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Expressió diferencial determinant del fenotip metastàtic en un model d'adenocarcinoma de pulmó humà

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CAPÍTOL IV

Identificació de l'expressió gènica de la Semaforina E a cèl·lules metastàtiques d'adenocarcinoma de pulmó humà mitjançant la tècnica del *differential display* d'RNA

El fenotip metastàtic de les cèl·lules tumorals és el resultat d'una sèrie d'esdeveniments genètics que alteren l'expressió de l'RNA i les proteïnes de les cèl·lules normals. Donades les diferències d'expressió relacionades amb aquest fenotip prèviament identificades a l'adenocarcinoma de pulmó humà (capítols I, II i III), el nostre darrer objectiu ha estat la identificació d'altres gens que s'expressen diferentment entre les cèl·lules metastàtiques i les no metastàtiques i que es puguin utilitzar com a factors pronòstic.

En el present capítol hem utilitzat la tècnica del *differential display* per a comparar els patrons d'expressió d'RNA característics d'ambdues línies cel·lulars. S'ha confirmat mitjançant anàlisi per Northern blot, l'expressió diferencial de tres cDNAs. Dues d'aquestes seqüències, que corresponen a un possible factor d'*splicing* i a un factor relacionat amb la proliferació respectivament, presenten una expressió disminuïda a les cèl·lules metastàtiques respecte de les no metastàtiques. Hem trobat un increment considerable de l'RNAm la Semaforina E humana a les cèl·lules metastàtiques. Aquest gen, recentment identificat, codifica per una proteïna que ha estat relacionada amb diferents mecanismes tant de supervivència cel·lular com d'immunossupressió. Aquests resultats indiquen un possible paper de la Semaforina E a la disseminació metastàtica de les cèl·lules d'adenocarcinoma de pulmó humà.

Identification of semaphorin E gene expression in metastatic human lung adenocarcinoma cells by mRNA differential display

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Synopsis: Human lung adenocarcinoma cell lines HAL-8Luc and HAL-24Luc differ in their metastatic potential. HAL-8Luc cells metastasize to lungs when injected either intravenously or intramuscularly in mice while HAL-24Luc cells do not. The differential display method is used to identify genes differentially expressed between the two cell lines and the findings are extensively discussed.

Background: Lung cancer is the leading form of cancer in most countries and metastasis is the main cause of death in oncological patients. The metastatic phenotype of tumor cells is the result of genetic events altering the RNA and protein expression of normal cells. Our objective was to identify genes expressed differentially between metastatic and nonmetastatic human lung adenocarcinoma cells that might be used as prognostic factor.

Methods: The differential display technique was used to compare the RNA expression patterns distinguishing metastatic (HAL-8Luc) and nonmetastatic (HAL-24Luc) human lung adenocarcinoma cells, two genetically close cell lines.

Results: Differential expression of three cDNAs was confirmed by Northern blot analysis. Two sequences corresponding to a putative splicing factor and a proliferation-related factor cDNAs were underexpressed in the metastatic cells relative to the nonmetastatic ones. Interestingly, we found that human semaphorin E mRNA was several fold overexpressed in the metastatic cells. This recently identified gene encodes a protein whose expression has been related to several cell survival mechanisms as well as to immunosuppression.

Conclusion: Our results point to the relevance of semaphorin E in metastatic spread of human lung adenocarcinoma cells.

INTRODUCTION

Lung cancer is the leading cause of cancer death in most countries. In several areas, the annual incidence rates among males exceed 100 per 100.000 and adenocarcinoma is a major form of this pathology (Blot & Fraumeni, 1996). Much of the malignant neoplasms lethality is directly attributable to the tumor ability to develop secondary growths at distal

organs (Woodhouse *et al.*, 1997). It is thus of great clinical importance to identify novel genes associated with the processes of invasion and metastasis that could be targets for therapeutical intervention.

The differential display method developed by Liang P. and Pardee A.B. is ideally suited to compare altered gene expression between eukaryotic cells (Liang *et al.*, 1993; Liang & Pardee, 1992). This technique, based on the PCR amplifications of expressed RNA sequences, has already been succesfully used to identify genes differentially expressed in metastatic cells of diverse origins such as mammary carcinoma (Liu *et al.*, 1997), brain tumors (Sehgal *et al.*, 1997), medullary thyroid carcinoma (Musholt *et al.*, 1997) or melanoma (Duncan *et al.*, 1998; Lee & Welch, 1997).

HAL-8Luc and HAL-24Luc are human lung adenocarcinoma cell lines characterized by their different metastatic potential (Martin-Satue *et al.*, 1998). When injected either i.v. or i.m. in athymic mice HAL-8Luc cells developed lung metastasis while HAL-24Luc cells did not metastasize. Both cell lines were derived from a common parental cell line (Inufusa *et al.*, 1991) and have, consequently, a close genetic background thus being a convenient system to study changes in gene expression that could be related to their metastatic behaviour. We have previously described that enhanced expression of α(1,3)-fucosyltransferase genes in the metastatic HAL-8Luc cells, compared with HAL-24Luc cells, contributes to the adhesive capacity of these cells to the activated endothelium and correlates with their lung colonization potential (Martín-Satué *et al.*, 1999; Martin-Satue *et al.*, 1998). We show here for the first time the use of the differential display method to identify genes differentially expressed in these two cell lines that could be related to human lung adenocarcinoma cell invasiveness.

MATERIALS AND METHODS

Cell Culture

Human lung adenocarcinoma cell lines HAL-8Luc (metastatic) and HAL-24Luc (nonmetastatic) (Martin-Satue *et al.*, 1998) were mantained in RPMI 1640 (Bio-Whitaker, Verviers, Belgium) supplemented with 10% (v/v) heat-inactivated FBS (Bio-Whitaker), 2mM L-glutamine (Bio-Whitaker), penicillin (100 units/ml), streptomycin (100 μg/ml) and 300 μg/ml geneticin (G418; Life Technologies, Inc., Gran Island, NY) in a humidified atmosphere of 5% CO_2 at 37°C.

mRNA Isolation and Primers

mRNAs were extracted from confluent HAL-8Luc and HAL-24Luc cell cultures by using the PolyATtract System 1000 kit from Promega (Madison, WI). Oligonucleotide primers were obtained from Pharmacia Biotech Europe GmbH (Barcelona, Spain).

Downstream primers were: $T_{11}AA$ and $T_{12}AG$. 10-mer arbitrary primers were: MM1, 5′-CATAAGCAGG-3′; MM2, 5′-TACGATGACG-3′; 1374, 5′-GTGGCCGAGG-3′.

Differential Display

Differential display reverse transcription-technique (DDRT) was performed according to Liang P. et al (Liang *et al.*, 1993). From 100 to 700 ng of mRNA were reverse-transcribed with 300 units of MMLV reverse transcriptase (Promega, Madison, WI) in the presence of 2.5 μM of anchored downstream primers either $T_{11}AA$ or $T_{12}AG$ in a 20 μl reaction. 2 μl of this reverse transcription were PCR amplified in the presence of [α-^{33}P]-dATP with the matching downstream primer and either of the 10-mer upstream primers MM1, MM2 or 1374, using the following cycling parameters: 94°C for 30 sec, 40°C for 2 min and 72°C for 30 sec, for 40 cycles, followed by 5 min at 72°C. A 3.5 μl aliquot PCR product was mixed with 2 μl of loading dye and incubated for 2 min at 95°C before loading onto a 6% DNA sequencing gel. Electrophoresis were conducted for about 2.5 hours at 55W constant power. The gels were dried and exposed to Hyperfilm MP (Amersham, UK). Fragments of cDNA corresponding to bands that were differentially displayed between sets of cells were recovered from the gel by boiling the gel segment in dH_2O for 15 min. The eluted DNA was reamplified using the same set of primers and the same cycling parameters used to generate the original PCR product. The amplified fragments were cloned into the pCRII vector using the TA Cloning kit (Invitrogen, San Diego, CA), and sequenced using the T7 Sequenase 2.0 DNA sequencing Kit (Amersham, UK). Sequences were compared to known nucleotide sequences in the GenBank using the Basic Local Alignment Tool (BLAST) program.

Northern blots

3 μg poly(A)$^+$RNA from HAL-8Luc and HAL-24Luc cells as well as 2 μg of RNA molecular weight markers (United States Biochemical, Cleveland, Ohio) were fractionated by electrophoresis in a 1.2% denaturing agarose gel containing 1.8% (v/v) formaldehyde and transferred to nylon membranes (Hybond-N$^+$; Amersham, UK). The membranes were hybridized with ^{32}P-labelled cDNA probes in a solution containing 50% (v/v) formamide, 5x SSC, 50mM sodium phosphate buffer pH 6.5, 250 μg/ml sheared salmon sperm DNA, 10x Denhart's solution and 10% dextran sulphate overnight at 42°C. Following hybridization the membranes were washed twice for 10 min at RT in 2x SSC, 0.1% SDS and once in 0.2x SSC, 0.1% SDS for one hour at 68°C. Finally the membranes were autoradiographed by exposure to Hyperfilm MP (Amersham, UK). Blots were stripped and reprobed with human β-actin cDNA as a control for RNA integrity and loading consistency. Band intensity was analyzed with the Molecular Analyst/PC 1.4 software (BioRad, CA) and calibrated by comparison with the β-actin bands.

RESULTS

To identify genes showing altered expression in the metastatic human lung adenocarcinoma HAL-8Luc cells, we compared mRNA expression by differential display using poly(A)$^+$ RNA extracted from HAL-8Luc cells and their nonmetastatic counterpart HAL-24Luc. Three 10-mer arbitrary primers were used in combination with either $T_{11}AA$ or $T_{12}AG$ downstream primers. Figure 1 shows typical DDRT-PCR amplifications obtained using two separate primer combinations. Differentially expressed PCR products are indicated. Once reamplified and cloned, the PCR identified products were used as probes in Northern blot analysis for confirmation of differential expression and full-length sequenced (Table I). We analyze in this work three of such PCR products: MS3, a 274 bp-sequence overexpressed in the metastatic cells and the 6.6.5 (342 bp) and 18.1.5 (330 bp) sequences, both underexpressed in the same cells. cDNAs corresponding to these sequences were isolated from the gel, reamplified and cloned.

Northern Blot Analysis

To further establish by an independent procedure that the candidate sequences were differentially expressed, mRNA from metastatic and nonmetastatic cells was analyzed by Northern blot using as probes the cDNA clones derived from the differential display analysis (Fig. 2).

Significantly different expression levels for the three sequences were confirmed: probe 6.6.5 identified two transcripts of 4 and 1.9 kb respectively whose expression was reduced in HAL-8Luc cells (3- and 1.5-fold respectively); probe 18.1.5 hybridized with a 2.1 kb message whose expression appeared also diminished (1.5-fold) in those cells. Finally, the MS3 sequence that was 7-fold overexpressed in the metastatic compared with the nonmetastatic cells, recognized a 5.2 kb message.

DNA Sequence Analysis

Differentially expressed cDNAs were full-length sequenced and compared to GenBank sequences (Table I). Sequence MS3 was identical to human semaphorin E mRNA (GenBank acc. nr.: AB000220); sequence 18.1.5 was 98.5% homologue to a 54 KDa protein mRNA (GenBank acc. nr.: U02493) and the 6.6.5 sequence showed a 93.3% homology with a proliferating cell-associated mRNA from *Rattus norvergicus* intestinal epithelium (GenBank acc. nr.: U21718).

DISCUSSION

Cancer metastasis requires a long series of sequentially interrelated steps, the failure or insufficiency of anyone of which may abort the process. In broad sense, besides the need

to avoid the immune system, these steps include 1) progressive growth of neoplastic cells after the initial transforming event, 2) establishement of a tumor neocapillary network from the surrounding host tissue, 3) entry to the circulation, 4) adhesion of tumor cells that survive the circulation to the capillary beds of organs, 5) extravasation and 6) proliferation within the organ parenchyma (Fidler, 1990). Each of these steps is in itself the result of a complex series of events that result from the alteration of normal gene expression. The differential display method is ideally suited for the detection of changes in gene expression that may result in the metastatic phenotype of tumor cells.

We describe the identification by differential display of three genes differentially expressed between the metastatic human lung adenocarcinoma cell line HAL-8Luc and the nonmetastatic but genetically close HAL-24Luc cells. The 6.6.5 and 18.1.3 mRNA sequences were underexpressed in HAL-8Luc cells while the MS3 mRNA sequence was overexpressed in the same cells.

Sequence 18.1.5 is 98.5% homologue to the human mRNA sequence encoding $p54^{nrb}$ (for nuclear RNA-binding protein, 54kDa). Although the function of this protein remains unknown, it contains two RNA recognition motifs and shares high homology with human splicing factor PSF. $p54^{nrb}$, as well as PSF, was identified in HeLa cells and may probably function as a general and/or regulatory splicing factor (Dong *et al.*, 1993). As it has been previously reported, alternative splicing of a single gene may lead to unbalanced expression of some mRNA isoforms related to neoplastic progression and metastatic promotion (De Rossi *et al.*, 1997; Harwood *et al.*, 1996; Siebert & Huang, 1997).

Sequence 6.6.5 shares high homology with a proliferating cell-associated mRNA from rat intestinal epithelium (Sykes & Weiser, 1992). To date, the corresponding protein has yet to be identified. However, the high homology shared by the human 6.6.5 sequence and its rat homologue suggests the existence of a new and highly conserved family of proliferating factors in epithelial cells. It is conceivable that altered expression of the activating and/or inhibiting factors implicated in the highly regulated process of cell proliferation would contribute to tumor metastasis. In this context, it has been shown that decreased expression of the human KAI-1 metastasis-suppressor gene resulted in the progression of human prostate and possibly lung and breast cancers as well (Dong *et al.*, 1997).

Sequence MS3 was identical to the recently identified human semaphorin-E (H-sema E) mRNA. The semaphorin gene family codes for transmembrane and secreted proteins of ~750 aa in length characterized by a ~500 aa extracellular domain termed the semaphorin (sema) domain which is conserved between invertebrates and vertebrates [20]. The best characterized function of this protein family is the repulsive guidance of nerve axons during development (Bagnard *et al.*, 1998; Kolodkin *et al.*, 1992; Luo *et al.*, 1993; Matthes *et al.*, 1995; Puschel *et al.*, 1995) but its function in non-neural systems is largely

unknown. There are indications that mouse semaphorin D and E are involved in the histogenesis of tissues other than neural (Puschel *et al.*, 1995), and several reports indicate that semaphorins also participate in diverse cell survival mechanisms (Hall *et al.*, 1996; Yamada *et al.*, 1997). Interestingly, homologues to the sema domain of semaphorins have been found in two poxvirus-encoded proteins involved in the immune suppression of the host (Ray *et al.*, 1992), what has lead to the especulation that semaphorins may function as natural suppressants of the immune system (Luo *et al.*, 1993).

H-sema E is a secreted protein. Transfection of semaphorin-E conferred drug-resistant phenotype to cisplatin-sensitive human ovarian cancer cells, probably by acting as an autocrine factor bound to undetermined cell surface receptor(s) (Yamada *et al.*, 1997). In addition, the same authors described aberrant overexpression of H-sema E in 33.3% of recurrent squamous cell carcinomas, including those of pulmonary origin, removed at autopsy after extensive radiochemoterapy. These results suggest the participation of H-sema E in the carcinogenesis and progression of certain human tumors. Increased expression of H-sema E has also been related to the pathogenesis of rheumatoid arthritis, where it has been suggested this protein has a role as regulator of inflamatory processes (Mangasser-Stephan *et al.*, 1997; Seki *et al.*, 1998).

Based on these previous findings we would like to suggest that in human lung adenocarcinoma cells sema E may be involved in tumor cell survival mechanisms, participating in the immunological regulation of tumor spread by helping to avoid or subvert immune surveillance. This is further reinforced by our observation of a noticeable absence of lymphocytes in intramuscularly generated HAL-8Luc tumors, in contrast with the high lymphocyte infiltration observed in the HAL-24Luc derived primary tumors (10). These results support a role of sema E in the metastatic phenotype of tumor cells as immunological regulator of tumor progression.

CONCLUSSIONS

In summary, our results demonstrate that mRNA differential display is a valuable tool for identification of potential metastasis-related genes in this human lung adenocarcinoma system. Our results also reinforced recent studies pointing to the usefullness of sema-E as a prognostic factor in human lung adenocarcinomas.

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FIGURES and TABLE

Figure 1. mRNA differential display gel patterns from metastatic (*8*) and nonmetastatic (*24*) human lung adenocarcinoma cells using two different primer combinations: T_{11}AA-1374 (*A*) and T_{12}AG-MM1 (*B*). Differentially expressed cDNA fragments are indicated by arrows.

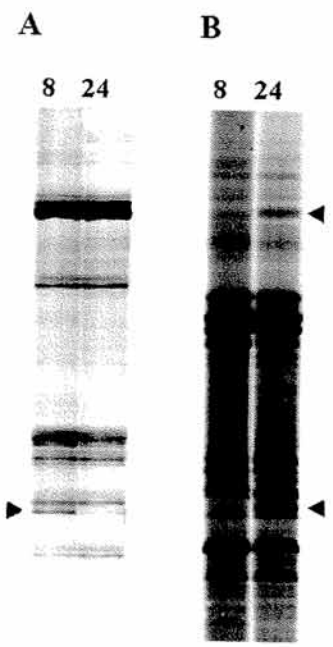
A
8 24
B
8 24

Figure 2. Northern blot analysis of metastatic (*8*) and nonmetastatic (*24*) human lung adenocarcinoma cell mRNA using the *18.1.5, 6.6.5* and *MS3* sequences as probes. 3 μg poly(A)$^+$ RNA were electrophoresed, blotted, and hybridized with the ^{32}P-labelled 18.1.5, 6.6.5 and MS3 cDNA probes as described in "Materials and Methods". After a first autoradiographic exposure, the probes were stripped out, and the blots were rehybridized with the β-actin probe (*β-act*). Band sizes are indicated in kilobases at the *left* of each set.

The bargraphs represent the relative expression level of each mRNA species in the respective cell lines. Relative mRNA levels were calculated after correction of gel loading differences using the β-actin gene expression as standard.

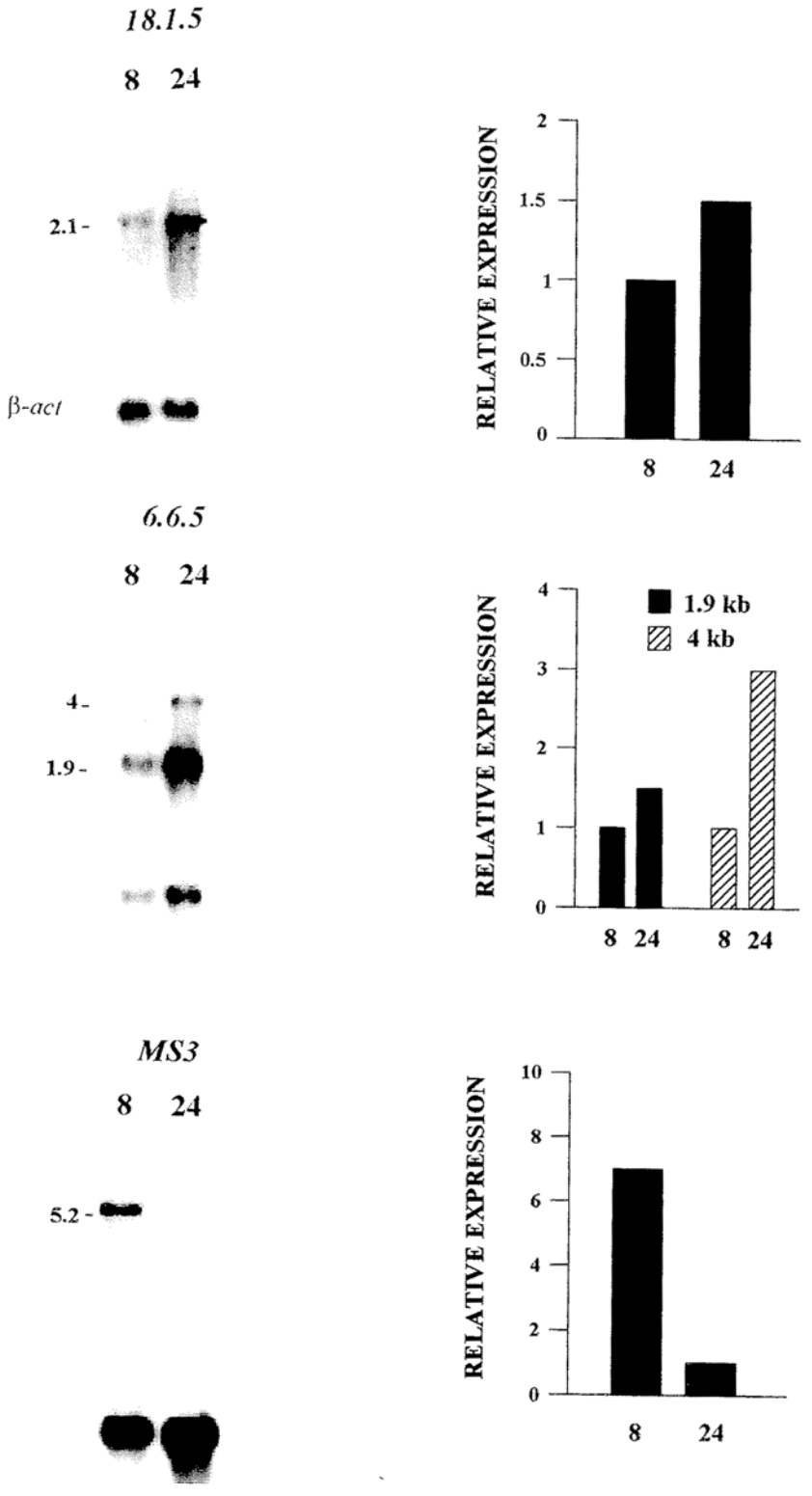
18.1.5
8 24
2.1 -
β-act
RELATIVE EXPRESSION
2
1.5
1
0.5
0
8 24
6.6.5
8 24
4 -
1.9 -
RELATIVE EXPRESSION
4
3
2
1
0
1.9 kb
4 kb
8 24 8 24
MS3
8 24
5.2 -
RELATIVE EXPRESSION
10
8
6
4
2
0
8 24

Table 1. Summary of clones differentially expressed between metastatic and nonmetastatic cell lines.

Clone	Relative expression in metastatic cells	Northern Blot Confirmation	Identity. Homology
18.1.5	Reduced	Confirmed	Human 54 kDa protein mRNA (U02493). 325/330, 98.5%
16.1.3	Reduced	Not confirmed	Human mRNA for β-actin (X00351). 240/240, 100%
18.3.1	Reduced	Confirmed	Human mRNA for fibrillarin (M59849). 283/294, 96.2%
16.1.4	Reduced	Not confirmed	Not determined
17.4.1	Augmented	Not confirmed	Not determined
17.2.1	Reduced	Not detectable	Unknown
17.3.1	Augmented	Not confirmed	Not determined
6.6.5	Reduced	Confirmed	Rattus norvegicus clone c426 intestinal epithelium cell-associated mRNA (U21718). 319/342, 93.3%
136.1.4	Augmented	Not detectable	Unknown
136.3.4	Augmented	Not confirmed	Not determined
MS3	Augmented	Confirmed	Human semaphorin E mRNA (AB000200). 274/274, 100%
MS1.8	Augmented	Not detectable	Unknown
MS2.4	Augmented	Not confirmed	Homo sapiens chromosome 17, clone HCIT542B22 (AC004253). 156/166, 93%
MS4.6	Augmented	Not confirmed	Homo sapiens mitochondrial genoma (V00662). 166/174, 95%
MS7.2	Reduced	Not confirmed	Bovine gamma globin gene (M63452). 171/184, 92%