Gene profile analysis of colorectal cancer cell lines by cDNA macroarray

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Abstract. In order to identify high-risk groups of patients with cancer, understanding the mechanisms of invasion and metastasis from the viewpoint of gene expression is required; in particular, the changes in gene expression as tumours gain the ability to metastasise. Using a rat model of metastatic colorectal cancer (CRC), we determined the expression profiles of CRC cells that metastasised to the liver and the lung. In the hepatopulmonary metastasis model, metastasising ability increased with successive transplanted cell line generations. No significant differences in cellular proliferation ability were seen between the original cell line and succeeding metastatic cell lines. Analysis using cDNA macroarray showed that metastasising ability was associated with increased expression of integrin ß4, γ-catenin, Smad 7, Bax, Bcl-2, c-fos and TGF-α, and a particularly marked increased expression of TGF-ß, PDGFb, Cdk4 and Rho B. Expression of both Rho GDIß and Gelsolin was reduced. These results suggest that high metastasising ability does not derive from a single gene, but rather through accumulated changes in the expression of several different genes.

Introduction

Under the banner of 'tailor-made medicine', we now expect diagnosis and treatment to be suited to the individual patient. The first stage is preparing a profile for each disease, because if we understand their genetic expression profiles we can then classify diseases on this basis. A disease cannot be identified from the expression profiles of one or two genes, but its characteristics can be elicited from the pattern of expression of hundreds or thousands of different genes.

Expression profiling using DNA chips (i.e. DNA microarrays) has attracted much interest in recent years, but for the present study we chose to use DNA macroarrays as the easier method at present. We chose as the target disease group 'malignant tumours', because of their high prevalence and poor cure rates. Using a rat model of metastatic colorectal cancer (CRC) we investigated changes in gene expression as the tumours gained the ability to metastasise, in order to elucidate the mechanisms of invasion and metastasis from the gene expression standpoint.

Materials and methods

Establishment of a metastatic cell line. We developed a cell line with the ability to metastasise to the liver by repeatedly injecting a $2x10^6/100-\mu l$ cell suspension of a rat CRC cell line (ACL-15) into the anterior mesenteric vein (a branch of the portal vein) of F344 rats, and then harvesting cells from the hepatic metastases. We confirmed the formation of pulmonary metastases after introduction of hepatic metastatic cells through the caudal vein, and we repeatedly harvested cells from the pulmonary metastases. Both procedures were repeated for four generations, establishing cell lines with the ability to metastasise to both the liver and lung.

Orthotopic transplant model. The cell lines with the ability to metastasise to both organs were orthotopically transplanted under the caecal serosa, and the animals were monitored for development of hepatic and pulmonary metastases, which were then sampled.

Monitoring of cellular proliferation. Formation of metastases became more active with each generation. We identified changes in cellular characteristics in order to determine if this was caused by increased cell proliferation or by changes in metastasising ability, such as cellular adhesiveness.

DNA macroarray. We extracted mRNA from cell lines undergoing the process of attaining the ability to metastasise, and compared the expression profiles of 434 genes using a cDNA macroarray, the Gene Navigator™ cDNA array system (Toyobo Co. Ltd, Osaka, Japan). Initial total mRNA extraction was performed using the Isogen acid-guanidium-phenol-chloroform (AGPC) kit (Nippon Gene Co. Ltd, Toyama, Japan). Treatment with DNase digested any remnant strands of DNA. Finally, mRNA was retrieved using the MACS mRNA isolation kit (Daiichi Pure Chemical Co. Ltd, Tokyo, Japan).

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Table I. Upregulated genes.

Gene	Class
Bax	Tumour suppressor gene/apoptosis
Bcl-2	Tumour suppressor gene/apoptosis
Bcl-X (Bcl-ß)	Tumour suppressor gene/apoptosis
Cdk6 (PLSTIRE)	Cell cycle protein
Skp2 p45	Cell cycle protein
c-fos	Regulatory transcription factor
CBP (CREB-binding protein)	Regulatory transcription factor
TGF-α	Growth factor and hormone
HSC70	Signaling intermediates
TINP-2	Signaling intermediates
Ran	GDP/GTP binding proteins
Ran BP-1	GDP/GTP binding proteins
ARF1	GDP/GTP binding proteins
ARF5	GDP/GTP binding proteins
ARF6	GDP/GTP binding proteins
CD4	Lymphocyte signaling
γ-catenin (plakoglobin)	Cell adhesion protein
Integrin ß4	Cell adhesion protein
Smad7 (MADH7)	Regulatory transcription factor
GM2 activator protein	Other
AMF-R (autocrin motility factor receptor)	Membrane receptor

Table II. Upregulated genes: high expression.

Gene	Class
TDAG 51	Tumour suppressor gene/apoptosis
Lamin A	Tumour suppressor gene/apoptosis
Cdk4 (PSK-13)	Cell cycle protein
Cdc37 homolog	Cell cycle protein
ER α (estrogen receptor α)	Steroid receptor
Erk1	Protein kinase
TGF-ß	Growth factor and hormone
PDGF b	Growth factor and hormone
PI3 kinase p110 α	Signaling intermediates
Rho B	GDP/GTP binding proteins
Rho C	GDP/GTP binding proteins
GSTP1 (glutathion S-transferase P1)	Enzyme

Results

Development of a metastatic cell line. Through reimplant-ation of successive generations of cell lines that had acquired the ability to metastasise to the liver and the lung, a marked increase in metastasis formation was seen in rats transplanted with the fourth generation compared with the original cell line.

Table III. Downregulated genes.

Gene	Class
Rho GDIß	GDP/GTP binding proteins
Angiopoietin-1	Growth factor and hormone
Sin (Efs)	Signaling intermediate
Granzyme B	Tumour suppressor gene/apoptosis
Gelsolin	Tumour suppressor gene/apoptosis

Cellular proliferation. When we examined cell proliferation ability using [^{3}H]-thymidine uptake, no significant difference was seen between the original strain and daughter strains for metastasising ability. We therefore investigated changes in metastasising ability by determining gene expression profiles, thereby determining how the cellular characteristics had changed.

Gene expression profiles. Examination of the cDNA macro-array results for the original cell line and cell lines metasta-sising to the liver, the lung, or both, revealed increases and decreases in the expression of a variety of genes during the process of acquiring metastasising ability (Fig. 1).

First, compared with the original cell line, in the metasta-sising strains there was increased expression of 21 genes; in particular, regulatory cell adhesion proteins, including γ-catenin and integrin ß4; transcription factors such as Smad 7; and growth factors such as TGF-α (Table I).

Second, in the metastasising strains there was markedly increased expression of 12 genes: growth factors, including TGF-ß and PDGFb; cell cycle proteins such as Cdk4 and Cdc37; and GDP/GTP binding proteins, including Rho B and Rho C (Table II).

Third, in the metastasising strains there was reduced expression of 5 genes, including tumour suppressor genes related to apoptosis, such as Granzyme B and Gelsolin (Table III).

Fourth, expression profiles were compared between groups to determine if quantitative analysis of differences in expression levels was possible with macroarays, unlike with microarrays (Fig. 2). Marked changes were detected for 9 genes in the pulmonary metastatic cell line, with increased expression of 8 genes (TDAG 51, Lamin A, Cdk4, Cdc37 homolog, Erk1, PI3 kinase p110 α, Rho B, and Rho C), and decreased expression of 1 gene (Gelsolin), confirming the quantitative significance of this method.

Discussion

Cancer research in recent years has seen a variety of approaches to the elucidation of the clinical picture in cancer patients. In particular, the greatest clinical problem, the degree of malignancy, derives from a tumour's ability to metastasise. Because the initial stage of destruction of the basement membrane is an important part of the mechanism of cancer invasion and metastasis, previous studies have investigated the constituents of the extracellular matrix, including laminin, fibronectin and type IV collagen (1,2). In
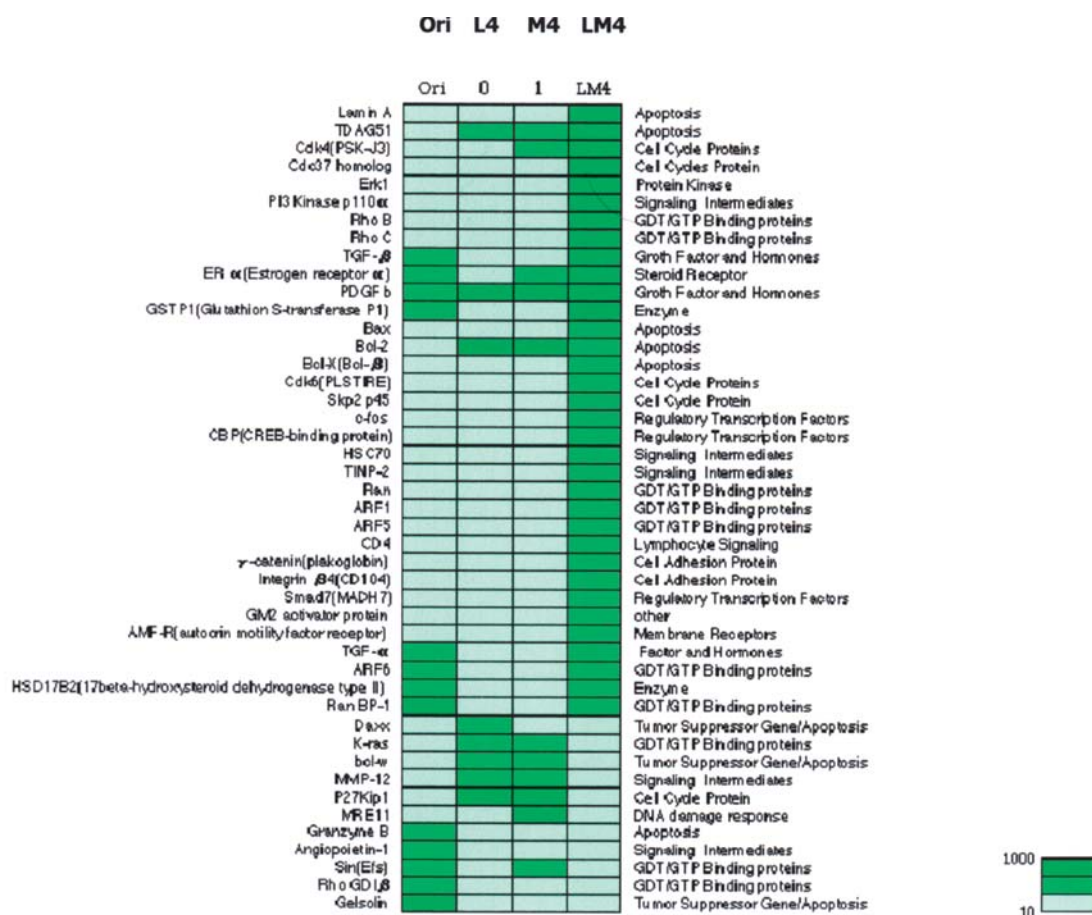
Figure 1. Comparison of the original cell line (Ori), hepatic metastasis line (L4), pulmonary metastasis line (M4), and the cell line tending to metastasise to both the liver and lung (LM4). The darker the green, the higher the level of expression. Relative indices from 10 to 1000 are indicated by the three shades.
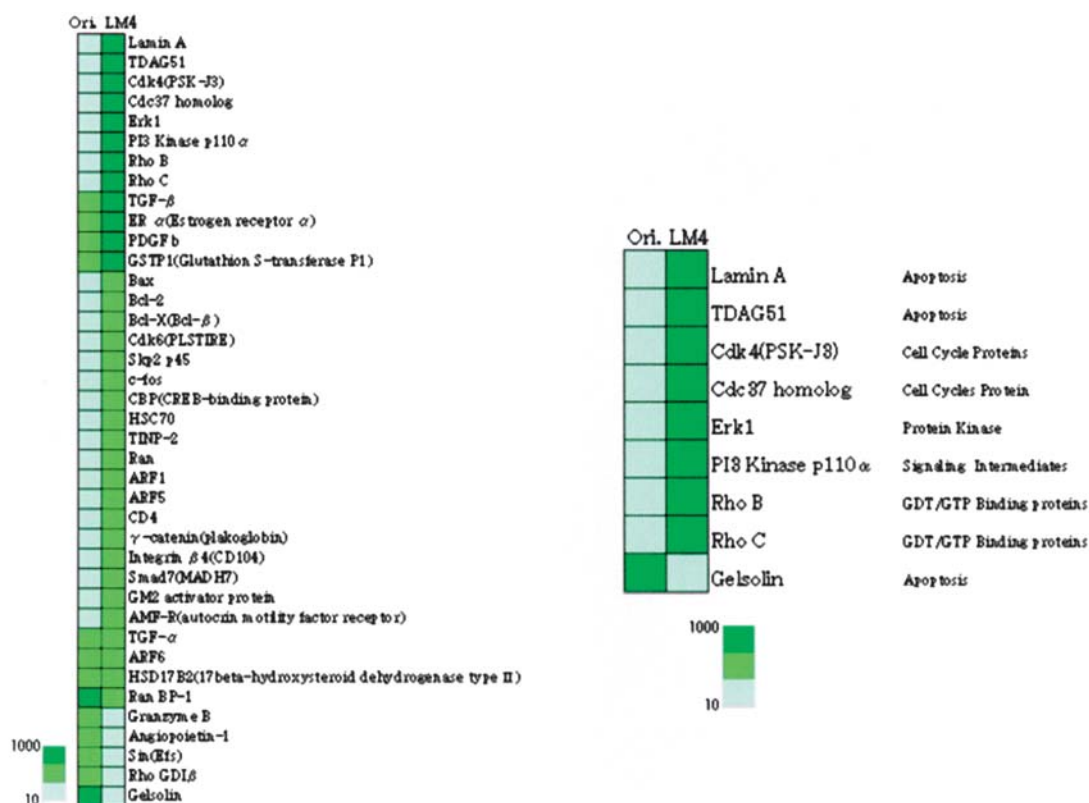

Figure 2. Comparison of the gene expression profiles of the two groups. Differences in the level of expression between the original cell line (Ori), injected into the rat colon, and the pulmonary metastasis cell line of hepatocellular origin (LM4) are shown as shades of green: the darker the green, the higher the level of expression. Relative indices from 10 to 1000 are indicated by the three shades.

clinical studies, laminin has been identified, by both serological and immunohistochemical approaches, as a significant predictive factor for the progression of CRC. However, clinical studies that have begun after metastases developed have failed to elucidate the mechanisms of acquiring metastasising ability, necessitating studies using basic metastatic models. Prior to the present study, we spent considerable time establishing such a model, and conducted basic studies of combination therapies with angiogenesis inhibitors on cellular adhesiveness (3). Further experimentation was required before we succeeded in developing a model that reproduced both hepatic and pulmonary metastases (this model will be described in detail elsewhere). Clinically, CRC in humans usually metastasises to the liver before the lung, although in some cases pulmonary metastases without hepatic metastases have occurred. Since this does not follow the usual pattern (i.e. that cancer cells travel from the lung parenchyma via the inferior vena cava to the lung), studies of metastasising ability at the cellular level have become necessary.

For the present gene profile study, rather than use a DNA chip (DNA microarray) (4-14), we chose a DNA macroarray for its superior ease of use and ability to make quantitative measurements (15-19). The former holds from several hundred to several tens of thousand dot-shaped mRNA measuring probes arranged on a 1 to 2-cm square plate, whereas the latter has a similar number of dot-shaped mRNA measuring probes, which, as the name suggests, are visible to the naked eye, arranged on a filter ~10x15 cm in size. DNA chip cluster analyses are generally graded as increased, unchanged or decreased, whereas macroarrays allow for a more detailed analysis of the level of expression, and are considered to provide better quantitative results than DNA chips. Production of expression profiles using DNA macroarrays does not require the expensive equipment needed for microarrays, and can be performed using standard equipment found in general laboratories (20-25). Although results obtained using macro-arrays are superior from the quantitative point of view, often more genes can be analysed using DNA chips (26-30).

In this study, we identified nine mRNA genes with different levels of expression from only two different cell lines: the original cell line metastasising to the liver and a fourth-generation cell line metastasising to the lung. This level of variation in the expression profile between cell lines derived from the same source (i.e. CRC) (31-35) suggests that many different mRNA genes with different levels of expression will be found in studies of different diseases with different conditions. In our experience, it is difficult to identify different levels of gene expression in cancers at different stages, even in the same organ. Many mRNA genes show different levels of expression between hormone-dependent and -independent cancers, so different approaches can yield widely differing results (36,37).

We did not find a difference in the expression of 395 mRNA genes between cancer cells metastasising to the lung and to the liver, which left 39 genes as the subject of analyses. In other words, of 434 genes, 91% were 'undetected' and this result is meaningful in itself. We examined the original cell line, and the cell lines metastasising to the liver, the lung, and both the liver and lung, and quantitatively determined increases and decreases in the expression of several genes as

part of the process of acquiring metastasising ability. In future studies, we intend to concentrate on the 9 genes thus identified, with the aim of identifying mRNA specific to the lung or liver for possible clinical application as genetic markers and thus identification of high-risk groups of patients.

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