

Identification of osteopontin as the most consistently over-expressed gene in intrahepatic cholangiocarcinoma: Detection by oligonucleotide microarray and real-time PCR analysis

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Abstract

AIM: To investigate the molecular pathways involved in human cholangiocarcinogenesis by gene expression profiling.

METHODS: Oligonucleotide arrays (*Affymetrix* U133A) were used to establish a specific gene expression profile of intrahepatic CCC in comparison to corresponding non-malignant liver tissue. To validate the expression values of the most overexpressed genes, RT-PCR experiments were performed.

RESULTS: Five hundred and fifty-two statistically differentially expressed genes/ESTs (221 probes significantly up-regulated, 331 probes down-regulated; $P < 0.05$; fold change > 2 ; $\geq 70\%$) were identified. Using these data and two-dimensional cluster analysis,

a specific gene expression profile was obtained allowing fast and reproducible differentiation of CCC, which was confirmed by supervised neuronal network modelling. The most consistently overexpressed gene (median fold change 33.5, significantly overexpressed in 100%) encoded osteopontin. Furthermore, an association of various genes with the histopathological grading could be demonstrated.

CONCLUSION: A highly specific gene expression profile for intrahepatic CCC was identified, allowing for its fast and reproducible discrimination against non-malignant liver tissue and other liver masses. The most overexpressed gene in intrahepatic CCC was the gene encoding osteopontin. These data may lead to a better understanding of human cholangiocarcinogenesis.

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Key words: Cholangiocarcinoma; Oligonucleotide arrays; Osteopontin; Cell cycle regulation; Gene expression

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INTRODUCTION

Cholangiocarcinoma (CCC) is the second most common primary hepatic malignancy next to hepatocellular carcinoma (HCC), arising from cholangiocytes of intra- or extrahepatic bile ducts. In contrast to HCC, cholangiocarcinomas are, in most cases, adenocarcinomas and relatively hypovascularised. Two principal forms of these tumors are recognized based on the clinical presentation^[1]. The most common form is a highly desmoplastic cancer with a growth pattern characterized

by periductal extension and infiltration, leading to an early onset of jaundice as one of the first clinical signs of this disease (perihilar and distal extrahepatic CCC). The other form of CCC grows as a mass lesion within the liver, very often leading to the misdiagnosis of hepatocellular carcinoma or metastatic adenocarcinoma (intrahepatic CCC).

Despite recent progress in various imaging modalities the neoplasm lesion is, in most cases, advanced by the time of diagnosis. Thus, curative treatment options by surgical resection are limited, leading to a poor prognosis with median survival times of only three to six months, especially in patients with intrahepatic CCCs^[2,3].

Recent epidemiologic studies have shown an increasing incidence of CCC in western countries^[4,5]. In most patients with CCC, no risk factors for cholangiocarcinogenesis can be identified. However, established risk factors for ductal cholangiocarcinoma include diseases leading to chronic inflammation of the bile ducts, such as infections with *Clonorchis sinensis*, *Opisthorchis viverrini* (liver flukes) and chronic viral hepatitis, chronic intrahepatic lithiasis, primary sclerosing cholangitis (PSC), congenital diseases (Caroli disease, congenital choledochal cysts) and exposure to the radiopaque medium thorium dioxide (Thorotrast)^[6-8]. In particular, patients with PSC have an increased risk for cholangiocarcinogenesis and epidemiologic studies have shown a lifetime risk for CCC of approximately 1.5% per year of disease^[9].

Despite this knowledge about the major role of chronic inflammation in cholangio-carcinogenesis, little is known about the molecular pathways involved in CCC that lead to uncontrolled cell growth, downregulated apoptosis and, as a result, to tissue invasion.

Therefore, it is important to identify the genes involved in these molecular pathomechanisms, as this may shed light on the specific tumor biology and help to establish prognosis patterns as well as specific markers for screening in patients with a known increased risk for CCC (for example, patients with PSC). At present, the most commonly used markers in CCC include tumor antigens or products, such as CA 19-9, cytokines (for example, interleukin-6), (epi-)genetic lesions (for example, *K-ras* and p53 mutations), and metabolic products as lactate and proteases (for example, trypsinogen-2). However, none of these have been proven to be a useful diagnostic tool with high specificity and sensitivity for CCC^[9].

Today, high-throughput techniques using cDNA or oligonucleotide arrays for the simultaneous and fast analysis of thousands of genes are available, potentially leading to the identification of new markers and subclassification of various human carcinomas^[10-12]. In the present study oligonucleotide arrays (HU 133A, *Affymetrix*) containing more than 20 000 genes and expressed sequence tags (ESTs) were used to analyze gene expression in intrahepatic CCC tissues. The aim was first to detect specific genes (by comparison with genes expressed in non-malignant liver tissue) that may be helpful for discrimination between non-malignant and different malignant liver masses, and to establish a specific diagnostic genetic profile of intrahepatic CCC for clinical

routine, and second, to elucidate the genetic pathways that are typically altered in cholangiocarcinogenesis.

MATERIALS AND METHODS

Acquisition of samples

Surgical specimens were obtained from 10 patients who underwent surgical treatment with curative intention for intrahepatic CCC between 2003 and 2004 (Table 1). In 8 cases, the adjacent corresponding non-malignant liver tissue was also acquired for microarray analyses. No patient received preoperative or adjuvant chemo- or radiotherapy.

Samples were resected after appropriate informed consent was obtained and the genetic analysis of the tumor and liver tissue was conducted in accordance with the guidelines of the Declaration of Helsinki (1995) and approved by the local ethic committee. Samples of sufficient weight (> 400 mg) from malignant and corresponding non-malignant liver tissue were excised and snap-frozen in liquid nitrogen within 20 min after excision. Until RNA isolation, samples were stored constantly at -80°C.

Histopathological evaluation

The various architectural patterns and cytological variants of cholangiocarcinoma and especially hepatocellular may complicate the differentiation of primary liver tumors and even that between primary liver tumors and metastases. Therefore, pathological reports with tumor typing, staging (performed using UICC criteria) and grading as well as clinical data were obtained for each tissue sample. Hematoxylin-eosin staining was performed for detection of features like bile canalicular structure and Mallory hyaline bodies. Additional histochemical and immunohistochemical stainings with a panel of antibodies were used routinely (HEP-PAR-1, CK7, CA19-9, CD10, CEA, AFP; Figure 1A and B) in all resected tissue biopsies to confirm the histogenesis of cholangiocellular carcinoma and to exclude hepatocellular carcinoma or metastases. In particular, CK7 is used as an important marker for CCC, as it is significantly overexpressed in CCC compared with HCC and most cases of other adenocarcinoma^[13].

Oligonucleotide array analysis

Sample preparation: Samples were processed with only minor modifications according to the Affymetrix GeneChip® Expression Analysis Manual (Santa Clara, CA/ USA). For the extraction of total RNA frozen samples were homogenized in Trizol (Invitrogen, USA). The total RNA yield for each sample was 200-400 µg. After washing with DEPC-treated water (Ambion, USA), total RNA was cleaned using RNEasy mini kits (Quiagen). The amount of total RNA was measured photometrically using a BIO-Photometer (Eppendorf; OD1 E260 = 40 µg/mL).

Preparation of labeled cRNA and hybridization to oligonucleotide arrays: For transcription of total RNA into cDNA, 8 to 10 µg of total RNA was used for cRNA preparation using the SuperScript Choice system (Invitrogen, USA). First-strand cDNA synthesis was primed using a T7-(dT24) oligonucleotide primer

Table 1 Patient demographics and histopathological data

Patient No.	Sex	Age	Histology	Stage	Grading
1	F	57	Adenocarcinoma	pT3pNxpM0	G3
2	F	65	Adenocarcinoma	pT3pN0pM0	G2
3	F	68	Adenocarcinoma	pT2pNxpM0	G2
4	M	74	Adenocarcinoma	pT3pN1pM0	G3
5	F	62	Adenocarcinoma	pT3pN0pM0	G2
6	F	47	Adenocarcinoma	pT3pNxpM0	G2
7	M	73	Adenocarcinoma	pT3pN0pM0	G2
8	F	71	Adenocarcinoma	pT2pNxpM0	G2
9	M	52	Adenocarcinoma	pT3pN2pM0	G2
10	M	65	Adenocarcinoma	pT3pNxpM0	G1

with an RNA polymerase promoter site added to the 3' end. After second-strand synthesis, *in vitro* transcription was performed in the presence of biotin-11-CPT and biotin-16-UTP (Enzo Diagnostics) to produce biotin-labeled cRNA. After fragmentation of the cRNA products (20 µg at 94°C for 35 min) to lengths of 35-200 bp, the samples were added to a hybridization solution to a final cRNA concentration of 0.05 mg/mL. Hybridization was performed by incubation (18-20 h) of 200 µL of the sample to an Affymetrix human GeneChip (Hu 133A) containing 22283 probe sets for known genes/ESTs and stained with streptavidin-phycoerythrin. A Gene Array scanner G2500A (Hewlett Packard, ID) was used to scan the arrays according to procedures described in the Affymetrix manual.

To validate the relative change in gene expression of the most consistently overexpressed gene in all tumor samples (osteopontin, OPN), further analysis with real-time PCR using the LightCycler® system (Roche Diagnostics, Mannheim, Germany) was performed in 8 samples (tumor tissue *vs* corresponding non-malignant liver tissue).

Gene-specific primers corresponding to the coding region were designed using OLIGO software and were obtained from Biomers.net (Ulm, Germany; forward primer OSTEO: 696 U; GGACAGCCGTGGGAAGG, reverse primer OSTEO 810 L; TCAATCACATCGGAATGCTCA). Preliminary experiments were performed to test for the specificity of product formation, determine annealing temperatures, and check for $T_m(\text{Product-Primer Dimer}) > 3^\circ\text{C}$. Validation experiments were performed within a fluorescence signal window excluding primer-dimer formation. The correct PCR efficiency for each target was determined by constructing relative standard curves using five-point half-logarithmic RNA dilutions from one sample. Template concentrations were given arbitrary values of 1, 0.316, 0.1, 0.0316 and 0.01. RT-PCR reactions were performed using the LC RNA Amplification Kit containing SYBR Green I, (Roche Diagnostics; Mannheim, Germany). Amplification was followed by melting curve analysis. Relative values for the initial target concentration in each sample were determined using Light Cycler software 3.5. Crossing points (C_p) were computed using the "2nd derivative maximum method", part of the software above. The relative change in gene expression was computed by pairwise comparison of tumor samples

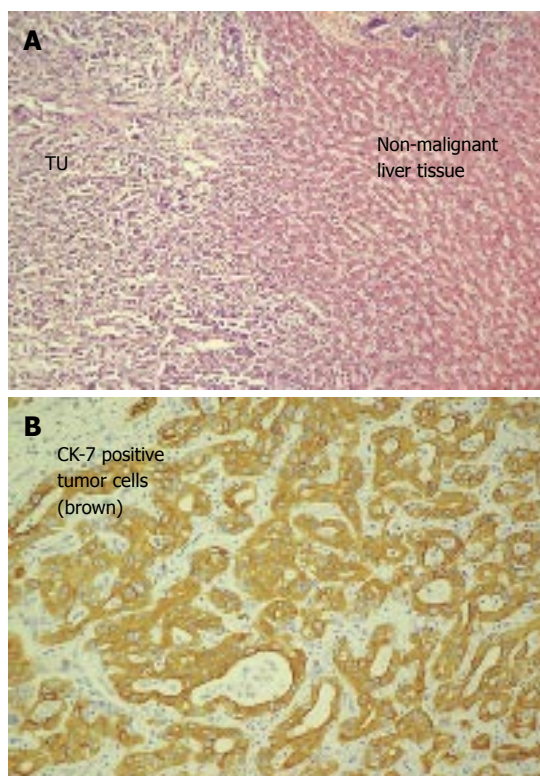


Figure 1 A: HE staining of infiltrative intrahepatic cholangiocarcinoma (Patient No. 6; left tumor tissue with cells of a moderate (G2) differentiated adenocarcinoma, right non-malignant liver tissue); B: Detection of cytokeratin 7 in intrahepatic cholangiocarcinoma (same patient as in A) using immunohistochemistry. Cytokeratin 7 was detected in over 80% of all analyzed CCCs strongly overexpressed (14.6-fold change).

to samples of adjacent normal tissue for each patient.

Statistical analysis

The statistical analyses and presentation of the obtained data were performed in accordance with the MIAME criteria and will be published on the web page (www.matri-gene.de/geneprofiles/CCC).

Preliminary data analysis: Raw data analysis was conducted using the software of the Affymetrix microarray suite (MAS *vs* 5.0.1). MAS produced an expression value plus an index parameter indicating positive or negative detection (present call index) for each of the 22283 probe sets (known genes/ESTs) on the array. Statistical analysis and post-processing were performed using GeneSpring (*vs* 6.1; Silicon Genetics, Redwood City, CA) and Gene-Explore (*vs* 1.1; AppliedMaths, Saint-Martens-Latem, Belgium) software. Mismatch probes acted as specific controls on each array and allowed the direct subtraction of both background and cross-hybridization signals. Only Chip results with scaling factors of 0.5 to 1.8 were accepted for further analyses. Expression values were log₂ transformed on the basis of the signal log ratio, given by the comparison of two array results between tumor and non-tumorous tissues.

A *P*-value cut off of < 0.05 (*t*-test) and a fold change difference of ≥ 2 in 70% or more of all analyzed samples were considered to be significant.

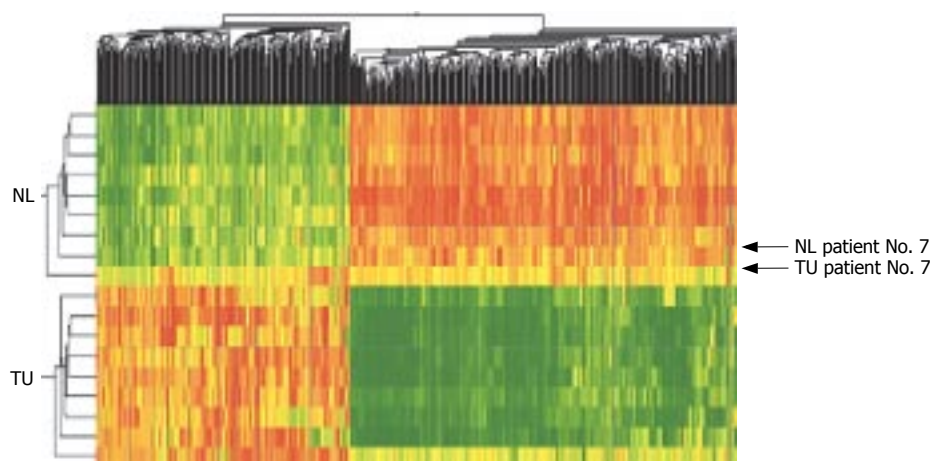


Figure 2 Two-dimensional cluster analysis of CCC (TU) and corresponding non-malignant liver tissues (NL) using 552 dysregulated genes/ESTs (red: overexpressed genes, green: downregulated genes; Pearson's correlation). Dysregulation was defined as different genetic expression in > 70% of all probes with a fold change > 2.0. Horizontal lines show all dysregulated genes in one singular tissue probe, vertical lines show the expression of one gene in all analyzed tumor and non-malignant tissue probes. Using cluster analysis a fast differentiation between tumor tissue and non-malignant tissue was possible in 90% of cases (tumor tissue of Patient No. 7 showed many genetic similarities to non-malignant liver tissue).

Second analysis step - Hierarchical clustering and supervised analysis: To identify differentially expressed genes in CCC, normalized expression data on statistically different genes were used for a paired data analysis by hierarchical clustering with gene trees/experiment trees using either Pearson's correlation or standard correlation algorithms (Genespring *vs* 6.1; GeneExplore *vs* 1.1).

The class predictor program (supplied by GeneSpring 6.1) was used for supervised learning and training (neuronal training) to identify unknown probes as tumor or non-tumorous tissue. A list of the most predictive genes was made for each class and an equal number of genes with the lowest *P*-value using Fischer's exact test were taken from each list. To make a prediction, the class predictor uses the k-nearest-neighbour method.

To detect statistically differentially expressed genes in well and poor differentiated intrahepatic CCC (genetic subclassification), a 1-way ANOVA test was performed. In a second step significantly dysregulated genes ($P < 0.05$) in tumor samples were used for a weighted two-dimensional clustering.

Identification of significantly altered metabolic pathways involved in cholangiocarcinogenesis:

To identify specific signaling or metabolic pathways involved in cholangiocarcinogenesis data on significantly differentially expressed genes were transferred to a public domain software program (GenMAPP 2.0 beta[©]Gladstone Institutes, 2000-2004).

RESULTS

Gene expression profiling of intrahepatic CCC

Primary chip data were screened for RNA quality by 5' to 3' degradation. Of the 22283 probes (genes/ESTs) present on the chip, on average, 43.3% (SD \pm 4.53) and 39.0% (SD \pm 3.48) of genes were expressed in CCC and corresponding non-malignant tissue samples.

Based on the primary data an algorithm was developed to identify and rank the most consistently up- and downregulated genes in CCC compared with non-malignant tissue. All statistically different genes ($P < 0.05$) with a fold change of at least a 2-fold increase or decrease in 70% or higher percentage of the CCC tumor samples

were used to generate a databank of 552 genes/ESTs. Of these, 221 were upregulated and 331 were downregulated. This set of data was used for two-dimensional clustering. Ninety percent of all tumor samples showed a consistent pattern of up- and downregulated genes; only 1 case (No. 7) showed characteristics of both tumor and non-tumor tissue (Figure 2).

To further test the robustness of this cluster, a supervised learning method (SLM) based on neuronal networking was applied. An optimal prediction with subsequent cross-validation was obtained using all significantly dysregulated genes/ESTs and 8 neighbors; using this, all 10 tumor samples could be correctly identified. This resulted in a positive prediction value of 90% ($P < 0.001$) and a negative predictive value of 100% ($P < 0.00023$) of the Gene cluster with the expression pattern of the 552 probes (Table 2).

Using these data, even a specific and fast discrimination between intrahepatic CCC and other malignant liver tumors (HCC, metastases; data not shown), with a high predictive value ($P < 0.001$), was possible by two-dimensional cluster analysis and SLM.

Dysregulated genes and specifically involved metabolic pathways in intrahepatic CCC

CCC gene expression profile data were imported into GeneMapp software to identify specific changes in molecular pathways. Of the 552 dysregulated genes, a total of 364 genes could be assigned to specific metabolic or signaling pathways. Most of the consistently and strongly overexpressed genes were related to cell cycle regulation and DNA replication (15 genes, including *ribonucleoside-diphosphate reductase M2*, *calgizarrin*, *calcyclin*, *BUB1B*) or intracellular signaling (15 genes, including *CD24* and *MARCKS*), genes encoding transcription factors (6 genes, such as *SOX9*), or genes involved in nuclear organization and nucleic metabolism (13 genes, such as *thymidylate synthetase*). Most of the other up-regulated genes could be attributed to gene families, such as genes coding for extracellular matrix and cell adhesion molecules (37 genes, for example, *OPN*, *ADAM9*, *thymosin beta-10*, *integrin alpha-6*), cytoskeleton structure (16 genes, such as *tropomyosin2*, *cytokeratin 7* and *19*) or protein biosynthesis (4 genes).

Table 2 Statistical results obtained using a supervised neuronal training method for discrimination between CCC and non-malignant liver tissue

Condition	True value	Prediction	P value
Pat. No. 1, non-malignant	Normal liver tissue	Non-malignant	0.00023
Pat. No. 2, non-malignant	Normal liver tissue	Non-malignant	0.00023
Pat. No. 3, non-malignant	Normal liver tissue	Non-malignant	0.00023
Pat. No. 4, non-malignant	Normal liver tissue	Non-malignant	0.00023
Pat. No. 5, non-malignant	Normal liver tissue	Non-malignant	0.00023
Pat. No. 6, non-malignant	Normal liver tissue	Non-malignant	0.00023
Pat. No. 7, non-malignant	Normal liver tissue	Non-malignant	0.00023
Pat. No. 8, non-malignant	Normal liver tissue	Non-malignant	0.00023
Pat. No. 1, CCC	CCC	Malignant	0.00103
Pat. No. 2, CCC	CCC	Malignant	0.00103
Pat. No. 3, CCC	CCC	Malignant	0.00103
Pat. No. 4, CCC	CCC	Malignant	0.00103
Pat. No. 5, CCC	CCC	Malignant	0.00103
Pat. No. 6, CCC	CCC	Malignant	0.00103
Pat. No. 7, CCC	CCC	Malignant	0.16100
Pat. No. 8, CCC	CCC	Malignant	0.00103
Pat. No. 9, CCC	CCC	Malignant	0.00103
Pat. No. 10, CCC	CCC	Malignant	0.00103

Statistical results of the ability to differentiate between normal and malignant liver tissue (CCC) using a supervised neuronal training method. In all cases, a fast and correct differentiation was possible with a high positive ($P < 0.001$ in 90%) and negative (100%, $P < 0.00023$) predictive value.

According to an upregulation of genes involved in cell cycle regulation, most of the genes encoding proteins involved in cellular apoptosis (7 genes, such as growth-arrest specific protein 2, *CIDE-B*) were found to be down-regulated in intrahepatic CCC. Furthermore, a significant suppression of genes encoding proteins important for metabolic pathways like amino acid metabolism (39 genes), carbohydrate and fat metabolism (13 and 11 genes) or electron transport (27 genes) was noted. For clarity, only the 41 most consistently dysregulated (increased or decreased) genes ($P < 0.001$; 4-fold change in 100%) are listed in Table 3.

Separation of well and poorly differentiated intrahepatic CCC by 2-dimensional cluster analysis

For subclassification of intrahepatic CCC in relation to histopathological differentiation (grading) a weighted two-dimensional clustering of the tumor samples was performed. All expressed genes/ESTs in the tumor samples (43.3%, approx. 9650 genes/ESTs) were grouped according to the histological grading ranging from 1 to 3 and then ranked according to their significantly differential expression values. Differentiation of the gene expression profiles of tumor samples, according to the histopathological grading of 1 and 3, identified a total of 136 dysregulated genes ($P < 0.05$ and two-fold change upregulation or downregulation in ≥ 70), which were used for a weighted two-dimensional cluster analysis (Figure 3). Interestingly, upregulation of specific cell surface antigens such as cytokeratins (for example, cytokeratin 6, 7, 13 and 15) and specific membrane proteins (such as EMP1, EVA1 and proteoglycan 2) was detected in well differentiated tumor samples (G1, G2) when compared with more dedifferentiated tumor samples. In contrast, G3-tumor samples showed a relative overexpression of genes

involved in G-protein signaling and genes involved in transcription (Table 4).

Results of RT-PCR for detection of dysregulated OPN expression

The most consistently overexpressed gene (median fold change 33.5) in all analyzed intrahepatic CCC samples was that encoding the glycoprotein OPN.

To validate this observation the expression levels of OPN were confirmed by quantitative RT-PCR in 16 tissue samples (malignant and corresponding non-malignant tissue from 8 patients) using the LightCycler[®] system. In general, the changes in OPN expression levels detected by microarray reflected the results obtained by RT-PCR. However, the dynamic range of real-time PCR results was greater than that of the microarray data (Figure 4).

DISCUSSION

The poor response of cholangiocarcinoma to therapy, along with the concerning worldwide increase in morbidity and mortality^[4,5], highlights the need for increased efforts to understand the etiology and pathogenesis of this primary liver cancer.

Previous studies have examined the potential role of known genes involved in human carcinogenesis to elucidate the molecular pathways involved in cholangiocellular carcinoma, for example, members of the type I family of growth factor receptors (EGF-R, c-erbB-2)^[14-16], (proto-) oncogenes (c-met, c-Ki-ras, Cyclin D)^[17-19], dysregulated tumor-suppressor genes (p53, pRB, p16INK4a, p21*WAF1*)^[20,21], and apoptosis-related genes (bcl2, FAS-L, BAX)^[22,23]. Other studies have shown microsatellite instability and loss of heterogeneity (LOH) as contributing factors in the development of CCC^[24,25].

Excepting the actual data there are only two other reports using gene expression analysis to detect dysregulated genes and prognostic markers in cholangiocarcinogenesis^[26,27]. The present study identified a high number of differentially expressed genes and ESTs in CCC (43.6 vs 39.01%, $P > 0.05$). Based on a relatively conservative arbitrarily chosen algorithm, a gene expression profile of 552 dysregulated genes (221 genes upregulated, 331 genes downregulated) for CCC was generated. This profile was validated based on a neuronal training method by its ability to differentiate the CCC profile from the expression profiles of corresponding and non-corresponding non-malignant liver tissues. This method achieved a fast and reproducible differentiation between CCC and non-malignant liver tissue in all samples, as well as between CCC and other malignant tissues (HCC, liver metastases; data not shown). One tumor sample (No. 7) also showed characteristics of non-malignant liver tissue in that the tissue expressed a number of genes that were more frequently expressed in non-diseased human liver than in CCC samples. The sample was attributed to the non-malignant tissue group in two-dimensional hierarchical clustering. A potential reason for this could be a putative contamination of tumor sample with normal liver tissue, especially owing to the fact that no microdissection of tumor tissue was used. However, using a supervised neuronal training method to

Table 3 Most significantly dysregulated genes (> 4-fold change in 100% of all cases) in intrahepatic cholangiocarcinoma - relation to known cellular functions and metabolic pathways (using the GenMAPP® software)

Swiss prot	GenMAPP ID	Gene ID	Dysregulated in %	Name	fc inc (median)	fc dec (median)
Cell adhesion (cell-cell/cell-matrix)-extracellular matrix						
OSTP_HUMAN	P10451	SPP1	100	Osteopontin precursor (Bone sialoprotein 1/SPP-1 1)	33.5	
Q13443	Q13443	ADAM9	100	A disintegrin and metalloproteinase domain 9 (meltrin γ)	10.4	
TYBO_HUMAN	P13472	TMSB10	100	Thymosin beta-10	5.3	
ITA6_HUMAN	P23229	ITGA6	100	Integrin alpha-6 precursor	4.5	
Signal transduction - G protein signaling						
CD24_HUMAN	P25063	CD24	100	Signal transducer CD24 precursor	11.8	
IQG1_HUMAN	P46940	IQGAP1	100	Ras GTPase-activating-like protein IQGAP1	9.1	
ECT2_HUMAN	Q9H8V3	ECT2	100	ECT2 protein (Epithelial cell transforming 2 oncogene)	6.3	
MAC5_HUMAN	P29966	MAC5	100	Myristolated alanine-rich C-kinase substrate (MARCKS)	4.6	
Q9BUV5	Q9BUV5	INSIG1	100	Similar to insulin induced gene 1		5
Cell cycle - DNA replication						
RIR2_HUMAN	P31350	RRM2	100	Ribonucleoside-diphosphate reductase M2 chain	16.2	
S111_HUMAN	P31949	S100A11	100	Calgizzarin (S100C protein)	15.5	
O00496	O00496	IPL	100	Tumor suppressing subtransferable candidate 3	14.1	
S106_HUMAN	P06703	S100A6	100	Calcyclin (Growth factor inducible protein 2A9)	6.9	
Transcription						
SOX9_HUMAN	P48436	SOX9	100	Transcription factor SOX9	4.1	
NRI3_HUMAN	Q14994	NR1/3	100	Orphan nuclear receptor NR1/3		6.8
Cell motility - cytoskeleton						
SPT2_HUMAN	O53291	SPINT2	100	Kunitz-type protease inhibitor 2 precursor (Hepatocyte growth factor activator inhibitor type 2)	5.8	
Amino acid metabolism						
BHMT_HUMAN	Q93088	BHMT	100	Betaine-homocysteine S-methyltransferase		48.5
METL_HUMAN	Q00266	MAT1A	100	S-adenosylmethionine synthetase alpha and beta forms		38.5
ATTY_HUMAN	P17735	TAT	100	Tyrosine aminotransferase		27.0
SPYA_HUMAN	P21549	AGXT	100	Serine-pyruvate aminotransferase		22.0
HUTH_HUMAN	P42357	HAL	100	Histidine ammonia-lyase		16.7
CGL_HUMAN	P32929	CTH	100	Cystathione gamma-lyase (Gamma-Cystathionase)		9.9
GLSL_HUMAN	Q9UI32	GA	100	Glutaminase, liver isoform, mitochondrial precursor		9.5
GAMT_HUMAN	Q14353	GAMT	100	Guanidinoacetate N-methyltransferase		8.4
SDHL_HUMAN	P20132	SDS	100	L-serine dehydratase		7.1
Carbohydrate/monosaccharide metabolism/catabolism						
KPY2_HUMAN	P14786	PKM2	100	Pyruvate kinase, M2 isozyme	26.7	
ALFA_HUMAN	P04075	ALDOA	100	Fructose biphosphate aldolase A (Lung cancer antigen)	16.3	
PPCC_HUMAN	Q16822	PCK1	100	Phosphoenolpyruvate carboxykinase, cytosolic		16.7
Q9H277	Q9H277	Q9H227	100	Cytosolic beta-glucosidase		7.6
Electron transport						
ACDB_HUMAN	P45954	ACADSB	100	Acyl-CoA dehydrogenase, branched chain specific		14.1
CPA6_HUMAN	X13929	CYP2A6	100	Cytochrome P450 2A6		11.9
CP12_HUMAN	P05177	CYP1A2	100	Cytochrome P450 1A2		10.0
Protein metabolism/proteolysis						
BAE2_HUMAN	Q9Y5Z0	BACE2	100	Beta secretase 2 precursor	10.5	
Q9UJ28	Q9UJ28	GALNT7	100	UDP-GalNAc: N-acetylgalactosaminyltransferase 7	5.4	
CATC_HUMAN	P53634	CTSC	100	Dipeptidyl-peptidase I precursor	4.7	
GLMT_HUMAN	Q14749	GNMT	100	Glycine N-methyltransferase		49.5
Transport						
CHLR_HUMAN	P17516	AKR1C4	100	Chlordecone reductase		22.0
APF_HUMAN	Q13790	APOF	100	Apolipoprotein F precursor		15.5
Lipid binding/metabolism						
Q9Y2P5	Q9Y2P5	Q9Y2P5	100	Very-long-chain AcylCoA synthetase homolog 2		23.7
VLCS_HUMAN	O14975	FACVL1	100	Very-long-chain AcylCoA synthetase		16.6
MMSA_HUMAN	Q02252	MMSDH	100	Methylmalonate-semialdehyde dehydrogenase		8.1

reanalyze all of the obtained array data, correct assignment to malignant and non-malignant tissue groups was possible for all samples (including No. 7).

Previous studies have shown a correlation between differentially expressed genes and tumor progression or tumor dedifferentiation in various human tumors^[28,29]. In the present study we used two-dimensional cluster

analysis with all expressed genes in the tumor samples (> 9000 genes/ESTs) for the detection of statistically significantly differentially expressed genes in relation to tumor differentiation (grading). This method detected 136 differentially expressed genes when tumor samples with low (G1) and high (G3) grading were compared. Because of the small number of analyzed tumor

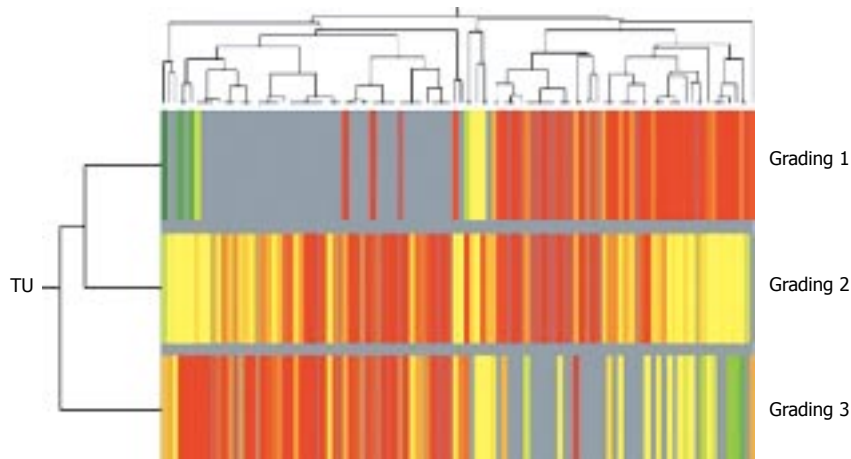


Figure 3 Subclassification of intrahepatic CCC using two-dimensional cluster analysis of 136 different regulated genes/ESTs (dysregulation in more than 70% of all probes, fold change of genetic expression > 2.0; Pearson's correlation) in relation to histopathological findings (well to poor differentiated tumor tissue). Horizontal lines show all dysregulated genes in one singular tumor tissue probe, vertical lines show the expression of one gene in all analyzed G1-, G2- and G3-tumors. Overexpressed genes are coloured red, downregulated genes are shown in green).

Table 4 Expression levels of dysregulated genes in intrahepatic cholangiocarcinoma in relation to tumor differentiation according histopathological criteria (grading 1 to 3)

Probe set	Grading 1	Grading 2	Grading 3	Gene function
204857_at	3.09	4.14 (2.9-5.8)	No data	Hs. MAD1 (mitotic arrest deficient, yeast, homolog)-like 1
211571_s_at	3.49	0.98 (0.7-1.3)	1.411	mRNA for proteoglycan PG-M (V3), proteoglycan 2
209016_s_at	3.93	1.25 (0.3-6.7)	0.96 (0.92-1.0)	Hs. similar to keratin 7 (KRT7)
201325_s_at	4.27	4.5 (3.0-6.7)	1.08	Hs. epithelial membrane protein 1 (EMP1), mRNA
203779_s_at	5	No data	No data	Hs. epithelial V-like antigen 1 (EVA1), mRNA
211796	7.82	1.53 (1.1-1.9)	1.16	Hs. T cell receptor beta chain (TCRBV13S1-TCRBJ2S1) mRNA
204734_at	7.97	8.5 (6.3-11.4)	No data	Hs. keratin 15 (KRT15), mRNA
209126_x_at	10.26	8.254	No data	Hs. keratin 6 isoform K6f (KRT6F) mRNA
207935_s_at	19.94	10.4	No data	Hs. keratin 13 (KRT13), mRNA
217109_at	22.75	No data	No data	Hs. partial mRNA for sv7-MUC4 apomucin
205157_s_at	34.79	10.36	No data	Hs. keratin 17 (KRT17), mRNA
209070_s_at	No data	3.188	7.54 (6.9-8.2)	Hs. regulator of G-protein signaling 5, mRNA
204338_s_at	No data	18.98	128.5	Hs. regulator of G-protein signaling 4 (RGS4), mRNA
203638_s_at	No data	1.14 (0.7-2.3)	4.16 (2.7-6.4)	Hs. FGF receptor 2, keratinocyte growth factor receptor
208343_s_at	No data	1.36 (1.1-1.8)	2.45 (1.2-4.9)	Hs. CYP7A promoter binding factor mRNA, nuclear receptor 5
207846_at	No data	No data	1.414	Hs. POU domain, class 1, transcription factor 1 (Pit1)

No data: No significant (< 2-fold change) or consistent (< 70% of all cases) change in the expression level of the gene in this subgroup of CCC (G1-, G2- or G3-tumors).

samples (one highly differentiated tumor (G1) among 10 samples) it was not possible to create an unambiguous clear correlation between all dysregulated genes and the histopathologic grading. However, an obvious trend towards a higher expression of specific cell surface proteins (EMP1, EVA1, proteoglycan2) and intermediate filaments (cytokeratin 6, 7, 13, 15, 17) in well-differentiated tumors was observed, whereas samples of high-grade intrahepatic CCC had an elevated expression of genes involved in G-protein signaling and nuclear transcription. Pathological alterations to the chemical composition, molecular structure, or spatial arrangement of the liver matrix will ultimately lead to specific changes in the intermediate filament pattern in human cholangiocytes. It can be assumed that the dedifferentiation of tumor cells in CCC results in pathological alterations of cell surface antigens, cell-to-cell and cell-to-matrix attachment, followed by a switch from physiological to pathological cell-activation. More investigations are needed to elucidate the role of dysregulated genes in tumor progression with histomorphological tumor dedifferentiation in cholangiocarcinogenesis.

To further validate the microarray data, real-time PCR

was performed. The gene encoding OPN was identified as the highest and most consistently overexpressed gene (33.5-fold change) in all analyzed tumor samples. The expression profile determined by real-time PCR correlated well with the resulting ratios carried out with the gene expression data of the analyzed CCC. However, as has been shown previously for other similar comparisons^[30,31], the dynamic range of real-time PCR was about 1.5-fold to 10-fold higher than that of array analysis.

Using oligonucleotide arrays we were able to identify genes already known to be dysregulated in other human cancers^[32,33]. However, a significant number of the genes identified to show a significant alteration in their expression values, have not been known to be involved in cholangiocarcinogenesis until now. By incorporating the expression data into GenMAPP software, a specific pattern of signaling and metabolic pathways as well as changes in cell cycle regulators specific for CCC could be generated. For example, changes in apoptosis-regulating genes consistent with the decreased cell death rate typical of tumor cells were identified. Generally, the changes in cell cycle metabolism are well in line with a pattern of increased cell growth. However, the major focus of this

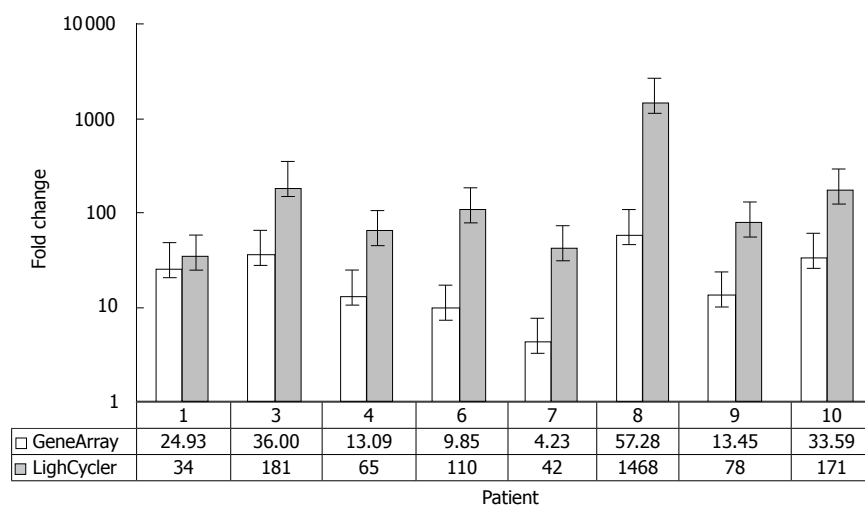


Figure 4 Comparison between expression values of osteopontin in 8 intrahepatic CCCs estimated by RT-PCR (LightCycler®System; grey) and gene expression data (HU 133A, Affymetrix; white). The detected changes in osteopontin expression levels measured by RT-PCR reflected very well the changes in gene expression between tumor and non-malignant liver tissue obtained by microarray analysis. The results of RT-PCR revealed larger changes than the microarray data in all cases.

study was to establish a specific genetic profile of CCC for differentiation against non-malignant liver tissue and other malignant liver tumors (HCC, metastases) for clinical routine. This approach intentionally did not take into consideration the different origins of the analyzed tissue probes (tumorous epithelium cells *vs* non-malignant transformed hepatocytes). Thus, it remains a possibility that our observations of the downregulation of genes involved in specific metabolic pathways, such as amino acid metabolism, may be a consequence of the different origin of malignant and non-malignant tissue rather than a specific feature of cholangiocarcinogenesis. Nevertheless, our own unpublished data revealed similarities between the genetic profiles of other malignant liver tumors (HCC, metastases of adenocarcinomas) and CCC with a downregulation of hepatocyte-specific metabolic functions and an upregulation of genes well known to be involved in human carcinogenesis.

The gene encoding OPN, a secreted adhesive glycoprotein was identified as the most consistently overexpressed gene in all CCC samples (see above). OPN has been shown to be overexpressed in excessive amounts in a variety of human carcinomas and its expression level has been linked to the recurrence of metastasis or to clinical progression^[34-36].

OPN is not a typical oncogene; it is mutationally activated in cancer cells, but it is regulated by a variety of stimuli, such as the Wnt/Tcf signaling pathway, steroid receptors, growth factors, TNF-alpha and transcription factors, such as AP-1 and Ras proto-oncogene^[37]. OPN is a ligand of CD44, binds to alphaV-containing integrins and has an important role in malignant cell attachment and tumor invasion^[38,39]. Potential mechanisms of OPN in human carcinogenesis include the regulation of different signaling cascades through activation of various kinases^[40]. Thus, OPN seems to be a potent anti-apoptotic factor *via* inhibition of caspase 3 activation^[41]; it also induces increased activation of promatrix metalloproteinase-2 (pro-MMP2, mediated by nuclear factor kappaB) and secretion of urokinase-type plasminogen activator^[42].

Using gene expression profiling, OPN has been shown to be one of the highest overexpressed genes in hepatocellular carcinoma (HCC) and it has been shown

the expression of OPN correlates with earlier recurrence, poorer prognosis and metastasis in HCC^[43,44]. To the best of our knowledge, there is only one report on OPN in human cholangiocarcinogenesis^[45]. Interestingly, Terashi *et al* observed a correlation between low OPN levels and tumor aggressiveness, whereas another group found high levels of the glycoprotein in CCC in a rat model system^[46]. Therefore, it remains to be shown whether OPN is a potential prognostic marker and target for anticancer treatment in CCC.

In conclusion, the present study represents one of the first attempts to establish a gene expression pattern of intrahepatic CCC by genetic comprehensive analysis. Based on the analysis of a subgroup of 364 genes, a specific pattern of changes in metabolic and signaling pathways, as well as receptors, cell cycle changes and alterations in apoptosis could be identified. The observed changes are consistent with some of the known characteristics of CCCs in particular and tumors in general. The gene expression profile appears to be useful as a diagnostic tool, especially in terms of differentiation from other liver masses as well as for the subclassification of intrahepatic CCC in comparison to histopathological findings.

Conclusively, besides a number of known overexpressed tumor-related genes in intrahepatic CCC, we describe here for the first time other strongly and consistently dysregulated genes in cholangiocarcinogenesis, which are well known to be involved in other human cancers. Although our data at present do not support a correlation between the expression of these genes and tumor stage for CCC, this gene expression pattern is in line with the usually observed bad clinical prognosis of this cancer. Another evolving application based on the profile described in this report and the observation of OPN as the most consistently overexpressed gene might be the identification of novel therapeutic targets and related genes predicting survival and outcomes of therapeutic modalities.

COMMENTS

Background

Cholangiocarcinoma (CCC) is the second most common primary hepatic malignancy with increasing incidence in western countries. Despite recent progress

in various imaging modalities, intrahepatic CCC is in most cases advanced by the time of diagnosis. Aggravating circumstances leading to a poor prognosis with median survival times of only three to six months are the limited curative treatment options by surgical resection and the low effect of palliative chemotherapy in CCC. Therefore a better understanding of human cholangiocarcinogenesis is strongly needed to establish new diagnostic markers and therapeutic approaches.

Research frontiers

Despite the advances in genomic profiling of many human malignancies in recent years, there have only been a few reports of gene expression analysis in CCC. In this study, oligonucleotide arrays were used to detect differentially expressed genes and to establish a specific genetic profile of intrahepatic CCC. To subclassify between well and poorly differentiated tumors, additional analyses were performed.

Innovations and breakthroughs

A total of 41 genes that were strongly deregulated (upregulated or downregulated in 100%, $fc > 4$) in CCC were detected by gene expression analysis. For the first time, a subclassification of intrahepatic CCC in relation to malignant differentiation using oligonucleotide arrays was performed. The gene encoding osteopontin (OPN) was the most overexpressed gene with a high fold-change of 33.5, which was confirmed by RT-PCR.

Applications

Besides a number of known overexpressed tumor-related genes in intrahepatic CCC, other strong and consistently dysregulated genes were described for the first time in human cholangiocarcinogenesis. Another evolving application based on the profile described in this report and the observation of OPN as the most consistently overexpressed gene might be the identification of novel therapeutic targets and related genes predicting survival and outcomes of therapeutic modalities.

Peer review

The manuscript by Hass *et al* describes a microarray analysis of genetic changes in human cholangiocarcinoma samples compared to non-tumor tissue. The analysis revealed very intriguing data with respect to the overexpression of a number of genes, the most consistent of which is OPN, and further analysis with respect to tumor grade was performed.

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