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Identification of differentially expressed genes in pulmonary adenocarcinoma by using cDNA array

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No clear patterns in molecular changes underlying the malignant processes in lung cancer of different histological types have been found so far. To identify critical genes in lung cancer progression we compared the expression profile of cancer related genes in 14 pulmonary adenocarcinoma patients with normal lung tissue by using the cDNA array technique. Principal component analyses (PCA) and permutation test were used to detect the differentially expressed genes. The expression profiles of 10 genes were confirmed by semi-quantitative real-time RT–PCR. In tumour samples, as compared to normal lung tissue, the up-regulated genes included such known tumour markers as *CCNB1, PLK, tenascin, KRT8, KRT19* and *TOP2A*. The down-regulated genes included *caveolin 1* and *2*, and *TIMP3*. We also describe, for the first time, down-regulation of the interesting *SOCS2* and 3, *DOC2* and *gravin*. We show that silencing of *SOCS2* is not caused by methylation of exon 1 of the gene. In conclusion, by using the cDNA array technique we were able to reveal marked differences in the gene expression level between normal lung and tumour tissue and find possible new tumour markers for pulmonary adenocarcinoma.
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Introduction

Lung cancer causes about 1 million deaths per year worldwide, which is more than any other malignant disease. The majority of lung cancers can be classified into three main histological types: adenocarcinoma, squamous cell carcinoma (SCC), and small cell lung cancer (SCLC). Although each of these types share common characteristics, all are thought to arise at least partially from different sets of mutations and gene expression changes. Indeed, clear differences between the different lung cancer types have been described at both epidemiological and molecular levels, for instance amplification of 3q and deletion of 3p are more common in squamous cell carcinoma than in adenocarcinoma (Björkqvist *et al*., 1998a; Wikman *et al*., 2000). Furthermore, whereas SCC is the most common form of lung cancer among Caucasian males, adenocarcinoma is the predominant lung cancer form among women, non-smokers and among most Oriental populations. Even though AC is not linked to smoking as strongly as SCC and SCLC, there is evidence that the subgroup of K-*ras* (mutation) positive adenocarcinomas could be linked with cigarette smoking and more aggressive tumour type (Husgafvel-Pursiainen *et al*., 1993; Keohavong *et al*., 1996).

Tobacco smoking is clearly the most important cause of all lung cancer. As even the heaviest smokers do not necessarily contract lung cancer, individual differences in the capacity to metabolise tobacco carcinogens and differences in the DNA repair are anticipated to modify individual susceptibility to the disease (Bartsch *et al*., 2000). A series of genes involved in cell cycle regulation, cell growth, apoptosis, cell motility, and invasion are assumed to be crucial for tumour progression and metastasis (Wilkinson and Millar, 2000). However, for the moment there is no biomarker available that can be used for early detection or prognosis of lung cancer.

New cDNA microarray techniques allow fast detection of the expression of thousands of genes simultaneously. We have successfully used the microarray technique for detecting altered expression patterns in several forms of cancer, including malignant mesothelioma, sarcomas and leukaemia (Wolf *et al*., 2000; Aalto *et al*., 2001; Kettunen *et al*., 2001). In this study we describe the use of cDNA array technology for the detection of specific expression patterns for pulmonary adenocarcinomas, and describe genes that are differentially expressed in primary lung tumours as compared to normal lung tissue. In addition, we describe for the first time the abnormal expression of a few genes in lung tumours.

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Results

Two different statistical methods were used for analysing the expression profiles. PCA analysis assigns to each gene a score based on the principal component of its expression level in the patients, whereas the permutation test estimates the degree of difference in the expression between the patients and the reference subjects.

Several genes were differentially expressed in lung tumour tissue compared to normal lung. Both the permutation test and the PCA analysis gave mostly similar results. Out of the 50 most up-regulated or 50 down-regulated genes calculated applying either method, 59 genes were common to both methods, and a total of 141 genes appeared on either list (all scores and gene names are available on request). Out of the 25 most up-regulated genes, 15 were found using both analyses, whereas 11 were common among the down-regulated genes. Table 1 presents these genes, their function and chromosomal location. Figure 1 shows all

Table 1 Genes found deregulated in both the PCA analysis and the permutation test

Gene/protein	*Code*[a]	*PCA*	*G-score*	*Function*	*Crom. location*	*GenBank accession*	*Reference*
Up-regulated							
HMG1 (high mobility group protein)	D08a	10.78	0.96	Chromatin protein, activated during cell proliferation	6p21	M23619	1,2
DPI and *DPII* (desmoplakin I & II)	D10g	9.21	0.90	Part of desmosomes, attaches IF to plasma membrane	6p24	M77830	3
nm23-H2S (nucleoside diphosphate kinase B)	A09b	8.38	0.90	Transcription factor, binds to c-*myc* promoter	16	L16785	4,5
CCNB1 (G2/mitotic-specific cyclin B1)	A05i	8.00	0.96	Cell cycle control, G2 to M transition	5q12	M25753	6,7
HDGF (hepatoma-derived growth factor)	E04b	7.99	0.94	Mitogenic growth factor	Xq25	D16431	8
KRT8; *CK8* (cytokeratin 8)	F03g	7.64	0.92	Intermediate filament of cytoskeleton	12q13	M34225	9
histone H4	D08b	7.35	0.87	Core histone protein	6p21.3	X67081	10
MMP12 (matrix metalloproteinase 12)	E11i	6.95	0.93	Matrix-degrading enzyme	11q22.2–22.3	L23808	11
TOP2A (DNA topoisomerase II alpha)	C12e	6.83	0.95	DNA double-strand endonuclease and ligase	17q21–22	J04088	12
14-3-3 protein sigma; stratifin	B03m	6.77	0.95	Cell cycle control, up-regulated in lung tumours	1p36–p35	AF029082	12,13
PLK (polo-like kinase 1)	A02k	6.51	0.91	Activator of CCNB1	16p12.1–12.2	U01038	7,14
IGFBP3 (insulin-like growth factor-binding protein 3 precursor)	E02f	6.50	0.96	Negative modulator of IGFs and growth regulator of tumour cells	7p14–p12	M31159	15,16
MIF (macrophage migration inhibitory factor)	E07h	6.28	0.91	Positive regulator of tumour growth	22q11.2	M25639	17
TN-C (tenascin)	F11a	5.84	0.88	Extracellular matrix hexameric glycoprotein	9q33	X78565	18
RRM2 (ribonucleotide reductase)	F07e	5.83	0.92	Supplies deoxyribonucleotide precursors to intranuclear replication enzymes	2p25–p24	X59618	19
Down-regulated							
TIMP3 (tissue inhibitor of metalloproteinase 3)	A08g	−12.54	−0.83	Inhibitor of metalloproteinases	12q22.1–13.2	Z30183	11
COPEB (DNA-binding protein CPBP)	D03b	−11.58	−0.95	Regulator of TATA-box less genes	10p15	U44975	20,21
gravin	F09g	−9.70	−0.91	Scaffold pre-assembly protein for protein kinase A and C	6q24	M96322	22,23
TYROBP/DAP12 (DNAX activation protein 12)	D08k	−9.68	−0.90	Transmembrane adapter protein of CD3zeta family	19q13.1	AF019562	24
CAV1 (caveolin 1)	B04k	−8.24	−1.00	Tumour suppressor gene, pre assembly of signal complexes in caveolae	7q31.1	Z18951	25,26
BMPR2 (bone morphogenetic protein 4 type II receptor precursor)	D11i	−8.11	−0.94	Serine/threonine kinase; universal regulator of development	2q33	Z48923	27,28
AHR (aryl hydrocarbon receptor)	C09m	−7.97	−0.89	Receptor for xenobiotics; regulator of bax	7p21-p15	L19872	29
chitinase precursor	D05n	−7.71	−0.90	Glykosylhydrolase, defence against some pathogens	1q31-32	U58514	30,31
EGR alpha/TIEG (early growth response alpha)	F06n	−6.81	−0.95	Zinc-finger transcription factor	8q22.2	S81439	27,28,30
nuclear protein C-193	D09b	−6.30	−0.93	Cytokine inducible protein, homologue of muscle ankyrin repeat protein	10q23	X83703	32
CAV2 (caveolin 2)	B03k	−6.12	−0.93	Scaffold pre-assembly of signal complexes	7q31.1	U32114	25

[1]Reeves and Nissen, 1995, [2]Xiao *et al*., 1997, [3]Bornslaeger *et al*., 1996, [4]Caligo *et al*., 1995, [5]Lee *et al*., 1997, [6]Pines and Hunter, 1992, [7]Toyoshima-Morimoto *et al*., 2001, [8]Everett *et al*., 2001, [9]Nhung *et al*., 1999, [10]Smith, 1991, [11]Fassina *et al*., 2000, [12]Laronga *et al*., 2000, [13]Nakanishi *et al*., 1997, [14]Smith *et al*., 1997, [15]Yu and Rohan, 2000, [16]Jaques *et al*., 1997, [17]Nishihira, 1998, [18]Sethi *et al*., 1999, [19]Zhou *et al*., 1998, [20]Ratziu *et al*., 1998, [21]Kojima *et al*., 2000, [22]Diviani and Scott, 2001, [23]Feliciello *et al*., 2001, [24]Wilson *et al*., 2000, [25]Engelman *et al*., 1998, [26]Razani and Lisanti, 2001, [27]Tuder *et al*., 2001, [28]Hefferan *et al*., 2000, [29]Boot *et al*., 1998, [30]Dohr *et al*., 1997, [31]Matikainen *et al*., 2001, [32]Chu *et al*., 1995. [a]Clontech array gene-specification number

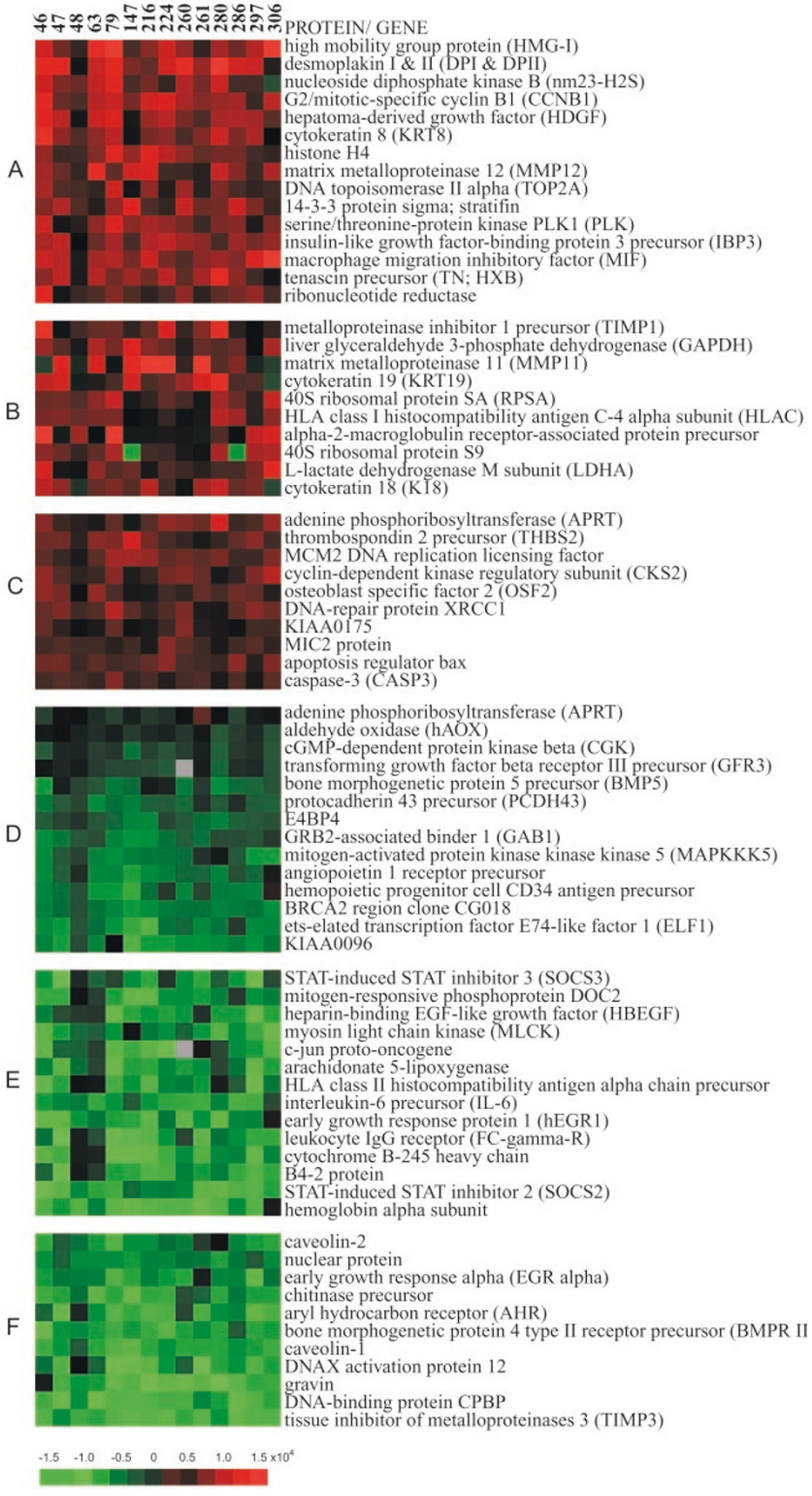

Figure 1 The gene expression profile of the 25 most over- and under-expressed genes in 14 patients calculated with either PCA analysis or permutation test. Names of the genes appear on the right-hand side and the samples studied are listed at the top. The brightness of colour correlates with the degree of 'normalized expression difference' as shown at the bottom of image. Group **A** and **F** are genes found significant in both analyses, whereas groups **B** and **E** are genes which were significant in PCA analysis, and **C** and **D** in permutation test only. The normalized expression difference was calculated by first subtracting the background value from each spot. Thereafter the adjusted intensity of each spot of the reference (mean of four) was subtracted from the corresponding intensity in the patient array. Finally, from these new values the average intensity difference of all the spots in one array was subtracted from each gene to get the normalized expression difference

74 genes, which were found aberrantly expressed with either method.

The genes expressed abnormally encode proteins with a wide variety of functions. Nevertheless, three main groups of genes could be identified to be most often deregulated, i.e. proteins involved in (a) cell cycle regulation, (b) matrix maintenance and degradation, and (c) cell motility and structure. Several classical oncogenes and tumour suppressor genes were also found deregulated, including *cyclin* B1 (*CCNB1*), *polo-like kinase 1* (*PLK*), *topoisomerase 2A* (*TOP2A*), *caveolin 1* (*CAV1*) and *macrophage migration inhibitory factor* (*MIF*). Some of the known lung tumour markers could only be detected with one of the methods. These markers included up-regulation of *DNA replication licensing factor MCM2* (with permutation test), up-regulation of *cytokeratin 18* and *19* (with PCA), and down-regulation of *death-associated protein-kinase 1* (*DAPK*1, PCA). In addition, we found deregulation of genes that have not previously been described to be abnormally expressed in lung tumours, including *suppressor of cytokine signalling*-2 and 3 (*SOCS2* and *3*, PCA), *high mobility group 1 protein* (*HMGIY*, both analyses), *gravin* (both analyses) and *DOC2* (PCA).

Permutation tests were also used for assessing differences within the cancer patient group; however, categorization by tumour stage or grade did not reveal any clear differences or clustering. The results were most likely hampered by our relatively small sample number and uneven grouping, for instance only stage 2 and 3 tumours were included in the study.

To confirm the gene expression differences found by using the cDNA array, semi-quantitative RT–PCR analyses on LightCycler were performed for 10 different genes (*MMP*11, *TIMP*3, *TOP*2*A*, *CAV*1, *COPEB*, *CCNB1*, *DOC*2, *SOCS*2, *HDGF*, and *PLK*) (Table 2, Figure 2). The results for all 10 genes were confirmed. However, generally the differences LightCycler showed in the gene expression levels between the cases and references were slightly larger than in the cDNA array. This is consistent with the notion of LightCycler being a more sensitive method. Then again, one has to bear in mind that different methods were used for normalization.

The methylation status of *SOCS2* exon 1 was investigated in five tumours with low *SOCS2* mRNA expression. The methylation of CpG sites was studied by complete sequencing after sodium bisulphite treatment, amplification and cloning of exon 1. No methylation was detected in five investigated tumours with low/no *SOCS2* expression.

Discussion

In the present study we used cDNA arrays to identify aberrantly expressed genes in human adenocarcinomas of the lung, by investigating 14 well characterized primary tumour samples and four references. Our objective was to find genes whose expression explains the two groups (tumour vs normal) as well as possible. As none of the available methods can be considered to be a superior tool for array data analysis, several different statistical methods have generally been applied. The two statistical methods we used emphasise somewhat different features of the expression measurements. On the whole, both methods find the genes that have the most obvious connection with the disease. However, both methods also find genes that may be overlooked by the other method. The cDNA array results were verified by randomly choosing five up- and

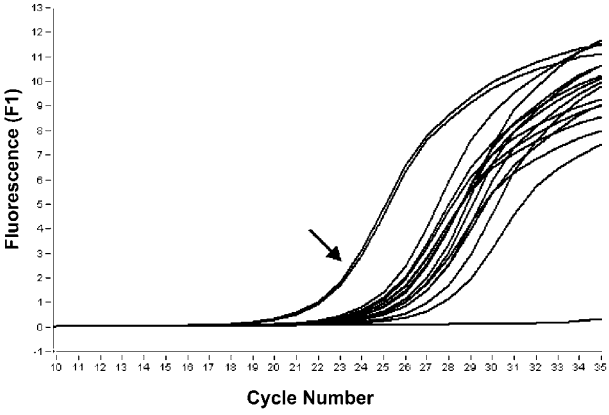

Figure 2 Differences in gene expression of the *CAV1* gene among cancer patients and references were confirmed by semi-quantitative real-time RT–PCR. Both duplicates of references (arrow) enter the exponential phase of PCR amplification (cycle 22) before the cases (cycles 24–30), indicating under-expression of the *CAV1* gene in tumours

Table 2 Comparison of the expression results of 10 genes produced by the array and semi-quantitative RT–PCR

	MMP11		*PLK*		*CCNB1*		*TOP2A*		*HDGF*	
	mean dif. (range)[a]	*nr sig*[b]	*mean dif.*	*nr sig.*	*mean dif.*	*nr sig.*	*mean dif.*	*nr sig.*	*mean dif.*	*nr sig.*
Array	1.95 (0.3–3.6)	7/14	8.01 (0.7–15.4)	9/14	4.54 (1.4–7.1)	11/14	4.99 (2.5–8.2)	5/14	2.99 (1.5–4.4)	10/14
RT–PCR	20.1 (0.9–78.2)	9/14	18.7 (0.6–77.8)	11/14	14.9 (0.9–53.5)	10/14	7.37 (0.2–27.6)	6/14	2.71 (0.9–6.0)	3/14
	CAV1		*COPEB*		*TIMP3*		*SOCS2*		*DOC2*	
	mean dif. (range)	*nr sig.*	*mean dif.*	*nr sig.*	*mean dif.*	*nr sig.*	*mean dif.*	*nr sig.*	*mean dif.*	*nr sig.*
Array	0.31 (0.1–0.9)	9/14	0.50 (0.2–0.9)	6/14	0.60 (0.2–0.9)	5/14	0.44 (0.07–1.1)	7/14	0.62 (0.2–1.4)	5/14
RT–PCR	0.07 (0.02–0.2)	11/14	0.29 (0.04–1.1)	8/14	0.47 (0.05–1.3)	7/14	0.10 (0.01–1.1)	7/14	0.76 (0.1–2.0)	6/14

[a]Mean difference (signal intensities or relative concentrations) between tumour and normal reference. [b]Significance in arrays = signal intensity difference > 5000 and ratio > 2; significance in RT–PCR analyses = fivefold difference in after normalization to *Phospholipase 2A*

five down-regulated genes for semi-quantitative RT–PCR.

Normal lung tissue from four different individuals was used to minimize the chance of findings that do not reflect tumour characteristics. The reference samples were, therefore, chosen so that they would be as similar as possible with the cancer patients regarding gender, age and smoking status. We found, indeed, some variation in gene expression profiles between the references (data not shown), but the statistical methods used excluded any genes with high intra-individual variation among controls. Pure primary peripheral epithelial cells would have been an alternative reference, but it was not possible to obtain adequate amounts of high quality RNA from pure primary epithelial lung cells. Neither did our tumour samples consist exclusively of cancer cells (minimum 50%), but of a mixture of stromal and cancer cells.

Recently two extensive studies on different histological types of lung cancer using high-density microarrays were published (Bhattacharjee *et al*., 2001; Garber *et al*., 2001). In both of these studies the different types of lung cancer could clearly be separated according to gene expression profiles by hierarchical clustering. Different survival for patients in different clusters was also demonstrated. Besides, a number of smaller array studies have also been published. They are conducted mostly on cell culture level and investigate different aspects of lung cancer, including metastatic potential and classification (Anbazhagan *et al*., 1999; Wang *et al*., 2000; Hellmann *et al*., 2001; Chen *et al*., 2001; Gemma *et al*., 2001). Comparison of results from different array studies is difficult, because the references used and sets of genes studied have not been similar.

Up-regulated genes

The growth and metastasis potential of a tumour is thought to be highly dependent on its interactions with surrounding extracellular matrix and neighbouring cells. Therefore, not surprisingly, we found several genes involved in cell motility, adhesion and regulation to be deregulated among cancer patients.

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes with over 20 members that break down proteins in the extracellular matrix. MMPs are regulated by specific inhibitors, known as the tissue inhibitors of metalloproteinases (TIMPs). Both the MMPs and TIMPs have been studied extensively in various cancers as they are thought to be involved in carcinogenic processes like angiogenesis and metastasis (Fassina *et al*., 2000). Several different *MMP* genes have indeed been found highly expressed in very many different cancer types, including lung cancer (Nawrocki *et al*., 1997; Thomas *et al*., 2000).

We found *MMP11* (only in PCA analysis), *MMP12* and *TIMP1* (PCA) up-regulated, whereas *TIMP3* was found down-regulated in the tumours. *MMP11* has been found up-regulated in several lung cancer studies (Nawrocki *et al*., 1997; Karameris *et al*., 1997), whereas the expression of the less known *MMP12* has to our knowledge not been studied in human lung tissue. The importance of *MMP12* in human lung has, however, been shown by its role in the development of emphysema caused by cigarette smoke (Hautamäki *et al*., 1997). Silencing of *TIMP3* by DNA methylation has been detected in lung, gastric and pancreatic tumours (Kang *et al*., 2000; Ueki *et al*., 2000; Zochbauer-Müller *et al*., 2001). In our data *TIMP3* was significantly less expressed among the cases, while *TIMP1* was expressed at a significantly higher level among the cases. In two studies a correlation between elevated *TIMP1* expression and shortened survival has, indeed, been found among lung cancer patients (Fong *et al*., 1996; Ylisirniö *et al*., 2001). Therefore, TIMP1 and TIMP3 seem to have opposite roles in carcinogenic processes.

Cytokeratins are a large structurally related family of intermediate filament (IF) proteins found on epithelial cells. Three different cytokeratins, KRT8, KRT18 (PCA) and KRT19 (PCA) were found up-regulated in our tumours. KRT19 fragment levels in serum, assayed in the blood as CYFRA 21.1, have been widely used as a tumour marker for non-small cell lung cancer (NSCLC) (Dohmoto *et al*., 2000). Similarly, intact KRT8 peptides in NSCLC patient's serum may correlate with advanced disease (Pendleton *et al*., 1994), whereas KRT18 can be used to distinguish AC from SCC (Nhung *et al*., 1999).

The highest PCA score was found for the *high-mobility-group protein Y* (*HMGI-Y*). HMGI is a member of the non-histone chromatin HMG protein gene family. *HMGI* is expressed in rapidly dividing cells and has been localised to metaphase chromosomes (Reeves and Nissen, 1995). Interestingly, rearrangements of *HMGI* gene are found in pulmonary hamartomas, causing an activation of the gene (Kazmierczak *et al*., 1999; Xiao *et al*., 1997). In lung cancer these rearrangements have not been studied, but the chromosomal location 6p21 has been found amplified in Finnish lung cancer patients (Björkqvist *et al*., 1998b). Another gene found over-expressed in our tumours was *histone H4*, also located at 6p21.

Over-expression of *cyclin B1* has been reported in various tumour types. Cyclin B1 (CCNB1) is a cell cycle control protein that is required for passage through G2 and mitosis (Pines and Hunter, 1992). In NSCLC high levels of *cyclin B1* have been associated with a significantly shorter survival (Soria *et al*., 2000). Polo-like kinase (PLK/STPK13) is involved in targeting cyclin B1 to the nucleus (Toyoshima-Morimoto *et al*., 2001). No *PLK* expression has been detected in normal non-dividing cells but, similarly to *cyclin B1*, it is commonly over-expressed in human cancers (Holtrich *et al*., 1994). One study has shown elevated *PLK* mRNA expression in most NSCLC tumours and, similar to *cyclinB1*, high *PLK* expression has been associated with poor survival (Wolf *et al*., 1997). Consistent with previous findings, the majority of cases had a high expression of both *CCNB1* and *PLK*. Furthermore, in our samples the expression of *PLK* and *cyclin B1* were highly co-regulated (correlation coefficient 0.92).

A novel finding was the over-expression of *hepatoma-derived growth factor* (*HDGF*) among the tumour samples. HDGF is a heparin-binding protein with mitogenic activity in hepatoma cells, fibroblasts and vascular smooth muscle cells (Everett *et al*., 2001). Interestingly, HDGF has also been implied to play a role in murine lung and in type II cell differentiation and proliferation (Cilley *et al*., 2000). In our samples *HDGF* was strongly expressed in most of the tumour samples, whereas only faint expression was seen in the references when studied in the array. However, in real-time PCR the difference was not as clear, perhaps due to a non-optimal choice of primers for the PCR.

Other genes that we and others have found over-expressed in lung tumours, include the *macrophage migration inhibitory factor* (*MIF*), *topoisomerase 2A* (*TOP2A*), *DNA replication factor MCM2* (permutation test) and *tenascin-C (TN-C/HXB)*. MCM2 has been considered a promising lung tumour marker and perhaps even a marker for premalignant lesions (Tan *et al*., 2001), whereas tenascin has been proposed as a stromal marker for lung cancer (Kusagawa *et al*., 1998). Also *MIF* mRNA and protein levels have been found to be elevated in lung adenocarcinoma specimens (Kamimura *et al*., 2000) and TOP2A inhibitors are widely used as chemotherapeutic agents in lung cancer treatment (Kellner *et al*., 2000).

Down-regulated genes

Among the down-regulated genes we found several potential tumour suppressor genes involved in negative regulation of cell cycle, signal transduction or inhibition of matrix degradation.

One of the most interesting findings was the down-regulation of *SOCS2* and *3*. The suppressors of cytokine signalling (SOCS) family of proteins act as inhibitors of the JAK–STAT signal transduction pathway (Krebs and Hilton, 2001). *SOCS1* has recently been shown to function as a tumour suppressor gene in hepatocellular carcinoma. Silencing of the expression of *SOCS1* is caused by methylation of exon 1 (Kishimoto and Kikutani, 2001).

SOCS2 and *3* have been shown to be expressed in normal human lung, whereas *SOCS1* is expressed only very weakly (Minamoto *et al*., 1997). As far as we know, no studies have been conducted on lung tumours. In our study both *SOCS2* and *3* were found down-regulated in the tumour samples. Lower expression of *SOCS2* was verified by real-time PCR analyses. Therefore *SOCS2* and *3* could function as potential tumour suppressor genes in lung carcinogenesis.

As the silencing of *SOCS1* has been shown to be caused by methylation of the first exon, we investigated if this would also be the case for *SOCS2*. *SOCS2* silent exon 1 is also highly GC rich, but it is considerably shorter than *SOCS1* exon 1. No methylation of this sequence was, however, detected among the five investigated tumour samples. Further characterization of *SOCS2* and *3* in AC are needed to evaluate their diagnostic, prognostic and therapeutic potential.

Three different so-called scaffold proteins, *CAV1*, *CAV2* and *gravin*, were down-regulated among our tumour samples. Caveolins, the major integral membrane components of caveolae, can functionally regulate the activity of G-proteins, Src-like kinases, protein kinase C-alpha, and Ras-related GTPases by generating pre-assembled signalling complexes (Engelman *et al*., 1998; Razani and Lisanti, 2001). The *CAV1* expression is lost in several tumours including lung cancer (Racine *et al*., 1999). Racine *et al*. (1999) have detected *CAV1* and *2* expression in normal bronchial cell lines, *CAV1* was silenced in lung cancer cell lines, whereas CAV2 was expressed. We found also a clear signal of *CAV1* and *2* in normal tissue and no signal of *CAV1* in tumours. However, in contrast to the results of Racine *et al*. (1999) *CAV2* was also down-regulated in our tumour samples. The discrepancy could stem from the differences in studying primary tumours and cell lines.

The third scaffold protein that was down-regulated in our samples, gravin/AKAP250, belongs to the family of cyclic AMP-dependent kinase-anchoring proteins (AKAPs) (Feliciello *et al*., 2001). Gravin forms part of a scaffold coordinating the location of, at least, protein kinase A and protein kinase C (Diviani and Scott, 2001). *Gravin* expression has been described to be lost in prostate tumours (Xia *et al*., 2001). As far as we know, no reports have been published on the expression of *gravin* in lung tissue. Very low *gravin* expression was found in our tumour samples, whereas all four references clearly expressed gravin mRNA.

Another novel finding was the down-regulation of *DOC2* in lung adenocarcinomas (verified by real-time RT–PCR). The *mitogen-responsive phosphoprotein DOC2/DAB2*, it has been suggested, is an essential component of the TGFbeta signalling pathway (Hocevar *et al*., 2001). Silencing of the gene has been described in ovarian, choriocarcinoma, pancreatic, prostate, and mammary carcinomas (Fulop *et al*., 1998; Huang *et al*., 2001; Mok *et al*., 1998; Tseng *et al*., 1999).

A less well-known gene, down-regulated in our samples, was *DNA-binding protein CPBP* (*COPEB/Z9f*) (also verified by real-time RT–PCR). COPEB belongs to the family of Krüppel-like transcription factors, suggested to be involved in tissue repair by activating urokinase plasminogen activator (uPA) and TGF beta (Kojima *et al*., 2000; Kim *et al*., 1998).

In conclusion, by using the cDNA array technique we could reveal marked differences in the gene expression level between normal lung and lung adenocarcinomas. Furthermore, we could find aberrant expression of previously undescribed cancer-related genes, such as the highly interesting *SOCS2* and *SOCS3* genes. Gene expression profiling with microarray was shown to be a good and fast screening method for detecting new interesting genes and pathways. More vigorous molecular studies, especially on the protein level are, however, needed in order to assess the potential of these genes as diagnostic and prognostic markers of lung cancer.

Materials and methods

Study subjects

All cases were Finnish Caucasians with histologically confirmed primary pulmonary adenocarcinoma. All the samples have been examined and classified according to the histological type and grade by WHO standards (1999) by the same pathologist (S Anttila). Histologically verified normal whole lung tissue from patients operated for a tuberculoma (one), intra bronchial granuloma (one) and lung cancer (two) were used as reference. The patient characteristics are given in Table 3.

Detailed information of the patients' work and health history as well as of their smoking habits and survival data are recorded. All the patients have been personally interviewed and their consent to take part in the study and to use their tissue has been obtained. An ethical review board of the Department of Thoracic and Cardiovascular Surgery of the Helsinki University Central Hospital has approved the study protocol.

RNA isolation

Fresh snap-frozen lung tissue samples were cut in a cryotome; the first and last slice were put on a slide and stained with hematoxylin and eosin. A pathologist (S Anttila) examined each sample and only samples with more than 50% of tumour cells were chosen. Total RNA from about 100 mg of lung tissue was isolated with Ultraspec™ RNA isolation system (Biotecx Laboratories Inc., Houston, TX, USA) according to the manufacturer's instructions. The RNA was treated with DNAse I according to the Atlas cDNA Expression Array's user manual (Clontech Laboratories Inc., Palo Alto, CA, USA) and the integrity and yield of RNA was verified on a 1% agarose gel and by spectrophotometry.

cDNA array hybridization and image processing

Atlas Human Cancer Gene Filter 1.2 including 1176 tumour relevant genes was used for the cDNA array experiments (a list of the spotted genes is available at http://atlasinfo.clontech.com/bioinfo/). 3.5 μg of total RNA was reverse transcribed into cDNA with labelled [^{33}P]dATP using the Clontech cDNA array labelling kit. Purification of the probe, hybridization (68ºC, overnight) and washings were done according to the manufacturer's instructions.

Arrays were exposed to a Fuji BAS-MP 2040S intensifying screen (Fuji, Kanagawa, Japan) for 2–4 days and scanned at 16-bit and 50 micron resolution with Bio-Imaging Analyzer (BAS-2500, Fuji). The images were analysed and the expression levels determined using AtlasImage 2.0 software (Clontech).

Table 3 Main characteristics of the study population

	Reference n = *4*	*AdenoCa.* n = *14*
Female/male	1/3	2/12
Age (mean)	56.8	60.4
Range	48–70	35–75
Grade I	–	0
II	–	4
III	–	10
Smoking non	1	1
current	3	13
PY[a] (mean ± s.d.)	47.6 ± 2.9	41.6 ± 18.6
Smoking years (mean ± s.d.)	44.7 ± 7.1	38.0 ± 13.8
Asbestos (exp/non exp.)	2/2	6/8

[a]PY = pack years; exp = exposed

Statistical analyses

The raw expression data obtained with AtlasImage 2.0 were analysed with two complementary statistical techniques, the principal component analysis and the permutation test, in order to find genes with abnormal expression.

Principal component analysis Principal component analysis (PCA) is a linear signal decomposition technique, which in contrast to multivariate least-squares regression, allows for noise in the predictor variables as well as a predictor variable. Technically, PCA finds a set of orthogonal directions in the data space so that the variance of the data is maximal along these directions; the first direction (principal component, PC) explains the most variance, the second PC explains the most of the remaining variance etc. (Hand *et al*., 2001). PCA has previously been demonstrated to be a good method for analysing array data (Hilsenbeck *et al*., 1999; Armstrong *et al*., 2002).

We computed the mean expression for each gene among the normal lung samples and subtracted this from the expression levels of the tumour samples. Next we normalized this data to obtain mean and unit variance of zero and estimated the principal components. The data was then projected onto the first PC. The result is one number for each gene; we call this a PCA score. Those genes, whose expression level differs consistently among the tumour samples compared to the mean of references, have a high absolute PCA score. The sign of the PCA score corresponds to lower or higher expression level in tumour samples compared to the references.

Permutation test The other statistical technique we used was the permutation test in order to quantify how different two groups of measurements are. The primary interest was in the patient/control grouping, but we also examined other groupings by tumour stage and grade. The standard *t*-test is difficult to apply when the sizes of two groups are significantly different (e.g., 14 patients and four references) and cannot be assumed to have similar variance. A permutation test trades computational effort for solving these difficulties.

On a general level, the permutation test for assessing how much two groups differ is performed by computing a test statistic (difference of the means) for both the actual data and for several (in our case, 10 000) permuted versions of the data. In these permuted versions, new random groups are selected from all the measurements. When the difference between the groups occurs by chance, a similar or higher degree of difference will be observed for most of the permuted data, but when the difference is statistically significant, the randomised groups will usually exhibit a lower degree of difference. This gives an overall measure of confidence in the results.

To put this in a mathematical formula, the expression measurements in the first group are $x_1, x_2, \ldots, x_r$, and the measurements in the second group are $y_1, y_2, \ldots, y_s$. Then the groups have means $\bar{x} = (x_1 + \ldots + x_r)r$ and $\bar{y} = (y_1 + \ldots + y_s)/s$, and variances $\sigma_x = \sum_{i=1}^{r}(x_i \quad \bar{x})^2/(r \quad 1)$ and $\sigma_y = \sum_{i=1}^{s}(y_i \quad \bar{y})^2/(s \quad 1)$, respectively. Denoting by f the standard normal cumulative distribution function, we compute $p_i = f(|x_i \quad \bar{y}|/\sigma_y)$ and $q_i = f(|y_i \quad \bar{x}|/\sigma_x)$. The final

Table 4 Primers and annealing temperatures used in the real-time RT–PCR

Gene	*Primer*	*T (°C)*[a]	*PCR length (bp)*
PL2A	F: (2418-2436) 5′-tcc ttt gct tgc atc cca c-3′ R: (2551-2527) 5′-aag gca gac aat gac aga cca-3′	62	133
CAV1	F: (495-515) 5′-acc cac tct ttg aag ctg ttg-3′ R: (633-613) 5′-gaa ctt gaa att ggc acc agg-3′	62	138
COPEB	F: (312-331) 5′-caa ctt aga gac caa cag cc-3′ R: (484-466) 5′-tca gtt ccg gag aag atg g-3′	60	172
CCNB1	F: (661–680) 5′-acc tac tgg gtc ggg aag tc-3′ R: (1013-994) 5′-tct ccg aag gaa gtg caa ag-3′	60	352
DOC2	F: (537-555) 5′-ggg cat ttg gtt acg tgt g-3′ R: (704-684) 5′-ctt tgc tgg ctt cct cta tc-3′	62	167
HDGF	F: (1846-1867) 5′-cct ctc cac aca gtg ctc aa-3′ R: (2008-1990) 5′-ggg aag gaa gca gaa tgg ag-3′	60	162
MMP11	F: (1963-1982) 5′-cct gca tct gtc tgc ctt ct-3′ R: (2159-2140) 5′-gct ttg gag gat agc agt gc-3′	63	196
PLK	F: (1377-1396) 5′-ctc aac acg cct cat cct c-3′ R: (1531-1511) 5′-gtg ctc gct cat gta att gc-3′	66	154
SOCS2	F: (263-282) 5′-ctc gca ttc aga cta cct ac-3′ R: (447-429) 5′-gct tac tcc ttg cac atg tg-3′	58	184
TIMP3	F: (464-484) 5′-cta cct gcc ttg ctt tgt gac-3′ R: (603-584) 5′-act ctc ggt acc agc tgc ag-3′	62	139
TOP2A	F: (301-322) 5′-cta gtt aat gct gcg gac aac a-3′ R: (526-506) 5′-cat ttc gac cac ctg tca ctt-3′	63	225

[a]PCR annealing temperature; b.p. = base pair

score is $G = \text{sign}(\bar{x} \quad \bar{y}) \cdot (\prod_{i=1}^{r} p_i)^{1/r} \cdot (\prod_{i=1}^{s} q_i)^{1/s}$. This score (g-score) assesses the probability that each set of measurements would have arisen from a normal distribution that is estimated from the opposite set. We performed actual permutation test on these scores and an empirical *P*-value was estimated for each gene as the frequency of more extreme values of the score.

Real-time semi quantitative RT–PCR

RT–PCR quantification was used to verify the array data. Eight hundred nanograms of template RNA was used in a single RT reaction round according to the manufacturer's instructions using AMV-RT enzyme (1st strand cDNA synthesis kit; Roche Diagnostics Corp., Indianapolis, IN, USA).

The expression levels of 10 genes were verified by using the LightCycler technique (Roche Diagnostics GmbH, Mannheim, Germany) (Table 4 and Figure 2). 1/10 or 1/20 cDNA dilutions were used depending on the expression level of the genes. All four references were used in each run separately as well as a pool. Standard curves were obtained by doing serial dilutions of at least two samples in each run. The housekeeping gene *Phospholipase* 2A (*PL2A*) was chosen as a reference, due to its constant expression at moderate level in the cDNA array experiment and each PCR result was normalised against *PL2A*.

PCR analyses were performed in 10 μl volumes in glass capillaries (Roche Diagnostics) using the LightCycler Fast-start DNA Master SYBR green kit (Roche). 0.5 mM of each primer (TIB MolBiol, Berlin, Germany) (Table 4) and 2.5 mM $MgCl_2$ (2.0 mM for PLK1) were used in each PCR run. The cycling conditions were as follows: initial denaturation at 95°C for 7 min following by 35–45 cycles with denaturation at 95°C for 0 s, annealing at 58–66°C (see Table 4) for 8 s, and elongation at 72°C for 9 s, (for *CCNB1* 12 s), with a ramping rate of 20°C s^{-1}. To verify the amplification specificity, melting curve analyses were performed using an initial denaturation at 95°C for 10 s, annealing at 55°C for 20 s followed by slow heating of the samples to 95°C at a ramping rate of 0.1°C s^{-1} with continuous fluorescence detection. The Second Derivative Maximum method provided by the LightCycler software was used to estimate the concentration of each sample.

SOCS2 methylation status

The methylation status of *SOCS2* in five tumour samples and their corresponding normal lung tissue were evaluated by using sodium bisulphite treatment with subsequent sequencing of exon 1 of the gene. Treatment of genomic DNA with sodium bisulphite was performed according to Suzuki *et al*. (2000). In brief, 2 μg of genomic DNA was denaturated in 0.2 M NaOH for 10 min, freshly prepared 10 mM hydroquinone and 3 M sodium bisulphite (pH 5.0) was added, and the DNA was incubated at 50°C overnight. The modified DNA was purified using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and subsequently, a 380 base pair fragment containing exon 1 was amplified (forward primer: 5′-gtt gag gag gtt gtt tgg tg-3′; reverse 5′-cca cac aaa ctt aat tct cc-3′). The produced PCR product was run on an agarose gel and the right bands were cut out and purified with a QIAGEN gel extraction kit. The purified product was cloned into a pGEM-T Easy vector according to the manufacturer's instructions (Promega, Madison, WI, USA). SOCS2 exon 1 was sequenced from 5–10 clones of each sample with an ABI-Prism 310 sequencer (Applied Biosystems) using ABI Prism BigDye^{TM} terminator cycle sequencing Ready Reaction Kit 2.0 (Applied Biosystems, Warrington, UK).

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