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An expression signature of phenotypic resistance to hepatocellular carcinoma identified by cross-species gene expression analysis

Maddalena Frau¹, Maria M. Simile¹, Maria L. Tomasi¹, Maria I. Demartis¹, Lucia Daino¹, Maria A. Seddaiu¹, Stefania Brozzetti², Claudio F. Feo³, Giovanni Massarelli⁴, Giuliana Solinas¹, Francesco Feo¹, Ju-Seog Lee⁵, and Rosa M. Pascale¹

¹Department of Clinical and Experimental Medicine, Division of Experimental Pathology and Oncology, University of Sassari, Sassari, Italy

²Department of Surgery “Pietro Valdoni”, University of Rome “La Sapienza”, Rome, Italy

³Department of Clinical and Experimental Medicine, Division of Surgery, University of Sassari, Sassari, Italy

⁴Department of Clinical and Experimental Medicine and Oncology, Division of Morbid Anatomy, University of Sassari, Sassari, Italy

⁵MD Anderson Cancer Center, University of Texas, Huston, TX, USA

Abstract

Background and aims—Hepatocarcinogenesis is under polygenic control. We analyzed gene expression patterns of dysplastic liver nodules (DNs) and hepatocellular carcinomas (HCCs) chemically-induced in F344 and BN rats, respectively susceptible and resistant to hepatocarcinogenesis.

Methods—Expression profiles were performed by microarray and validated by quantitative RT-PCR and Western blot.

Results—Cluster analysis revealed two distinctive gene expression patterns, the first of which included normal liver of both strains and BN nodules, and the second one F344 nodules and HCC of both strains. We identified a signature predicting DN and HCC progression, characterized by highest expression of oncosuppressors *Csmd1*, *Dmbt1*, *Dusp1*, and *Gnmt*, in DN, and *Bhmt*, *Dmbt1*, *Dusp1*, *Gadd45g*, *Gnmt*, *Napsa*, *Pp2ca*, and *Ptpn13* in HCCs of resistant rats. Integrated gene expression data revealed highest expression of proliferation-related *CTGF*, *c-MYC*, and *PCNA*, and lowest expression of *BHMT*, *DMBT1*, *DUSP1*, *GADD45g*, and *GNMT*, in more aggressive rat and human HCC. *BHMT*, *DUSP1*, and *GADD45g* expression predicted patients' survival.

Conclusions—Our results disclose, for the first time, a major role of oncosuppressor genes as effectors of genetic resistance to hepatocarcinogenesis. Comparative functional genomic analysis

allowed discovering an evolutionarily conserved gene expression signature discriminating HCC with different propensity to progression in rat and human.

Keywords

Hepatocarcinogenesis; Genetic predisposition; Gene expression profiling; Oncosuppressor genes; Prognostic markers

1 Introduction

Numerous low-penetrance genes control inherited predisposition to rodent and human HCC [1]. Genetically resistant BN rats, subjected to diethylnitrosamine/2-acetylaminofluorene/partial hepatectomy treatments of “resistant hepatocyte” protocol [2], display lower incidence of slow proliferating DN and HCCs than susceptible F344 rats [3]. Accordingly, cell cycle, iNos/IKK/NF- κ B axis, Ras/Erk signaling, and Mybl2 are strongly deregulated in DN and HCCs in F344 rats, but undergo smaller/no changes in BN rats [3–5]. Interestingly, analogous alterations of cell cycle and signaling pathways occur in human HCC, with a subtype with better prognosis (based on survival length; HCCB), and a subtype with poorer prognosis (HCCA) resembling HCC of resistant and susceptible rats, respectively [5,6].

The use of microarray technologies to evaluate global gene expression in HCC provides a snapshot of the transcriptional state of healthy or diseased tissue, to identify common aberrant molecular pathways involved in human hepatocarcinogenesis, molecular signature of metastases, molecular subclasses of HCC, prediction of HCC outcome [7–9]. The studies of gene expression profiles during rat liver carcinogenesis are scanty. Analysis of liver tissue, containing early and persistent liver nodules and HCC, showed the dysregulation of numerous genes potentially linked to progression [10]. Recently, a cluster of 1,308 differentially expressed genes versus normal liver was identified in persistent preneoplastic lesions of F344 rats, whereas remodeling lesions exhibited only 156 differently expressed genes [11].

In the present research we evaluated gene expression profiles of DN and HCC, chemically induced in rats with different susceptibility to hepatocarcinogenesis, to identify gene expression traits affected by inherited predisposition to HCC and molecular events, linked to the progression and prognosis of preneoplastic and neoplastic lesions, contributing to determine a phenotype resistant to hepatocarcinogenesis. We also comparatively evaluated gene expression data sets from rat and human HCC to identify gene expression signatures reflecting similar phenotypes during HCC progression in the two species, and to enhance the possibility, by integrating independent data sets, to identify key regulatory elements during HCC progression.

2 Materials and methods

2.1 Animals and treatments

F344 and BN rats were fed, housed, and treated according to “resistant hepatocyte” protocol [2]. Rats were killed by bleeding through thoracic aorta, under ether anaesthesia. DN (32 weeks) and HCCs (45–50 and 60–67 weeks for F344 and BN rats, respectively) were

collected. Histological (HE staining), histochemical (silver staining of reticulin) and immunohistochemical (glutamine synthase immunostaining) criteria were used, in addition to morphology, to classify liver lesions according to the published criteria [12–14].

2.2 Human tissue samples

Five normal livers and 60 HCCs were used. Supplementary Table S1 shows patients' clinicopathologic features. Liver tissues were archival surgical samples kept at -80°C immediately after collecting, provided by the Departments of Surgery of the University "La Sapienza" of Roma, and the University of Sassari, Italy. Informed patients' consent and Institutional Review Board approval was obtained at these Departments.

2.3 Microarray analysis

Total RNA from 4 rat livers, 8 pools of 5–10 DNs/rat, and 10 single HCCs, from each strain, was reversed, fluorescently labeled with Cy3 or Cy5, and hybridized. Fluorescence intensities of spots were normalized to average intensity of housekeeping genes. Genes with expression ratio at least 2-fold different from that of reference samples were selected. Hierarchical cluster analysis was performed as described [15].

2.4 Quantitative real-time reverse-transcription polymerase chain reaction

Quantitative Real-time RT-PCR (qPCR) reactions and quantitative evaluations were as published [4].

2.5 Western blot analysis

Hepatic tissue samples were processed as reported, and membranes were probed with specific primary antibodies [3].

2.6 Statistical analysis

Differences between means of qPCR and Western determinations were analyzed by Tukey-Kramer or Mann-Whitney tests, and correlation coefficients (R) were determined by multiple regression analysis. Overall survival was estimated according to Kaplan-Meier and Log-rank (Mantel-Cox) test. Survival predictivity was estimated by Cox method. Microarray results were analyzed by parametric Student's t-test and the False Discovery Rate (FDR). $P < 0.05$ was considered significant.

Additional methodological information is available in "Supplementary Materials and Methods".

3 Results

3.1 General findings

Thirty-two weeks after initiation, 160–370 DNs were collected from 8 F344 and BN rats (15–50 DNs/rat). DNs from single rats were pooled and split in half. One half was processed for morphological analysis, and the other half for microarray analysis (cf. Supplementary Materials and Methods). Histologic analysis showed that all DNs of F344 and BN rats were high-grade and low-grade nonremodeling lesions, respectively (Supplementary Figure S1).

This was considered, bona fide, to basically reflect the situation of the correspondent half of DN pools used for microarray analysis. High-grade DNs exhibited prevalently small hepatocytes with high nuclear: cytoplasmic ratio, hepatocytes in nests or pseudogland formation, and cytoplasmic basophilia, low-grade DNs were constituted by clear-cell/eosinophilic hepatocytes (Figure S1). These features are close similar to those of analogous lesions of human liver [13]. Moreover, similarly to human DNs, rat DNs were distinguished from HCC for the presence of a preserved reticulin fibers array, absent in HCC, and for the absence of the early HCC marker glutamine synthase [14], which instead was present in the cytoplasm on HCC cells (Figure S1). Forty-five-50 weeks after initiation, 8 moderately differentiated (ES grade III) and 2 poorly differentiated HCC (ES grade IV) were collected from F344 rats. Three well-differentiated (ES grade I) and 7 moderately differentiated HCC (ES grade II/III) were collected at 60–67 weeks from BN rats.

3.2 Identification of deregulated genes in DN and HCC

Gene expression profiles showed 105 upregulated and 94 downregulated genes in F344 HCC (threshold cut-off: 2; $P < 0.0001$; Table S2), with respect to normal liver, 70% of which were also upregulated, and 46% were downregulated in F344 DN, respectively. Most genes involved in signal transduction and HCC progression were upregulated in DN and/or HCC, whereas tumor growth inhibitors *Gadd45g*, *Gnmt*, *Dusp1*, and *Dmbt1*, were downregulated in DN and/or HCC of F344 rats. In HCC of BN rats 126 genes were upregulated and 88 were downregulated (threshold cut-off: 2; $P < 0.0001$; Table S3). Upregulated genes included different growth-related and signal transduction genes. Notably, growth inhibitors such as *Dmbt1*, *Dusp1*, *Fath1*, *Gadd45g*, *Gnmt*, *Klf6*, and *Pp2ca* were upregulated in BN nodules and/or HCC. Changes in gene expression regarded 23 upregulated and 26 downregulated genes in BN nodules, only 55% of which were also deregulated in BN HCC.

3.3 Unsupervised hierarchical clustering of gene expression reveals two distinct classes of DN in rats with different susceptibility

Evaluation of gene expression profiles of F344 normal liver versus BN liver showed about 2-fold higher expression of *Gng10* and *Rapgef2* in BN than F344 rat liver ($P < 0.001$; $n=4$), and 2.2–3.1 fold higher expression of *Cyp7b1*, *Decr1* and *Gsta2* in F344 liver ($P < 0.001$, $n=04$). This indicates the existence of close similar expression patterns between F344 and BN normal livers. Therefore, direct interstrain comparison of DN and HCC expression profiles was made, using BN normal liver as a reference.

Unsupervised hierarchical cluster analysis of gene expression data from normal livers, DN, and HCC of F344 and BN rats revealed two distinctive gene expression patterns, the first of which included normal liver of F344 and BN rats and DN of BN rats, and the second one DN of F344 rats, and HCC of both strains (Fig. 1). When the data were analyzed using high statistical stringency, expression of 91 DN genes was significantly different between BN and F344 rats ($P < 0.001$; Figure S2), whereas no interstrain difference in expression of these genes occurred in normal livers. Sixty-nine known genes, among the 91 annotated genes differentially expressed, exhibited a wide range of functions according to GeneOntology database (Table S4). Most genes involved in “metabolic process”, “response to stimulus”, “response to xenobiotics”, “oxidative stress”, “signal transduction”, and “cell proliferation”

were more expressed in F344 than BN DN, whereas oncosuppressors and cytochrome P450 isoforms were more expressed in BN than F344 DN. Interstrain comparison for gene expression in HCC showed significant differences for 55 genes ($P < 0.001$; Figure S3), most of which, involved in “metabolic process”, “cell proliferation” and “signal transduction”, and all oncosuppressors were more expressed in BN than F344 HCC (Table S5). Only ~20% of genes differently expressed with respect to normal liver exhibited a uniform behavior in DN and HCC of BN rats, whereas about 70% of genes showed a uniform pattern in F344 rat lesions. The BN/F344 expression ratios of genes differently expressed in DN and HCC (Tables S4 and S5), were plausible with the expression ratios of these lesions compared to correspondent normal livers in each strain, (Tables S2 and S3), supporting the validity of interstrain comparative analysis.

3.4 Cell proliferation and cell survival genes

Since a striking difference between DN and HCC developing in susceptible and resistant rats concerns their capacity to progress [3,5] we selected, on the basis of a public database search, genes differently expressed in nodules and HCCs, more specifically related to cell proliferation and cell survival. 70% of cell cycle- and cell proliferation-related genes, and all genes related to cell differentiation, oxidative stress, and ubiquitination were significantly more expressed in DN of F344 than BN rats (Table 1). In contrast, tumor growth inhibitors, including *Gnmt*, *Csmd1*, *Dmbt1*, and *Dusp1*, were more expressed in DN of BN than F344 rats. Remarkably, in BN HCC highest expression of numerous cell cycle- and cell proliferation-related genes, was associated with a highest expression of tumor growth inhibitors *Bhmt*, *Dmbt1*, *Dusp1*, *Gadd45g*, *Gnmt*, *Napsa*, *Pp2ca*, and *Ptpn13* (Table 2).

3.5 Validation of microarray analysis

Expression of 9 randomly chosen genes, involved in cell proliferation and cell survival, was made by qPCR on 6 normal livers, 15 DN and 14 HCCs, including the lesions used for microarray analysis, from each strain. These samples consisted of low-grade and high-grade DN of F344 and BN rats, respectively, 3 poorly-differentiated (ES grade IV) and 11 moderately-differentiated HCCs (ES grade II/III) of F344 rats, and 3 well-differentiated (ES grade I) and 11 moderately-differentiated (ES grade II/III) BN HCC. qPCR analysis showed relatively low variance of liver lesions from both strains, supporting a low heterogeneity of rat liver lesions, and roughly confirmed the results of microarray analysis (Fig. 2a). Upregulation of *Anxa5*, *c-Myc*, *Igfbp3*, *Ctgf*, and *Igfbp1* occurred in DN and HCC from both strains, with highest values in F344 rat lesions. *Bhmt* downregulation occurred in DN and HCC from both strains, with significantly lower values in HCC of F344 than BN liver. A progressive decrease in *Gnmt*, *Dusp1*, and *Dmbt1* expression from normal liver to DN and HCC of F344 rats, contrasted with significantly lower decrease in *Gnmt*, and sharp increase in *Dusp1* and *Dmbt1* in BN rat lesions. The validity of microarray analysis was further supported by a close correlation of expression data of qPCR and microarray analyses of *c-Myc* and *Dusp1* expression (Fig. 2b). Western blot analysis of *c-Myc*, *Gnmt*, *Bhmt*, *Dusp1*, and *Pp2A* proteins, performed on 5 HCCs of both strains (ES grade III), reproduced the results of qPCR and/or microarray analyses (Fig. 2c).

3.6 Comparison of gene expression pattern of subclasses of rat and human liver lesions

We next examined the possible value of the rat model of hepatocarcinogenesis to assess the significance of a susceptible/resistant phenotype for human disease. We thus performed a comparative functional genomic approach [7,11] by integrated analysis of 28, 25, and 35 human non-tumor surrounding liver (SL), HCCB and HCCA, respectively, and the rat liver lesions. This approach is based on the hypothesis that due to the maintenance of regulatory elements in evolutionarily related species, gene expression traits related to similar phenotypes could be conserved in different species [7]. In favor of this hypothesis, numerous studies demonstrated that cross-comparison of gene expression data of human and rodent tumors can identify aberrant phenotypes reproducing the evolutionarily conserved signaling pathways [11,16–18]. A higher number of human lesions were used for this analysis, due to their high heterogeneity (Table S1) with respect to relatively homogeneous rat lesions.

Unsupervised hierarchical analysis of 6,132 genes, common to rat and human liver (Fig. 3a) showed two distinct subtypes of gene expression patterns. Human liver tissues were separated into subgroups previously recognized (nontumor, HCCA and HCCB) [15]. BN nodules and HCCs, and F344 DNs clustered with HCCB, while all F344 DNs and HCCs clustered with HCCA (Fig. 3a). The expression signatures of rat lesions were well stratified with respect to those of human HCC, confirming the existence of low heterogeneity of rat lesions, and excluding the fortuity of differences between strains and lesion types. In line with clustering of rat liver lesions, proliferation and apoptosis indices of lesions were about 2-fold lower and 2-fold higher in BN than F344 lesions, respectively (Fig. 3b, c). Analogous differences in proliferation and apoptosis indices exist between HCCB and HCCA [6]. Notably, the analysis of HCCA:HCCB expression ratios showed differences, between human HCC subtypes, analogous to those found between BN and F344 rat HCC for *BHMT*, *DMBT1*, *DUSP1*, *GADD45g*, *GNMT*, *CTGF*, *c-MYC*, and *PCNA* genes (Table 2). To confirm these data, qPCR analysis of these genes was performed in human HCC, used for the microarray analysis, and the results were integrated with clinicopathological features. Two clinically relevant distinct subgroups, showing different degrees of downregulation of *BHMT*, *DMBT1*, *DUSP1*, *GADD45g*, and *GNMT* genes, inhibitory of HCC development, and of upregulation of *CTGF*, *c-MYC*, and *PCNA* genes, favoring HCC growth (Fig. 4a). Highest downregulation of inhibitory genes and upregulation of growth-favoring genes was associated with significantly shorter patients' survival (Fig. 4b). Cox' analysis of the predictivity of patients' survival, on the basis of gene expression levels, showed significant predictivity for *BHMT* (hazard ratio: 0.0143, 95% CI: 0.021–0.097, $P=0.046$), *DUSP1* (hazard ratio: 4.767, 95% CI: 1.293–17.568, $P=0.019$), and *GADD45g* (hazard ratio: 0.0996, 95% CI: 0.012–0.81; $P=0.032$).

4 Discussion

Expression profiling revealed, in accordance with previous work [10,11], upregulation of genes involved in response to oxidative stress, cell proliferation, angiogenesis, and signal transduction, and downregulation of oncosuppressor genes, in DN and HCC of F344 rats. Upregulation of few proliferation-related genes in slowly progressing DN of BN rats

supports the hypothesis that resistant rat HCCs derive from rare nodules overcoming the block of progression by still unknown mechanisms, thus explaining low HCC incidence and multiplicity in BN rats [3]. Accordingly, interstrain comparison of DN and HCC expression traits allowed identification of two different DN subtypes, in resistant and susceptible rats, recognizable by differences in expression of relatively few genes involved in cell proliferation and survival. More expressed in F344 than BN nodules were: *c-Myc*, whose amplification is critical for progression of rat [5] and human [19] DN and HCC, *Ctgf*, *Akap9*, *Klf12*, and *Bzrp*, implicated in HCC aggressivity, *Gpx2*, *Nqo1*, *Ddit4l* protecting against oxidative stress, *Enc1*, a downstream β -Catenin target, and *Igfbp3*. *Igfbp3* may be bidirectional in terms of tumor behavior: it may inhibit cell proliferation and induce apoptosis, or activate proliferation-related pathways and inhibit apoptosis in different experimental conditions [20]. Only few proliferation-related genes were more expressed in BN than F344 DN. They include *G0s2* that favors G0-G1 progression [21] whose effect, however, may be contrasted by upregulation of *p16INK4a* [3] and WAF/KIP family, *p130*, and *RASSF1A* cell cycle inhibitors [22] in BN lesions.

Differently from DN, gene expression features of HCC showed interstrain overlapping, especially for PKC and MAPK signaling genes. Nevertheless, implication of partially different signaling pathways in HCC of genetically resistant and susceptible rats is suggested by higher expression of growth-related *Pdffa* and its receptors, *Prkci*, *Rin3*, *Cxcl12*, *Fgfr*, *Lcn2* and *Pld1*, in BN than F344 HCC, whereas higher *Ctgf*, *Igfbp1*, *Igfbp3*, *Pcna*, *c-Myc* and *P2ry2* expression occurred in F344 than in BN tumors. Further, our data suggest, for the first time, that genes inhibiting crucial steps of the signal transduction network and cell cycle (Figure S4) are involved in the determination of resistant phenotype. Tumor growth inhibitors such as *Csmd1* [23], *Gnmt* [24], *Dusp1* [25], and *Dmbt1* [26] were more expressed in BN than F344 DNs, and *Bhmt* [27], *Gnmt*, *Dusp1*, *Dmbt1*, *Gadd45g*, [28], *Napsa* [29], *Ptpn13*, [30], and *Pp2A* [31] catalytic subunit α , were more expressed in BN than F344 carcinomas. *Gnmt* and *Bhmt* are involved in the maintenance of high hepatocyte S-adenosylmethionine (SAM) level which controls hepatocyte growth by interfering with DNA methylation, genomic stability, and gene expression [24]. SAM accumulation, consequent to *Gnmt* knockdown, and SAM depletion consequent to downregulation of *Bhmt* and *Mat1A* genes are two conditions associated with fast HCC progression [24,27]. *GNMT* and *DMBT1* are considered susceptibility genes for liver [32] and mammary [33] tumors, respectively. Rat *Dmbt1* is located at chromosome 1q37, in the *Hcs3* susceptibility locus controlling rat hepatocarcinogenesis [1]. These findings and the upregulation of cell cycle inhibitors, in BN rat lesions [22], strongly suggest a role of oncosuppressors in the determination of the resistant phenotype.

Interstrain differences in DNA synthesis, apoptosis, cell cycle activity, and expression of several cell survival and cell death genes only occur in autonomously growing DNs and HCCs, being absent in early preneoplastic foci of F344 and BN rat liver [3–6,22]. These observations rule out the possibility that interstrain differences in expression of proliferation-related genes, in DN and HCC, are linked to hepatocarcinogenesis initiation. Nevertheless, it cannot be excluded that gene expression signatures partly reflect interstrain differences in developmental stage, and loss of liver function in liver lesions. This, however,

is not the case of HCC, because the majority of tumors from both strains, used for microarray and qPCR analyses, and all HCC examined by Western blot were moderately differentiated, ES grade III tumors. Differences in gene expression could instead reflect interstrain differences in the progression capacity of preneoplastic and neoplastic lesions, as indicated by late development of moderately-differentiated HCC and absence of poorly-differentiated HCC in BN rats.

A major challenge of the comparison of animal models with human tumors is represented by the different conditions for tumor induction. However, to meet this challenge, previous research established the usefulness of comparative functional genomics to evaluate rodent models for human liver cancer [7,11,16] and other cancers [17,18], indicating that molecular pathways associated with specific cancer phenotypes are evolutionarily conserved [7]. Accordingly, a body of evidence indicates that the upregulation of growth factor receptors, MAPK, IKK/NF- κ B, JAK/STAT, WNT/FZD, and Pi3K/AKT signaling pathways, and cell cycle key genes, and the downregulation of cell cycle inhibitors, are correlated with the progression of both human and rodent HCC prognostic subtypes [reviewed in 5, 34–36].

In keeping with these observations, integrated cross-comparison of human HCC with rat HCC, performed here, identified a gene signature, discriminating rat and human lesion subtypes differently prone to progression, including upregulation of *CTGF*, *c-MYC*, and *PCNA*, and downregulation of *BHMT*, *DMBT1*, *DUSP1*, *GADD45g*, and *GNMT*. qPCR analysis of expression of these genes in human HCC identified clinically relevant distinct prognostic subgroups of human HCCs. Furthermore, *BHMT*, *DUSP1*, and *GADD45g* were identified as predictors of patients' survival. Various observations support the connection between the deregulation of most of above genes and HCC progression. According to recent results, *CTGF* downregulation blocks HCC cross-talk with the stroma and HCC progression [37]. *c-MYC* silencing inhibits the growth of human and rat HCC cell lines [38] and is associated with remodeling of preneoplastic lesions [39]. Functional experiments indicate that in vitro growth of HCC cells is reduced by *DUSP1* overexpression, and enhanced by *DUSP1* inhibition [25], and *GADD45g* transfection in HepG2 cells causes G2/M arrest through induction of P38 and JNK kinase pathways [28]. Impaired expression of *GNMT* and *BHMT* has been found in the pre-neoplastic cirrhotic liver and in human HCC tissues [40]. In agreement with this we observed a decrease in *Gnmt* and *Bhmt* mRNA levels in F344 DN and HCC and BN HCC. These findings suggest that impairment of *GNMT* and expression in preneoplastic lesions may favor HCC development. Accordingly, *GNMT* knockdown in mice leads to HCC development [24]. *BHMT* is involved in SAM synthesis [27]. SAM inhibits HCC development [24]. However, the effects of manipulation of BHMT levels on HCC cell proliferation are unknown and await further evaluation. *DMBT1* is one of the putative suppressor genes which frequently lacks expression in different kind of tumors, such as glioblastoma, and lung, esophageal, colorectal, prostatic, and breast cancer [26,41]. Low *DMBT1* expression occurs in intrahepatic cholangiocarcinoma [42] and, according to present results, in HCC. However, the oncosuppressor activity of *DMBT1* for HCC has not been demonstrated as yet.

At present few expression studies analyzed the role of gene expression signatures on overall survival after surgical HCC resection. Two prognostic subgroups with different survival

were identified by a 406-gene expression signature [15], and a patients subgroup with short survival was characterized by a 111-Met regulated genes signature [43]. A gene expression profile resembling that of fetal hepatoblasts allowed identifying a patients subgroup with particularly short survival [44]. In a recent study, a 186-gene expression profile failed to yield a significant association with survival of HCC patients, whereas profiles of the non-tumorous SL were highly correlated with survival [45]. Patients populations in this study had early HCC, whereas other studies discovering outcome-predicting tumor-derived signatures [15,43,44] analyzed patients which tended to have more advanced disease. It was hypothesized [45] that late recurrence, typical of small early HCC [46], could result from new primary tumors arising in a damaged SL rather than from the proliferation of residual cells derived from original tumor [45]. These observations emphasize the importance of outcome-predicting signatures derived from early stages of hepatocarcinogenesis. In our rat model a signature characterized by higher expression of cell proliferation genes and lower expression of oncosuppressors discriminated fast-proliferating and progressing DN from slowly-proliferating DN, unable to progress to HCC. Few studies focused on the molecular signature of human DN. A 240-gene signature was reported to discriminate low-grade DN, high grade DN and grade 1 HCC in HBV patients [47]. A study on liver lesions from HCV cirrhotic patients, led to the identification of 12 genes differently expressed between early HCCs and DN [47]. A 3-gene set including *GPC3*, *LYVE1*, and *Survivin* had elevated discriminative accuracy [48]. Functional analysis identified the *MYC* oncogene as a plausible driver gene for malignant conversion of human DN [19], in accordance with observations on rat DN [38 and present results], indicating that the deregulation of *MYC* driven signaling underlies the progression of DN and HCC in humans and rodents.

In conclusion, our results disclose, for the first time, a major role of oncosuppressor genes as effectors of genetic resistance to hepatocarcinogenesis. Furthermore, by applying comparative functional genomic analysis, we discovered an evolutionarily conserved gene expression signature discriminating HCC phenotypes with different propensity to progression in rat and human, and suggest that the comparative rat model of hepatocarcinogenesis may help identifying prognostic subgroups of human HCC and novel putative prognostic markers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Anxa5	Annexin5
Bhmt	Betaine-homocysteine methyltransferase

BN	Brown Norway
Bzrp	Benzodiazepine receptor, peripheral
Cxcl12	Chemokine, cxc motif, ligand 12
Ctgf	Connective tissue growth factor
Csmd1	CUB and SUSHI multiple domain protein 1
Cyp7B1	Cytochrome P450 7B1
Decr1	2,4-dienoyl CoA reductase 1
DN	Dysplastic nodule
Dmbt1	Deleted in malignant brain tumors 1
Dusp1	Dual specificity phosphatase 1
Enc1	Ectodermal-neural cortex 1
ERK	Extracellular signal-regulated kinase
F344	Fisher 344
Fath1	Fat tumor suppressor homologue 1
Fgfr	Fibroblast growth factor receptor
FoxM1	Forkhead box M1B
G0s2	G0-G1 switch gene 2
Gadd45b	Growth arrest and DNA-damage-inducible-
Gadd45g	Gadd45- γ
Gnmt	Glycine N-methyltransferase
Gng10 G	protein gamma 10
Gpx2	Glutathione peroxidase 2
Gsta2	Glutathione-S-transferase, alpha2
HCC	Hepatocellular carcinoma
HCCA	HCC with poorer prognosis
HCCB	HCC with better prognosis
Igfbp	Insulin-like growth factor binding protein
Klf6	Kruppel-like suppressor 6
Lcn2	Lipocalin 2
Mat1A	Methyl adenosyltransferase 1 ^o
Napsa	Napsin A
Nqo1	NAD(P)H dehydrogenase, quinone 1

P2ry2	Purinergic receptor P2Y
Pcna	Proliferating cell nuclear antigen
Pckdbp	Protein kinase δ binding protein
Pdgf-, α	Platelet-derived growth factor- α
Pp2A	Protein phosphatase 2 $^{\circ}$
Prkci	Protein kinase C, ι
Ptpn13	Protein tyrosine phosphatase, non-receptor, type 13
qPCR	Quantitative real-time reverse-transcription polymerase chain reaction
Rin3	Ras and Rab interactor 3
Rapgef2	Rap guanine nucleotide exchange factor 2
SAM	S-adenosylmethionine

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Fig. 1. Unsupervised hierarchical cluster analysis of gene expression patterns of rat liver tissues. Microarray experiments with 4 normal livers, 8 DN, and 10 HCCs, per each strain, were made with Agilent Rat 4130A or B. Out of 22,575 gene features, 1,362 gene features, showing more than 2-fold difference compared to median expression value in more than 4 arrays, were selected for cluster analysis. Expression values were Log 2 transformed before clustering. Rows represent individual genes and columns represent each tissue,

Fig. 2.

(a) qPCR analysis of *Anxa5*, *Myc*, *Igfbp3*, *Gnmt*, *Ctgf*, *Igfbp1*, *Bhmt*, *Dmbt1*, and *Dusp1* expression in normal liver (C), dysplastic nodules (N) and HCC (H) of F344 and BN rats. Results are means $\pm$ SD of 6 normal livers, 15 DNs, and 14 HCCs per each strain of differences between target gene and RNR-18 expression, named Number Target (NT). $NT = 2^{-Ct}$, $Ct = (CT(target) - CT(RNR-18))$. Tukey-Kramer test: N and H vs. C, BN vs. F344, at least $P < 0.05$ for all genes tested. (b) Correlation analysis of *c-Myc* and *Dusp1* expression determined by microarray and quantitative RTPCR, on 4 normal livers, 8 DNs and 10 HCCs per each strain. (c) Left panel: representative Western blot of *c-Myc*, *Gnmt*, *Bhmt*, *Dusp1*, and *Pp2A*. Right panel: Optical densities of the peaks normalized to β -actin values and expressed in arbitrary units. Data are means $\pm$ SD of 3 normal livers, 5 DNs, and 5 moderately-differentiated HCCs from F344 and BN rats. Tukey-Kramer test: (*) N and H vs. C, at least $P < 0.05$. (†) F344 vs. BN, $P < 0.001$

Fig. 3.

(a) Unsupervised hierarchical cluster analysis of integrated 26 human surrounding non-tumorous liver, 25 HCCB, and 35 HCCA, and 36 rat liver lesions. Orthologous genes with an expression ratio differing at least 2 from the reference in one of the data sets were selected for hierarchical analysis (6,132 genes). (b) Labeling index (percentage of 2-bromo-3-deoxyuridine incorporating nuclei; LI) and apoptotic index (percentage of apoptotic bodies; AI) of DN and HCC of F344 and BN rats. 3,000 hepatocytes per lesion were counted, and results are means $\pm$ SD of 8 DN, and 10 HCCs per each strain. (c) Left panels: representative propidium iodide staining of HCC from F344 and BN rats. Arrows indicate nuclear changes representing apoptosis. Right panel: percentage of apoptotic cells in HCC (AI). Data are means $\pm$ SD of 5 HCC per each strain. Tukey-Kramer test: (*) BN versus F344, $P < 0.001$ for LI and AI of both hematoxylin/eosin- and propidium iodide stained sections.

Fig. 4.

(a) qPCR analysis of *BHMT*, *DMBT1*, *DUSP1*, *GADD45*, *GNMT*, *CTGF*, *c-MYC*, and *PCNA* expression in human HCC with poorer (A) and better (B) prognosis. Results are differences between Number Target (NT) of HCC and mean value of 5 normal livers. $NT = 2 - Ct$, $Ct = (CT(target) - CT(RNR-18))$. Box plots show the dispersion of the results of 35 HCCA and 25 HCCB, Negative values on y-axes indicate decrease with respect to normal liver. Mann-Whitney U-test: HCCA vs HCCB, $P < 0.0001$ for all genes tested. (b) Kaplan-Meier survival plots of distinct subgroups of human HCC identified by integrated gene expression data. Mantel-Cox statistical analysis: differences between survival plots, $P < 0.0001$.

Table 1BN:F344 expression ratio of proliferation-related genes in liver and dysplastic nodule^a

Gene symbol	Fold change^b	Parametric P value
Angiogenesis		
Crp	2.418	0.0009200
Signal transduction and cell proliferation		
G0s2	3.226	0.0005702
Ogn	2.244	0.0006244
Akap9	0.408	0.0007998
Anxa5	0.329	0.0001257
Bzrp	0.236	0.0000772
Ctgf	0.253	0.0005681
Igfbp1	0.164	0.0005096
Igfbp3	0.193	0.0005136
Kif12c	0.271	0.0005952
Myc	0.282	0.0004274
Pcna	0.526	0.0004472
Tumor suppression		
Csmd1	3.728	0.0003821
Dmbt1	4.872	0.0003829
Dusp1	3.699	0.0004473
Gnmt	4.366	0.0000336
Cell differentiation		
Enc1	0.418	0.000415
Oxidative stress		
Ddit4l	0.326	0.0005055
Gclm	0.175	0.0002086
Gpx2	0.200	0.0000745
Nqo1	0.152	0.0005225
Response to xenobiotics		
Gstm1	0.280	0.0004301
Yc2	0.117	0.0285714
Ubiquitination		
Ubd	0.304	0.0285714

^aFold change: BN:F344 expression ratio.^bCf. Supplementary Table S4 for FDR values, Unigene cluster, and gene description.

Table 2BN: F344 expression ratio of proliferation-related genes in HCC^a

Gene symbol	Fold change^b	Parametric P value
Angiogenesis		
Cd74	2.625	0.0008470
Signal transduction and cell proliferation		
Cxcl12	2.739	9.23E-05
Fgfr2	3.086	0.0001610
Fgfr3	3.112	0.0006580
Lcn2	6.327	0.0001370
Pdgfa	2.669	0.0004882
Pdgfra	2.042	0.0002280
Pdgfrb	3.543	0.0027180
Pld1	3.576	2.02E-05
Prkci	2.943	0.0018750
Rin3	3.668	0.0065990
Timp2	3.478	0.0004776
Anxa5	0.174	0.0007729
Ctgf ^c	0.553	0.0008840
Kif12	0.271	0.0005950
Igfbp1	0.059	2.14E-05
Igfbp3	0.059	2.14E-05
Myc ^c	0.192	0.0003327
Pcna ^c	0.562	0.0003670
P2ry2	0.439	0.0006650
Oxidative stress		
Nfe2l2	2.658	0.0004460
Tumor suppression		
Bhmt ^c	2.502	0.0006456
Dmbt1 ^c	18.607	1.42E-05
Dusp1 ^c	3.303	0.0008400
Gadd45g ^c	3.339	0.0003570
Gnmt ^c	3.477	0.0007309
Napsa	4.161	0.0001218
Pp2ca	2.249	0.0003772
Ptpn13	8.024	1.52E-05

^a Fold change: BN:F344 expression ratio.^b Cf. Supplementary Table S4 for FDR values, Unigene cluster, and gene description.

^cBN:F344 ratio concurrent with HCCB:HCCA expression ratio found by integrated gene expression analysis

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