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Novel candidate targets of Wnt/β-catenin signaling in hepatoma cells

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Abstract

The activity of β-catenin/TCF, the key component of Wnt signaling pathway, is frequently deregulated in HCC, resulting in the activation of genes whose dysregulation has significant consequences on tumor development. Therefore, identifying the target genes of Wnt signaling is important for understanding β-catenin-mediated carcinogenesis. We analyzed the transcriptome profile of human hepatoma cell lines using cDNA microarrays representing 15,127 unique, liver-enriched gene loci to identify the target genes of β-catenin-mediated transcription ($p<0.005$). This analysis yielded 130 potential Wnt-associated classifier genes, and we found 33 of them contain consensus TCF-binding sites in presumptive transcriptional regulatory sequences. These genes were, then, tested for their Wnt-dependence of expression in experimental models of Wnt activation. Genes such as *RPL29*, *NEDD4L*, *FUT8*, *LYZ*, *STMN2*, *STARD7* and *KIAA0998* were proven to be up-regulated upon Wnt/β-catenin activation. Gene ontology analysis of the 33 candidate genes indicated the presence of functional categories relevant to Wnt pathway such as cell growth, proliferation, adhesion and signal transduction. In conclusion, we identified a number of candidate Wnt/β-catenin target genes that can be useful for studying the role of altered Wnt signaling in liver cancer development, and showed that some of them might be direct targets of Wnt signaling in hepatoma cells.

Keywords: Wnt/β-catenin; Target genes; HCC; cDNA microarray

Introduction

Hepatocellular carcinoma (HCC) accounts for an estimated half million deaths annually (Murray and Lopez, 1997). The etiological factors of HCC has been well established, and includes hepatitis B or C virus (HBV or HCV) infection, exposure to chemical carcinogens including aflatoxin B1 (AFB1), cigarette smoking, or heavy alcohol consumption (Schafer and Sorrell, 1999). Recently, somatic mutations in the Wnt signaling components, especially those of *CTNNB1* (β-catenin) and *AXIN1*, were also found to be important in hepatocarcinogenesis (Satoh et al., 2000). However, the spectrum of the genes that link these etiological factors to the malignant transformation of hepatic cells remains poorly defined.

The Wnt signaling pathway can lead to oncogenesis when aberrantly activated (Peifer and Polakis, 2000). The protein β-catenin plays a critical role in this process (Nelson and Nusse, 2004). A number of downstream target genes of Wnt/β-catenin signaling have been reported, which play critical roles in carcinogenesis by affecting cell growth and cell cycling (*c-MYC*, *CCND1*, c-*Jun*, fra-1, Gastrin, *WISP-1*, *ITF-2*), cell survival (Id2, *MDR1*, *COX2*), and invasion and tumor dissemination (*MMP7*, *LAMC2*, VEGF) (http://www.stanford.edu/~rnusse/pathways/targets.html) (Giles et al., 2003). Most of the known Wnt targets have been identified in colon cancers but the Wnt signaling is likely to regulate distinct subsets of target genes during the tumorigenesis of different tissues. For example, ectopic expression of an activated form of β-catenin in the liver

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of transgenic mice resulted in hepatic hyperplasia but the level of *CCND1* and *c-MYC* mRNA did not change significantly (Cadoret et al., 2001). Instead, it has been suggested that other target genes are likely involved in the proliferation of hepatocytes in response to dysregulation of Wnt/β-catenin signaling. Therefore, identifying hepatocyte-associated Wnt targets can have important implications in understanding the contribution of deregulated Wnt signaling to the neoplastic change of hepatocytes.

We have performed a comprehensive gene expression profiling of 9 human hepatoma cell lines using high-density cDNA microarray chips, and report here on the identification of a subset of novel candidate target genes of Wnt/β-catenin pathway that might be implicated in HCC development. We also provide experimental evidence suggesting that many of these genes are indeed regulated by the Wnt/β-catenin signaling.

Materials and methods

Cell culture and culture conditions

Hep3B (HCC), PLC/PRF/5 (HCC) and HepG2 (hepatoblastoma) were cultured in DMEM (JBI, Daegu, Korea) supplemented with 10% heat-inactivated FBS (Hyclone, Logan, UT) and antibiotic–antimycotic in 5% CO_2–air atmosphere at 37 °C. Other HCC cell lines such as SNU-182, SNU-354, SNU-368, SNU-387, SNU-449, and SNU-475 were maintained in RPMI 1640 (JBI) supplemented as above. Cancer cells such as SNU-16 (stomach), Raji (lung), CaSki (cervical), Y-79 (retinoblastoma), MCF-7 (breast), A431 (colon), HCT116 (B lymphoma), Ovk-4 (ovary), and Jurkat (T lymphoma) were cultured following the prescriptions in ATCC (Manassas, VA) or Korean Cell Line Bank (KCLB, Seoul, Korea).

cDNA microarray analysis

cDNA microarrays were fabricated in our core facility using a robotic arrayer (PixSYS 5500, Cartesian; Ann Arbor, MI). The 10 K microarray [Gene Expression Omnibus (GEO), http://www.ncbi.nlm.nih.gov/geo, Platform accession: GPL2911] contains 10,336 genes obtained from Incyte Unigene (Incyte, Wilmington, DE), while the 14 K and 24 K microarrays (GEO Platform accession: GPL2912 and GPL2913) containing 14,080 and 24,288 genes, respectively, derived from KUGI unigene library (http://kugi.kribb.re.kr). Total RNA extracted form the hepatoma cells were labeled with Cy-5, while the reference RNA pooled from 9 cancer cells (SNU-16, Raji, CaSki, Y-79, MCF7, A431, HCT116, Ovk-4 and Jurkat) were with Cy-3 using 3DNA Expression Array Detection Kit (Genisphere, Hatfield, PA). Hybridization of cDNA was performed at 65 °C for 16 h in a hybridization chamber (Array Chamber X; GenomicTree, Daejon, Korea). Arrays were scanned at 10 μm resolution in Scannarray 5000 (Packard, Billerica, MA). The signal intensity was calculated photometrically by GenePix Pro v4.0 (Axon Instruments, Whipple Road Union City, CA). We used the robust scatter-plot smoother lowess from the statistical software package R to perform a local A-dependent normalization as described (Chen et al., 2003). The relative expression value (*M*) was calculated as the log ratio of Cy-5/Cy-3, i.e., $\log_2$(Cy-5/Cy-3). If the signal-to-background ratio was lower than 1.4, the feature was assessed as a null value to reduce bias. The filtered

Table 1
Primer sequences used for cloning and PCR

Gene	Forward primer	Reverse primer
For ΔN87β-cat construction		
ΔN87β-cat	CGGGATCCGCCATGGCTCGAGCTCAGAGGGTAC	ATGGACCATAACTGCAGCTTTATTA
For RT-PCR		
OXA1L	TCTGGTGGTTCCAGGATCTC	GTGTCGTGCCAGGGATACTT
RPL29	CTTTGCCAAGAAGCACAACA	AGCCTGAGCTGGAACTGAAG
FUT8	ATCCCAGGTCTGTCGAGTTG	GCCTCAGGATATGTGGGGTA
STMN2	GCAATGGCCTACAAGGAAAA	CTCTGCCAATTGTTTCAGCA
LYZ	CTCATTGTTCTGGGGCTTGT	ATCACGGACAACCCTCTTTG
NEDD4L	TCGGTGATGTGGATGTGAAT	ACCCCTTCAAATCCTTGAGC
S100A11	ATCGAGTCCCTGATTGCTGT	GAGGAAGGAGTCATGGCAAG
TPM2	GGACAGAGGATGAGGTGGAA	TCCTTCAGCTTCTCCTCCAA
YWHAB	AGCAGGCTGAGCGATATGAT	CAGGCTACAGGCCTTTTCAG
GABPB2	TGCTGATGTACACACGCAAA	TGCTGACCCCCTGAACTAAC
BCAS2	CTGCTCGACAACCAATTGAA	CCAGCTGTGAGTTGCATGTT
STARD7	ATGCCCTGGTAATCAAGCTG	CCACTGGAAACCATCCAACT
KIAA0998	TCGCCTGATGAAAGACAGTG	CTCCTCCAGTTCAGCCTCAC
CTNNB1	GGTCCTCTGTGAACTTGCTCAG	GCCACCTGATTGCTGTCACCTG
c-MYC	GCAAGACTCCAGCGCCTTCTCTC	TGACACTGTCCAACTTGACCCTCTT
BIRC5	CCCTGCCTGGCAGCCCTTTCT	GGCAGCCAGCTGCTCGATGGC
LEF1	CCAGCTATTGTAACACCTCA	TTCAGATGTAGGCAGCTGTC
CCND1	TGGAACACCAGCTCCTGTGCTG	GCGCGTGTTTGCGGATGATCTG
Actin	CAAGAGATGGCCACGGCTGCT	TCCTTCTGCATCCTGTCGGCA