Targeting H3K27 trimethylation epigenome for liver cancer prevention: abridged secondary publication

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KEY MESSAGES

1. In hepatitis B virus (HBV) endemic areas such as Mainland China and Hong Kong, hepatocellular carcinoma (HCC) is a common manifestation of chronic HBV carriers. Cancer genome sequencing studies have demonstrated that epigenome disruption is a major hallmark of HCC.
2. The HBV X protein (HBx) has been shown to up-regulate the polycomb protein enhancer of zeste homolog 2 (EZH2), which catalyses tumour suppressor gene silencing via histone H3 lysine 27 trimethylation (H3K27me3). The identification of genomic repertoire of H3K27me3 targets and the polycomb recruiters for H3K27me3 deposition provide insights into molecular carcinogenesis and development of novel therapeutic strategies.
3. Using an integrated high-resolution genome-wide approach, we have characterised the HBx-deregulated H3K27me3 epigenome in HBx-transgenic HCC model. Our integrative study demonstrates that Ying yang 1 overexpression contributes to EZH2 recruitment for H3K27me3-mediated repression of tumour-suppressive protein–coding and microRNA genes, thereby enhancing nuclear factor-kappa B signalling in hepatocarcinogenesis.
4. Despite the survival improvement in HCC patients receiving multi-kinase-targeted inhibitor, the outcomes are still far from satisfactory. Given the availability of potent EZH2 inhibitors and their significant effects in reactivating tumour suppressor genes in HCC cells, further testing is warranted to determine if it is an effective chemopreventive strategy in patients with HBV-associated precancerous lesions. Targeting H3K27me3 epigenome for HCC prevention might benefit large populations of chronically HBV-infected patients.

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Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer death in China and the fifth most frequent malignancy worldwide. Most HCC cases are associated with cirrhosis secondary to chronic hepatitis B virus (HBV) infection. Around 12% of the population of China are estimated to be HBV carriers. Although targeted therapy using tyrosine kinase inhibitor has shown clinical efficacy, no specific oncogene addictions are yet known for HCC.

One remarkable finding of cancer genome sequencing is the repeated discovery of somatic driver mutations in genes that encode chromatin-remodelling factors, which regulate the epigenome (ie the totality of chemical modifications to the genome that do not involve a change in the nucleotide sequence). As much as 50% of HCCs are estimated to harbour mutations in different chromatin regulators. This suggests that aberration in chromatin remodelling, which leads to epigenome disruption, is a hallmark of HCC.[1] In addition to somatic mutations, malfunction of chromatin regulators can be caused by transcriptional deregulation in cancer. Enhancer of zeste homolog 2 (EZH2) is the catalytic subunit of the polycomb-repressive complex 2 that represses gene transcription through histone H3 lysine 27 trimethylation (H3K27me3).[2] *EZH2* gene mutations are discovered in haematological cancers, and aberrant mis-expression of EZH2 is noted in a variety of solid tumours including HCC.[3] The HBV X protein (HBx), a promiscuous *trans*-activator involved in hepatocellular neoplastic transformation, can up-regulate EZH2 gene expression.[3,4] Although EZH2 causes H3K27me3-mediated silencing of Wnt antagonists to promote β-catenin-dependent hepatocarcinogenesis,[2,3] the complete genomic repertoire of H3K27me3 targets and their deregulated signalling pathways in HCC remain largely unknown. To determine the epigenetic connection between chronic HBV infection and hepatocarcinogenesis, we used an HBx-transgenic (TG) HCC model

to characterise the H3K27me3 epigenome using an integrated high-resolution genome-wide and bioinformatics approach. Our findings reveal new epigenetically-silenced microRNAs (miRNAs) that contribute to key oncogenic signalling activation in HCC development.

Methods

Liver tissues from both HBx homozygous TG and C57BL/6 wild-type male mice were collected for immunoprecipitation of H3K27me3-bound DNA followed by high-throughput DNA sequencing (ChIP-seq) and RNA-seq.[5] The animal study was approved by the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong.

Human HCC cell lines were transfected with small-interfering RNAs using HiPerfect (Qiagen).[3,4] For measuring the activities of cancer-related transcription factors, cells were transfected with the cancer 10-pathway reporter constructs (Qiagen) followed by dual luciferase reporter assays (Promega).[5] For validation of miRNA targets, wild-type or mutated construct was cloned into the vector pGL3-basic (Promega) followed by transfection and dual luciferase reporter assays.

Quantitative RT-PCR, western blot, and immunohistochemistry in human HCC tissue microarray were performed as previously described.[3-5]

Patients who underwent hepatectomy for HCC at the Prince of Wales Hospital, Hong Kong were included. All patients gave written informed consent on the use of clinical specimens for research purposes. This study was approved by the Joint Chinese University of Hong Kong – New Territories East Clinical Research Ethics Committee.

Data were expressed as mean ± standard deviation with triplicate experiments. Independent Student's *t*-tests were used for in vitro experiments. Differences in gene expression levels in clinical samples was analysed using paired *t*-tests. Kaplan Meier survival analysis was used to determine the disease-free survival rate; the difference was compared by log-rank Mantel-Cox test.

Results

We used ChIP-seq and RNA-seq to investigate the genome-wide H3K27me3-bound promoters and the concurrently down-regulated genes in normal and tumour-bearing liver tissues from wild-type and HBx-TG mice (Fig 1a). Western blot analysis showed that the normal livers from wild-type mice contained low levels of EZH2 and H3K27me3. By contrast, the liver tissues from ageing HBx-TG mice exhibited increasing expressions of these proteins and peaked in the tumour tissues of 18-month-old TG mice (Fig 1b). By integrating the ChIP-seq and RNA-seq results, we revealed 1359 and 1027 H3K27me3-occupied and repressed genes in tumour (TG-18m-T) versus normal liver (WT-3m-NL) and adjacent non-tumour (TG-18m-NT), respectively (Fig 1c). Among the 611 targets common in both gene lists, at least 20 have been reported to be TSGs (Fig 1d). For example, potential H3K27me3-mediated silencing of *glycine N-methyltransferase (gnmt)* was a prominent tumour suppressor in murine and human HCCs (Fig 1e).

Quantitative RT-PCR demonstrated that 16 potential TSGs were significantly down-regulated in murine tumours compared with control tissues (Fig 1f). Pharmacological inhibition of EZH2 and H3K27me3 in four human HCC cell lines further demonstrated that nine of the 16 genes were indeed epigenetically regulated (data not shown). To investigate the clinical relevance of our findings, we examined their gene expressions in 12 pairs of human HBV-associated HCC specimens, and six genes were significantly down-regulated in tumours compared with adjacent non-tumour counterparts (Fig 1g). These data illustrate that EZH2 epigenetically silences TSGs via H3K27me3 during HCC development.

To elucidate the transcription factors that mediate polycomb targeting in HCC, we performed motif analysis of the tumour-specific H3K27me3-bound regions and identified a highly significant centrally-enriched motif (P<5E-6) for YY1. Western blot analysis of the wild-type and HBx-TG tissues showed concordant up-regulation of YY1, EZH2, and H3K27me3 in HCC tissues, thus supporting the involvement of YY1 in H3K27me3 modification (Fig 1b). Down-regulation of YY1 remarkably reduced the global levels of H3K27me3 in HCC cell lines (Fig 2a). Using 50 pairs of human HBV-associated HCCs, quantitative RT-PCR demonstrated that YY1 and EZH2 overexpression was detected in 68% and 84% of HCCs (Fig 2b) and positively correlated (P<0.0001, Fig 2c). Consistently, both protein expressions were significantly up-regulated in HCCs as shown by immunohistochemistry using a tissue microarray containing 194 cases (P=9E-5 and P=2.4E-44, respectively, Fig 2d). Kaplan–Meier analysis showed that advanced-stage (II-III) HCC patients with concomitant YY1/EZH2 up-regulation were significantly associated with shorter disease-free survival (hazard ratio=1.875, 95% confidence interval=1.008-3.488, P=0.047, Fig 2e).

Given the extensive H3K27me3 modification in the HCC genome, we focused on aberrant epigenetic control of miRNAs because of their biological significance in signalling deregulation. ChIP-seq demonstrated enriched H3K27me3 occupancy in 16 miRNA loci in HBx-induced HCC tumours compared with the adjacent non-tumour tissues (Fig 3a). Notably, 15 of them have been reported

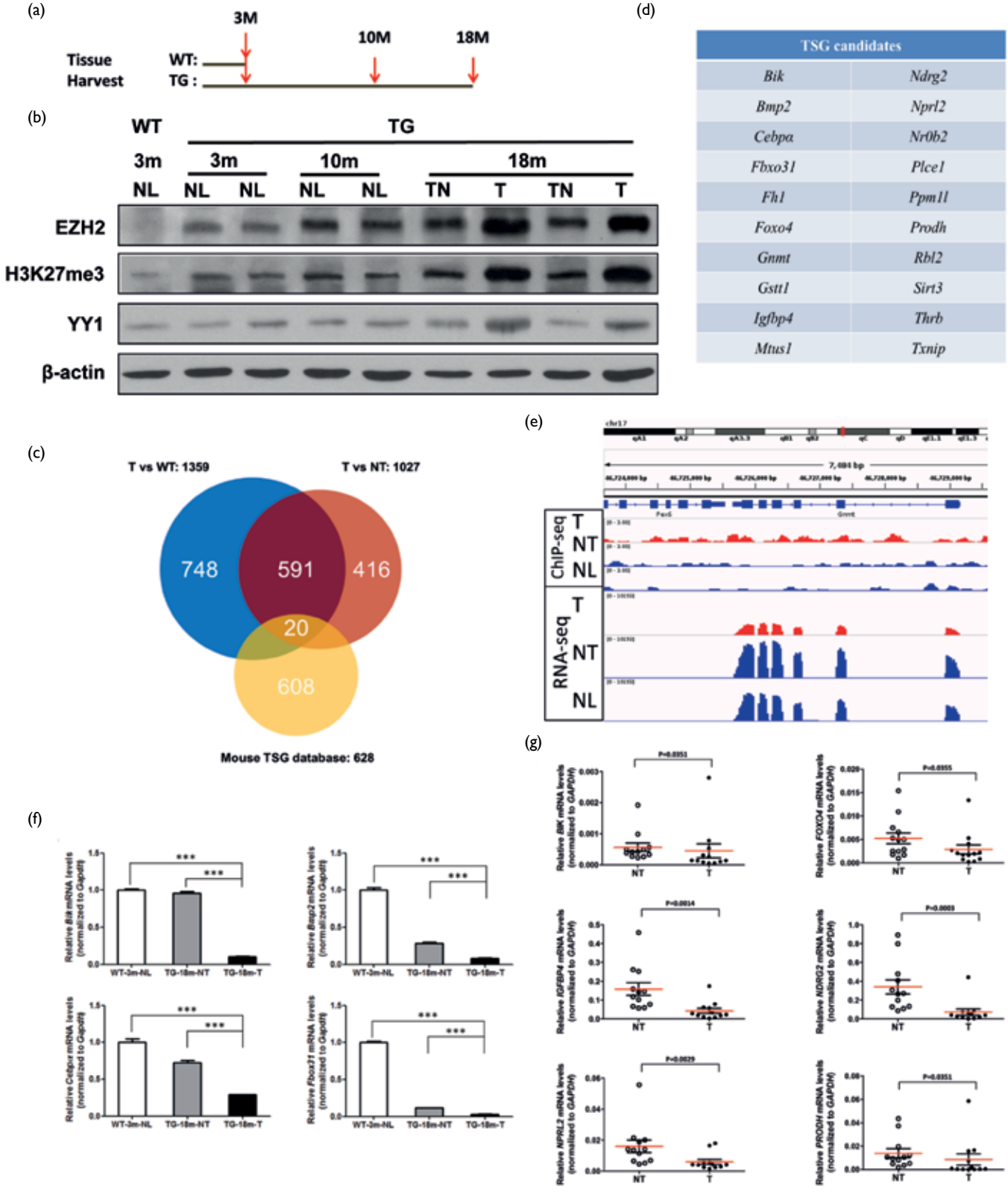

FIG 1. Deregulation of H3K27 trimethylation and gene repression in an HBx-TG mouse model: (a) Schematic diagram showing the collection of liver tissues from wild-type and HBx-TG mice at different time-points. (b) Western blot analysis of the normal (NL), adjacent non-tumour (NT), and tumour (T) tissues. (c) Venn diagram showing the integration of ChIP-seq, RNA-seq and mouse TSG database: 1359 H3K27me3-occupied and repressed genes in tumour (TG-18m-T) versus normal liver (WT-3m-NL); 1027 genes in tumour (TG-18m-T) versus adjacent non-tumour (TG-18m-NT); and 628 mouse TSGs from a published database. (d) 20 epigenetically-regulated TSGs in murine HBx-TG model. (e) Representative diagram showing the ChIP-seq and RNA-seq signals mapped to a candidate gene, Gnmt, in liver tissues of WT-3m-NL, TG-18m-NT and TG-18m-T mice, respectively. Quantitative RT-PCR analysis of the candidate genes in the (f) original murine tissues and (g) human HBV-associated HCCs. **P<0.01; ***P<0.001.

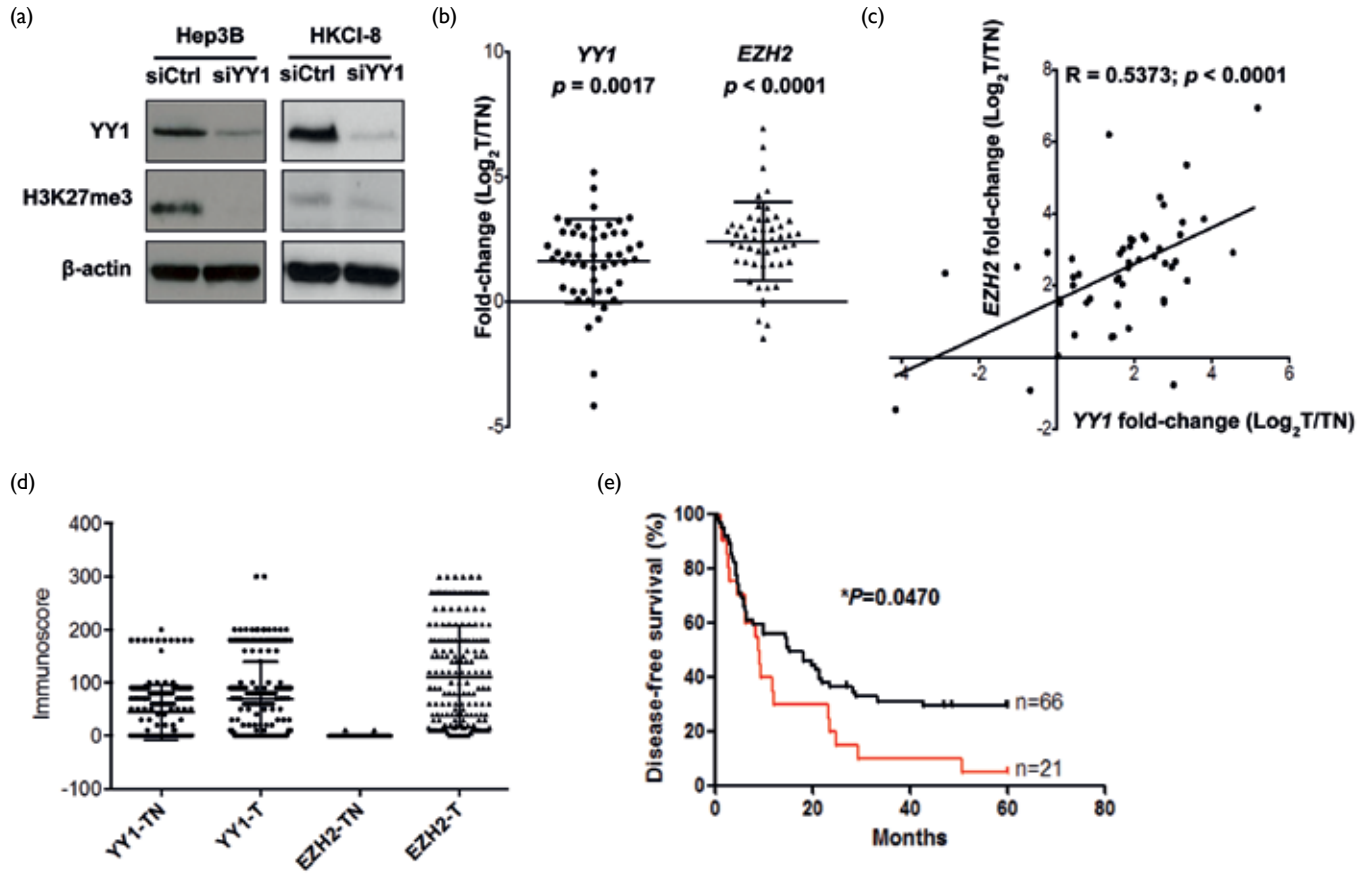

FIG 2. Significant association between YY1 and H3K27me3 in HCC: (a) Western blot showing that knockdown of YY1 reduced H3K27me3 levels in Hep3B and HKCI-8 HCC cells. (b) Up-regulation of *YY1* and *EZH2* mRNA in 68% and 84% of human HCCs, respectively. (c) *YY1* and *EZH2* gene expressions exhibiting a significant positive correlation. (d) Immunoreactivities of YY1 and EZH2 are significantly higher in human HCC tumours (T) than in tumour-adjacent liver tissues (TN). (e) Survival of advanced-stage HCC patients with concurrent up-regulation (high activation) of YY1 and EZH2 is significantly shorter than those with low activation.

to function as TSG in human cancers (Fig 3b). We found that knockdown of YY1 not only decreased its own occupancy but also EZH2 and H3K27me3 in the upstream regions of the *miR-9-1* and *miR-9-2* loci (P<0.05, Fig 3c), resulting in *miR-9* transcription in HCC cells (P<0.01, Fig 3d).

Using a luciferase reporter array that allows simultaneous measurement of ten transcription factor activities, we found that YY1 knockdown significantly reduced NF-κB signalling by ~50% in HCC cells (P<0.01, Figs 3e, 3f). Consistently, down-regulation of YY1 suppressed the p50 and p65 protein expressions (Fig 3e). Using TarBase 6.0 database, we found that the target genes of the 16 H3K27me3-occupied miRNAs were enriched in the regulation of the IκB kinase/NF-κB cascade (P=0.00156). We experimentally confirmed the direct repression of *NFKB1* by *miR-9* in HCC cells (Fig 3g). Concordantly, *miR-9* overexpression reduced the protein levels of p50 and p65 (Fig 3h) and NF-κB transcriptional activity (Fig 3i). Collectively, these data suggest that epigenetic silencing of miRNAs by YY1 activates NF-κB signalling in HCC cells.

Discussion

Genome-wide mapping of polycomb binding sites in different cell types and disease states has revealed an unexpected diversity. The identification of the PRC2 recruiters for H3K27me3 deposition provides additional insights into molecular pathogenesis and development of novel therapeutic strategies. Using an HBx transgenic model, HBV-associated HCC cell lines, and clinical specimens, we provide evidence to support the notion that YY1 acts as a crucial mediator of H3K27me3 modification in HCC. Of particular clinicopathological significance, we found that concomitant up-regulation of YY1 and EZH2 is associated with shortened survival in patients with advanced HCC. Multiple tumour-suppressive protein-coding and miRNA genes are concordantly silenced by H3K27me3, the latter of which

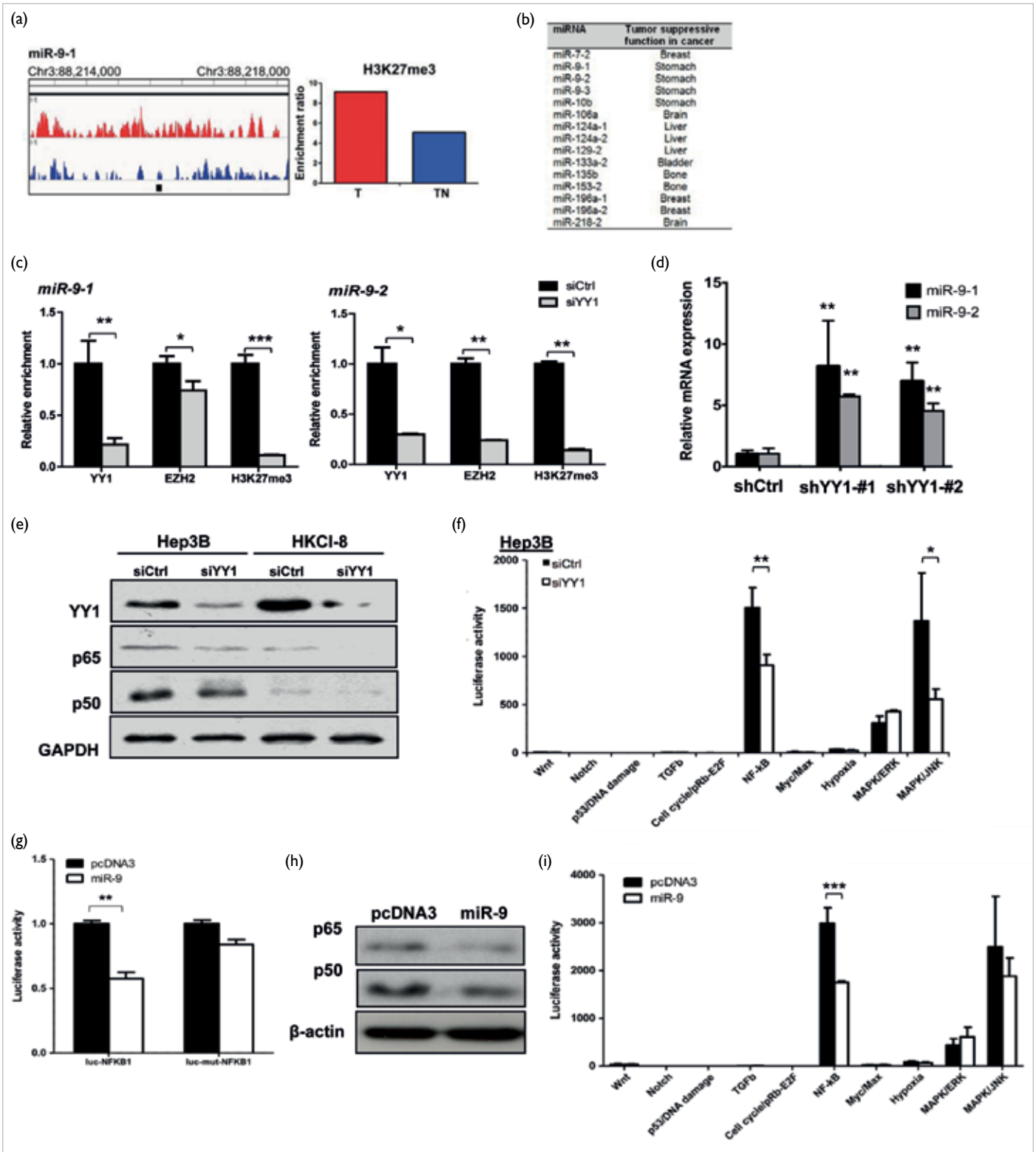

FIG 3. Epigenetic silencing of miRNA by YY1 activates NF-κB signalling in HCC cells: (a) H3K27me3 enrichment at miRNA loci in HCC compared with non-tumour tissues as revealed by ChIP-seq. (b) List of H3K27me3-enriched miRNAs in HBx-TG model showing tumour suppressive functions in human cancers. (c) Quantitative ChIP-PCR analysis of YY1, EZH2, and H3K27me3 occupancy in the promoter regions. (d) Quantitative RT-PCR analysis of *miR-9-1* and *miR-9-2* upon YY1 knockdown in Hep3B cells. (e) Western blot analysis of p65 and p50 upon YY1 knockdown in Hep3B and HKCI-8. (f) The activities of ten cancer-related transcription factors upon YY1 knockdown were measured by luciferase reporter assays. (g) Luciferase reporter assays confirmed the direct physical interaction between miR-9 and NFKB1 3'UTR in Hep3B. (h) Western blot analysis of p65 and p50, and (i) luciferase reporter assay in Hep3B upon transfection with miR-9 mimics. * P<0.05; ** P<0.01; *** P<0.001.

contributes to constitutive NF-κB activation in HCC cells. These findings not only unveil a novel master regulator of PRC2 recruitment in HCC but also shed mechanistic insight into the anti-neoplastic action of EZH2-targeted therapy.

Activation of NF-κB, a master regulator of inflammation and cell survival, is a frequent and early event in human HCC of both viral and non-viral aetiologies. The carcinogenesis function of NF-κB is supported by genetically-modified mouse studies. We showed that YY1 overexpression contributes to NF-κB activation in HCC, which is at least partially mediated by epigenetic deregulation of miRNAs. To our knowledge, this is the first evidence to support a chromatin regulation of NF-κB signalling via miRNAs in HCC.

This project has improved our understanding on the establishment and functional significance of H3K27me3 epigenome in HBV-associated HCC. Our integrative study demonstrates that YY1 overexpression contributes to EZH2 recruitment for H3K27me3-mediated repression of tumour-suppressive protein-coding and miRNA genes, thereby enhancing key oncogenic signalling pathways in hepatocarcinogenesis.[4,5] With the advancement of genome editing technology, liver cancer cell-specific targeting of YY1 could be a novel therapeutic option. Given the promising preliminary data from ongoing clinical trials of specific EZH2 inhibitors in different haematological and solid tumours (NCT01897571, NCT02082977, NCT02601950), the strategy of targeting H3K27me3 epigenome for HCC prevention and treatment might benefit large populations of chronically HBV-infected patients.

Funding

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Disclosure

The results of this research have been previously published in:

1. Tsang DP, Wu WK, Kang W, et al. Yin Yang 1-mediated epigenetic silencing of tumour-suppressive microRNAs activates nuclear factor-κB in hepatocellular carcinoma. J Pathol 2016;238:651-64.

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