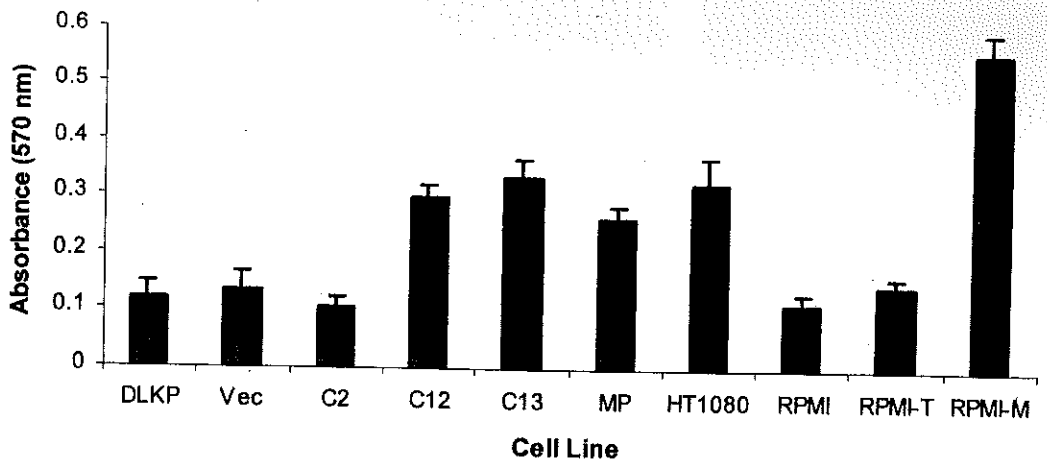


Figure 4(B) *Motility assays: Results following elution of crystal violet, and absorbance read at 570 nm, from inserts illustrated in Figure 4(A). Results represent means +/-SD for at least 3 experiments.*

pREB9 plasmid vector (Vec) was isolated as a negative control.

RT-PCR analysis. Transcription of galectin-3 mRNA was assessed in DLKP, Vec, C2, C12, C13, MP, RPMI and it's variants RPMI-Melphalan and RPMI-Taxol, by RT-PCR. DLKP parent cells, all newly constructed DLKP variant cell lines, and the RPMI cell types, expressed human galectin-3 mRNA (Figure 1). Whereas levels of galectin-3 mRNA transcripts in C2 were apparently comparable to those in DLKP and Vec, C 12, C 13 and MP over-expressed galectin-3 mRNA (Figure 1(A)). Analysis of the RPMI variants indicated that RPMI-Taxol cells under-express galectin-3 mRNA, whereas RPMI-Melphalan cells and the RPMI parent cell line express similar levels of galectin-3 transcripts (Figure 1(B)). Amplification of ß-actin (383 bp) as endogenous control, in all cases, indicated that the mRNA was of sufficient quality and that the RT and PCR reactions were conducted successfully.

Western blot analysis. Single bands (approximately 30 kDa) corresponding to galectin-3 protein were detected in all DLKP and RPMI variants. Galectin-3 protein expression levels were greater in MP, C12 and C13 compared to DLKP parent cells (Figure 2(A)), whereas C2 had decreased levels of this protein (Figure 2(B)). Galectin-3 protein levels were over-expressed by RPMI-Melphalan, compared to RPMI-Taxol and the parent (RPMI) cell line (Figure 2(C)).

Proliferation rates of cell lines. Doubling-times were determined for all DLKP and RPMI variants (Table I). Doubling times did not differ greatly between the cell lines analysed and apparently did not correlate with galectin-3 expression levels. It is interesting to note, however, that although the doubling-time for MP was similar to the other DLKP variants, the lag-time before exponential growth was longer for MP than the other cell lines included.

Chemosensitivity of galectin-3 expressing cells. The sensitivity of the galectin-3 transfectants (Vec, C2, C 12, C 13 and MP) to a range of chemotherapeutic drugs was determined and compared to DLKP parent cell line drug sensitivity (Table II). Whereas galectin-3 over-expression did not affect cell sensitivity to adriamycin and taxol, cells over-expressing galectin-3 (in particular the MP cells) tended to be more resistant to carboplatin.

Adhesion assays. Results obtained from the adhesion assays suggest that galectin-3 over-expression supports DLKP adhesion to ECM, fibronectin and laminin. Adhesion to collagen IV did not correlate with galectin-3 expression levels (Figure 3). As previously described by Liang *et al.* (25), and supported by results from this study, RPMI-Melphalan was more adhesive to all substrates than RPMI and RPMI-Taxol.

Motility and invasion assays. Motility (Figures 4(A) and (B)) and invasion (Figures 5(A) & (B)) assays suggest that galectin-3 expression levels in DLKP cells correlate with cell motility and invasiveness *i.e.* galectin-3 over-expression renders the cells more motile and invasive through ECM, *in vitro.* Similarly, RPMI-Melphalan, which over-expresses galectin-3, compared to RPMI and RPMI-Taxol, is apparently more motile and invasive than RPMI and RPMI-Taxol (Figure 4 and Figure 5). Results from the *in vitro* invasiveness analysis of the positive control cell line HT1080 confirm that the conditions for these assays were correct. Results from the inserts (Figure 4(A)-h and Figure 5(A)-h)

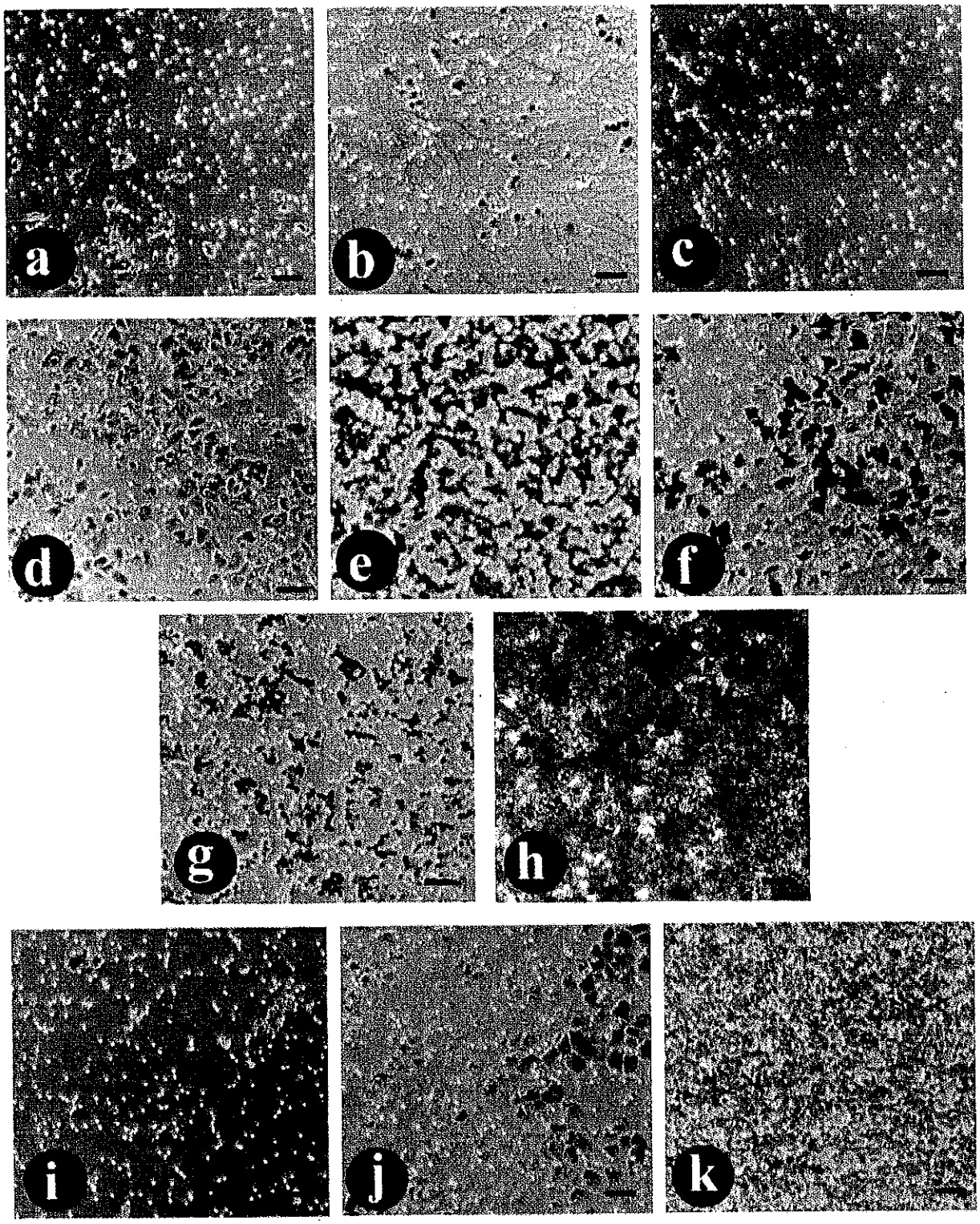


Fig. 5(A) *Invasion assay of (a) DLKP, (b) Vec, (c) C2, (d) C12, (e) C13, (f) MP, (g) HT1080, (i) RPMI, (j) RPMI-Taxol and (k) RPMI-Melphalan cells. (h) DLKP "unwiped" insert shown as a control. Scale bar: 200 μm.*

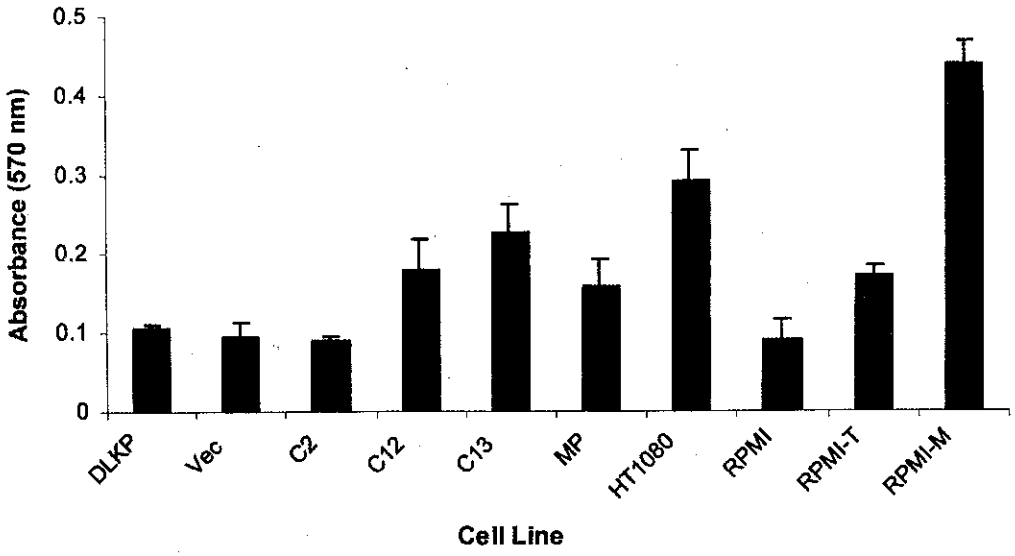


Figure 5(B) *Invasion assays: Results following elution of crystal violet, and absorbance read at 570 nm, from inserts illustrated in Figure 5(A). Results represent means +/-SD for at least 3 experiments.*

which were unwiped (see Materials and Methods) support the assumption that only cells which have migrated remain attached to the membrane and are stained with crystal violet, as shown in Figures 4 and 5 (a-g & i-k).

Discussion

To date, analysis of a range of normal and tumour cell types have implicated galectin-3 in cell growth, differentiation, adhesion, and tumour progression. Although galectin-3 up-regulation is associated with progression of certain tumour types (for example, colorectal cancer), while galectin-3 down-regulation correlates with progression of other tumour types (including breast and ovarian neoplasms), the relevance of galectin-3 expression in lung and nasal carcinomas has not yet been reported. In the present study we have investigated the constitutive expression levels of galectin-3 in DLKP (a non-small cell lung carcinoma cell line) and we have assessed it's significance with respect to cell proliferation, adhesion, motility, invasion and chemosensitivity, by modifying the expression levels of galectin-3 in these cells. RPMI-2650 (nasal carcinoma) variants with different patterns of invasiveness, MDR and integrin expression were also investigated, to establish if galectin-3 may be involved in expression of their different phenotypes.

Galectin-3 was found to be constitutively expressed by both DLKP and RPMI parent cell lines. Transfection of galectin-3 cDNA into DLKP cells resulted in a MP and clonal populations (C12 and C13) over-expressing and under-expressing (C2) galectin-3. In agreement with reports from studies of transfected breast carcinoma cell lines (7), but unlike results reported from Jurkat cell studies (3), differing levels of galectin-3 expression (mRNA and protein) did not significantly affect the DLKP proliferation rate. Similarly, the doubling times for RPMI-Melphalan, shown in this study to significantly over-express galectin-3 protein, did not differ greatly from that of RPMI and RPMI-Taxol.

Analysis of cell chemosensitivity indicated that galectin-3 expression levels in DLKP were not associated with induction of an MDR phenotype. Although galectin-3 over-expression in DLKP MP cells was associated with increased resistance (approximately 2.4-fold) to carboplatin; in general, galectin-3 over-expression apparently did not protect cells from drug-induced death. A direct evaluation of galectin-3 association with drug resistance in the RPMI variants cannot be deduced from this study. Both RPMI-Melphalan and RPMI-Taxol express MDR phenotypes, compared to the RPMI parent cell line. However, different fold-resistance levels to each drug studied have been reported for these cell lines (25) *i.e.* RPMI-Melphalan, which overexpresses galectin-3, is more resistant than RPMI-Taxol to VP-16, melphalan, cisplatin and cadmium chloride, whereas RPMI-Taxol has greater fold-resistance than RPMI-Melphalan to adriamycin, vincristine, vinblastine, taxol (paclitaxel) and 5-fluorouracil.

Cell adhesion, motility and invasion of basement membrane are critical steps in tumour metastasis. There are, however, conflicting reports on the role of galectin-3 in tumour metastasis. In agreement with studies on breast carcinoma cell lines reported by Choi Kim *et al.* (23) and Matarrese *et al.* (7), in this study we have shown, for the first time, that galectin-3 over-expression in lung tumour cells is associated with enhanced adhesion to ECM proteins, cell motility and *in vitro* invasiveness. Galectin-3 involvement in metastasis is further supported by our finding of constitutively

over-expressed levels of galectin-3 in RPMI cells expressing an adhesive, motile and invasive phenotype *i.e.* RPMI-Melphalan.

In conclusion, we have demonstrated that galectin-3 over-expression is associated with adhesion, motility, and *in vitro* invasiveness in lung (DLKP) and nasal (RPMI-2650) carcinoma cell types. These observations suggest a significant role for galectin-3 in human lung and nasal cancer metastasis using cell line models and support a need for further studies of lung and nasal metastatic tumour biopsy material to evaluate the clinical significance of galectin-3 in metastases of human lung and nasal carcinomas.

Acknowledgements

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