

Role of DLC1 tumor suppressor gene and MYC oncogene in pathogenesis of human hepatocellular carcinoma: Potential prospects for combined targeted therapeutics (Review)

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Abstract. Hepatocellular carcinoma (HCC) is the third leading cause of cancer death, and its incidence is increasing worldwide in an alarming manner. The development of curative therapy for advanced and metastatic HCC is a high clinical priority. The HCC genome is complex and heterogeneous; therefore, the identification of recurrent genomic and related gene alterations is critical for developing clinical applications for diagnosis, prognosis and targeted therapy of the disease. This article focuses on recent research progress and our contribution in identifying and deciphering the role of defined genetic alterations in the pathogenesis of HCC. A significant number of genes that promote or suppress HCC cell growth have been identified at the sites of genomic reorganization. Notwithstanding the accumulation of multiple genetic alterations, highly recurrent changes on a single chromosome can alter the expression of oncogenes and tumor suppressor genes (TSGs) whose deregulation may be sufficient to drive the progression of normal hepatocytes to malignancy. A distinct and highly recurrent pattern of genomic imbalances in HCC includes the loss of DNA copy number (associated with loss of heterozygosity) of TSG-containing chromosome 8p and gain of DNA copy number or regional amplification of protooncogenes on chromosome 8q. Even though 8p is relatively small, it carries an unusually large number of TSGs, while, on the other side, several oncogenes are dispersed along 8q. Compelling evidence demonstrates that DLC1, a potent TSG on 8p, and MYC oncogene on 8q play a critical role in the pathogenesis of human HCC. Direct evidence for their role in the genesis of HCC has been obtained in a mosaic mouse model. Knockdown of DLC1 helps MYC in the induction of hepatoblast transformation *in vitro*, and in the development of HCC *in vivo*. Therapeutic interventions, which

would simultaneously target signaling pathways governing both DLC1 and MYC functions in hepatocarcinogenesis, could result in progress in the treatment of liver cancer.

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1. Introduction

Genomic instability and several other hallmarks of cancer underline the complexity of neoplastic disease (1). During the process of cancer development, the expression output of a variety of genes can be modulated by mutations, amplifications, deletions, and chromosome translocations; these changes accumulate in time and materialize in the appearance of an incipient abnormal cell with the capacity to perpetually proliferate (2). Advances in cytogenetics and molecular biology have led to the establishment of the genetic basis of neoplasia and to the recognition that chromosomal alterations affect genes critical in the pathogenesis of human cancer. The detection of recurrent chromosomal alterations has greatly facilitated the identification of genes important in oncogenesis and provided the basis for development of highly efficient cancer therapeutic agents. The best known examples are the development of Imatinib and Herceptin therapies for chronic myelocytic leukemia (CML) and breast cancer, respectively. Imatinib was consequential to identification of a bcr-abl fusion protein resulting from specific

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chromosome translocation between chromosomes 9 and 22 in CML, whereas development of Herceptin followed the detection of recurrent HER/ Neu (ErbB2) amplification on abnormal chromosomes in breast cancer cells (3,4). Both examples underline the importance of searching for gene alterations at the sites of recurrent chromosome abnormalities.

Epigenetic changes are equally important to the process of cancer development. In many types of cancer, tumor suppressor genes (TSGs) are frequently downregulated or silenced by promoter hypermethylation or histone deacetylation (5). In the past few years a new trend emerged in cancer therapy with focus on functional alterations mediated by epigenetic mechanisms. Therapeutic approaches that reverse the adverse effect of epigenetic modifications have already been developed, such as Vidaza and Decitabine, both potent DNA methyltransferase inhibitors, or Vorinostat, a histone deacetylase inhibitor. These therapeutics are applied either alone or in combination (6,7).

2. Hepatocellular carcinoma, a common cancer worldwide

Human hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, accounting for 90% of all liver neoplasias. It is the third leading cause of cancer death, and its incidence is increasing in several countries, including the United States (8,9). Infection with hepatitis B (HBV) and C viruses (HCV), consumption of aflatoxin-contaminated foods and/or alcohol, and exposure to other chemical carcinogens have been implicated in the etiology of HCC (8). In the multistep process of HCC development, both the chemical carcinogens and the oncogenic viruses can cause DNA damage, which is manifested at the chromosome level as deletions, duplications, and translocations, frequently affecting the structure and expression of cancer-related genes. With the use of a combined approach based on integration of molecular cytogenetics and molecular biology, our investigations focused on identification and characterization of recurrent chromosome alterations that have led to the discovery of new cancer-related genes as well as to the detection of alterations in known ones. Our group and many others searched for recurrent and specific genomic and gene alterations in HCC and examined both the karyotypes and the quantitative profiles of genomic imbalances and gene expression of cell lines or primary tumors. A recent article underlining the role of TSGs as metabolic regulators stated that cancer therapy is increasingly shifting toward therapeutics based on genetic alterations displayed by cancer cells (10).

Here we summarize the progress and our contribution toward uncovering genomic and related gene alterations as targets for therapeutic interventions, and focus on the role of two genes, MYC oncogene and deleted in liver cancer 1 (DLC1) gene, in hepatocarcinogenesis.

In our early studies, we identified novel nonrandom chromosome rearrangements and a distinct pattern of multiple recurrent DNA copy-number gains and losses, some of which mimicked alterations frequently seen in other neoplasias and those potentially specific for HCC (11,12). Such analyses significantly expanded the map of genomic changes in HCC and led to the identification of genes that may play an important role in the pathogenesis of HCC. We examined the karyotypes of a number of HCC cell lines and, by using approaches such as spectral karyotyping (SKY), comparative genomic hybridiza-

tion (CGH), and array-based CGH (aCGH) analyses, we were able to identify nonrandom chromosomal alterations and recurrent breakpoints in balanced and unbalanced rearrangements, as well as to precisely map recurrent genomic amplifications and deletions (11,12). It became apparent that not all the genomic sites were equally affected by gain or loss of DNA and that the regions known as fragile sites (FSs) were most frequently involved. In the past several years, molecular and cytogenetic evidence for cancer-specific translocations, amplification of oncogenes, deletion of TSGs, and viral integration at FSs firmly implicated these regions of fragility and recombination in cancer development (13). A half of all microRNA genes, whose transcripts (mRNAs), may act as either oncogenes or TSGs, and whose expression is deregulated by amplification, deletion, mutation, and epigenetic modifications in a variety of cancers, are located within or near FSs (14).

3. Genomic deletion and tumor suppressor genes

Molecular cytogenetic analysis of HCC cell lines revealed nonrandom deletions and unbalanced translocations of chromosomes 1 and 3, with the breakpoints clustered at regions 1p36 and 3p14-21, close to the loci of p73 and FHIT TSGs, respectively (11). This report was the first to describe unbalanced translocations of chromosomes 1 and 3 with the breakpoints nonrandomly involving these loci in HCC. Unlike in balanced abnormalities, unbalanced alterations may result in loss of genes. Cytogenetic abnormalities of chromosome 1 are common in HCC, and several lines of evidence implicate alterations of specific sites on its short arm in the pathogenesis of HCC (15,16). Region 1p36 is a region of fragility and recombination and is suspected to harbor multiple TSGs. Because several oncogenes and at least two cell senescence genes are located on chromosome 1, it has been postulated that structural alterations and the consequential imbalance of chromosome 1 may be important for gene dosage in certain types of cancer, including HCC (12). Recently, using an approach based on integrated genomic data of DNA copy number and gene expression profiles, researchers identified several potential driver genes on chromosome 1 in HCC (17).

In addition to 1p36, another site of recurrent translocation involves the region 3p14-21 where the FHIT gene is located. The FHIT gene is expressed in normal hepatocytes but is either abnormally expressed or inactivated in HCC cells. We detected recurrent chromosome 3p rearrangements, a decrease or absence of FHIT mRNA expression, intragenic deletions, and an absence of protein expression. These observations strongly suggested that FHIT alterations might be pathologically relevant to HCC. Chemical carcinogens or HBV integration at FRA3B may initiate a background of genetic instability early in the process of hepatocarcinogenesis (18). In HCC cell lines, we also identified recurrent DNA loss on the short arm of chromosome 3 at sites other than 3p21 (12).

Among other candidate TSGs, located at regions of deletion in 3p in various cancers, is the TMEM7 gene, which encodes a transmembrane protein (19). This gene is expressed specifically in the liver, and its protein shares substantial sequence homology with human and mouse 28-kDa interferon- α (IFN- α) response modifier protein. We investigated the role of TMEM7 in the development of human HCC and demonstrated that, in the absence of genomic deletion and mutation, the downregulation

or silencing of the gene is due to aberrant DNA hypermethylation and histone deacetylation. Ectopic expression of TMEM7 inhibited HCC cell proliferation, colony formation, and cell migration *in vitro*, and reduced tumor formation in nude mice. Treatment of two highly invasive HCC cell lines with IFN- α significantly increased TMEM7 expression and inhibited cell migration. These observations implicate loss of TMEM7 expression in hepatocarcinogenesis, and suggest that modification of TMEM7 expression by IFN- α may have potential therapeutic relevance in a subset of HCC (20).

During the neoplastic process, certain tumor cells acquire resistance to the antiproliferative signaling of TGF β . We examined a human HCC cell line sensitive to TGF β 1 (Hep3B-TS), and its derivative, Hep3B-TR, rendered resistant to TGF β 1 by step-wise exposure to the agent (21). SKY and aCGH analysis showed that the TGF β resistance resulted from a loss of TGF β receptor II (TGF β RII) gene, which occurred when the only remaining apparently normal chromosome 3 in Hep3B-TR cells, underwent microdeletion encompassing the site of the gene. A comparative differential gene expression analysis of the above-mentioned cell lines, using an oligonucleotide microarray, identified six genes in Hep3BTR cells that are downstream targets of the TNF gene, suggesting that loss of TGF β RII triggered the activation of the tumor necrosis factor network known to be regulated by the TGF β 1 pathway. Functionally, loss of TGF β RII in cells resistant to TGF β 1 significantly enhanced cell migration and anchorage-independent growth *in vitro*, and also increased *in vivo* tumorigenicity compared with parental sensitive cells (21).

Deletion and loss of heterozygosity (LOH) at specific regions of the long arm of chromosome 16 are common in several forms of cancer, including HCC (8). We have focused on region 16q24 that harbors tumor suppressor gene WWOX, which spans FRA16D, the second most common FS (22). The status of WWOX genomic DNA, as well as of the transcribed RNA and translated protein, was examined in HCC cell lines, and recurrent alterations of the gene have been identified. Loss of DNA copy number confined to band 16q23 was detected by CGH in several cell lines (12). Although homozygous deletions of WWOX were not detected, WWOX mRNA was either absent or reduced in 60% of the cell lines examined. The detection of aberrant RT-PCR products of WWOX transcripts, with deletion of exons 6 to 8, correlated significantly with altered WWOX expression. All of the cell lines showing downregulation of WWOX mRNA, also had a reduced or undetectable level of WWOX protein. Furthermore, in a majority of the HCC cell lines, the overall amount of WWOX protein was markedly reduced or undetectable in comparison with that of a normal liver. These results show that WWOX is frequently altered in HCC, and therefore implicate it in hepatocarcinogenesis (23). Because carcinogenic agents preferentially target common FSs, it is possible that breakage of WWOX locus at FRA16D and of FHIT gene at FRA3B occurs concomitantly in certain HCCs.

4. Genomic overrepresentation and oncogenes

In our CGH analysis of HCC cell lines, several regions of recurrent DNA copy-number gains have been identified (12). The detection of two regions of DNA overrepresentation on 11q13 and 5q31, both overlapping with the locations of common FSs, led us to take a closer look at two genes, EMS1 and SMAD5,

that reside at/or close to those chromosomal spots. Region 11q13 harbors EMS1 oncogene and FRA11H. We found that EMS1 is amplified in primary HCC and overexpressed in HCC cell lines in the absence of gene amplification. This oncogene encodes cortactin, a cortical actin-associated protein that is a substrate for the tyrosine kinase Src and contributes to reorganization of the actin cytoskeleton. Alterations of EMS1 that lead to the overproduction of cortactin may thus be important in the development of HCC. EMS1 amplification and overexpression are indicative of an unfavorable prognosis in several cancers and may have similar prognostic implications in liver cancer (24). The other earlier-mentioned minimal region of DNA copy-number gain in HCC, identified at chromosome 5q31, overlaps with the location of FRA5C and with the locus of the SMAD5 gene (25). Deletions at this location, unbalanced translocations with breakpoints near the SMAD5 locus, recurrent formation of isochromosome 5q resulting in selective loss of 5p and gain of 5q, and intrachromosomal amplification of SMAD5 at FRA5C have been detected; all point to this locus as relevant in the development of HCC. High-level amplification of SMAD5 at FRA5C is one of the few examples of gene amplification at a common FS mediated by breakage-fusion-bridge cycles (13). These observations show that SMAD5 undergoes gain in copy number, high-level amplification, and overexpression rather than loss of expression, suggesting that it does not function as a TSG in HCC (25).

5. Genomic and related cancer gene alterations on chromosome 8 in hepatocellular carcinoma

The genes thus far identified in our studies, which promote or suppress tumor cell growth, most likely represent only a fraction of possible future therapeutic targets. In spite of wide heterogeneity of genomic alterations in HCC, certain chromosomes or chromosomal sites are more commonly deleted or amplified, which results in deregulation of critical genes that, ultimately, may trigger transformation of normal hepatocytes into malignant phenotype. The best example of the above, perhaps, are genomic alterations of chromosome 8 in HCC. This chromosome shows a distinctive pattern of both recurrent of DNA copy number loss on 8p, and gain on 8q (12). Due to the high frequency of large genomic deletion and LOH in HCC and several other cancers, chromosome 8p has been long suspected to carry TSGs. Moreover, LOH on chromosome 8p is among the most common alterations in HCC and is associated with liver dysplastic nodules and primary and metastatic HCC (26-32). Chromosome 8p contains two FSs and is rich in candidate and validated TSGs, some of which have been implicated in the pathogenesis of HCC (33,34). Region 8p22 alone harbors six TSGs (Table I). Among the candidate and bona fide TSGs implicated in HCC are PDGFRI, DLC1, LFIRE/ HFREP-1, SFRP1, MSRA, and newly identified SCARA5 gene (35-41).

Liver fibrinogen-related gene-1, LFIRE-1/HFREP-1, specifically expressed in normal human liver tissue, is recurrently downregulated or inactivated and has an antiproliferative effect on HCC cells both *in vitro* and *in vivo* (37).

SFRP1, a secreted Wnt antagonist, and SCARA5, a plasma membrane protein, which activate the focal adhesion kinase (FAK) signaling pathway and inhibit the tyrosine phosphorylation cascade of the FAK-Src-Cas pathway, are strong

Table I.

A, Candidate or bona fide tumor suppressor genes on chromosome 8p

Name	Location	Type of cancer
MSRA	p23	Liver
DLC1	p22	Liver and other cancers
N33	p22	Prostate
PDGFRL	p22	Liver
MTUS1	p22	Liver and other cancers
LFIRE-1	p22	Liver
LZTS1	p22	Various cancers
TRAIL-R1	p21	Various cancers
TRAIL-R2	p21	Various cancers
DBC2	p21	Breast
RHOBTB2	p21	Breast
DOK	p21	Lung
SORBS3	p21	Liver
SHRBS3	p21	Liver
SCARA5	p12-11.1	Liver
NRG1	p11-12	Breast, pancreas
PROSC	p11	Liver
FGFR	p11	Myeloproliferative syndrome
TACC	p11	Breast
SFRP1	p11	Liver, breast
MOZ	p11	Acute myeloid leukemia

B, Protooncogenes on chromosome 8q

Name	Location	Type of cancer
MOS	q11	Lung and other cancers
MDTH	q22.1	Breast
MYC	q24	Liver and other cancers
HSF1	q24.3	Liver
LYN	q13-qter	Ewing's sarcoma

candidate tumor suppressor genes due to their antiproliferative effect on HCC cells (39,41). Since its isolation, SFRP1 gene was predicted to be a TSG because of its location at 8p11-12, a site of LOH in HCC (42). However, in many types of cancer, including HCC, SFRP1 has been found to be silenced primarily by hypermethylation, and not by genomic deletion (39,43). In a few instances, restoration of SFRP1 expression resulted in reversal of the malignant phenotype (39,43). However, one study reports that SFRP1 is upregulated in metastatic renal cell carcinoma and that it contributes to the invasiveness of the tumor cells (44). The same researchers and others had reported that SFRP1 is frequently downregulated in primary renal cell carcinoma (45). The probability that SFRP1 downregulation contributes to hepatocarcinogenesis (39) is rather high, given the role and importance of Wnt signaling in the pathogenesis of HCC (46,47). A study demonstrating the suppressive effect of SCARA5 on tumor cell proliferation *in vitro* and on metastasis *in vivo* (41), indicates that this gene also may play an important role in initiation and development of HCC.

Other characterized TSGs such as N33, TRAIL-R1, TRAIL-R2, LZTS2, hBD-1, DBC2, DOK, and NRG1, are also located on 8p, but none of them was examined in HCC. Three new genes located on 8p, SORBS3, SHRBS3 and PROSC, have been characterized quite recently and their function will be discussed later in the text, within the context of TSG role in HCC. A number of oncogenes are located on 8q, but only MYC is closely associated with the development of HCC (Table I). Among all cancer-related genes on chromosome 8, DLC1 and MYC play a major role in hepatocarcinogenesis, and evidence for their involvement in the development of HCC is presented next.

6. DLC1 a potent tumor suppressor gene in hepatocellular carcinoma

The DLC1 gene was isolated from a chromosomal fragment deleted in a human HCC, and was suspected to be a TSG (36). Since its isolation, the interest in this gene has grown considerably worldwide. Progress in understanding of its tumor-suppressive function in multiple cancers, particularly in HCC, will be discussed here. Two closely related genes, DLC2 and DLC3, also were isolated and were found to be frequently deregulated and to elicit oncosuppressive effects in HCC (48,49). Until now, three DLC1 isoforms, called variants 1, 2, and 3 (or α , β and γ), were isolated (50). Most recently, a new isoform 4 (DLC1-i4) was identified and shown to suppress tumor cell growth, with its functional promoter regulated by p53 (51).

In recent years, DLC1 has emerged as a major tumor suppressor gene and a strong candidate metastasis suppressor gene in HCC and other cancers (50,52-55). DLC1 is also a contributing factor in processes affecting normal functions, or disorders unrelated to cancer. DLC1 is vital for normal development because null mutations in the gene result in embryonic lethality in mice and flies. It is also involved in insulin signaling and the pathogenesis of coronary spastic angina and is among four genes that negatively regulate periodontal ligament cell proliferation (56-59).

The DLC1 gene is located on chromosome 8p22, a region (Table I) of recurrent LOH in HCC (36). Although region 8p22 does not correspond to an FS, its propensity for deletion is similar to that of the most unstable and vulnerable FSs. Downregulation or inactivation of TSGs has profound consequences in the genesis and progression of cancer, and DLC1 is one of the most frequently deregulated genes in the cancer genome. Downregulation or inactivation of the DLC1 gene in many forms of cancer, is commonly mediated at the genomic level by heterozygous and homozygous deletion, and at the transcriptional level by aberrant promoter methylation or histone deacetylation (50). Initially, we detected lack of DLC1 expression in over 40% of human primary HCC and in 90% of HCC cell lines (36). These results were independently confirmed, as 40% of HCC samples had no detectable DLC1 expression (60). More recently, using representational oligonucleotide microarray, heterozygous deletions of DLC1 were found in 59 of 86 primary HCCs. Furthermore, deletions of DLC1 occurred in other cancers more frequently than of some other well-known TSGs (53). In contrast with the early observation that mutations in the coding region of DLC1 are rare in HCC, recent genome-wide sequencing analyses of several cancers have identified missense mutation of DLC1 (61-64). In an early study of HCC cell lines, we identified a number of single

nucleotide polymorphisms in the DLC1 genomic DNA sequence, which may be associated with an increased risk for development of HCC (61). Only recently, a comprehensive genotyping analysis of a large number of cases in the Chinese population provided convincing evidence for an association between DLC1 polymorphism and susceptibility to HCC. Furthermore, evidence shows that the DLC1 polymorphism and risk for HCC development are consistent with its biological function and strongly suggest that the DLC1 polymorphism may directly confer susceptibility to HCC (65).

Promoter hypermethylation appears to be associated with human cancer at least as frequently as is disruption of TSGs by mutation or deletion. TSGs that are downregulated or silenced by promoter hypermethylation are often located in genomic regions that are frequently deleted in cancer (13,66). Despite a high rate of deletion, DLC1 is predominantly silenced or downregulated by epigenetic mechanisms, frequently by promoter methylation (50,67,68). An expression profile of the three DLC1 isoforms - variants 1, 2, and 3 - in normal and malignant hepatic tissue, showed that only variant 1 was silenced by promoter methylation; variants 1 and 2 (but not variant 3) localize at focal adhesions and suppress stress fiber formation and *in vitro* HCC cell proliferation (69). DLC1 variant 1 is not the only one silenced by promoter methylation. As recently demonstrated with the newly identified isoform 4, DLC-i4 is expressed in normal tissues and normal immortalized cell lines but is significantly downregulated in a high number of nasopharyngeal, esophageal, gastric, breast, colorectal, cervical, lung carcinoma cell lines as well as in primary tumors. Methylation of DLC1-i4 was detected in 38% of primary HCCs (51).

In addition to deletion, mutation, and promoter methylation, DLC1 can be downregulated or silenced by other mechanisms. DLC1 did not escape the action of certain miRNAs that target TSG expression (70). A new study of significant interest and with implications for the role of HCV in chronic liver disease and HCC, has demonstrated that efficient HCV replication requires miR-141-mediated suppression of DLC1. In primary human hepatocytes, HCV infection stimulated cell proliferation that was reversed by transduction of DLC1 (71).

DLC1 encodes a RhoGAP protein that catalyzes the conversion of active GTP-bound Rho GTPase (Rho) to the inactive GDP-bound form (Fig. 1). DLC1-mediated inhibitory effect of cell growth and tumorigenicity is primarily due to its RhoGAP's ability to inactivate the Rho proteins - RhoA, RhoB, and RhoC, and to some degree Cdc42, but not Rac - although RhoGAP-independent mechanisms also contribute to its antioncogenic activity (72-80). Rho GTPase activity is frequently deregulated in human cancers, and experimental evidence implicated Rho GTPase signaling in promoting growth and invasiveness through morphologic and functional cellular alterations such as cell shape, actin cytoskeleton remodeling and focal adhesion organization, as well as cell migration and invasion (50,81,82). Because the activity of Rho GTPases is elevated in many human cancers, the ability of RhoGAPs to downregulate Rho proteins may attenuate or inhibit tumorigenic and/or metastatic process (83-85). The importance of altered Rho GTPase signaling in the genesis and progression of HCC has been previously covered in detail (85). Special attention was given to DLC1, whose loss of function was considered a driving event in the promotion and progression of HCC. DLC1 also was recog-

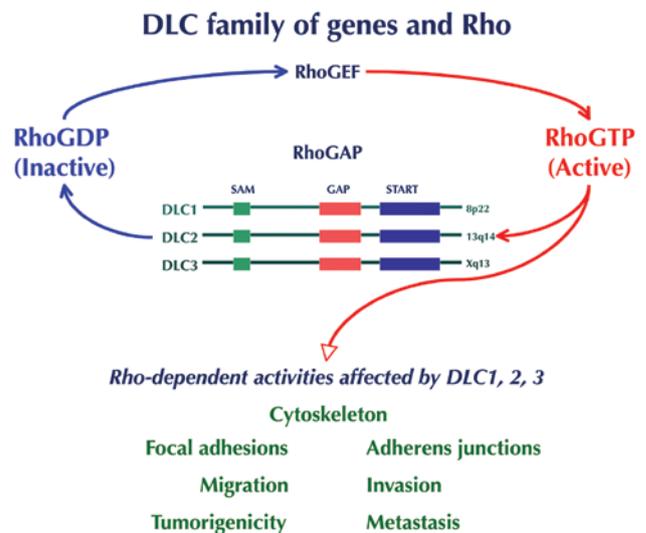


Figure 1. Small RhoGTPases alternate between their active (RhoGTP) and inactive (RhoGDP) forms. Activation is controlled by guanine nucleotide exchange factors (GEFs), whereas deactivation is mediated by GTPase activating proteins (GAPs) Rho effector proteins, which include DLC protein family, play an important role in processes that affect cell morphology, motility and tumorigenesis.

nized as the best example of a RhoGAP alteration in HCC (85). Most recently, in an integrative genomic and transcriptomic profiling of HCC from 76 patients with HBV infection designed to identify survival-related driver genes, DLC1 and five other genes on 8p were found deleted in patients with poor outcome. Interestingly, three of these genes, SORBS3, SHRBS3 and PROSC act as TSGs and suppress HCC *in vitro* and *in vivo* (86).

Indeed, highly recurrent alterations of DLC1 have been found in solid tumors and hematologic malignancies, and a meta-analysis of microarray experiments lists DLC1 as the fifth of the top-50 genes implicated in multiple cancers (87).

DLC1-transduced HCC cells display a diffuse cytoplasmic localization of the protein (72,88), whereas in other cells DLC1 protein co-localizes with the tips of actin stress fibers and in focal adhesions, which is critical for cell migration (72,75,88-90). Evidence that DLC1 functions as a TSG in HCC comes from experiments in which DLC1 cDNA was ectopically expressed in cells with disabled endogenous gene expression. In several independent studies, restoration of DLC1 expression invariably resulted in inhibition of cell proliferation, colony formation, and cell migration *in vitro*, and it reduced the development of tumors after xenografting of HCC cells in athymic nude mice (60,72,74,88). Two well-characterized cell lines, derived from patients with aggressive HCC, and negative for endogenous DLC1 activity, were used to test the antiproliferative effect of the DLC1 ectopic expression. Restoration of DLC1 expression resulted in inhibition of *in vitro* cell proliferation and *in vivo* tumorigenicity, as well as in induction of apoptosis associated with cleavage of caspase 3 and a reduced level of the antiapoptotic Bcl-2 (88). Subsequent studies with prostate cancer showed that tumor cells acquire sensitivity to DLC1-induced apoptosis after treatment with HA14-1, a Bcl-2 inhibitor (76). It has been demonstrated that subcellular locations of DLC1 protein determines different manifestations of its antioncogenic action; in cytoplasm DLC1 functions as an

inhibitor of tumor cell proliferation and migration, whereas in the nucleus it acts as an inducer of apoptosis (91).

In the past few years, evidence supporting the metastasis suppressor function of DLC1 has been generated with breast cancer, and HCC cells (77,92,93). The role of DLC1 in metastatic process will be covered in a separate article.

A yeast two-hybrid screening was invaluable to further dissect complex molecular machinery underlying DLC1 functions. While the multidomain structure of DLC1 merely indicated its capacity to interact with other molecules, the yeast two-hybrid analysis identified several potential binding partners of DLC1. Those binding partners were later confirmed in human cells, allowing the examination of consequences of their interactions with DLC1. Such was the case with the tensin family of focal adhesion proteins, which serve as a link between the actin cytoskeleton and the cytoplasmic tails of integrins, whose relationship with DLC1 has been thoroughly investigated in different types of tumor cells (94-100). In human lung cancer cells, it was demonstrated that DLC1 binds to SH2 and PTB domains of the tensin proteins and, most importantly, that cooperation between DLC1 RhoGAP action and its tensin-binding activity is required for suppression of cancer cell migration, although the two functions are not interdependent (75).

Another group reported that tensin 2 and DLC1 form a complex, which binds to caveolin-1 (Cav-1), a structural component of caveolae. Such binding brings DLC1 in close proximity to locally enriched RhoGTPases and facilitates the inactivation of Rho proteins through the RhoGAP activity of DLC1 and with repercussions at the level of cytoskeleton. Interaction of this kind would constitute a potential new mechanism of DLC1 action in hepatocytes, through cytoskeleton reorganization (94). Cav-1 functions as a TSG, and recently Cav-1 interaction with the START domain of DLC1 was identified and shown to contribute to the tumor suppressor activity of DLC1 (101). We also detected DLC1 interactions with p120RasGAP, α -catenin, and S100A10 proteins in human cells, and assessed their influence on DLC1's tumor suppressor function (102-104). The interaction of DLC1 with p120RasGAP protein, which is implicated in cross-talk between Ras and Rho, not only provided relevant data for the negative modulation of DLC1 tumor suppressor activity but may, also, lead to potential clinical interventions (102). The interaction was mapped to the RhoGAP catalytic domain of DLC1 and the SH3 domain of p120RasGAP, and resulted in a dramatic reduction of DLC1's RhoGAP activity *in vitro*. Moreover, overexpression of p120RasGAP in colon carcinoma cells that harbored mutant Ras and thus were resistant to the negative regulation of Ras by p120RasGAP, increased the level of endogenous active Rho in a DLC1-dependent manner and antagonized the growth-suppressive effects of DLC1. These data show that p120RasGAP can promote the growth of cells containing mutant Ras, and are consistent with evidence suggesting that it might represent a valid therapeutic target for tumors carrying a mutant Ras, which account for approximately 30% of all tumors, and for which an efficient cancer treatment to block Ras function did not translate in clinical interventions (55). Thus, p120RasGAP-targeting therapy might be effective also in a subset of HCC with mutated Ras (102).

In contrast with the negative effect of interaction with p120RasGAP on DLC1 tumor suppressor function, prelimi-

nary evidence indicates that DLC1 interaction with α -catenin enhanced DLC1 anti-oncogenic activity (103).

We reported DLC1 interaction with S100A10, an inflammatory protein and a key cell surface receptor for plasminogen that regulates pericellular proteolysis and tumor cell invasion. DLC1 and annexin 2 share the same binding site at the C-terminus of S100A10. DLC1 binding to S100A10 caused an dose-dependent decrease in steady-state S100A10 expression by displacing it from annexin 2 and making it accessible to ubiquitin-dependent degradation. This process attenuated plasminogen activation and was accompanied by inhibition of *in vitro* cell migration, invasion, colony formation, and anchorage-independent growth of aggressive lung cancer cells. DLC1 binding to S100A10 did not affect DLC1's RhoGAP activity. Thus, this study unraveled a novel GAP-independent mechanism that contributes to the tumor suppressor activity of DLC1 (104).

Although transcriptional regulation of DLC1 through genetic and epigenetic alterations was discussed in detail, only recently have posttranslational modifications been reported, mediated by phosphorylation of a serine-rich domain, an unstructured region of DLC1 gene, and shown to negatively regulate the anti-oncogenic function of DLC1 (93,105). Unstructured domains are preferred sites for posttranslational modifications, including phosphorylation, that target multiple sites in the DLC family proteins (50,106). In an earlier study, Ser322 of rat DLC1 was found to be phosphorylated in rat adipocytes after insulin treatment, and posttranslationally, rat DLC1 has been shown to be phosphorylated by Akt kinase (57). This finding set the stage for investigations of the role of Akt phosphorylation of DLC1 on suppression of tumorigenicity and metastasis in HCC (93).

The 14-3-3 protein is known to function as a phosphorylation-dependent adaptor protein that facilitates interactions with other proteins (107). It is interesting that activated protein kinase D (PKD) phosphorylates DLC1 and in turn facilitates interaction with 14-3-3 protein isoforms. Binding of 14-3-3 protein inhibited DLC1 RhoGAP activity and prevented nucleocytoplasmic shuttling and, thus, may impede the DLC1 apoptotic function (91,108). As a follow-up to this study, the same group provided evidence showing that PKD phosphorylation of Ser 807 within the GAP domain negatively regulates DLC1 function (105).

In summary, the role of DLC1 is rather complex, and, as recognized by others, intricacies of the DLC1 tumor suppressor function and its modulation by multiple and elaborate interactions should stimulate interest and new research in this TSG (109). DLC1 meets the criteria for a gatekeeper gene because of its inhibitory effect on tumor cell growth and cell death (110). Given the unusually high number of TSGs on the short arm of chromosome 8, the inhibitory effect of DLC1 on tumor cell growth may compensate for loss of function of other TSGs. Evidence presented here confirms DLC1 as an important gene in the development and progression of HCC.

7. MYC, a major oncogene in cancer

MYC protooncogene, the human cellular homologue of the avian myelocytic leukemia virus, a retroviral oncogene, is a transcription factor that regulates cell proliferation, controls

the expression of many genes, and is implicated in the pathogenesis of a plethora of human solid tumors, leukemias and lymphomas, as well as in animal tumors. MYC is a powerful cancer-promoting gene but, paradoxically, also shows oncosuppressive activity by inducing apoptosis and cell senescence. Since its isolation 27 years ago, MYC has commanded interest worldwide, and nearly 20,000 studies on virtually every form of cancer have been published. A recent comprehensive article reviewed the progress in understanding MYC function; the mechanisms contributing to the induction of cell transformation, apoptosis, gene deregulation, and control of expression; and the identification of MYC target genes (111). Therefore, we will briefly outline the general aspects of MYC's role in cancer development and present our encounter with this gene in various cancers, particularly in HCC.

Abnormal MYC expression is a common denominator in cancer, and its activation is mediated by insertional mutagenesis, chromosomal translocations and gene amplification, but not by mutations in the coding sequence (111). Insertional mutagenesis, the process of insertion of new sequence(s) into a normal gene, frequently occurs during integration of retroviral proviruses into the host genome, resulting in chimeric sequences of both viral and cellular origin. Retroviruses are obligate mutagens because their life cycle require integration into the host's chromosomal DNA (up to 8% of human genome is of retroviral origin), an event that, in addition to structural alterations at the site of impact, also may activate adjacent cellular oncogenes (111-114). MYC activation by retroviral insertion was the first demonstration of such phenomena (115). Unlike retroviruses, the integration of certain DNA viruses into chromosomal DNA, being not required for viral persistence, was long considered rather an exception, than a rule. Abundant evidence for integration of several oncogenic DNA viruses into the cellular genome, obtained from a variety of human cancers, has challenged this view. As already mentioned, their integration, apparently, is not a random event, because they preferentially target FSs, which frequently encompass the location of growth-regulatory genes (116). Integration of oncogenic DNA viruses, such as of human papillomavirus (HPV) in most invasive genital cancers, Epstein-Barr virus (EBV) in infected lymphoblastoid cells or Burkitt's lymphoma (BL), and adeno-associated virus (AAV) targeting MYC locus, have been well documented (13). The most recent evidence of specific viral integration at FS was the localization of simian virus 40 (SV40) in SV40-immortalized cells, at region 1q21.1, which corresponds with the location of a common FS (FRA1F). SV40's insertion in the cell's genome caused deregulation of adjacent genes involved in senescence and apoptosis and, thus, played a critical role in cellular immortalization (117).

The specificity of viral integration is fundamental to determining the biological significance of this phenomenon in various pathologic conditions, including cancer. The introduction of viral genetic material and its interaction with the cellular genome may contribute to the induction of cell transformation, the maintenance of the transformed phenotype, or tumor progression (113,114,118). Undoubtedly, the most conclusive evidence for the effect of viral integration at FSs in cancer development was provided in a study of young patients with X-linked severe combined immunodeficiency, when three of ten children developed monoclonal acute lymphoblastic leukemia-

like lymphoproliferation after gene therapy that used murine leukemia virus-derived vector as a gene delivery vehicle. It has been unequivocally demonstrated that in two of those patients (and probably in all three) the activating vector integrated within FRA11E region, near the transcription start site of the LMO2 oncogene (119), thus causing high levels of transcription and, consequently, of LMO2 protein.

Chromosomal translocations, isochromosomes, double minute chromosomes (DM), and abnormally banded or homogeneously stained regions (HSRs) are also responsible for MYC deregulation in a variety of cancers. Chromosomal translocations are common in leukemias and lymphomas, and the best known examples are reciprocal translocations in BL involving the MYC locus at FRA8C to any of the three immunoglobulin genes on chromosome 2, 14, or 22, resulting in MYC gene deregulation. Juxtaposition of MYC with immunoglobulin loci was causally related to development of lymphomas (120). In addition to the reciprocal translocation t(8;14), in two well-characterized BL cell lines we identified two more complex translocations, t(8;14;18) and t(7;8;14). These novel rearrangements, a manifestation of genomic instability, resulted in transposition of MYC sequences in a new genomic context and, together with duplication of chromosome 1q, which is the second most common alteration in BL, may have contributed to the acquisition of an invasive tumor phenotype (121).

An example of an increased copy number of MYC on normal and rearranged chromosome 8 in HCC is shown in Fig. 2.

Whereas abnormal isochromosomes cause a modest increase in MYC copy number, DM and HSR may generate high-level amplification, commonly found in human solid tumors (122,123). HSR may contain two or more amplified genes, frequently oncogenes. In breast cancer cells, for example, using DNA fibers hybridized with genomic probes, we detected high-level amplification not only of MYC but of ERBB2, as well (124). Nonrandom chromosome alterations resulting in amplification of MYC gene also have been identified in normal human mammary cells neoplastically transformed by the controlled introduction of SV40LT, hTERT, and H-RAS genes, thus implicating MYC in the early stages of neoplastic development (125).

8. Critical role of MYC in the pathogenesis of human and mouse hepatocellular carcinoma

Evidence from genetic analyses of human early liver lesions, primary and metastatic liver tumors, tumor-derived cell lines, and from different rodent models, unequivocally demonstrated that MYC deregulation is a critical alteration in the initiation and progression of HCC. A recent gene expression profiling of cirrhotic and dysplastic nodules and early HCC, identified MYC as a plausible driver gene and a central regulator of malignant transformation in initial stages of hepatocarcinogenesis (126). In primary and advanced HCC and in derived cell lines, MYC is commonly overexpressed because of genomic amplification. Using a conventional CGH analysis in HCC cell lines, we detected a DNA copy number increase throughout the 8q arm, and the minimal common region of amplification confined to band 8q24.1, which corresponds with the site of FRA8C and with location of MYC locus (12). In this study and in several others, the CGH analyses did not establish a correlation between genomic imbalances and the etiology of HCC (127). It

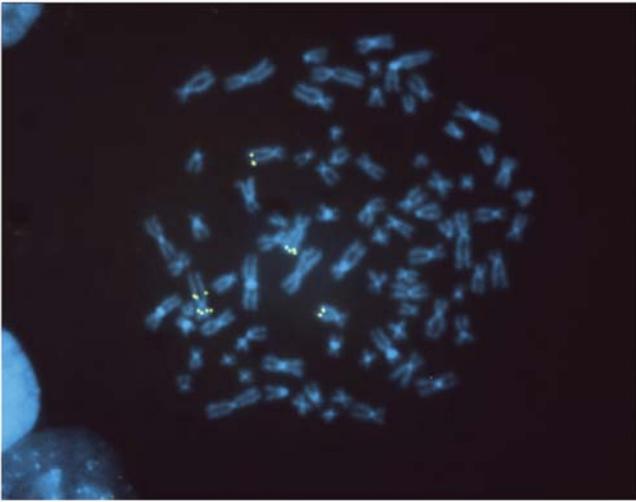


Figure 2. Metaphase from HCC cell line 7703K, with an abnormal chromosomal complement, was hybridized *in situ* with a genomic MYC probe. Multiple fluorescent signals for MYC gene, indicative of gene amplification, are located at normal and abnormal chromosome 8.

is interesting that a CGH analysis and gene expression profiling established both the copy-number gains and MYC overexpression in viral and alcohol-related HCC, but not in cryptogenic, nonalcoholic, steatohepatitis-induced HCC (128).

In an earlier minireview article, describing chromosome-mediated alterations of MYC in various cancers, we discussed the impact of viral integration in hepatocarcinogenesis, and stressed the finding that the woodchuck hepatitis virus (WHV) is known to integrate near MYC locus and cause enhanced MYC expression (114). HBV has a genomic structure similar to that of WHV, and both viruses exhibit similarities to retroviruses in terms of integration and requirements of RNA intermediate and reverse transcriptase for replication. It is possible that HBV integrates at FRA8C and activates the MYC gene (129). However, due to HBV's capacity to cause secondary genomic rearrangements and virus relocation, the localization of HBV integration sites in HCC may not represent the initial site of integration (114). Protooncogenes other than MYC often are amplified in HCC because of HBV integration (130). Mutagens-carcinogens also target FSS and induce genomic and gene alterations (131). It is possible that damage inflicted at FRA8C may explain the high frequency of MYC overexpression in viral and alcohol-related HCC (128), in addition to the ability of HBV's X protein to produce such an effect (132).

The development of mouse models for HCC provided an ideal experimental approach for the detection of cellular and genetic alterations and the discovery of candidate cancer genes relevant to human hepatocarcinogenesis. Several new mouse models for HCC have been developed in our laboratory, among which MYC single-, and MYC-TGF α double-transgenic mice underscore the critical role of the MYC gene in hepatocarcinogenesis (133).

Such models offer the opportunity to detect lesions associated with the initial stages of neoplastic development, and identify alterations related to the acquisition of malignancy or to tumor progression. A conventional cytogenetic analysis revealed that at 10 weeks of age, the level of structural chro-

mosomal aberrations was nearly tenfold higher in MYC-TGF α hepatocytes than in wild-type hepatocytes, and that, as a sign of genomic instability, early dysplastic lesions display breakage on several chromosomes, whose locations correspond with regions of tumor susceptibility (134). Also, it has been postulated that increased generation of reactive oxygen species might be responsible for the extensive chromosomal damage and acceleration of hepatocarcinogenesis characteristic of MYC-TGF α mice.

To determine whether vitamin E (VE), a potent antioxidant, is able to protect against the chromosomal damage triggered by oxidative stress caused by overexpression of c-myc and TGF- α transgenes, the incidence of chromosomal and chromatid aberrations was examined in primary hepatocyte cultures established from 10-week-old MYC-TGF α mice maintained on either a control diet, or a VE-supplemented diet. VE supplementation markedly decreased the frequency of chromosomal damage at the early stage of hepatocarcinogenesis before the development of morphologically defined preneoplastic and neoplastic lesions (135). The cytogenetic constitution of tumors developed in MYC-TGF α transgenic mice has been analyzed using conventional G-banding analysis and FISH with several chromosome painting and single-copy gene probes. Cells from all such cytogenetically characterized tumors maintained ability to form tumors after reinoculation in nude mice. The most frequently observed stable alterations involved chromosomes 1, 4, 6, 7, and 12. A correspondence existed between the location of breakpoints in stable alterations of these chromosomes, and the regions of fragility identified in the early stage of hepatocarcinogenesis, thus strongly suggesting the importance of these nonrandom changes in both initiation and progression of neoplasia (136). The location of MYC transgene was identified at chromosome 5G-ter; interestingly, in all tumors and derivative HCC cell lines, a balanced translocation t(5;6) was identified, with the breakpoint near the site of MYC transgene integration. This is the first balanced translocation found in either human or mouse liver tumors (137,138). Significantly, near the balanced translocation t(5;6), we characterized a deletion that inactivated a gene whose human homolog, GTF2IRD1, encodes a widely expressed, multifunctional helix loop helix transcription factor that binds to pRb and is one of the 16 genes present in approximately 1.6 Mb interval commonly deleted in Williams-Beuren syndrome (WBS) (139). Therefore, the c-myc transgenic mice carrying the t(5;6) translocation represent, also, the first 'knockout' of one of the genes present in the WBS critical region. The Gtf2ird1-null mice exhibited growth retardation and craniofacial and neurologic abnormalities similar to clinical symptoms in WBS patients (140).

Our combined SKY and aCGH analysis of several cell lines derived from HCC developed spontaneously in MYC transgenic mice, identified recurrent chromosome rearrangements and genomic imbalances. Among genomic imbalances, partial or complete gain of chromosomes 15 and 19 and loss of chromosomes 4, 9, and 14 were the most common alterations. These alterations are also recurrent in HCC developed in other transgenic mouse models, in mouse spontaneous HCC, in derivative cell lines, and in preneoplastic liver lesions induced by chemical carcinogens. Overall, these results demonstrate selective, nonrandom genomic changes associated with development of HCC (141).

Among those changes, gains of chromosomes 15 and 19 are particularly important. Mouse chromosome 15 bears large regions of homology with human chromosome 8q, and it is likely that increased copy number of chromosome 15 might enhance the effect of MYC in development of HCC, by providing more copies of the gene. DM and HSR have been found in a variety of solid tumors and hematologic malignancies but not in adult HCC or in HCC developed in animals (137,138). In a cell line derived from HCC developed in MYC transgenic mice, we detected a recurrent gain of chromosome 19 and DM derived from chromosome 19. At the site of DNA amplification are located the MYCS and MX11 genes, both known to interfere with MYC gene activity, and the mouse RelA gene that promotes cell survival by inhibiting apoptosis and allowing MYC to drive proliferation of transformed cells (142).

Other related studies demonstrated that coexpression of MYC and TGF- α enhances the development of HCC through the disruption of the pRb/EF2 pathway and that TGF- α might function as a survival factor for neoplastic cells, thereby accelerating the neoplastic process (143). In a different mouse model, it has been demonstrated that MYC inactivation induced regression of invasive HCC resulting in differentiation into hepatocytes and biliary cells forming bile duct structures. This suppressive effect was accompanied by a loss of expression of the tumor markers and an increase in expression of liver cell and liver stem cell markers (144).

9. Modulation of DLC1 and MYC in hepatocellular carcinoma: prospects for combined pharmacologic interventions

There is no question that downregulation or silencing of DLC1 gene and/or amplification or upregulation of MYC gene are major contributing factors in the pathogenesis of human and murine HCC. A highly recurrent pattern of genomic imbalances in HCC predicts that both DLC1 and MYC would be deregulated in a large number of cases and that such synchronization may be consequential in hepatocarcinogenesis. This was demonstrated in an *in vitro/in vivo* reconstitution mouse model for HCC (53). In this model, genetically modified liver progenitor cells lacking p53, transduced with a retrovirus expressing MYC and with another expressing a DLC1 shRNA, were transplanted into the liver of syngeneic mice (53,145). DLC1 knockdown aided MYC in the induction of HCC in mice, and tumors thus developed are similar to aggressive human HCC. Ectopic overexpression of DLC1 in HCC cells expressing oncogenic Ras and containing increased GTP-bound RhoA, abolished the tumor formation. Conversely, suppression of RhoA inhibited growth of DLC1-negative HCC cells, showing that loss of DLC1 and RhoA activation has a similar effect in hepatocarcinogenesis. The results of this study provide conclusive evidence that loss of DLC1, combined with an oncogenic stimulus, promotes HCC *in vivo* and that this oncogenic process is associated with activation of RhoA, which is a key consequence of loss of DLC1 tumor suppressor activity (53,54).

Given the fact that many types of cancer accumulate multiple genetic alterations over time, the treatment of cancer by targeting a single damaged gene was originally considered unlikely to be effective. However, numerous experiments

subsequently showed that silencing of an oncogene or activation of a TSG can be sufficient to abolish cell tumorigenicity (146). Despite the accumulation of a multitude of genetic alterations, tumor cells may depend only on a single oncogenic pathway to provide critical competitive advantage for their survival and unlimited multiplication. This phenomenon, known as 'oncogene addiction,' was described more than a decade ago (147). Only in the past few years, however, has this concept received renewed interest, and its potential to translate into effective targeted cancer therapy and the need for future investigations have been eloquently advocated (148). Suppression of such a critical oncogenic pathway may result in the death of tumor cells without any harmful consequences in normal cells. Likewise, reintroduction of the wild-type of TSGs, which are frequently inactivated in cancer cells, referred to as 'tumor suppressor hypersensitivity,' prevents tumor growth by inducing cell death or cell cycle arrest, and in many instances, it yields a complete response without toxicity to normal cells. These are fundamental requirements for an efficient cancer therapy.

It is well documented that DLC1 and MYC are deregulated in a large fraction of HCC. In a preclinical study designed to provide ground for potential tailored therapeutics, transplanting tumor tissue from HCC patients into nude mice has generated a number of explant models. The analyses of gene copy number, gene mutation, mRNA expression, and protein expression profiles demonstrated that transplanted tumors preserve the genotypic alterations of the original tumors. Consistent with the concept of oncogene addiction and tumor suppressor hypersensitivity, amplification of MYC and cyclin D1 oncogenes and deletion of DLC1 and FHIT TSGs were detected in different individual models, as well as in corresponding patients' HCC tissues (149).

Novel therapeutic intervention options targeting the DLC1 pathway in HCC have been discussed and the clinical therapeutic efficacy of various agents was rigorously evaluated in several articles (53,54,85,150). Therapeutic interventions based on DLC1 activity require a full understanding of signaling pathways affected by loss of its function (54). The inhibition of RhoA pathway and Rho kinase (ROCK), a downstream effector of Rho, tops the options for therapeutic interventions (53,54,85,150). Currently, a major effort is underway to develop small molecule Rho kinase inhibitors to treat various disorders, including cancer. An effective therapeutic agent, Y-27632 displays distinct antimetastatic activity in HCC (151,152). Wf-536, a more potent derivative of Y-27632, also inhibited the metastasis of melanoma *in vivo* but has not yet been tested in HCC (153,154). Results with a new potent and selective ROCK inhibitor that is superior to Y compounds will be reported soon (Channing Der, personal communication).

A number of compounds that restore DLC1 expression, extend the half-life of its protein, or mimic its function, in different cancers, also might be effective in therapy for HCC. The ability of flavone to restore DLC1 expression and, consequently, to suppress metastatic breast cancer cell proliferation is not an isolated example (155). Other agents, such as all-trans retinoic acid and peroxisome proliferator-activated receptor γ (PPAR γ), significantly elevated DLC1 expression in cancer cells (50). Morelloflavone, a biflavonoid, also inhibited tumor growth and angiogenesis of prostate cancer xenografts in mice by targeting RhoA and Rac1 GTPases (156). A newly developed synthetic flavone derivative, 6f, induces apoptosis

mediated through death receptor and mitochondria-dependent pathway in human HCC (157). Thiazolidinedione, a synthetic activator of PPAR γ , inhibits the growth of PPAR γ -expressing human colon cancer cells by inducing terminal differentiation and a marked increase in p21 abundance (158). Because epigenetic modifications are the main cause of DLC1 down-regulation and inactivation, therapy based on the ability of DNA methyl-transferase and histone deacetylase inhibitors (HDAC) to restore DLC1 expression in cancer cells has been highlighted as an attractive therapeutic approach (54). In HCC cells, a combined treatment involving suberoylanilide hydroxamic acid, a powerful HDAC inhibitor already used in clinical trials, and DLC1 transduction had a synergistic inhibitory effect on tumor cell proliferation and anchorage-independent growth (159). Other unrelated agents, such as ursodeoxycholic acid (UDCA), a hydrophilic bile acid, inhibited *in vitro* HCC cell proliferation by increasing the half-life of DLC1 protein and reducing RhoA activity (160). It remains to be seen whether UDCA has a similar antiproliferative effect in xenografted HCC in mice.

It is disappointing that after many years of intense effort and despite major advances in understanding the regulation and function of the MYC gene, the development of cancer therapeutics that would target MYC itself remains elusive (111). Future pharmacologic interventions targeting MYC should be guided by a new strategy that takes into account the differential MYC roles of either tumor promoting or tumor suppressing, depending on its level of expression, and also should consider modulation of the two opposite functions of MYC oncoprotein (161,162).

An informative review article outlined the modest progress in development of MYC targeted therapeutics in HCC. For example, small MYC inhibitors showed an antiproliferative effect on HCC cells *in vitro* and, furthermore, sensitized those cells to chemotherapeutic agents, but they were not equally effective *in vivo* (163). Quarfloxin CX-3453, which targets MYC expression through a four-stranded DNA structure, reached clinical trials for neuroendocrine carcinoma (164). Overall, there are few treatment options for advanced HCC. The development of Sorafenib, a multikinase inhibitor that marked a breakthrough in understanding of the disease, has shown remarkable survival benefits in patients with advanced HCC (165,166). Quarfloxin has the potential to be effective in therapy for HCC because, like Sorafenib, it inhibits vascular endothelial growth factor or may enhance the antitumor effect of Sorafenib (163).

Numerous studies suggest that statins, the cholesterol-lowering drugs, have an antiproliferative effect on cancer cells. Significantly, an interesting recent study demonstrated that inhibition of HMG-coenzyme A reductase by Atorvastatin blocks MYC phosphorylation and activation, leading to suppression of mouse and human HCC cell proliferation *in vivo* and *in vitro*. Suppression of MYC phosphorylation was, most likely, mediated through the inhibition of GTPases in the Rac pathway *in vitro* (167). In a clinical trial, treatment with Pravastatin improved survival in patients with HCC (168). Regarding the effects of statins, there is a significant link with DLC1 gene, which in its COOH-terminal regions harbors a START domain, thought to be important in cholesterol binding (36,169,170). As discussed earlier, Cav1, a cholesterol transporter, interacts with DLC1 START domain and contributes to the tumor suppressor

activity of DLC1 (101). It is possible that statins may affect normal START function.

In summary, given the limitations of single-agent therapy, a combination of molecular therapies that target different pathways is expected to be more effective in treatment of HCC (166). Cancer therapeutics targeting gain and loss of function of oncogenes and TSGs, respectively, could lead to the identification of novel genotype-selective antitumor agents (171). In that respect, a combined therapeutic approach, targeting both MYC and DLC1 signaling networks, have realistic potential to improve the treatment of liver cancer.

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