Systemic Genome Screening Identifies the Outcome Associated Focal Loss of Long Noncoding RNA PRAL in Hepatocellular Carcinoma

Chuan-chuan Zhou,1* Fu Yang,1* Sheng-xian Yuan,2 Jin-zhao Ma,1 Feng Liu,1 Ji-hang Yuan,1 Feng-rui Bi,1 Kong-ying Lin,2 Jian-hua Yin,3 Guang-wen Cao,3 Wei-ping Zhou,2 Fang Wang,1 and Shu-han Sun1

Systemic analyses using large-scale genomic profiles have successfully identified cancer-driving somatic copy number variations (SCNVs) loci. However, functions of vast focal SCNVs in “protein-coding gene desert” regions are largely unknown. The integrative analysis of long noncoding RNA (lncRNA) expression profiles with SCNVs in hepatocellular carcinoma (HCC) led us to identify the recurrent deletion of lncRNA-PRAL (p53 regulation-associated lncRNA) on chromosome 17p13.1, whose genomic alterations were significantly associated with reduced survival of HCC patients. We found that lncRNA-PRAL could inhibit HCC growth and induce apoptosis in vivo and in vitro through p53. Subsequent investigations indicated that the three stem-loop motifs at the 5’ end of lncRNA-PRAL facilitated the combination of HSP90 and p53 and thus competitively inhibited MDM2-dependent p53 ubiquitination, resulting in enhanced p53 stability. Additionally, in vivo lncRNA-PRAL delivery efficiently reduced intrinsic tumors, indicating its potential therapeutic application. Conclusions: lncRNA-PRAL, one of the key cancer-driving SCNVs, is a crucial stimulus for HCC growth and may serve as a potential target for antitumor therapy. (HEPATOLOGY 2016;63:850-863)

Somatic copy number variations (SCNVs) are extremely common in cancer,1,2 and, in some cases, focal SCNVs have led to the identification of cancer-causing genes and have suggested specific therapeutic approaches.3-5 Genomic analyses of cancer samples by cytogenetic studies,6 array-based profiling,7 and, more recently, by targeted exome capture8 have identified recurrent SCNVs that are associated with cancer. However, an important challenge is to identify the oncogene and tumor suppressor gene targets of driver SCNVs (which often encompass unknown genes) and to elucidate the functional roles of SCNVs. In the past two decades, great progress has been achieved in the identification of candidate hepatocellular carcinoma (HCC)-related protein-coding and microRNA genes in abnormal chromosome

Abbreviations: ANOVA, analysis of variance; CCK-8, cell-counting kit-8; ChIRP, chromatin isolation by RNA purification; CNVs, copy number variations; dChIRP, domain-specific chromatin isolation by RNA purification; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HBV, hepatitis B virus; HBx, HBV X protein; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HSC90, heat shock protein 90; IHC, immunohistochemistry; kb, kilobases; lncRNA, long noncoding RNA; lncRNA-PRAL, p53 regulation-associated lncRNA; mRNA, messenger RNA; nt, nucleotide; Ops, oligonucleotide pools; qPCR, quantitative real-time PCR; RNA-FISH, RNA fluorescence in situ hybridization; SCNVs, somatic copy number variations; siRNA, small interfering RNA; XAF1, XLAP-associated factor 1.

Received August 5, 2015; accepted December 10, 2015.

Additional Supporting Information may be found at onlineibrary.wiley.com/doi/10.1002/hep.28393/suppinfo.

This work was supported by grants from the National Natural Science Foundation of China (grant nos.: 81301831 and 81472691), the National Key Basic Research Program (973 project) (2015CB554004) from the Ministry of Science and Technology of China, Key Program (no. 81330037) from National Natural Science Foundation of China, and the State key infection disease project of China (2012ZX10002010 and 2012ZX10002016).

*These authors contributed equally.

Copyright © 2015 by the American Association for the Study of Liver Diseases.

View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep.28393

Potential conflict of interest: Nothing to report.
Although genome-wide copy number analyses have identified a large number of HCC phenotype-associated SCNVs in intergenic regions of the human genome, it is difficult to explain the pathogenesis of these SCNVs because they cannot be directly linked to changes in protein content or function.  

Long noncoding RNA (lncRNA) is a class of noncoding functional RNA that has recently attracted great research interest. Indeed, roles of lncRNAs as tumor suppressors and oncogenic drivers have appeared in prevalent types of cancers, such as prostate cancer and HCC. Furthermore, recent studies show that some lncRNAs that locate at genomic fragile sites or abnormal regions are associated with cancer phenotype, suggesting that these breakpoint-associated lncRNAs might be important in the development and progression of human cancer as cancer-causing genes. Therefore, linking cancer-associated SCNVs to lncRNAs will provide independent support for functional implications and lead to a greater understanding of cancer pathogenesis.

To address these challenges, we reanalyzed the copy number profiles of 286 HCC tissues and matched non-tumor liver tissues and identified regions in the HCC genome that have undergone recurrent high-level focal amplifications or deletions. By integrating copy number profiles with lncRNA expression signatures derived from our previous study, we identified differentially expressed lncRNAs within the aberrant genome regions. Of these, p53 regulation-associated lncRNA (lncRNA-PRAL) was significantly underexpressed with recurrent genomic deletions in HCC. And lncRNA-PRAL genomic alterations were correlated with poor prognosis of HCC patients. Using small interfering RNA (siRNA)-mediated knockdown, adenovirus-mediated overexpression and RNA pull-down, lncRNA-PRAL was found to be involved in p53-mediated HCC growth and apoptosis.

**Materials and Methods**

**HUMAN TISSUES**

All samples were collected with the informed consent of the patients and the experiments were approved by the ethics committee of Second Military Medical University (Shanghai, China). The human tissues are detailed in the Supporting Information.

**COPY NUMBER DATA ANALYSIS**

The raw copy number data were downloaded from Gene Expression Omnibus (GSE38323). The details of copy number data analysis are described in the Supplementary Information.

**DOMAIN-SPECIFIC CHROMATIN ISOLATION BY RNA PURIFICATION**

Chromatin isolation by RNA purification (ChIRP) was performed as previously described using the oligonucleotide pools listed in Supporting Table 6. RNA were extracted and quantified as described, using quantitative real-time polymerase chain reaction (qPCR) primers listed in Supporting Table 7. Western blottings were performed using p53 (ab26; Abcam, Cambridge, MA), heat shock protein 90 (HSP90; ab13495; Abcam), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; ab22556; Abcam), and β-actin (ab1801; Abcam) primary antibodies.

**TUMOR GROWTH ASSAYS IN VIVO**

The animal studies were approved by the institutional animal care and use committee of the Second Military Medical University. Additional details are described in the Supporting Information.

---

**ARTICLE INFORMATION:**

From the 1Department of Medical Genetics, Second Military Medical University, Shanghai, 200433, China; 2The Third Department of Hepatic Surgery, Eastern Hepatobiliary Hospital, Second Military Medical University, Shanghai, 200433, China; and 3Department of Epidemiology, Second Military Medical University, Shanghai, 200433, China

**ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:**

Shu-han Sun, Ph.D.,
Department of Medical Genetics, Second Military Medical University
800 Xiang-Yin Road
Shanghai 200433, China.
E-mail: sshsun@vip.sina.com
fax: (+86) 021-81871053.
IMMUNOPRECIPITATION

For whole-cell extracts, cells were lysed in buffer containing 50 mM of Tris-HCl (pH 7.5), 150 mM of NaCl, 1% Triton X-100, and cleared by centrifugation. Immunoprecipitation with anti-HSP90 and p53 antibodies in whole-cell extracts was performed as the Pierce Co-Immunoprecipitation Kit (Thermo, Rockford, IL) protocol described.

STATISTICAL ANALYSIS

All the statistical analyses were performed using SPSS software (version 17.0; SPSS, Inc., Chicago, IL). For comparisons, one-way analyses of variance (ANOVA), Fisher’s exact test, chi-squared test, Wilcoxon’s rank-sum test, Wilcoxon’s signed-rank test, and two-tailed Student’s t test were performed, as appropriate. Cumulative survival probability was evaluated using Kaplan-Meier’s method, and differences were assessed using the log-rank test. The SPSS software was also used to assess uni- or multivariate Cox’s proportional hazards regression analyses with the hazard ratios and P values indicated. No statistical method was used to predetermine sample size, and investigators were not blinded to allocation during experiments.

Results

ABERRANT lncRNAs AT CHROMOSOMAL BREAKPOINTS IN HCC

To find evidence of driver SCNVs in HCC genomes, we performed a data mining process using published data (GSE38323) and evaluated the frequency of SCNVs in HCC by subtracting those from paired nontumor liver tissues, in an effort to eliminate germline CNVs. A genome-wide view of segmented copy numbers revealed that most chromosomal arms undergo either copy number gain or deletion in a large proportion of the samples (Fig. 1A). Significantly, the genomic instability seems to be more remarkable in hepatitis B virus (HBV)-related HCC than in hepatitis C virus (HCV)- or alcohol-related HCC (Fig. 1B). Notably, the intergenic regions also harbor more SCNVs in HBV-related HCC than in HCV- or alcohol-related HCC (Fig. 1B). Among the overlapped SCNVs in HBV-, HCV-, and alcohol-related HCC, there are 43.2% that locate in the human genome intergenic regions (Fig. 1C). Considering an unanticipated and tremendous amount of noncoding sequences of human genome need to be transcribed, we further performed an association analysis of lncRNA expression microarray data (GSE27462) with the SCNVs data according to their precise chromosome locations. This analysis identified 73 differently expressed lncRNA genes in the minimal deletion or amplification regions of HCC (Supporting Table 8). After a preliminary screen by strand-specific qPCR and confirmation by genomic real-time PCR (representative results are shown in Supporting Fig. 1), we found 11 lncRNAs with genomic copy number gains or losses in HCC; among these, six lncRNAs were frequently gained and five were frequently lost (Table 1).

MOLECULAR CHARACTERS OF lncRNA-PRAL IN HCC

To investigate whether abnormalities of DNA copy number might result in deregulation of lncRNA expression, we analyzed the concordance between the lncRNA gene copy number and transcript level in cohort 1 HCC tissues (Supporting Table 1). The lncRNA-PRAL (NCBI no.: AK128092) was found to be the most consistently and markedly reduced on both RNA transcript (P = 0.000) and genome copy number levels (P = 0.000; Fig. 2A), had the highest correlation coefficient (R = 0.621; P = 0.000) in HCC (Fig. 2B), and was most correlated with outcome of HCC patients (Table 1). Then, we detected the lncRNA-PRAL genomic and transcriptional level in 41 normal liver tissues (distal normal liver tissue of liver hemangioma) and 22 hepatitis liver tissues (Supporting Table 2), compared with HCC tissues from cohort 1, to find that hepatitis tissues had a lower lncRNA-PRAL expression with genomic deletion, whereas from hepatitis liver to HCC, lncRNA-PRAL exhibited a focal loss and significant underexpression (Fig. 2C,D).

Our results indicated that lncRNA-PRAL was transcribed with a poly A tail (Supporting Fig. 2A). We next performed a rapid amplification of complementary DNA end analysis to identify the 5’ and 3’ ends of the lncRNA-PRAL transcript (Supporting Fig. 2B); the full-length lncRNA-PRAL sequence is presented in Supporting Fig. 3. The full-length transcription of lncRNA-PRAL was then validated by a northern blotting analysis (Supporting Fig. 2C). We applied RNA fluorescence in situ hybridization (RNA-FISH) to visualize the cellular localization and relative abundance of lncRNA-PRAL in HCCLM3 and SMMC-7721 cells. RNA-FISH demonstrated that lncRNA-PRAL...
FIG. 1. Pattern of copy number alterations in HCC. (A) The common chromosomal instability sites in HCC. Upper part of the figure indicates the common gain chromosome regions in HCC. Lower part of the figure indicates the deletion chromosome regions in HCC. (B) The mean sum of the SCNV's lengths in the four groups of patients with HCC (HBV male, male patients with HBV-related HCC, n = 172; HBV female, female patients with HBV-related HCC, n = 43; HCV male, male patients with HCV-related HCC, n = 23; Alcohol male, male patients with alcohol-related HCC, n = 17). (C) Percentage of the length of various SCNVs in the common SCNVs of four groups HCC patients (HBV male, HBV female, HCV male, and Alcohol male HCC patients). Gains-inter and Deletions-inter represent genomic fragments of SCNVs located in the region between protein-coding genes. Gains-mRNA and Deletions-mRNA represent genomic fragments of SCNVs located in the region containing protein-coding genes.
localizes to both cytoplasmic and nuclear regions (Fig. 2E). Furthermore, RNA from nuclear and cytoplasmic fractions was analyzed by qPCR, revealing that lncRNA-PRAL was diffusely distributed both in the nucleus and cytoplasm (Fig. 2F). We observed a substantially lower level of lncRNA-PRAL expression in hepatoma cells (HCCLM3, Huh7, Hep3B, and SMMC-7721) than immortalized hepatocytes (L02 and QSG-7701; Supporting Fig. 2D). Then, we performed the siRNA-mediated knockdown and adenovirus-mediated overexpression of lncRNA-PRAL to explore the pathophysiological significance in hepatic cancerous cell lines HCCLM3 and SMMC-7721 (Supporting Fig. 2E,F).

**HIGH EXPRESSION OF lncRNA-PRAL INHIBITS HCC CELL GROWTH IN VITRO AND IN VIVO**

Cell-counting kit-8 (CCK-8) assays indicated that cell proliferation was increased in HCCLM3 and SMMC-7721 cells when lncRNA-PRAL was silent (Fig. 3A), whereas the HCCLM3 and SMMC-7721 cells in which lncRNA-PRAL was overexpressed showed a lower degree of proliferation than the negative control cells (Fig. 3A). According to Annexin V/fluorescein isothiocyanate/propidium iodide dual staining, the proportion of apoptotic HCCLM3 and SMMC-7721 cells with lncRNA-PRAL overexpression was increased compared to control cells (Fig. 3B). Additionally, the HCCLM3 and SMMC-7721 cells in which lncRNA-PRAL was underestimated showed a lower degree of apoptosis than the negative control cells (Fig. 3B). However, lncRNA-PRAL cannot promote cell apoptosis in p53-deficient (Hep3B) or p53-mutant (Huh7) HCC cells (Supporting Fig. 4), whereas both HCCLM3 and SMMC-7721 are p53 wild-type cells.

To investigate the proapoptosis effect of lncRNA-PRAL in vivo, we transplanted HCCLM3 and SMMC-7721 cells overexpressing lncRNA-PRAL into the bilateral armpits of nude mice. We found that the growth of tumors from the lncRNA-PRAL-overexpressing cells was significantly reduced in comparison to the growth of control cells (Fig. 3C and Supporting Fig. 5A). Apoptosis regulation of lncRNA-PRAL was further confirmed by in situ terminal deoxynucleotidyl transferase dUTP nick end labeling assay of tumor tissue sections (Supporting Fig. 6). To further assess the biological therapeutic value of lncRNA-PRAL in vivo, we evaluated the therapeutic potential of adenovirus vector-mediated gene delivery.
FIG. 2. Molecular characters of IncRNA-PRAL in HCC. (A) The IncRNA-PRAL transcript expression level (RNA-T and RNA-L) and genomic copy number level (gDNA-T and gDNA-L) were determined by SYBR-green real-time PCR in HCC (T) and paired nontumor tissues (L) (n = 56). (B) Correlation between the IncRNA-PRAL expression levels and genomic DNA content in HCC tissue samples (n = 56). x, the relative expression level of IncRNA-PRAL; y, the relative IncRNA-PRAL genomic DNA content. real-time PCR analysis of IncRNA-PRAL transcript expression (C) and genomic copy number level (D) in 56 paired HCC tissue specimens with compared nontumor tissue in cohort 1, 22 hepatitis liver tissues, and 41 normal liver tissues. (E) RNA-FISH to detect IncRNA-PRAL in SMMC-7721 and HCCLM3 cells. Red, IncRNA-PRAL; blue, DAPI staining. Scale bars, 50 μm. (F) Agarose gel electrophoresis of real-time PCR products from cytoplasmic and nuclear RNA purification procedures. Strand-specific real-time PCR was performed using primers for IncRNA-PRAL (upper), human U2 snRNA (small nuclear RNA U2), and S14 (ribosomal protein S14) primers with either the cytoplasmic or nuclear RNA fraction isolated from HCCLM3 cells. The U2 snRNA and S14 primers were used to show the effective separation of the cytoplasmic and nuclear RNA. *P < 0.05; **P < 0.01; ***P < 0.001. Abbreviation: DAPI, 4',6-diamidino-2-phenylindole.
FIG. 3. Biological effects of lncRNA-PRAL in HCC cells. Knockdown of lncRNA-PRAL was mediated by transfection with siRNAs. Overexpression was mediated by transfection with an adenovirus vector system encoding the full-length lncRNA-PRAL. (A) Cell number was determined by CCK-8 assay, and the relative number of cells is shown in mean ± standard error. All assays were performed in triplicate and repeated at least three times. (B) After staining with Annexin V and propidium iodide, cell apoptosis in HCCLM3 and SMMC-7721 cells was analyzed using a flow cytometer. (C) Photographs of tumors were taken after transplanting the lncRNA-PRAL-overexpressing HCCLM3 cells and control cells into the bilateral armpits of nude mice for 3 weeks. Right two panels show the effect of lncRNA-PRAL on HCC tumor growth described by tumor weight and tumor weight/body weight ratio in the three groups. (D) Transfer of lncRNA-PRAL using the adenovirus vector system markedly inhibited tumor growth in the SMMC-7721 tumor model. Murine SMMC-7721 tumors were established (day 0). Intratumoral transfers of AV-PRAL, AV-CON, and AV-AS were performed on days 7, 12, 17, and 22 (10 mice per treatment group). Left panel shows the representative images of tumor growth viewed by the IVIS Lumina II system in each group 4 weeks after the subcutaneous tumor planting (n = 10). Middle panel shows the relative luciferase signal (mean ± standard deviation) captured in each group at different time points. Tumor volumes were detected as indicated in right panel. *P < 0.05. Abbreviations: AV-PRAL, adenoviral particles encoding lncRNA-PRAL; AV-AS, adenoviral particles encoding antisense lncRNA-PRAL; AV-CON, empty adenoviral vector.
of lncRNA-PRAL (AV-PRAL) in human HCC cell lines bearing a nude mouse subcutaneous model. Interestingly, tumor growth was significantly inhibited with injection of AV-PRAL (Fig. 3D and Supporting Fig. 5B). These data demonstrate that the adenovirus vector system provides an effective means to deliver lncRNA to HCC tissue, and in vivo administration of AV-PRAL may have considerable potential for HCC gene therapy.

**lncRNA-PRAL BINDS TO HSP90 AND REDUCES THE UBIQUITINATION OF P53**

Although most of the sequence of the lncRNA-PRAL overlaps in antisense with the 3′ untranslated region of XAF1 protein-coding gene, which is known to regulate apoptosis, we did not find any significant changes of XIAP-associated factor 1 (XAF1) messenger RNA (mRNA) or protein in lncRNA-PRAL over- or downexpressed SMMC-7721 and HCCLM3 cells (Supporting Fig. 7). These results indicate that lncRNA-PRAL-regulated cell apoptosis is independent of XAF1.

Because binding to specific protein is one important pattern for lncRNA to implement their molecular functions, we performed an RNA pull-down experiment to identify proteins that bind to lncRNA-PRAL (Fig. 4A). Mass spectrometry analysis of the differentially displayed band revealed that HSP90 was the main protein associated with lncRNA-PRAL (Supporting Table 3). We confirmed the association between lncRNA-PRAL and HSP90 by western blotting using the proteins isolated from the RNA pull-down assays (Supporting Fig. 8A). RNA immunoprecipitation was performed using a specific HSP90 antibody to ensure that lncRNA-PRAL could be specifically immune precipitated from cell lysates (Supporting Fig. 8B). The electrophoresis mobility shift assay (EMSA) results further confirmed that lncRNA-PRAL could directly bind to HSP90 (Fig. 4B) in vitro.

Because proapoptosis functions of lncRNA-PRAL were abolished in p53-mutant and -deficient cells, and meanwhile, p53 plays a key role in the regulation of cell apoptosis in cancer as one of the main tumor-related client proteins of HSP90, we detected the interaction of HSP90 and p53 protein in lncRNA-PRAL-overexpressed SMMC-7721 cells. By using a coimmunoprecipitation assay, we found the increased HSP90-p53 interaction in lncRNA-PRAL-overexpressed SMMC-7721 cells (Fig. 4C). The western blotting analysis showed that p53 was significantly increased in the SMMC-7721-overexpressing lncRNA-PRAL and decreased in the lncRNA-PRAL-underexpressed cells (Fig. 4D), whereas there was no significant association between altered lncRNA-PRAL expression and HSP90 protein levels (Fig. 4D). Enhanced p53 activity was confirmed by a luciferase reporter assay in the lncRNA-PRAL-overexpressing HCCLM3 cells (Supporting Fig. 8C). Furthermore, our data showed that lncRNA-PRAL overexpression induced up-regulation of apoptosis-related p53 target genes (BAX, PUMA, NOXA, and FAS) in SMMC-7721 cells (Supporting Fig. 8D). However, the transcript level of p53 did not change (Supporting Fig. 9). Interestingly, the ubiquitin modification of p53 in lncRNA-PRAL-overexpressed SMMC-7721 cells is more attenuated than controls (Fig. 4E). Considering that MDM2 is well-known to regulate p53 by its E3 ubiquitin ligase activity, we scanned the interaction of MDM2 and p53 when lncRNA-PRAL was overexpressed. Accompanied by the increased HSP90-p53 interaction, the p53-MDM2 interaction was decreased in lncRNA-PRAL-overexpressed SMMC-7721 cells (Fig. 4F). These results revealed that lncRNA-PRAL might enhance the interaction between p53 and HSP90, which opposes MDM2 induced p53 ubiquitination and degradation.

**THE THREE BASIC STEM-LOOP MOTIFS AT THE 5′ END OF lncRNA-PRAL ARE REQUIRED FOR THE ASSOCIATION BETWEEN HSP90 AND P53**

To monitor the ability of different truncated fragments of lncRNA-PRAL binding to HSP90, we predicted lncRNA-PRAL structure using the RNA Structure (version 5.3) and Vienna RNA Package (1.8.5). According to the predicted lncRNA-PRAL structure, we truncated lncRNA-PRAL into fragments S1, S2, S3, and S4 as shown in Supporting Fig. 10A. Our results revealed that the 1,180-nucleotide (nt) fragment (S1) at the 5′ end of lncRNA-PRAL mediated binding to HSP90 (Fig. 5A). Interestingly, a 2,583-nt fragment at the 5′ end (S2) including S1 could not bind to HSP90 (Fig. 5A). We next analyzed the RNA secondary structure and identified three basic stem-loop motifs (motifs A, B, and C) located close to one another in the S1 fragment (Supporting Fig. 10B). These motifs are also located close to one another in
FIG. 4. lncRNA-PRAL binds to HSP90 and up-regulates p53 protein levels. (A) SDS-PAGE gel showing the proteins specifically bound to lncRNA-PRAL compared to an antisense RNA. The band highlighted by the arrow was subjected to a mass spectrometry analysis and was identified as the HSP90 protein. (B) EMSA showing the interaction between lncRNA-PRAL and HSP90. The arrow indicates the shift in mobility induced by the biotin-labeled lncRNA-PRAL and HSP90 complex. Biotin-labeled lncRNA-PRAL alone was used as a control, and unlabeled lncRNA-PRAL was titrated to show dose-dependent competition. (C) Western blotting analysis of specific proteins immunoprecipitated with anti-HSP90 antibodies in lncRNA-PRAL-overexpressing SMMC-7721 cells. The level of p53 and HSP90 was detected and normalized to 1% input β-actin protein level. n = 3. (D) Protein levels of p53 and HSP90 were determined by western blotting analyses of lysates from SMMC-7721 cells under- or overexpressing lncRNA-PRAL. (E) Cell lysates from SMMC-7721 cells transduced by AV-PRAL, AV-AS, and AV-CON adenoviral particles were immunoprecipitated with a p53-specific antibody, followed by western blotting with an antibody to ubiquitin. Bottom panel depicts the input of the cell lysates. n = 2. (F) Western blotting analysis of specific proteins immunoprecipitated with anti-p53 antibodies in lncRNA-PRAL overexpressing SMMC-7721 cells. The level of MDM2, HSP90, and p53 was detected and normalized to 1% input β-actin protein level (n = 2). Abbreviations: IgG, immunoglobulin G; IP, immunoprecipitation; SDS-PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis.
FIG. 5. The three basic stem-loop motifs at the 5′ end of lncRNA-PRAL are the structural basis of its binding to HSP90. (A) The truncated versions of lncRNA-PRAL, S1 (1-1,181 nt), S2 (1-2,583 nt), S3 (1,162-3,606 nt), and S4 (2,564-3,606 nt) were produced according the predicted lncRNA-PRAL structure (Supporting Fig. 10A). Proteins isolated from the RNA pull-down assays were identified by western blotting analyses using specific anti-HSP90 or p53 antibodies. (B) The S1 fragment of lncRNA-PRAL was further truncated around the basic stem-loop motif to monitor the ability of shorter versions binding to HSP90 by western blotting. (C) Up: dChIRP oligonucleotide design strategy. Biotinylated antisense OPs are designed to tile motif A, B, C, and fragment S3 of lncRNA-PRAL. Light blue solid line, lncRNA-PRAL; dotted line, antisense oligonucleotide; green patch, possible binding protein. Down: Workflow of dChIRP. Whole HCCLM3 cells are cross-linked to preserve protein/nucleic acid interactions. Sonication is used to shear nucleic acids. Next, the mixture is subdivided into five equal samples. OP is added to each sample, then the biotinylated oligonucleotides, RNA targets, and cross-linked proteins are purified using magnetic streptavidin beads. (D) Fragments of lncRNA-PRAL are enriched >1,000-fold over the abundant GAPDH mRNA in dChIRP samples. LacZ ChIRP does not enrich for lncRNA-PRAL over GAPDH. Average of technical triplicates ± standard deviation shown. (E) The protein fraction from each lncRNA-PRAL dChIRP sample was analyzed by immunoblotting against HSP90, p53, and GAPDH. Motif A and C efficiently recovered HSP90 and p53. LacZ ChIRP recovered no detectable protein. GAPDH was not detected in any sample. β-actin was used as the input control. n = 2. (F) Western blotting analyses for total and nucleus p53 protein expression in HCCLM3 cells transfected with PRAL full-length vector, S1 fragment vector, S3 fragment (lncRNA-PRAL with S1 fragment deletion) vector, and null vector as negative control, respectively. Abbreviation: RT-PCR, real-time PCR.
the lncRNA-PRAL full-length transcript (Supporting Fig. 10A). However, those motifs are separate from one another in the S2 fragment (Supporting Fig. 10C), which may impede their association with HSP90. We further truncated the S1 fragment into shorter fragments that retained the basic stem-loop motifs (Supporting Fig. 10D) to monitor the ability of shorter versions binding to HSP90, and our results indicated that these shorter fragments lacked the ability for binding to HSP90 (Fig. 5B). In order to dissect the HSP90- and p53-binding domains of lncRNA-PRAL within its native cellular context, we further performed modified domain-specific chromatin isolation by RNA purification (dChIRP), a technique described previously. First, biotinylated antisense 20-mer oligonucleotides were designed according to the reported principle, and the oligonucleotides were divided into domain-specific oligonucleotide pools, such that pools target motif A, B, C, and fragment S3 of lncRNA-PRAL (Fig. 5C). We used a similar set of probes that targeted the lacZ mRNA as a negative control. Next, whole HCCLM3 cells were cross-linked to preserve protein-RNA interactions. Sonication was used to solubilize the fraction and shear nucleic acids. Oligonucleotide pools (OPs) were added to each sample and allowed to hybridize. Then, the biotinylated OP, hybridized RNA, and associated proteins were purified on magnetic streptavidin beads (Fig. 5C). To confirm that dChIRP could recover the intended fragments of lncRNA-PRAL, we purified the RNA fraction from the dChIRP samples and analyzed RNA recovery by qPCR using primers for motif A, B, C, fragment S3, and GAPDH that should not be enriched by lncRNA-PRAL dChIRP. We confirmed that lncRNA-PRAL dChIRP specifically retrieved lncRNA-PRAL, whereas LacZ ChIRP did not enrich for lncRNA-PRAL (Fig. 5D). Finally, the recovered material from each dChIRP sample was analyzed by immunoblotting. We found that motif C and motif A OP recovered p53 and HSP90, whereas the motif B and fragment S3 OPs recovered no detectable p53 or HSP90 (Fig. 5E). As negative controls, the LacZ OP recovered no proteins, and GAPDH was not detected in any sample. In general, the three basic stem-loop motifs at the 5′ end of lncRNA-PRAL are required for the association between HSP90 and p53. Then, we conducted the experiment to detect the function of the short versions of lncRNA-PRAL in p53 activation. Deletion of S1(S3), which mediates the HSP90 association, abolished the ability of lncRNA-PRAL in increasing the p53 protein levels both in total and nucleus protein, whereas S1 fragment could enhance the accumulation of p53 (Fig. 5F).

THE ASSOCIATION OF lncRNA-PRAL WITH HCC PROGNOSIS

To further investigate whether the lncRNA-PRAL genomic copy number deletion correlated with the survival of HCC patients, we investigated two independent cohorts of HCC patients: cohort 2 comprising 189 patients and cohort 3 comprising 102 patients (Supporting Table 1). Kaplan-Meier analysis revealed that low lncRNA-PRAL genomic DNA level in HCC tissues was significantly correlated with markedly reduced tumor-free survival and overall survival in HCC patients (Fig. 6A,B). Significantly, in the two independent HCC cohorts, the multivariate analysis confirmed that low lncRNA-PRAL genomic DNA level in HCC tissues was an independent predictor for the reduced tumor-free survival of HCC patients (Supporting Tables 4 and 5). Moreover, the integrated survival analysis with genomic level of lncRNA-PRAL, tumor number, and portal vein thrombus indicated a more efficient prognostic predict for HCC patients (Supporting Fig. 11). Taken together, the clinical data on our patients support the conclusion that genomic DNA deletion of lncRNA-PRAL locus is the potent genetic factor predisposing HCC patients with poor prognosis of the disease.

Tumor suppressor gene p53 has been described to be inactivated in HCC through various mechanisms. In order to verify the relationship between lncRNA-PRAL deletion and the level of p53 in clinic samples, we determined the level of p53 in HCC tissues using immunohistochemistry (IHC) prepared from cohort 2 patient samples without p53 deletion, R249S mutation, or HBV X protein (HBx) expression (n = 91). Among these patient samples, strong p53 staining, with a score of IHC intensity over 2 (+ +), was detected in 37 of 47 tumor specimens with higher lncRNA-PRAL genomic levels. In contrast, 27 of 44 tumor samples with lower lncRNA-PRAL genomic levels showed very low p53 levels with a score of IHC intensity less than 1 (+) (Fig. 6C). More important, lower lncRNA-PRAL genomic DNA levels in patients with lower p53 IHC score showed a much worse prognosis than higher lncRNA-PRAL genomic DNA levels in patients with higher level of p53 (Fig. 6D). These results demonstrated that genomic alterations of lncRNA-PRAL was a clinical risk factor and
FIG. 6. Copy number deletion of genomic IncRNA-PRAL correlates with poor survival in HCC patients. Shown are Kaplan-Meier survival curves of tumor-free survival and overall survival in cohort 2 (A) and cohort 3 (B), according to the genomic copy number level of IncRNA-PRAL in each tumor sample. (C) IHC of p53 in HCC samples without p53 deletion, R249S mutation, or HBx expression in cohort 2 (n = 91). Left: representative images showing different intensities of p53 staining. Scale bars, 100 μm. Lower panels represent magnified pictures (5×) of boxed area in the corresponding upper panels. Right: box plot graph showing the quantitative evaluation of p53 staining intensity from IHC analysis. Plot of a box plot (25%-75%) with whiskers to minimal and maximal of all the score data was used. The statistical differences between the two groups were analyzed by one-way ANOVA. **P < 0.01. (D) Tumor-free survival (left) and overall survival (right) between HCCs with lower and higher IncRNA-PRAL genomic DNA in cohort 2 HCC without p53 deletion, R249S mutation, or HBx expression. The median value in each cohort was chosen as the cut-off point. LINE1 (long interspersed element-1) as the genomic level internal control.
may serve as a combinative potential prognostic predictor with p53 for HCC patients.

Discussion

SCNVs are common events in HCC and can contribute to tumor progression. This is because regions harboring DNA changes can contain critical genes whose altered dosage contributes to the neoplastic process. Genomic instability detection, together with analyses of transcriptional expression levels and functional experiments, has identified candidate cancer genes in the development and progression of HCC. However, functional studies of individual genes selected from lists of candidate genes may be biased, particularly without the consideration of IncRNA function. Building upon the previous identified aberrantly expressed IncRNAs and SCNVs, we assigned the IncRNA genes to these common aberrant sites, identifying IncRNA genes in genomic gain and loss regions that frequently occur in HCC. Among these deregulated IncRNAs, IncRNA-PRAL on chromosome 17p13.1 is the most frequently deleted in HCC. Given the relatively small number of samples in the process of microarray, two independent cohorts of HCC patients were examined to confirm this finding. We analyzed the genomic regions harbored in the IncRNA-PRAL in SCNVs data. We found that the genomic location with IncRNA-PRAL deletion can be found in Chr17:0-22,403,245, with the average length of 2,506 kilobases (kb). And most of the copy number variations (CNVs; 87.8%) appeared from Chr17: 6,316,339-11,659,261 with an average length of 1,044 kb. Within this genomic region, accompanying with IncRNA-PRAL focal loss, genomic alterations of ak125534 can be found in 20.8% of samples, genomic loss of CR623485 can be found in 11.1% of samples, and ak091139 deletion can be found in 6.9% of samples. The presence of a deletion of IncRNA-PRAL can be used to predict tumor progression or response to treatment. It can also lead to the development of new tumor-fighting drugs.

The tumor suppressor protein, p53, is a transcription factor that induces growth arrest or apoptosis in response to a variety of stress signals. Inactivation of p53 is the most common event found in human cancer. Our results indicated that down-regulated IncRNA-PRAL may be the important factor to inactivate p53 in p53-wild HCC patients. Our results indicated that IncRNA-PRAL may up-regulate p53 protein level in hepatoma cells and reduce ubiquitination of p53 through the association of IncRNA-PRAL, HSP90, and p53. HSP90 has a more complex role in facilitating neoplastic transformation than simply by inhibiting apoptosis. The dynamic, low-affinity interactions of HSP90 with its client proteins, such as hormone receptors, transcription factors, and kinases, maintain these proteins in a latent, but readily activated, state. Using biophysical methods, a previous study indicated that the fine-tuned interplay between several HSP90 domains provides the interactions required for the efficient chaperoning of p53 into the nucleus. Additionally, p53 was reported to utilize an HSP90-dependent movement system for translocation to the nucleus along microtubule tracts driven by the cytoplasmic dynein motor protein. A common emerging theme among IncRNAs is that they form RNA-protein interactions to perform their functions by modulating chromatin-modifying complexes or by altering mRNA stability and likely by many additional unidentified mechanisms. The mechanism by which HSP90-associated IncRNAs affect HSP90 and its client protein complexes is an area that requires further study. The results presented here indicate that IncRNA-PRAL directly binds to HSP90, blocks ubiquitination of p53, and then promotes HCC cancer cell apoptosis in vitro and in vivo, findings that lead us to propose that HSP90-associated IncRNAs may provide many new options for cancer therapy.

REFERENCES

1) Hong SM, Vincent A, Kanda M, Leclerc J, Omura N, Borges M, et al. Genome-wide somatic copy number alterations in low-grade PanINs and IPMNs from individuals with a family history of pancreatic cancer. Clin Cancer Res 2013;18:4303-4312.
2) Li Y, Zhang L, Ball RL, Liang X, Li J, Lin Z, Liang H. Comparative analysis of somatic copy-number alterations across different human cancer types reveals two distinct classes of breakpoint hotspots. Hum Mol Genet 2012;21:4957-4965.
3) Fu L, Dong SS, Xie YW, Tai LS, Chen L, Kong KL, et al. Down-regulation of tyrosine aminotransferase at a frequently deleted region 16q22 contributes to the pathogenesis of hepatocellular carcinoma. HEPATOLOGY 2010;51:1624-1634.
4) Kwon SM, Kim DS, Won NH, Park SJ, Chwae YJ, Kang HC, et al. Genomic copy number alterations with transcriptional deregulation at 6p identify an aggressive HCC phenotype. Carcinogenesis 2013;34:1543-1550.
5) Tang YC, Amon A. Gene copy-number alterations: a cost-benefit analysis. Cell 2013;152:394-405.
6) Lau SH, Guan XY. Cytogenetic and molecular genetic alterations in hepatocellular carcinoma. Acta Pharmacol Sin 2005;26:659-665.
7) Guichard C, Amaddeo G, Imbeaud S, Ladeiro Y, Pelletier L, Maad IB, et al. Integrated analysis of somatic mutations and
focal copy-number changes identifies key genes and pathways in hepatocellular carcinoma. Nat Genet 2012;44:694-698.

9) Roessler S, Long EL, Budhu A, Chen Y, Zhao X, Jin J, et al. Integrative genomic identification of genes on 8p associated with hepatocellular carcinoma progression and patient survival. Gastroenterology 2012;142:957-966.e12.

10) Lonigro RJ, Grasso CS, Robinson DR, Jing X, Wu YM, Cao X, et al. Detection of somatic copy number alterations in cancer using targeted exome capture sequencing. Neoplasia 2011;13:1019-1025.

11) Ma NF, Wang K, Lim HY, Shi S, Lee J, Deng S, Xie T, et al. Genomic landscape of copy number aberrations enables the identification of oncogenic drivers in hepatocellular carcinoma. HEPATOLOGY 2013;58:706-717.

12) Gu DL, Chen YH, Shih JH, Lin CH, Jou YS, Chen CF. Targeted genes discovery through copy number alteration analysis in human hepatocellular carcinoma. World J Gastroenterol 2013;19:8873-8879.

13) Carriero C, Cimatti L, Biagioli M, Beugnet A, Zucchelli S, Fedele S, et al. Long non-coding antisense RNA controls Uchl1 translation through an embedded SINEB2 repeat. Nature 2012;491:454-457.

14) Massa PG, Rump A, Schulz F, Stricker S, Schulze L, Platzer K, et al. A misplaced IncRNA causes brachydactyly in humans. J Clin Invest 2012;122:3990-4002.

15) Ren S, Peng Z, Mao JH, Yu Y, Yin C, Gao X, et al. RNA-seq analysis of prostate cancer in the Chinese population identifies recurrent cancer-associated long noncoding RNAs and aberrant alternative splicings. Cell Res 2012;22:806-821.

16) Yang F, Zhang L, Huo XS, Yuan JH, Xu D, Yuan SX, et al. Long noncoding RNA high expression in hepatocellular carcinoma facilitates tumor cell migration and spreading through downregulating RhoGDIA. Nat Cell Biol 2010;12:390-399.

17) Pan T, Zeng W, Wang X, et al. A misplaced lncRNA causes brachydactyly in humans. RNA Biol 2013;10:1771-1777.

18) Zhu J, Liu S, Ye F, Shen Y, Tie Y, Zhu J, et al. Identification of a novel oncogene, amplified in liver cancer 1, within a commonly amplified region at 1q21 in hepatocellular carcinoma. HEPATOLOGY 2010;51:2019-2025.

19) Peng ZJ, Li YC, Lin CH, Jou YS, Chen CF. Revealing long noncoding RNA architecture and therapeutic potentials of virus-mediated CHD1L depletion. Gut 2011;60:534-543.

20) Wang K, Lim HY, Shi S, Lee J, Deng S, Xie T, et al. Genomic landscape of copy number aberrations enables the identification of oncogenic drivers in hepatocellular carcinoma. HEPATOLOGY 2013;58:706-717.

21) Chu C, Qu K, Zhong FL, Artandi SE, Chang HY. Genomic maps of long noncoding RNA occupancy reveal principles of RNA-chromatin interactions. Mol Cell 2011;44:667-678.

22) Quinn JJ, Ilik IA, Qu K, Georgiev P, Chu C, Akhtar A, Chang HY. Revealing long noncoding RNA architecture and functions using domain-specific chromatin isolation by RNA purification. Nat Biotechnol 2014;32:933-940.