

Differentially expressed genes in association with in vitro invasiveness of human epithelioid sarcoma

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Abstract

Aims—Differential display reverse transcription polymerase chain reaction (RT-PCR) was performed to identify genes associated with the invasive potential of human epithelioid sarcoma.

Methods—Two different clonal subpopulations, GRU-1A and GRU-1B, derived from the same human epithelioid sarcoma cell line GRU-1 and known to differ greatly in their invasive potential were compared by means of mRNA fingerprinting.

Results—Using a set of 10 arbitrary upstream primers and nine anchored oligo-dT primers, 22 candidate gene fragments were identified; differential expression was confirmed in four of these fragments by northern blot analysis. At the mRNA level, apoferritin light chain was predominantly expressed by the highly invasive cell line GRU-1A. In contrast, the mitochondrial gene M1, encoding cytochrome c oxidase I, and the TI-227H gene were expressed more strongly by the low invasive cell line GRU-1B. Furthermore, a novel gene fragment was identified and cloned that was preferentially expressed in the low invasive cell line GRU-1B, and therefore might have an inhibitory role in invasion. Consequently, this gene fragment was designated as expressed in low invasive sarcoma cells (ELISC-1).

Conclusions—A novel gene fragment (ELISC-1) and three known genes were identified as potential regulators of tumour invasiveness. Cloning of the entire sequence of ELISC-1 and subsequent investigations are required to establish its biological role.

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Keywords: invasion; epithelioid sarcoma; differential gene expression; heterogeneity

Metastasis is the major cause of death in patients with cancer. Many patients suffering from malignant tumours already have metastases at the time of diagnosis. It is of fundamental importance to elucidate the regulatory mechanisms underlying the complex process of metastasis to improve therapeutic strategies.^{1,2} The first step in metastasis formation is the process of invasion, during which tumour cells leave the primary tumour and actively infiltrate the surrounding tissue.¹ However, malignant tumour cells arising from the same original tumour often show a broad heterogeneity with respect to both morphology

and biological properties, such as invasion and metastasis. This intratumoral heterogeneity probably reflects different steps of progression of an individual tumour cell from a normal towards a malignant invasive and metastatic phenotype.³⁻⁵ These differences are probably paralleled by differences in the repertoire of genes expressed at a given time.

Recently, we successfully established an in vitro model for studying the mechanisms involved in the regulation of tumour cell invasiveness. The GRU-1 cell line was isolated from a human epithelioid sarcoma, which is a highly malignant soft tissue tumour, typically arising in the extremities of young adults and presenting an extremely aggressive clinical course with frequent metastasis and treatment failure.⁶ Subsequently, the clonal subpopulations GRU-1A and GRU-1B were isolated from GRU-1 and characterised with respect to morphological features, growth kinetics, drug sensitivities, cell motility, cell substrate adhesion, and in vitro invasiveness.⁷⁻⁹ In these studies, GRU-1A had a significantly higher invasive potential than GRU-1B.⁷ Because GRU-1A and GRU-1B cells have the same genetic background, but differ with respect to their invasive behaviour, differences in the pattern of gene expression should be responsible for the phenotypic diversity of both clonal cell lines.¹⁰ Hence, GRU-1A and GRU-1B provide a valuable system for the identification of candidate invasion related genes.

Differential display reverse transcription polymerase chain reaction (RT-PCR), originally introduced by Liang and Pardee in 1992,¹¹ provides a useful tool for identifying differences in gene expression between related cell populations, and has been applied successfully in an increasing number of studies investigating the molecular nature of cancer.^{12,13} A comparative analysis has shown that differential display RT-PCR has many advantages (identification of low abundant mRNA, need for only small amounts of starting RNA, and ability to identify increases and decrease in messages) compared with related techniques, such as electronic subtraction or subtractive hybridisation.¹⁴

In our present study, differential display RT-PCR was used to identify and isolate genes whose expression is associated with the invasive phenotype of two different clonal human sarcoma cell lines. At the RNA level, four different genes were found to be differentially expressed between the two cell lines. Apoferritin light chain was predominantly expressed by the highly invasive cell line GRU-1A, whereas cytochrome c oxidase I, TI-227H, and a novel

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gene fragment (ELISC-1) were more strongly expressed by the low invasive cell line GRU-1B.

Methods

CELL LINES AND CELL CULTURE

The clonal subpopulations GRU-1A and GRU-1B were isolated from the human epithelioid sarcoma cell line GRU-1 and have been characterised with respect to morphological features, proliferation kinetics, drug sensitivities, cell motility, cell-substrate adhesion, and in vitro invasiveness.⁷⁻⁹ All cell lines were maintained in Dulbecco's modified Eagle medium, supplemented with 10% fetal calf serum (both Gibco, Karlsruhe, Germany) and antibiotics. Cells were cultured at 37°C in an atmosphere of 5% CO₂.

DIFFERENTIAL cDNA DISPLAY

Total RNA was isolated from cells using the Qiagen RNeasy kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). Differential display was performed by DeltaTM RNA fingerprinting (Clontech Laboratories, Palo Alto, California, USA), essentially as described but with some modifications. Briefly, first strand synthesis was carried out with Moloney murine leukaemia virus (MMLV) reverse transcriptase using 2 µg of total RNA from both cell lines. PCR fingerprinting reactions were performed in a Perkin-Elmer Thermal Cycler 2400 (Perkin-Elmer, Norwalk, Connecticut, USA) using pairwise combinations of 10 arbitrary primers (25 mer) and nine anchored oligo-dT primers (30 mer). Cycling parameters were as follows: 94°C for five minutes, 40°C for five minutes, 68°C for five minutes for one cycle; 94°C for two minutes, 40°C for five minutes, 68°C for five minutes for two cycles; 94°C for one minute, 60°C for one minute, 68°C for two minutes for 25 cycles; followed by 68°C for an additional seven minutes. PCR products were run on a denaturing 6% polyacrylamide gel (SequaGel-6TM; Biozym, Oldendorf, Germany) and visualised by standard silver staining.¹⁵ Bands representing potentially differentially expressed mRNAs were excised from the gel, eluted in 0.1× TE, and reamplified using the same primers as for the first amplification under the following conditions: 94°C for two minutes; 94°C for one minute, 60°C for one minute, 68°C for two minutes for 20 cycles; followed by 68°C for an additional seven minutes. Subsequently, PCR products were purified for cloning procedures.

MOLECULAR CLONING

Reamplified PCR products were subcloned into the pGEM-T vector system (Promega, Madison, Wisconsin, USA). To obtain a representative selection of clones for each band, at least eight white colonies were picked, and inserts were sequence analysed using plasmid specific primers (SP6 and T7) in an ABI 310 automated sequencer (Perkin-Elmer Applied Biosystems, Foster City, California, USA). A computer search for homology was performed against NCBI GenBank data bases.

NORTHERN BLOT ANALYSIS

Total RNA was isolated as described above. Aliquots of 15 µg of total RNA were separated on a 1% agarose formaldehyde gel and blotted on to Hybond N nylon filters (Amersham, Little Chalfont, Buckinghamshire, UK). DNA probes of independent clones were generated by PCR from the respective cloned plasmid and radiolabelled by random sequence priming,^{16 17} using the rediprimeTM DNA labelling system (Amersham). Equal loading was confirmed by methylene blue staining of the membrane and hybridisation with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe. Membranes were prehybridised at 65°C in a solution containing 0.5M NaPi (pH 7.2), 7% sodium dodecyl sulphate (SDS), and 1mM EDTA (pH 8.0) and hybridised with labelled probes in the same buffer at 65°C overnight. Blots were washed to a final stringency of 0.1× saline sodium citrate (SSC), 0.1% SDS at 42°C, and exposed for four to 48 hours at -80°C on to a Kodak X-Omat film (Eastman Kodak, Rochester, New York, USA) with an intensifying screen. The mRNA expression levels of at least two independent experiments were measured by densitometric analysis, as described previously.⁷

SOUTHERN BLOT ANALYSIS

Southern blot analysis was performed for those probes for which we were unable to obtain a signal by northern blot analysis. cDNA was synthesised by reverse transcription of 4 µg of total RNA from each cell line, using an adapter ligated random primer (5'-CAC TGG ATC CTT GAG TTG AGG NNN NNN NNN NNN-3'), followed by low stringency PCR amplification using the same adapter (5'-CAC TGG ATC CTT GAG TTG AGG-3') for priming. PCR products were separated on a 2% agarose gel and transferred to a Gene-Screen Plus^R hybridisation membrane (NEN Research Products, Boston, Massachusetts, USA). Hybridisation was performed using essentially the same conditions as described for northern blot analysis. To confirm equal cDNA loading for each cell line, ethidium bromide staining of the gel was performed and, in addition, membranes were subsequently hybridised with a GAPDH probe.

Results

mRNA FINGERPRINTS

The repertoire of genes expressed in the GRU-1A and GRU-1B cell lines was compared. Using a set of 90 primer combination, 25 differences in the mRNA fingerprints were observed between the clonal human epithelioid sarcoma cell lines GRU-1A and GRU-1B. Of these, 11 bands were exclusively detected in the highly invasive cell line GRU-1A, whereas 14 bands were found exclusively in the low invasive cell line GRU-1B (fig 1). Despite repetitive efforts, including the use of alternative polymerases and purification of the eluates, only 20 of 25 PCR products could successfully be reamplified after recovering from the gel and were subjected to cloning procedures. Failure to recover the missing five

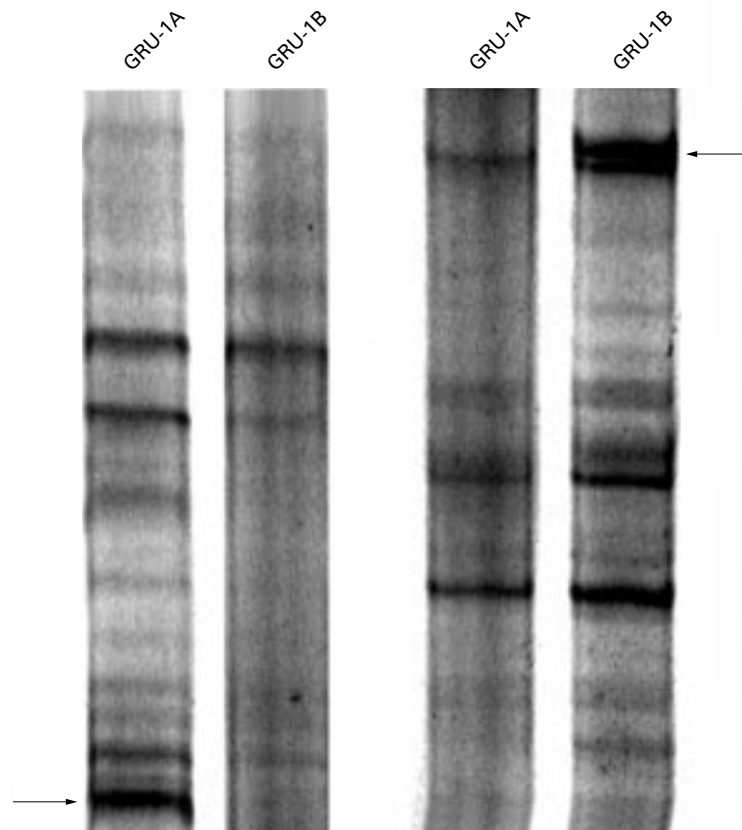


Figure 1 mRNA fingerprinting of GRU-1A and GRU-1B cells. First strand synthesis was performed with Moloney murine leukaemia virus reverse transcriptase using 2 µg of total RNA from both cell lines. PCR fingerprinting reactions were performed using pairwise combinations of 10 arbitrary primers (25 mer) and nine anchored oligo-dT primers (30 mer). PCR products were run on a denaturing 6% polyacrylamide gel and visualised by standard silver staining. Examples of bands differentially expressed between the highly invasive cell line GRU-1A and the low invasive cell line GRU-1B are indicated by arrows.

bands is probably the result of interfering components in the gel, as suggested by Konecny and Redinbaugh.¹⁸

SUBCLONING AND SEQUENCE ANALYSIS

Because a high rate of false positive bands is a well known drawback of differential display screening,^{19, 20} at least eight white colonies for each transformation procedure were picked to prevent missing the correct insert. A total number of 160 (20 × 8) clones was subjected to sequence analysis. Subsequently, clones were preselected for further investigation according to their redundancy and origin of the gene, thus excluding gene fragments encoding rRNA (16 clones from two bands) from further analysis. Table 1 summarises those gene fragments that because of their redundancy were regarded as representative for the respective bands. The mean number of different inserts for each cloning procedure performed for each band was 2.8, ranging from 1 to 4. Two gene fragments (27.1 and 28.1) originated from rRNA and therefore were excluded from further analysis. Thus, 20 independent clones, recovered from 18 different bands, were subjected to northern blot analysis or Southern blot analysis. Seven of these 22 gene fragments showed no significant homology to currently registered genes in the GenBank databases (table 1).

NORTHERN AND SOUTHERN BLOT ANALYSIS

To verify the differential expression of the gene fragments isolated by fingerprinting, northern and cDNA Southern blot procedures were performed as independent methods. Thus, we were able to detect signals in a total of nine blotting procedures. In contrast, for 13 gene fragments signals were not detectable despite repetitive attempts. We attribute this either to very low expression of the corresponding genes or to the shortness of the respective probes used, or a combination of both. At the mRNA level, no differences in expression between GRU-1A and GRU-1B were seen for five of the nine gene fragments that yielded a useful signal. However, four genes were preferentially

Table 1 Sequence analysis of the isolated cDNA clones and comparison with the GenBank database

Clone	Code	Cell line	Blast search	Homology	Blot
1	3.1	B	Human bac clone GS310A05 from 7q21	71%	NA
2	4.1	A	Human v-fos effector protein	100%	ND
3	5.1	B	None, putative novel fragment	–	NA
4	6.1	B	None, putative novel fragment	–	NA
5	9.1	A	None, putative novel fragment	–	NA
6	12.1	B	Human mitochondrial genes for several t-RNAs and 12S and 16S ribosomal RNAs	97%	ND
7	13.1	B	None, putative novel fragment	–	B
8	14.1	B	Human CpG DNA	100%	NA
9	19.1	B	Human mitochondrial DNA, fragment M1, encoding cytochrome C oxidase I	98%	B
10	22.2	A	Human SREBP-1	100%	NA
11	22.4	A	None, putative novel fragment	–	NA
12	25.2	B	None, putative novel fragment	–	NA
13	26.1	B	Type VI collagen alpha 3 chain	97%	ND
14	27.1	A	Human 28S ribosomal RNA	100%	NP
15	28.1	B	Human 28S ribosomal RNA	100%	NP
16	29.2	A	PEG3	97%	NA
17	36.1	A	None, putative novel fragment	–	NA
18	42.1	A	Human chromosome 16p13.11 bac clone CIT987SK-29B12	93%	NA
19	43.2	B	TI-227H	100%	B
20	51.1	A	Human ferritin light chain	100%	A
21	13.2	B	Human mitochondrion	91%	NA
22	26.4	A	Human hepatitis δ interacting protein A	100%	ND

Cell line refers to the clonal cell line from which the cDNA clones were amplified: A, GRU-1A; B, GRU-1B.

Blot refers to results obtained by northern blot analysis: ND, no difference in expression; NA, no blot result available; NP, no blot performed; A, predominantly expressed in GRU-1A; B, predominantly expressed in GRU-1B.

expressed by only one of the two cell lines (fig 2). For clones designated as 13.1, 19.1, and 43.2, significantly stronger expression was seen in the low invasive cell line GRU-1B than in the highly invasive cell line GRU-1A. In contrast, clone 51.1 was significantly more highly expressed by the GRU-1A cell line. Figure 3 gives the nucleotide sequences of gene fragments that were differentially expressed between the two cell lines. Differences in gene expression observed by northern blotting were confirmed by independent controls with additional blotting procedures, using RNA preparations of cells of a higher passage number (data not shown). In all cases, results were reproducible, indicating that the observed differences were stably conserved during prolonged cell culturing.

By means of sequence analysis, clone 51.1 was found to be 100% identical to human apoferritin light chain (BLASTN value, 4.6e-37). Northern blot analysis with the corresponding probe and measurement by densitometry revealed that the mean (SD) expression of this gene was 1.85 (SD, 0.12) times higher in GRU-1A than in GRU-1B (fig 2).

A computer search for clone 19.1 against the GenBank data base revealed that the identified insert of 201 bp was a fragment of human mitochondrial DNA, encoding cytochrome C oxidase I (BLASTN value, 2.8e-66; 100% identity). By means of northern blot analysis using the corresponding probe, two distinct signals were seen in both cell lines: one of about 1.5 kb and a second, albeit weaker one, of about 3.5 kb (fig 2). In contrast to apoferritin light chain, the mean (SD) expression of cytochrome C oxidase I was 3.34 (0.88) times higher in the low invasive cell line GRU-1B than in GRU-1A.

Clone 43.2 was found to be 100% identical (BLASTN value, 2.0e-29) to TI-227H. TI-227H was isolated as a novel gene fragment from the mouse melanoma cell line B16-F10 in an attempt to search for metastasis related genes by subtractive hybridisation.²¹ In our study, northern blot analysis revealed a strong signal in GRU-1B cells just below the 18S rRNA band, corresponding to mRNA of about 2 kb. By densitometry, the mean (SD) expression of TI-227H in GRU-1B cells proved to be 2.01 (0.16) times higher than in the GRU-1A cell line (fig 2).

No significant match to known genes in the GenBank database was found for a 207 bp gene fragment (clone 13.1), which is therefore probably a novel gene. Northern blot analysis revealed a single transcript of about 1 kb, the mean (SD) expression of which was 1.66 (0.19) times higher in the low invasive cell line GRU-1B than in the highly invasive cell line GRU-1A (fig 2). Therefore, we tentatively designated this gene as expressed in low invasive sarcoma cells (ELISC-1). The partial sequence has been released to NCBI GenBank and is available under accession number AF085351. Translation of the 398 bp sequence revealed an open reading frame of 49 amino acids running through the 3' end of this clone. The corresponding predicted amino acid sequence

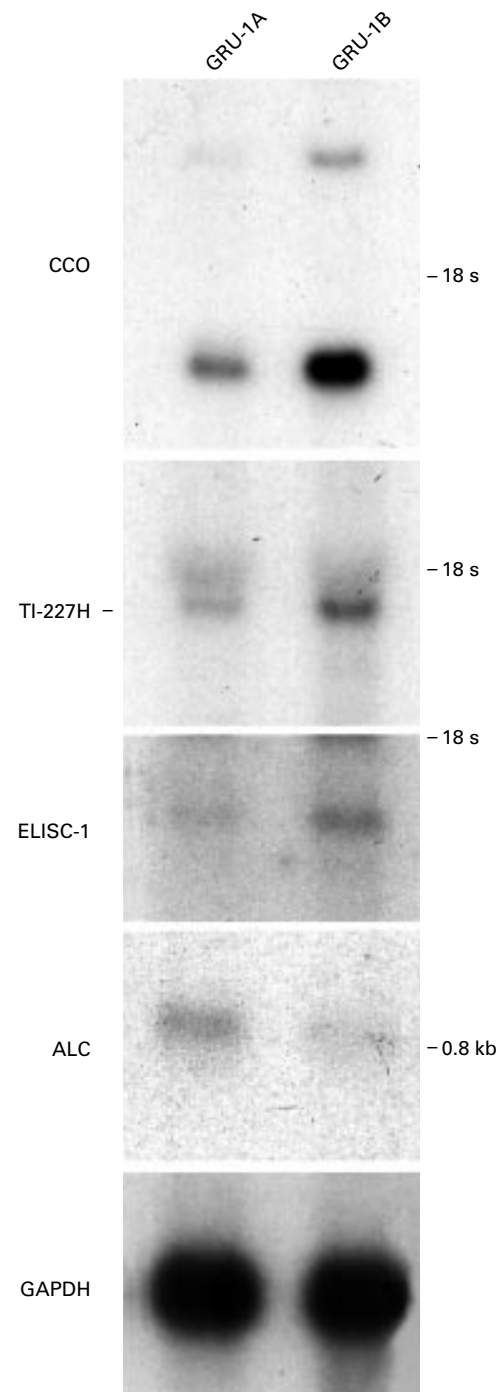


Figure 2 Differential expression of apoferritin light chain (ALC), cytochrome c oxidase I (CCO), TI-227H, and expressed in low invasive sarcoma cells (ELISC-1) between the highly invasive cell line GRU-1A and the low invasive cell line GRU-1B, as shown by northern blot analysis. Aliquots of 15 µg of total RNA were separated on a 1% agarose formaldehyde gel, blotted on to Hybond N nylon filters, and probed with DNA probes generated by PCR from the corresponding clone. Equal loading was confirmed by hybridisation with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe. Whereas ALC was preferentially expressed by GRU-1A, the expression of CCO, TI-227, and ELISC-1 was higher in GRU-1B.

origin suggests that increased expression of ferritin is a more general feature associated with the metastatic phenotype of tumour cells. In addition, it has been suggested that increased storage of total ferritin occurs in parallel with the development of hyper-resistance to photodynamic inactivation and H₂O₂ mediated cytotoxicity of tumour cells,^{25, 26} probably by acting as an intracellular scavenger for toxic iron metabolites. Taking these observations into account, one might speculate that high concentrations of ferritin provide an advantage for metastasising cancer cells in adopting a foreign environment.

A third gene that was differentially expressed between GRU-1A and GRU-1B was the mitochondrial M1 gene, encoding cytochrome c oxidase I. This gene was much more highly expressed in the low invasive cell line GRU-1B. Interestingly, a strong positive correlation between increased expression of mitochondrial genes and the grade of differentiation has been demonstrated for a variety of tumour and non-tumour cells of different histogenic origin—for example, HT-29 colon carcinoma cells and human fibroblasts.^{27, 28} We have shown previously that GRU-1B cells have a predominantly mesenchymal phenotype, whereas GRU-1A cells have a bidirectional differentiation pattern, which confers a combined mesenchymal-neural phenotype.⁸ It remains to be determined whether the known interclonal differences in differentiation and invasion are causally related to the newly identified difference in expression of cytochrome C oxidase I.

Finally, in the low invasive GRU-1B cell line we also found preferential expression of a gene that was identical to TI-227H. TI-227H was originally identified by subtractive hybridisation as a gene fragment that, at the mRNA level, was differentially expressed in variant mouse melanoma cell lines of the same primary tumour, but with different metastatic capacities.²¹ In this study, TI-227H was highly expressed in B16-F10 melanoma cells, which can only colonise the lung when directly inoculated into the blood vessels. In contrast, TI-227H was absent in B16-BL-6 cells, which are capable of spontaneously metastasising to the lung upon subcutaneous implantation. Because metastasis is initiated by the process of invasion, both these and our results suggest that TI-227H might have an invasion inhibitory function. When TI-227H was identified, no significant matches with known genes were found. In contrast, our search revealed significant homologies to mitochondrial genes for several t-RNAs (accession number V00710). This would explain the lack of an open reading frame for TI-227H described by Ishiguro *et al.*,²¹ and indicate a role at least in expression control. However, an RNA signal of about 2 kb seen for TI-227H by northern blotting using total RNA as a template is unusual for t-RNA. Although these conflicting results cannot be explained at present, it is remarkable that, while searching for invasion or metastasis related genes, TI-227H was independently isolated by means of two different technical

approaches (differential display RT-PCR versus subtractive hybridisation) and in two different tumour models (human sarcoma versus mouse melanoma). This strongly suggests a potential role for TI-227H in tumour invasion and metastasis.

When compared with other studies using differential display RT-PCR, both the total number of bands, differentially expressed in fingerprinting reactions, and the rate of real differences in expression as confirmed by independent blotting procedures (five of nine blotting procedures) are similar.^{29, 30} Moreover, similar rates of non-informative blotting procedures have also been reported by several other groups.^{31, 32} Despite repetitive attempts, in our present study, no interpretable signals could be obtained by northern blot analysis for a total number of 11 candidate gene fragments. This is probably the result of the scarcity of the respective mRNAs or suboptimal hybridisation procedures because these problems were only seen for the shortest fragments.^{29, 31, 32} In an attempt to overcome this problem, Southern blot analyses at the cDNA level were performed. Again, signals obtained by Southern blot analysis were only seen for gene fragments that had already been detected by northern blot analysis.

In conclusion, we have identified three known genes (apoferritin light chain, cytochrome C oxidase I, and TI-227H) and one novel gene (ELISC-1) that are differentially expressed between highly invasive GRU-1A and low invasive GRU-1B cells. Identification of the entire cDNA sequence of ELISC-1 and analysis of the role of all four genes in the regulation of invasiveness will be the subject of further investigations.

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