Central Role of c-Myc during Malignant Conversion in Human Hepatocarcinogenesis

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Abstract

Hepatocarcinogenesis is a multistage process in which precursor lesions progress into early hepatocellular carcinomas (eHCC) by sequential accumulation of multiple genetic and epigenetic alterations. To decode the molecular events during early stages of liver carcinogenesis, we performed gene expression profiling on cirrhotic (regenerative) and dysplastic nodules (DN), as well as eHCC. Although considerable heterogeneity was observed at the regenerative and dysplastic stages, overall, 460 differentially expressed genes were detected between DN and eHCC. Functional analysis of the significant gene set identified the MYC oncogene as a plausible driver gene for malignant conversion of the DNs. In addition, gene set enrichment analysis revealed global activation of the MYC up-regulated gene set in eHCC versus dysplasia. Presence of the MYC signature significantly correlated with increased expression of CSN5, as well as with higher overall transcription rate of genes located in the 8q chromosome region. Furthermore, a classifier constructed from MYC target genes could robustly discriminate eHCC from high-grade and low-grade DNs. In conclusion, our study identified unique expression patterns associated with the transition of high-grade DNs. In addition, the disease (8). Nevertheless, the exact sequence of the molecular events leading to malignant transformation in the preneoplastic hepatic lesions is yet to be discovered, and little is known about the possible involvement of different oncogenic pathways in this process.

In the current study, we analyzed gene expression differences between the consecutive stages of hepatocarcinogenesis to identify common regulatory mechanisms orchestrating malignant transformation. High-density microarrays were used to obtain expression profiles of regenerative LGDNs and HGDNs, as well as early HCCs (eHCC). Furthermore, a comparative functional genomics approach was applied to assess the transcriptional status of different oncogene-activated expression signatures. Our data show that induction of MYC target genes occurred ubiquitously during malignant conversion. Moreover, a genomic predictor constructed from cross-species conserved MYC-induced genes was able to accurately differentiate HGDN from eHCC with high accuracy. The predictor set was further validated using an independent set of DNs and eHCCs. This large-scale genomic profiling is the first study to uncover the potentially critical role of the MYC transcription signature activation in the malignant conversion of preneoplastic lesions in human hepatocarcinogenesis.

Materials and Methods

Collection of human DNs and eHCC samples. We collected biopsies of 49 nodular liver lesions, including 24 cirrhotic (regenerative) nodules (CN), 3 LGDNs, 12 HGDNs, and 10 eHCCs, which were located in explanted livers of 10 patients. The freshly removed livers were serially sectioned with 1-cm intervals, and any macroscopically identified nodules were dissected out and split in half. Tissue collected for histologic analysis was fixed in 6% neutral formalin and processed following standard procedures. The other half was immediately snap-frozen in liquid nitrogen–cooled isopentane, embedded in OCT, and stored at −80°C. Histologic classification of the lesions was determined by two expert pathologists (L.L. and T.R.), who evaluated each slide according to criteria established by the International Working Party (5). Microvascular invasion was considered present when tumor cells could be identified inside of the lumen. The clinical characteristics of lesions are summarized in Supplementary Table S1. The majority of the lesions arose in male patients (8 of 10) on ethanol-induced (4 of 10) cirrhotic background; other tumors were associated with chronic HCV nodules (LGDN) or high-grade DNs (HGDN; diameter < 10 mm; ref. 5). However, diagnosis of the small lesions is difficult, and the probability of malignant transformation could not be clearly determined (6). With the advancement of modern imaging techniques, an increasing number of atypical nodular lesions are encountered in cirrhotic patients, leading to a significant diagnostic and therapeutic challenge (7). Over the recent years, considerable effort has been made to decipher the molecular events of early hepatocarcinogenesis and to discover novel diagnostic markers specific for each of the consecutive phases of the disease (8).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/). Data deposition footnote: GEO accession number GSE12443.
(3 of 10) or HBV (2 of 10) hepatitis, and one case was associated with α1-antitrypsin deficiency. Four patients also underwent preoperative chemolipidization. The average size of LGDN and HGDN was 5.3 ± 1.5 and 9.3 ± 3.3 mm, respectively (P = 0.018), and the diameter of HCC was 15.6 ± 8.5 mm (P = 0.035 versus HGDN; Supplementary Fig. S1). The rate of cell proliferation progressively increased from LGDN to eHCC, although the difference in the number of MB-1–positive cells between eHCC and HGDN did not reach statistical significance (Supplementary Fig. S1). Vascular invasion with intrahepatic metastases was observed in one eHCC, whereas four HGDNs showed bilirubin and one HGDN showed iron pigment accumulation (Supplementary Table S1).

**RNA isolation, amplification, and microarray hybridization.** The nodular lesions were identified and manually macrodissected. From each lesion, 5 to 10 frozen sections (8-μm thick) were cut and lyzed immediately in RNA extraction buffer. Total RNA isolation, together with DNase digestion, was performed with RNA Easy Mini kit (Qiagen). Integrity of RNA was checked with gel electrophoresis. Total RNA (1 μg) was reverse-transcribed and linearly amplified with Aminoallyl MessageAmp II kit (Ambion). Aminoallyl-UTP–modified aRNA product (2 μg) was indirectly labeled with either Cy3 or Cy5 fluorescent dyes. Human Operon v2 oligonucleotide library containing 22K features representing expressed sequences was printed to glass arrays in the Advanced Technology Center (National Cancer Institute). aRNA probes were fragmented and hybridized to microarray slides, following standard procedures. All samples were hybridized against a common amplified reference RNA pooled from normal liver samples. Experimental duplicates were prepared following a reverse flour design. Arrays were scanned with a GenePix 4000B laser scanner, and image analysis was performed with GenePixPro v5 suite.

**Quantitative PCR analysis.** Real-time PCR quantification of mRNA levels of selected markers, HSPA1A, GPC3, FOXA1, HDAC2, MYC, and CSN5, was performed on total RNA samples isolated from the same lesions that were used for expression profiling. All ready-to-use human primer sets were obtained from SuperArray. The real-time quantitative PCR (Q-PCR) analysis was performed with ABI Prism 7900HT (Applied Biosystems) Thermal Cycler in a 96-well plate. Melting analysis of the PCR products was conducted to validate amplification of the specific product. Expression level of human glyceraldehyde-3-phosphate dehydrogenase was used as internal reference. Relative gene expression levels were calculated with 2^−ΔΔCt method (9).

**Tissue culture and small interfering RNA knockdown.** Three CSN5–specific small interfering RNAs (siRNA; CSN5-1, CSN5-2, and CSN5-3) were designed and tested for growth inhibition in HuH-7 and HepG2 cell lines. siRNA (15 nmol/L) were complexed with cationic lipids and added to 3 × 10^3 cells per well in 96-well plates. siRNA treatment was the most effective in blocking both HuH-7 and HepG2 cell proliferation (68 ± 6.0% and 77 ± 5.1%, respectively) at 4 d after transfection, as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Negative-control siRNA induced no significant inhibition of HuH-7 and HepG2 cell growth. Real-time reverse transcription–PCR analysis confirmed that treatment of HuH-7 cells with 15 nmol/L of CSN5-1, CSN5-2, and CSN5-3 siRNAs for 24 h resulted in 73 ± 5.1%, 80 ± 8.2%, and 74 ± 8.1% reduction of target mRNA, respectively, when compared with negative-control siRNA treatment. CSN5 mRNA expression in HepG2 cells at 24 h was inhibited by 45 ± 10.1%, 46 ± 9.7%, and 51 ± 14.3%, respectively. CSN5-2 siRNA was selected for further studies.

**Microarray data analysis.** First image spots with a diameter of <10 or >300 μm or a signal intensity below background intensity for any of the two fluorochrome channels were excluded. Only genes with at least four data points of six experiments in at least two experimental groups were selected for further data analysis. Gene expression values were normalized by microarray scaling and cross-validation performed for each spot, target, and reference intensity ratios were log transformed and averaged between duplicate experiments. Differentially expressed genes between histologic predefined samples groups were identified by performing a two-sample t test with a random variance model to estimate false discovery rate. Selection criteria for individual genes included a significance level of P < 0.001 in the univariate t test. Hierarchical cluster analysis based on Pearson correlation distance was performed with Cluster v2.11, and results were visualized with TreeView programs. For comparative genomic analysis, we selected genes represented on both the mouse and the human platform using curated mammalian orthologues from The Jackson Laboratory. Common genes between human and mouse oncogene-specific signatures and early neoplastic data set were matched by Locus Link ID. Nonparametric gene set enrichment analysis (GSEA) was performed with the GSEA tool developed by Broad Institute. Rank metrics were determined by performing 1,000 random permutations. We tested five class prediction algorithms, including compound covariate predictor, nearest neighbor, nearest centroid, support vector machines, and linear discriminator analysis from BRB-Array Tools to differentiate between eHCC and DN using mouse–derived MYC signature genes. The training set contained 10 eHCCs and 12 DN from our preneoplastic set; the independent validation set included 14 HBV-related eHCCs and 14 DN. To build an optimized classifier list, which could estimate the probability of the identity of a particular sample, we used a leave-one-out cross-validation approach. During the cross-validation step 1, sample was removed from the analysis and the remaining samples were used to identify the most differentially expressed genes between the groups. Based on the expression of these genes, identity of the left out sample was predicted with a given algorithm. This process was repeated until each sample was left out once. The number of genes in the classifier was varied to provide the highest correct prediction rate in the training set. To estimate accuracy of the prediction model, class labels were randomly permuted and the leave-one-out cross-validation process was repeated 1,000 times. The significance level is the proportion of random permutations that gave a cross-validated error rate no greater than the cross-validated error rate obtained with the real data.

**Results**

**Changes in global gene expression profiles during early hepatocarcinogenesis.** We performed detailed transcriptomic profiling of 49 liver lesions, including 24 CNs, 3 LGDNs, and 12 HGDNs, as well as 10 eHCCs. RNA, isolated from macrodissected lesions, was subjected to linear amplification and indirect fluorescent labeling and hybridized to oligonucleotide microarrays containing ~22,000 features representing ~19,000 unique tags. After data normalization, 12,994 features with at least 10 valid expression values across all samples were selected for further analysis. To assess the overall expression differences between the consecutive stages of early hepatocarcinogenesis, we performed hierarchical clustering with 1,632 genes showing at least a 2-fold regulation in >10% of the samples. This analysis revealed that most of eHCCs, with exception of two misclassified tumors, clustered together and could be readily differentiated from the remaining samples based on their distinctive expression patterns (Supplementary Fig. S2 and B). On the other hand, profiles from dysplastic and regenerative stages displayed considerable heterogeneity and were often closer to eHCC rather than to normal livers. Interestingly, the median variance of the expression levels calculated for the 12,994 genes was only slightly higher in the HCC (0.91) than in the dysplastic (0.08) and regenerative nodules (0.062). Also, there were no substantial differences between groups in the number of genes showing 2-fold regulations relative to normal liver reference (CN 231, DN 234, eHCC 267), suggesting that transcriptomic differences across the early stages were relatively small.

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3 http://rana.lbl.gov/EisenSoftware.html
4 http://jax.org/
5 http://oicr.org/
6 http://linus.nci.nih.gov/BRB-ArrayTools.html
Stage-related expression patterns identify early markers of neoplastic progression. To evaluate the presence of unique expression patterns associated with the consecutive stages of HCC formation, we performed a supervised class comparison analysis by classifying samples according to their histology. We focused primarily on two major transition points: (a) from regenerative nodule to LGDN and HGDN and (b) from dysplastic lesions to eHCC. Significant genes ($P < 0.001$) were selected using a two-sample $t$ test with a random variance model. Comparison of LGDN and HGDN with the regenerative nodules yielded 44 and 41 differentially expressed genes, respectively (Fig. 1A). Thirty-four of these genes had lower expression levels whereas 51 genes had higher expression levels in dysplastic livers compared with cirrhotic livers (Supplementary Table S2A). Functional classification of the significant gene sets based on Gene Ontology annotations showed that dysplastic lesions were characterized by overrepresentation of genes involved in cell-cell interactions ($ITGB4$, $CYR61$, $MADCAM1$, $DSG2$) and possessing electron transporter activity ($NDUFA1$, $RP11-217H1$, $NCF1$, $PDCL$, $PLOD1$), reaching observed/expected ratios (O/E) of 7.52 and 8.17, respectively (Supplementary Table S3A).

Figure 1. Expression patterns of genes differentially expressed between the consecutive stages of early human hepatocarcinogenesis. A, two-dimensional heat map represents profiles from 85 genes with significantly different expression levels between CNs and DNs. B, similar heat maps were constructed from the 460 genes differentially expressed between the dysplastic lesions and eHCCs. Progression of early hepatocarcinogenesis coincides with increase in the transcriptomic alterations between the consecutive stages. Genes were selected by setting the cutoff level in a univariate $t$ test at $P < 0.001$. Columns, individual samples; rows, genes. Each cell reflects the log$_{2}$-transformed expression level of an individual gene in a given sample, as indicated by the color code on the figure. The probability of having the same number of genes by chance was also determined with multivariate test after 1,000 random permutations and marked in the legends. C, gene connectivity network centered on the MYC transcription factor is identified by the PathwayAssist tool. Genes differentially expressed between the dysplastic lesions and eHCCs (Supplementary Table S2) were used to detect the dominant regulatory networks during malignant transformation. Although MYC itself is not differentially expressed (gray) between the two histologic categories, 48 of its immediate targets are either significantly more (red) or less expressed (green) in eHCC than in liver dysplasia. Gray arrows with blue rectangle, known transcriptional activation; purple arrows, direct binding between the connected nodes.
Transformation of DN into eHCC was marked by even more pronounced transcriptomic alterations (Fig. 1B). Class comparison identified 144 significantly different genes between LGDN and eHCC and 282 genes between HGDN and eHCC, with 34 genes being commonly significant in both comparisons. The differentially expressed set contained up-regulated (210) and down-regulated (248) genes in approximately equal abundance (Supplementary Table S2B). From the structural Gene Ontology categories (Supplementary Table S3B), actin cytoskeleton (ARPC3, ENAH, TMSB10, CCT3, PARVB, PLS1, ACTL6A, WASL; O/E, 2.35), microtubule (KPN2A, TUBA1B, TUBG1, BIRC5, TUBB3; O/E, 7.29), and ribosome (DAP3, RPL24, NPM1, MRPL12, MRPL13, MRPL15, MRPL18; O/E, 7.11)–related clusters were the most prominently deregulated, and the majority of these genes were more expressed in eHCC. The functional classification found overrepresentation of genes encompassing enzymes with monooxygenase activity (CYP51A1, CYP2C8, CYP4A11, CYP1A2, CYP4V2, CYP2C9; O/E, 5.09) or implicated in cholesterol, nucleotide, and ribonucleotide biosynthesis (Supplementary Table S3B).

To confirm the microarray results by an independent method and to identify sensitive early markers of malignant conversion in hepatocytic lesions, we validated expression levels of four differentially expressed genes (HSPA1A, GPC3, FOXM1, and HDAC2) by Q-PCR analysis (Supplementary Fig. S3A–D). The data obtained with microarray and PCR techniques were highly concordant, and all selected markers showed significantly (P < 0.05, Student’s t test) increased expression in eHCC versus dysplastic stage.

Pathway and gene set enrichment analyses commonly implicate MYC transcription factor as a potential regulator of malignant conversion. To reveal the dominant intracellular signaling networks in the malignant conversion of dysplastic liver lesions, we performed pathway analysis using 460 genes differentially expressed between DN and carcinomas. Using the PathwayAssist Analysis tool, several connectivity maps were constructed based on the previously reported interactions between members of the significant gene set. The analysis outlined an extensive regulatory network centered on the MYC gene (Fig. 1C). Overrepresentation of MYC targets among differentially expressed transcripts also favors that MYC activation might be essential for transition to eHCC.

To validate the regulatory role of MYC during malignant conversion, we further interrogated our human preneoplastic data set using independent MYC signatures. Previously, we obtained a prominent liver-specific MYC expression signature in a transgenic mouse model (10). In addition, we analyzed MYC signatures derived from human umbilical vascular endothelial cells (HUVEC) (11) and human breast epithelial cells (also including RAS target genes; ref. 12). Specificity of the many MYC target genes identified

Figure 2. Enrichment analysis of the MYC expression signatures in the eHCCs. Four MYC-regulated gene sets (two up-regulated and two down-regulated) were identified in MYC transgenic mice and human endothelial cells transfected with an adenosine-MYC vector. Heat maps representing gene expression in the early hepatocarcinogenesis data set (LEC) revealed that both the HUVEC-derived (A) and mouse liver-derived (B) MYC signatures show increased activation in the eHCC samples versus DN. The liver-specific MYC signature displayed similar activation (C) in an independent data set containing dysplastic lesions and early carcinomas from HBV-infected patients. Columns, samples; rows, log2-transformed expression ratios of individual genes, as defined by the color code on the figure. Colored bars, genes with significant core enrichment (orange). Up-regulation (pink) and down-regulation (dark blue) of the gene in the original signatures. Distribution and ES of the rank-ordered MYC up-regulated genes in HUVEC, LEC, and HBV-related data sets. Both up-regulated gene sets are significantly enriched in the eHCC compared with the DN.
in the HUVECs were validated with microarray, Q-PCR, and chromatin immunoprecipitation assays (11), whereas breast epithelial signature was successfully applied for the classification of human lung, breast, and ovarian carcinomas (12). Based on the list of curated homologous mouse and human UniGene clusters, we matched corresponding features from the different platforms. Next, the expression of six orthologous oncogene-specific signatures, including the MYC up-regulated and down-regulated gene sets identified in transgenic mice and in HUVECs, as well as RAS up-regulated and down-regulated gene sets detected in the breast epithelial cells, was assessed with GSEA (13). This nonparametric method calculates enrichment of a signature in a group of samples based on the location of its members in the rank-ordered gene list. We found that both HUVEC-derived [enrichment score (ES) = 0.5, \( P < 0.03 \)] and liver-specific (ES = 0.4, \( P < 0.05 \)) MYC up-regulated gene sets were significantly enriched in the eHCC versus the dysplastic samples (Fig. 2A and B). In contrast, neither MYC down-regulated sets (Supplementary Fig. S4A–C) nor RAS up-regulated genes (ES = 0.12, \( P < 1 \)) showed enrichment in any group (Supplementary Table S4A). Family-wise error rates \( P \) values and normalized ES (NES) calculated in the multivariate analysis also suggested enrichment of the MYC up-regulated sets in the carcinomas (mouse NES = 1.46, \( P < 0.15 \); HUVEC NES = 1.53, \( P < 0.08 \), although they did not reach significance probably due to the small size of the gene sets. In contrast, we could not observe apparent enrichment of any of the down-regulated sets. Furthermore, none of the oncogene-dependent signatures displayed enrichment when GSEA was performed to compare dysplastic versus regenerative nodules (data not shown). Together, these results argue convincingly that activation of MYC target genes has a decisive role in the malignant transformation of dysplastic lesions.

**MYC up-regulated gene expression signature can distinguish eHCC from dysplastic lesions.** To test the universal significance of our findings, we performed GSEA on an independent group of 14 DN and 14 eHC (grades 1 and 2) samples developed on a background of chronic HBV hepatitis (Fig. 2C). After overlapping the gene sets, we verified that MYC up-regulated gene sets were indeed enriched in eHCC versus hepatic dysplasia. However, the transgenic mouse liver–derived specific signature (ES = 0.47, \( P < 0.05 \)) displayed the most significant enrichment (Supplementary Table S4B).

We also tested whether the MYC signature genes could differentiate between hepatic dysplasia and eHCC in a prediction model. Because mouse liver–specific signature showed the most consistent enrichment pattern across all early neoplastic data sets, we used these genes to construct a MYC-dependent classifier. Five different supervised prediction algorithms, including compound covariate predictor, nearest neighbor, nearest centroid, support vector machines, and linear discriminant analysis methods were used to build a prediction model, following a leave-one-out cross-validation strategy. The training set included 10 eHC and 12 DN from our collection. The optimal classifier, producing the highest correct classification rate in the training set, contained 36 liver-specific MYC target genes (Supplementary Table S5A). Among the classifier genes, ribosomal structural proteins were most abundant (O/E = 3.2). The gene list could achieve 82% to 86% correct classification rate in the training set, depending on the algorithm (Supplementary Table S5B). We also applied the same classifier to the validation set of 14 DN and 14 HC (grades 1 and 2) associated with chronic HBV hepatitis. The prediction rates were somewhat lower in the validation set, but the compound covariate predictor (85%) and support vector machine (79%) could successfully classify eHCs from DN (Supplementary Table S5C).

These results not only confirmed the almost universal activation of the MYC signature at the eHCC stage but also strongly indicate that the MYC-driven expression signature could be used successfully for early cancer diagnosis.

**Figure 3.** Correlation of MYC signature with CSN5 expression and 8q amplification score. A, expression levels of CSN5 in human preneoplastic liver lesions and eHCC showed a nearly linear correlation (Pearson \( r = 0.7 \), \( P < 0.0001 \)) with a MYC signature score. A signature score for each sample was calculated from the correlation between expression of signature genes and canonical MYC signature. B, heat map shows the expression profile of the down-regulated genes (\( n = 83 \), \( P < 0.01 \)) by CSN5 knockdown treatment between CN, DN, and eHCC. Columns, mean; bars, SE (*, \( P < 0.001 \), two-sample Student’s \( t \) test).
The exact mechanism leading to aberrant activation of MYC and its target genes remains undetermined. In previous studies, frequent DNA copy number aberrations were identified in many cancers, including HCC (3). Therefore, we tested whether MYC activation in HCC was due to the copy number gain. The effect of regional copy number changes can be inferred by calculating the regional concordance of gene expressions. In this context, we calculated the gene expression differences between DN and eHCC for each cytoband. A total of 30 differentially expressed cytobands were identified ($P < 0.001$, two-sample $t$ test; Supplementary Table S6). As expected, many of the known regions with copy number alterations in HCCs (e.g., 1q21-22,6p21, 8q22-24, 11q11-14, 19q13, 20p12; Supplementary Table S6) were differentially expressed between DNs and eHCCs. In particular, 1q21-22 and 8q22-24 were overexpressed in eHCCs compared with DNs (Supplementary Table S6). The "early stage" copy number gains, especially 8q24, including MYC gene, may play a critical role in the malignant conversion of DN.

Although the mutation status of MYC is involved in cancer development and progression, no mutations in MYC genes were found in the 10 eHCC samples by sequencing analysis (data not shown). However, a recent study has revealed that CSN5, a member of COP9 signalosome, is linked to MYC activation and wound healing signature in breast carcinomas (14). We also observed that CSN5 expression was significantly correlated with the average expression level of the MYC signature ($\gamma = 0.72$, $P < 1 \times 10^{-5}$, Pearson's correlation test) implying its regulatory role in MYC activation (Fig. 3A). Thus, we examined the association of CSN5 with the malignant conversion of DN. We generated CSN5 regulating signature by inhibiting CSN5 gene expression using siRNA technology. The human HCC cell line HuH-7 was transfected with the CSN5 siRNA, and the differentially expressed genes were identified by gene expression profiling (for details, see Materials and Methods). Eighty-three genes, including CSN5, were identified as putative CSN5 target genes, which were significantly down-regulated by CSN5 knockdown ($P < 0.01$, two-sample $t$ test). The average expression levels of these genes were significantly elevated in eHCC compared with those in DN or CN ($P < 0.001$, two-sample $t$ test; Fig. 3B and C). Taken together, these data suggest that CSN5 and MYC activation, possibly caused by copy number gains at 8q, may play a driver role in the malignant conversion of DN to eHCC.

Discussion

To obtain a comprehensive picture of the global transcriptomic alterations during early steps of human hepatocarcinogenesis, we performed expression profiling of 49 nodular liver lesions ranging from regenerative and DNs to eHCC. Our data revealed unique expression patterns associated with the consecutive stages of hepatocarcinogenesis and identified potential markers, such as HSPA1A and CSN5, which may facilitate an early diagnosis of liver cancer. By using the most advanced pathway prediction and comparative genomics analysis tools, we showed that malignant transformation of preneoplastic liver lesions coincides with the universal activation of the MYC-regulated expression signature. Moreover, based on a set of liver-specific MYC target genes, we were able to construct a genomic prediction model that could successfully differentiate between DN and eHCC.

The role of MYC in hepatocarcinogenesis has been extensively investigated. Chromosome gains at MYC locus are among the most frequently reported genetic abnormalities in advanced human HCCs (3). Amplification of 8q22-24 region is also observed in 40% to 60% of eHCCs, although it only affects a small percentage of DNs (15). A cytogenetic tumor progression model constructed by Poon and colleagues determined that gains of 8q22-24 are among the earliest genomic events associated with HCC development (16). In animal models, MYC also proved to be a key contributor to hepatic carcinogenesis. Overexpression of MYC in mouse liver results in cellular dysplasia followed by tumor formation (3). Recently, the effect of MYC inactivation has been evaluated in different tetracycline-inducible transgenic systems (17). Inactivation of MYC in invasive HCCs led to sustained tumor regression with concomitant proliferation arrest, differentiation, and apoptosis of tumor cells (18). T-cell and B-cell lymphomas, as well as carcinomas of breast, skin, and pancreas, also displayed varying degrees of MYC oncogene dependence (17). Previously, using a comparative functional genomic approach, we established a clear molecular relationship between human HCCs and mouse liver tumors arising on a MYC transgenic background. In contrast, expression patterns of ciprofibrate-induced and Acox1-/- tumors displayed less similarities with human carcinomas (19). Thus, our current findings implicating MYC as a central mediator of human hepatocarcinogenesis are potentially consistent with a more universal tumorgenesis model wherein MYC activation is required for maintenance and expansion of transformed cells.

The average expression values of genes located in the 8q region were elevated in eHCCs and displayed a strong correlation with the presence of the MYC up-regulated signature. Intriguingly, neither we nor other investigators were able to detect a concomitant overexpression of the MYC gene itself (20). Alternatively, MYC activity is affected by posttranscriptional modifications affecting the half-life of the protein. Phosphorylation of the Thr-58 residue in the Myc box I domain targets MYC for ubiquitination and consequent proteasome-mediated degradation (21). Mutations of this region are frequently observed in Burkitt lymphomas and lead to stabilization of MYC, as well as to disruption of its proapoptotic function (22). However, it is unlikely that direct MYC mutations have a significant role in the early stages of hepatocarcinogenesis, as sequencing of MYC transcript in HCC samples did not reveal any genetic alteration. A member of the COP9 signalosome, CSN5 (alternative symbols are JAB1 or COP55), located at 8q13 locus, regulates activity of the ubiquitin ligase complex (21). Surprisingly, CSN5 proved to be an essential activator of MYC transcriptional activity in nontransformed breast epithelial cells by increasing its turnover (14). In advanced HCC, high expression levels of CSN5 significantly correlated with an increase in gene copy numbers (23). Importantly, using comparative genomics analysis, we showed that CSN5 overexpression occurred at the early stages of hepatocarcinogenesis and showed a significant association with the presence of the MYC-regulated expression signature (Fig. 3). These results are consistent with the notion that CSN5 plays an important role in liver cancer progression by a mechanism involving enhanced transcriptional activation of MYC targets. Fast-growing tumors were often characterized by tissue hypoxia, resulting in the activation of hypoxia-inducible factors HIF1 and HIF2. The interaction between the HIF and MYC transcription factors is well established and may alternatively explain aberrant activation of the MYC signature (24). Thus, under hypoxic conditions, expression of the MYC-regulated metabolic genes may provide tumor cells with a significant growth advantage. However, considering the relatively small size of the eHCCs investigated in this study, tissue hypoxia...
is more likely to be a contributing, rather than a major, cause of MYC activation.

Multiple approaches have been made to identify reliable tissue and serum markers that are able to differentiate between eHCC and other nodular lesions. Immunohistochemical studies validated HSPA1A (25) and CSN5 (26) as potential biomarkers of eHCC. Our results confirm these findings and indicate a regulatory connection with MYC. Thus, HSPA1A and CSN5 exemplify elements of the MYC signature that have already been determined in previous studies conducted on early hepatocarcinogenesis. We also constructed a 36–MYC gene–regulated classifier, which could predict the eHCC with 75% to 85% accuracy. Previously, other groups described that, in HBV-infected livers, a set of "grade-associated" genes could differentiate between LGDN and HGDN, as well as eHCC, with 100% accuracy (27). The discrepancy between the findings could be, in part, attributed to the smaller number and the different etiology of the LGDN analyzed in the current study. Still, during the validation of our model using independent samples sets, we showed that the 36 gene signature could be successfully applied to lesions with diverse etiologic background. However, the novelty of our approach goes beyond the description of a novel genomic classifier for eHCC. By using a comparative genomic approach, we provide comprehensive evidence for the central role of MYC in the malignant transformation of preneoplastic liver lesions.

In addition to the MYC signature, we also assessed the distribution of other oncogene-specific target gene sets, such as RAS and β-catenin. Surprisingly, neither RAS-induced nor β-catenin-induced genes showed a significant enrichment in the eHCC samples. Although considerable evidence supports the importance of RAS pathway in promoting liver carcinogenesis (28), RAS mutations, which frequently occur in other epithelial malignancies, are almost never detected in HCCs (29). Deregulation of the WNT/β-catenin pathway, however, is clearly a hallmark of eHCCs (30), although not detected at the initial stages (31). It is possible that these pathways do not take part in the earliest phase of liver malignant transformation. Alternatively, the lack of cross-species or cross-tissue conservation might be responsible for the lack of RAS and β-catenin signatures in the eHCC data set. We are also cognizant of the fact that transcriptional activity of MYC itself could be regulated by multiple pathways, including RAS/RAF/MPAK (32), JAK/STAT, and WNT/β-catenin (33–35) signaling, which may result in a significant overlap between the MYC and other oncogene-induced signatures. Similar observations have been made by Sansom and colleagues, who found that the inducible deletion of MYC in APC−/− intestinal epithelial cells leads to the loss of β-catenin expression signature together with the reversal of APC-deficient phenotype (36). Thus, the MYC signature might, in part, represent common effectors of proliferative stimuli with different origins. Indeed, a common induction of several cell cycle–related genes was observed in eHCCs in an independent report (37).

Previously, we successfully applied comparative functional genomic tools to identify subclasses of human HCC with progenitor cell origin (38) and with dominant activation of MET gene (39). Similar analysis conducted on atypical liver nodules confirms the power of this approach and shows a universal transcriptional activation of MYC target genes at the eHCC stage. In conclusion, the combination of genome-wide expression profiling with a comparative genomics approach not only produced a prediction model that could significantly facilitate early diagnosis of liver cancer but also permitted the identification of MYC as a central regulator of malignant transformation in early hepatocarcinogenesis. In the future, this strategy may provide a molecular classification system embracing the full spectra of preneoplastic and neoplastic liver lesions, the cellular origin of HCC, and may contribute to the identification of novel diagnostic and therapeutic targets.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 9/8/08; revised 12/10/08; accepted 1/2/09; published OnlineFirst 3/10/09.

Grant support: NIH intramural research program.

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We thank Dr. Joe W. Grisham for the thoughtful discussion of the manuscript.

References

Central Role of c-Myc during Malignant Conversion in Human Hepatocarcinogenesis


Cancer Res  Published OnlineFirst March 10, 2009.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-08-3357

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