Metastasis gene expression analyses of choriocarcinoma and the effect of silencing metastasis-associated genes on metastatic ability of choriocarcinoma cells

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Summary

Objective: Obtaining choriocarcinoma metastasis-associated genes and identifying the role and mechanism of VEGF-B in the progression of human choriocarcinoma. *Study Design:* (1) cDNA microarray technique was used to compare the transcriptional profiles between highly metastatic JEG-3 cells and lowly metastatic JAR cells; (2) An inhibitory effect of VEGF-B shRNA was demonstrated by RT-PCR; (3) The effect of VEGF-B shRNA on invasion of JEG-3 cells in vitro was detected by Matrigel invasion assay. *Results:* (1) In upregulated genes, 51 genes were correlated with the cell metastasis ability, and FN, MMP-2, uPA, CAV-1 and VEGF-B were the first five genes; (2) Afterwards transfected VEGF-B shRNA, VEGF-B mRNA expression decreased obviously; (3) VEGF-B shRNA transfection significantly downregulated invasion level of JEG-3 cells in vitro ($p < 0.05$). *Conclusion:* VEGF-B plays an important role in the metastatic capability of human choriocarcinoma. Reducing the expression of VEGF-B can help weaken the invasion ability of human choriocarcinoma.

Key words: cDNA microarray; RNAi; VEGF-B; Choriocarcinoma; Invasion; Metastasis.

Introduction

Choriocarcinoma develops from reproductive tissue cells which are very active. When these cells undergo cancerous changes, they grow and multiply very rapidly. A tumor forms and sheds cancer cells into the bloodstream at an early stage. The cancer cells in the bloodstream develop new cancers in other parts of the body, a process known as metastasis. If choriocarcinoma is not treated successfully, these tumors throughout the body can result in damage, and that can quickly lead to death. So how to weaken the invasive ability of choriocarcinoma cells is very crucial.

In a previous study [1] it was pointed out that JEG-3 was more metastasic than JAR. To gain insight into the alterations in metastasis-related gene expression that governs metastasis of choriocarcinoma, we first used a cDNA microarray technique to compare the transcriptional profiles between human choriocarcinoma JEG-3 cell lines (highly metastatic) and JAR cell lines (lowly metastatic). Afterwards vascular endothelial growth factor-B (VEGF-B) gene specific short hairpin RNA (shRNA) expressing plasmid was constructed and transfected into JEG-3 cells. Finally we investigated the effects of shRNA transfection on VEGF-B expression and the ability of invasion and metastasis in human choriocarcinoma cell lines JEG-3 (highly metastatic) in vitro.

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Material and Methods

Cells

JEG-3 and JAR human choriocarcinoma cell lines were acquired from American Type Culture Collection (ATCC, Manassas, VA).

cDNA microarray analysis

Briefly, first the total RNA was reverse transcribed into complimentary DNA (cDNA) using T7-promotor primer and MMLV reverse transcriptase. The cDNA was transcribed into complimentary RNA (cRNA), during which it was fluorescently labelled by incorporation of cyanine Cy5-CTP (JAR cell line) or Cy3-CTP (JEG-3 cell line). After purification, using the RNeasy mini kit (Qiagen), cRNA yield and Cy incorporation efficiency (specific activity) into the cRNA were determined using a NanoDrop Spectrophotometer (NanoDrop Technologies). cRNAs showing a yield > 825 ng and a specific activity of 8-20 pmol/μg cRNA were selected for further processing. Equal amounts of the exposed and negative control sample were competitively hybridized onto Agilent whole (14K) human oligonucleotide arrays in a hybridization oven at 60°C for 17 h. Slides were washed according to the manufacturer's instructions with washing buffers and finally dipped in stabilization and drying solution (Agilent Technologies) to protect them from environmental ozone. The arrays were scanned on an Agilent scanner (G2565BA) and further processed using Agilent Feature Extraction Software (Version 9.5.1). The software automatically finds and places microarray grids, rejects outlier pixels, accurately determines feature intensities and ratios, flags outlier pixels, and calculates statistical confidences. For the two-color microarrays, gProcessedSignal values from Agilent's Feature Extraction software were used as input into experimental analyses and includes additional preprocessing to adjust for possible dye bias within a microarray. Data used in

the two-color analyses was either the red and green ProcessedSignal or LogRatio values. Dye normalization included both linear scaling and Lowess normalization to a rank invariant set of microarray features. Further details on the data processing steps used to generate the Agilent two-color output can be found in the Agilent protocol GE2-v5_95_Feb07.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA were reverse transcribed in 30 ml of a solution (Fermentas), and then PCR was performed to detect the expression of VEGF-B mRNA in JEG-3 and JAR cells. Based on published DNA sequences of human VEGF-B and GAPDH gene, primers for VEGF-B (sense, 5'- GAG ATG TCC CTG GAA GAA CAC AG -3', antisense, 5'- AAA GCC ATG TGT CAC CTT CGC AG -3') and GAPDH (sense, 5'-GCT GGC GCT GAG TAC GTC GT-3', antisense, 5'-TGG GTG TCG CTG TTG AAG TC-3') were obtained. PCR was performed using a MJR PCR System (MJ Research Corp.). Aliquots (10 µl) of the amplification products were resolved by 1.5% agarose gel (Promega Corp.) electrophoresis and visualized by ethidium bromide staining, and the fragment size and signal intensity were analyzed by Genescan Analysis software and Genescan Genotyper software (Applied Biosystems).

Short hairpin RNA

The sequence targeted to 5'- AAA GGA CAG TGC TGT GAA GCC AGA C -3' in VEGF-B messenger RNA (mRNA) were designed without off-target effects. The sense and antisense strands of shRNAs were: 5'- AAA GGA CAG UGC UGU GAA GCC AGA CAA -3' (sense), 5'- AAU UUC CUG UCA CGA CAC UUC GGU CUG -3' (antisense). The negative-control mismatch sequence was: 5'- GAC TTC ATA AGG CGC ATG C -3'.

shRNA group: VEGF-B gene specific shRNA expressing plasmid is transfected into JEG-3 cells by lipofectamineTM 2000.

negative control group: negative-control mismatch sequence expressing plasmid is transfected into JEG-3 cells by lipofectamineTM 2000.

blank group: JEG-3 cells are treated by mixture of lipofectamineTM 2000 and free RPMI medium.

Briefly, JEG-3 cells were seeded in 60 mm dishes and plasmid-lipofectamine compounds were disposed. The plasmid-lipofectamine compounds were added into JEG-3 cells, and the final concentration of the shRNA-VEGF-B-plasmid was 200 µg/l. After six hours, the compounds were abandoned. The JEG-3 cells were incubated for an additional 48 h, selected with G418, and then lysed.

Matrigel invasion assay

Diluted 1:2 Matrigel (1.75 µg/l) (BD Biosciences, Beit-Ha'Emek, Israel) in serum free cell culture media was added to the upper chamber of a 24-well transwell plate, and incubated at 37°C 3-4 h for gelling. All cells were harvested from tissue culture flasks by Trypsin/EDTA, washed and resuspended in 0.1% FCS in DMEM medium and added to upper wells at a density of 10^5 cells/well in 100 µl medium, while 600 µl medium was added to the lower well. Plates were incubated at 37°C for 24 h, and then, the cells remaining on the upper surface of the membrane were removed with a cotton swab and the filters were fixed by 95% ethanol for 30 min. Cells that had invaded the lower surface of the filter were counted under an inverted microscope; 10 fields per well were counted. All experiments were performed in duplicate and the results from five separate sets of experiments were averaged.

Statistical analysis

Results are shown as mean ± SEM. Statistical significance of differences in mean values was assessed by using Student's t-test with SAS software (SAS Institute, Cary, NC). Differences among means were considered significant at p values of < 0.05.

Results

Gene expression profiles of the cell lines by cDNA microarray analysis

As shown in Figure 1, green spots represent downregulated genes of JEG-3 vs JAR, red spots represent upregulated genes, while indistinctive genes are represented as yellow spots. The results demonstrated that there were 216 genes and 105 expressed sequence tag (EST) which ratio (JEG-3(Cy3)/JAR(Cy5)) was > 2 and there were 334 genes and 88 EST with a ratio < 0.5. In all differential genes, there were 128 genes belonging to oncogenes, 51 genes belonging to metastatic genes, 44 genes belonging to energy metabolism-related genes, 39 genes belonging to angiogenesis-related genes, 25 genes belonging to protein synthesis-related genes, eight genes belonging to cell-cycle-related genes, eight genes belonging to cell proliferation-related genes, four genes belonging to cell apoptosis-related genes, and three genes belonging to cytoskeleton-related genes. Fibronectin (FN), matrix metalloproteinase-2 (MMP-2), urokinase-type plasminogen activator (uPA), Caveolin-1 (CAV-1) and VEGF-B show markedly increased expression of metastatic genes when compared to JEG-3 with JAR.

RT-PCR analysis of selected genes

As shown in Table 1 and Figure 2, the result of RT-PCR analysis for VEGF-B was in agreement with the microarray data, although the change in the expression level was not exactly the same.

Table 1. — *Expression of VEGF-B mRNA in JEG-3 and JAR.*

Group	Times	VEGF-B	*p*
JEG-3	5	1.862 ± 0.11	$p < 0.01$
JAR	5	0.479 ± 0.02	

Effect of VEGF-B shRNA on JEG-3 cells

Effect of VEGF-B shRNA on VEGF-B gene expression in JEG-3 cells.

As shown in Figure 3, there was an almost 4.3 fold decrease in VEGF-B mRNA in JEG-3 cells when these cells were treated with VEGF-B shRNA; the difference was significant ($p < 0.01$). While comparing the blank group with negative control group, we found that the difference was meaningless.

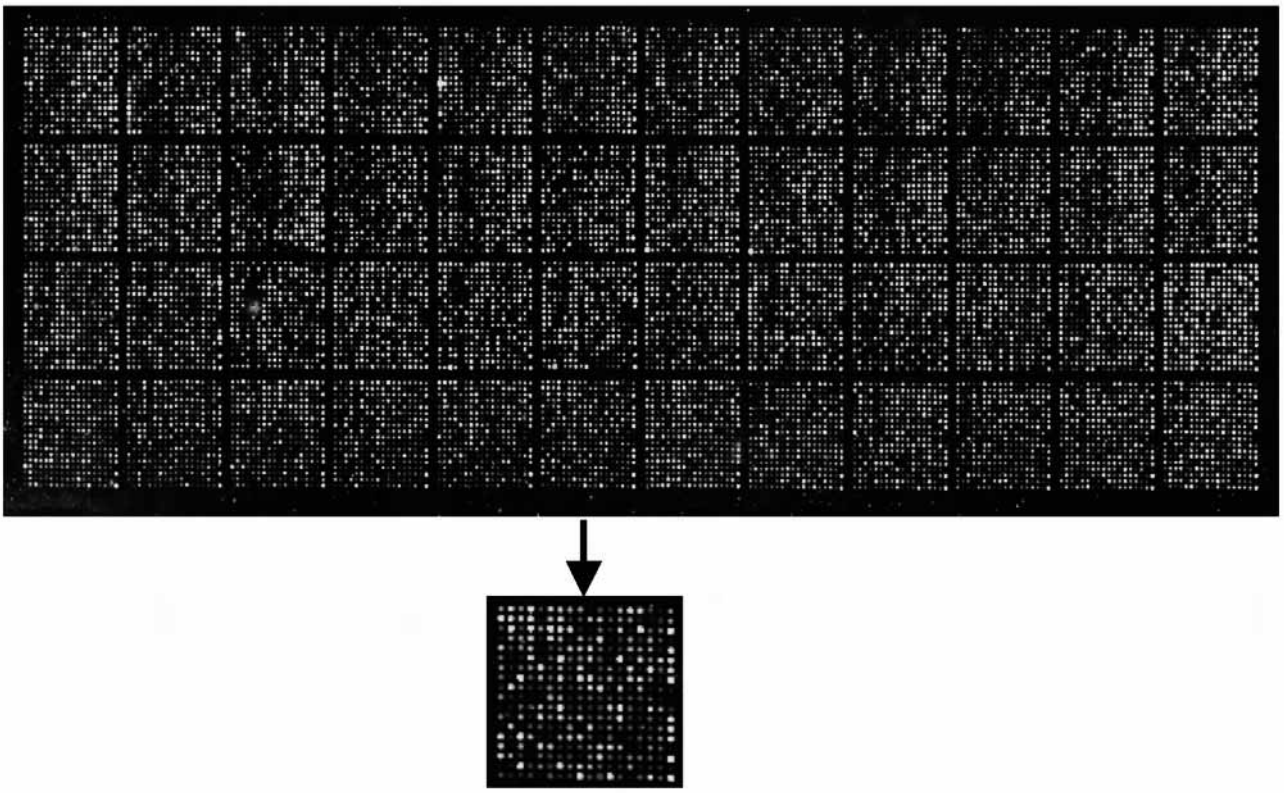

Figure 1. — cDNA microarray analysis.

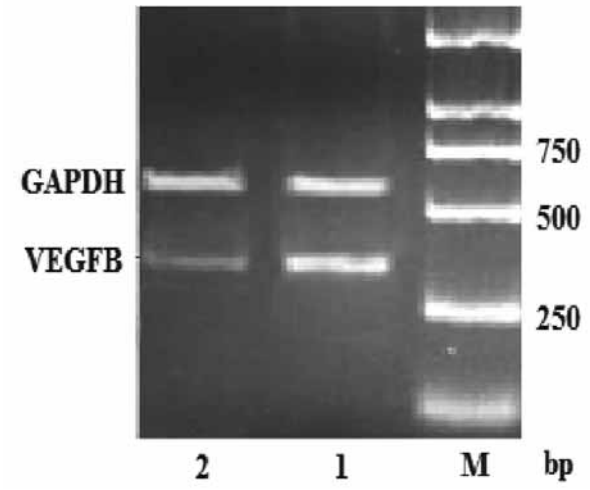

Figure 2. — Expression of VEG'F-B mRNA in JEG-3 and JAR 1: highly metastatic JEG-3 cells; 2: lowly metastatic JAR cells.

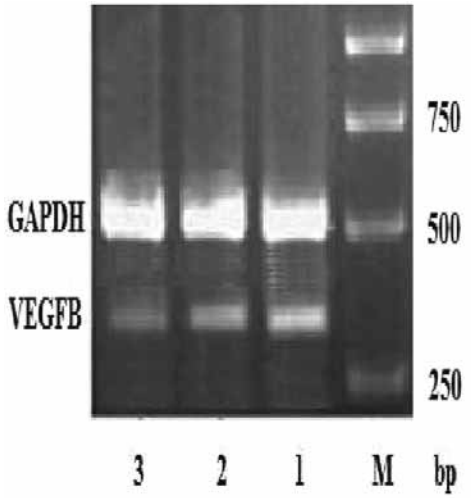

Figure 3. — Effect of VEG'F-B shRNA on VEGF-B gene expressionin JEG-3 cells M: Marker; 1: blank group; 2: negative control group: 3: shRNA group.

Effect of VEGF-B shRNA on invasion ability of JEG-3 cells.

As illustrated in Figure 5, transfection with VEGF-B shRNA induced an almost 2.1 fold decrease in JEG-3 cells migrated through Matrigel-coated filters, indicating that VEGF-B shRNA restrained choriocarcinoma cell invasion. At the same time, we also found that there were no differences between the blank group and negative control group.

Discussion

It is generally accepted that genetic alternations in tumor cells may endow a special subpopulation with the ability needed for invasion through the basement membranes and metastatic colony formation in distant organs; thus a comparative analysis of genetic alterations between highly metastatic cell lines and lowly metastatic cell lines and metastases would be helpful to unravel the

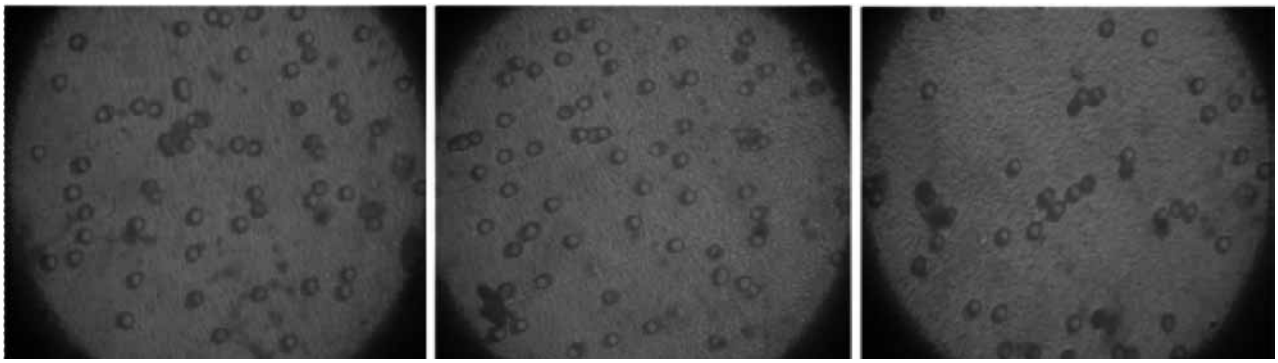

Figure 4. — Effect of VEGF-B shRNA on invasion ability of JEG-3 cells (HE x 400). A) blank group; B) negative control group; C) shRNA group.

mechanisms of metastasis. Abundant studies have indicated that a variety of genes, which encode those proteins involved in multiple processes of metastasis, such as heparanase [2], insulin growth factor-II [3] and interleukin-17 [4], may play crucial roles in invasion and metastasis in choriocarcinoma.

By cDNA microarray technique, we found that there were 51 known human metastasis-related genes when compared JEG-3 cells with JAR cells and the alteration in the expression of the first five genes, FN, MMP-2, uPA, CAV-1 and VEGF-B, is obvious.

Many publications now clearly link the expression of FN [5], MMP-2 [6, 7], uPA [8] and CAV-1 [9] to the invasive and metastatic properties of choriocarcinoma, but there is a lack of research to prove that these and VEGF-B are relevant to the metastasis of choriocarcinoma. In this study, we found that after transfected with VEGF-B shRNA, not only was the expression of VEGF-B mRNA in JEG-3 cells reduced, but also the invasion ability of JEG-3 cells was depressed, indicating that the expression of VEGF-B plays an important role in the metastatic capability of human choriocarcinoma, and inhibiting the expression of VEGF-B may contribute to weakening the invasive and metastatic ability of choricarcinoma cells.

Gunningham [10] measured the level of VEGF-B by ribonuclease protection assay and immunohistochemistry in 13 normal breast samples and 68 invasive breast cancers. He found that there was a significant association between VEGF-B and node status and the number of involved nodes. In a study of pancreatic carcinoma, Wey *et al*. [11] found out that VEGFR-1 appears to be expressed ubiquitously in pancreatic carcinoma cell lines, in which it induces signaling and promotes migration and invasion. Overexpression of VEGF-B in tumors may activate tumor cells bearing VEGFR-1 via an autocrine pathway. Agents targeting VEGF-B or its receptors may have a dual inhibitory effect on tumor growth by suppressing both angiogenesis and tumor cell function. All of these results are consistent with our study.

In Olofsson *et al's*. research [12], they pointed out that the binding of VEGF-B to its receptor on endothelial cells leads to increased expression and activity of urokinase type plasminogen activator (u-PA). Cancer cell invasion, both locally in primary tumors and in metastatic sites, involves extensive tissue remodeling that is performed by matrix-degrading proteinases. u-PA is a serine proteinase that can digest a broad spectrum of extracellular matrix (ECM) substrates including fibrin, fibronectin and laminin [13]. In addition, u-PA can activate other matrix-degrading proteinases including MMPs that can digest collagens and a variety of other matrix proteins [14]. This hints that VEGF-B can promote the invasion ability of choriocarcinoma cells just because it enhances the uPA pathway (an invasion and metastasis implicated pathway).

Choricarcinoma is a kind of disease that has strong invasive ability, so how to depress the invasive ability of choricarcinoma cells is a question that draws attention. The results of our study suggest that VEGF-B is the element that enhances the invasion of choricarcinoma cells; therefore, controling the expression of VEGF-B could result in depressing the invasive ability of choricarcinoma cells.

In our study, we found that shRNA-mediated silencing of VEGF-B not only significantly suppresses the invasion ability of JEG-3 cells, but also can inhibit the growth of hypodermal transplant tumor and the number of carmine nodes in lungs of nude mice. These results emphasize the potential clinical applications of VEGF-B inhibitors in choriocarcinoma.

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