precursors called pri-miRNAs, which are processed within the nucleus by Drosha, a ribonuclease III enzyme, in collaboration with Pasha (also known as DGCR8). The processing of pri-miRNAs produces ~70-nt-long molecules called pre-miRNAs, which fold into imperfect stem-loop structures. The pre-miRNAs are exported into the cytoplasm by the nuclear export protein Exportin-5 (XPO5). Once in the cytoplasm, the pre-miRNAs are released in a GTP-dependent manner and processed by another ribonuclease III, Dicer, in complex with the double-stranded RNA-binding protein TRBP to produce the functional 22-nt-long miRNA.

Several studies have provided evidence that dysregulated miRNA expression contributes to the initiation and progression of human cancers (Croce, 2009; Esquela-Kerscher and Slack, 2006; Esteller, 2011). Indeed, downregulation of a subset of miRNAs is a commonly observed feature of cancers, suggesting these molecules may act as tumor suppressors. The first report of altered miRNA expression in cancer was related to the frequent chromosomal deletion and downregulated expression of *miR-15* and *miR-16*, two miRNAs thought to target the antiapoptotic factor *BCL2* in chronic lymphocytic leukemia (Calin et al., 2002). Another example is let-7, which negatively regulates expression of Ras oncogenes; its downregulation in tumors is thought to contribute to activation of the Ras signaling pathway (Johnson et al., 2005).

Although the mechanism underlying miRNA dysregulation in cancer is not yet fully understood, recent studies have shown that epigenetic mechanisms play important roles in the regulation of miRNA expression. Epigenetic gene silencing due to promoter CpG island hypermethylation is one of the most common mechanisms by which tumor suppressor genes are inactivated during tumorigenesis. To date, approximately half of the classical tumor suppressor genes known to be mutated in familial cancer syndromes have been shown to be inactivated by promoter hypermethylation (Feinberg and Tycko, 2004; Jones and Baylin, 2002). In addition to classical tumor suppressors, increasing numbers of genes related to cell-cycle control, DNA repair, tumor invasiveness and the response to growth factors are being identified as inactivated by hypermethylation in malignancies (Jones and Baylin, 2007; Suzuki et al., 2008). Moreover, recent advances in microarray and sequencing technologies have enabled comprehensive analysis of the epigenome and miRNA expression in cancer cells, and as a result the list of miRNA genes silenced by methylation in cancer is rapidly growing (Lopez-Serra and Esteller, 2012) (Figure 1, Table 1). In this review, we will highlight the contribution made by epigenetic alteration of miRNA genes in cancer, and discuss their clinical application as biomarkers and therapeutic targets.

2. Screening methods to identify methylation of miRNA genes

The first evidence of an epigenetic mechanism involved in silencing miRNAs in cancer came from a pharmacological unmasking experiment. Using a miRNA microarray, Saito et al. analyzed the expression profiles of miRNAs in a T24

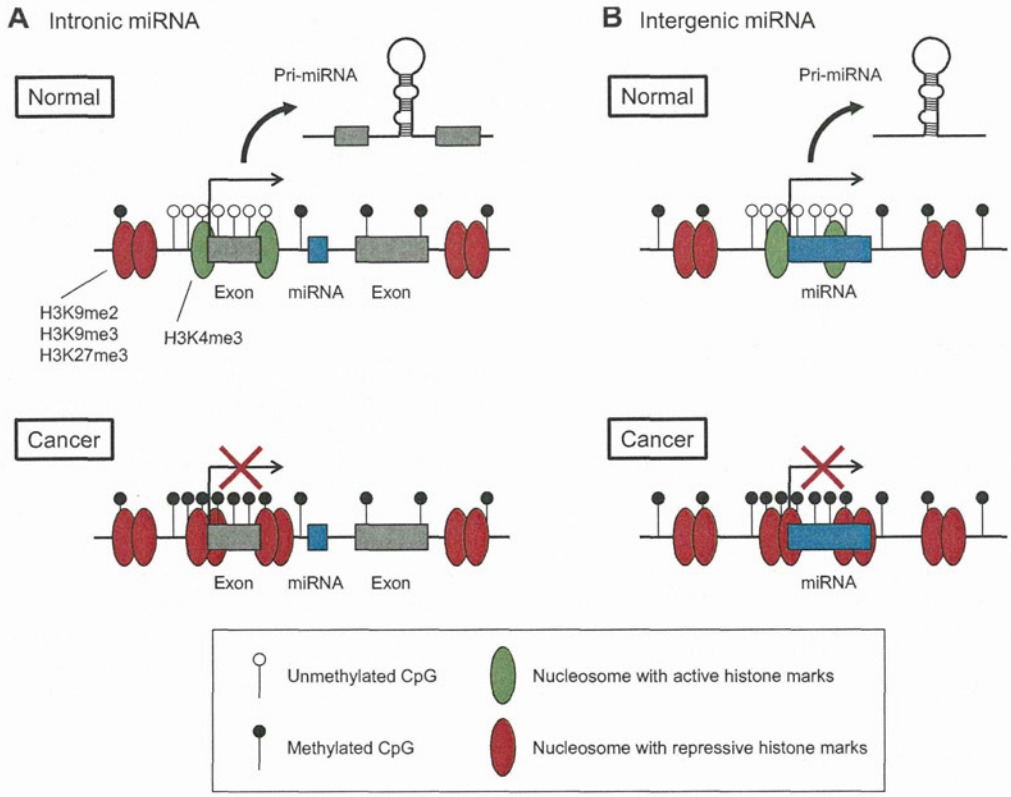

Figure 1 – Epigenetically silenced miRNA genes in cancer.

Table 1 – Aberrantly methylated miRNA genes in cancer.

Gene	Tumor type	Target gene
miR-1-1	Liver, colon	FOXP1, MET, HDAC4, ANXA2, BDNF
miR-9 family	Multiple types	FGFR1, CDK6, CDX2, E-cadherin
miR-10b	Stomach	MAPRE1
miR-34 family	Multiple types	MET, CDK4, CCNE2, C-MYC, CDK6, E2F3, Notch4
miR-124 family	Colon, stomach, liver, leukemia, cervix	CDK6, VIM, SMYD3, E2F6, IQGAP1, IGFBP7
miR-125b	Breast	ETS1
miR-127	Prostate, bladder, colon	BCL6
miR-129-2	Endometrium, colon, esophagus, stomach	SOX4
miR-132	Pancreas, prostate	TALIN2
miR-137	Head and neck, stomach, colon	CDK6, CDC42, LSD1
miR-143	Leukemia	MLL-AF4
miR-148a	Colon, head & neck, lung, breast, pancreas	TGIF2
miR-152	Endometrium, bladder, lung	DNMT1, E2F3, MET, RICTOR
miR-181c	Stomach	NOTCH4, KRAS
miR-193a	Liver, leukemia, bladder	SRSF2, KIT
miR-196b	Stomach	
miR-200 family	Colon, breast, lung, bladder	ZEB1, ZEB2
miR-203	Leukemia, liver, MALToma	ABL1, ABCE1, CDK6
miR-205	Bladder, lung	ZEB1, ZEB2
miR-218	Head and neck	RICTOR
miR-335	Breast	SOX4, TNC
miR-345	Colon	BAG3
miR-375	Esophagus, melanoma	IGF1R, PDK1
miR-512	Stomach	MCL1
miR-941	Colon	
miR-1224	Bladder	
miR-1237	Colon	
miR-1247	Colon	

bladder cancer cell treated with or without the DNA methyltransferase (DNMT) inhibitor 5-aza-2′-deoxycytidine (5-aza-dC) and the histone deacetylase (HDAC) inhibitor 4-phenylbutyric acid (4-PBA) (Saito et al., 2006). Of 313 miRNA genes evaluated, expression of 17 was upregulated by the drug treatment in T24 cells. Among them, *miR-127* is embedded within a CpG island, and its upregulation was associated not only with DNA demethylation but also with acetylation of histone H3 and trimethylation of histone H3 lysine 4 (H3K4me3), which are marks of active transcription. Experimental evidence confirmed that the proto-oncogene *B-cell lymphoma 6* (*BCL6*) is a target of *miR-127*, suggesting it can act as a tumor suppressor (Saito et al., 2006). Thereafter, miRNA microarray analysis revealed the epigenetic silencing of *miR-124* family genes in the HCT116 colorectal cancer (CRC) cell line and the same cell line in which both *DNMT1* and *DNMT3B* are genetically disrupted (Lujambio et al., 2007). The silencing of the *miR-34b/c* gene was also discovered in the same cells (Toyota et al., 2008), and a number of other methylated miRNA genes have been identified by screening for miRNAs upregulated by epigenetic drug treatment in cancer cells (Hashimoto et al., 2010; Kozaki et al., 2008; Lujambio et al., 2008; Saito et al., 2009).

Methylated miRNA genes have also been identified by analyzing genome-wide DNA methylation in cancer cells. For instance, through the combined use of methylated CpG island amplification (MCA) and CpG island microarray analysis, the methylation of *miR-9-1* was identified in pancreatic cancer (Omura et al., 2008). In addition, DNA methylation microarray analysis using the Infinium BeadChip revealed *miR-10b* as a target of DNA methylation in gastric cancer. Recently, Yan et al. performed a genome-wide methylome analysis entailing the deep sequencing of MBD (methylated DNA binding domain)-isolated DNA in HCT116 cells, and identified a variety of methylated genes, including *miR-941*, *miR-1237* and *miR-1247* (Yan et al., 2011).

As with protein-coding genes, epigenetic regulation of miRNA genes is tightly associated with histone modifications, among which trimethylation of histone H3 lysine 4 (H3K4me3) is a hallmark of active transcription, whereas di- or trimethylation of histone H3 lysine 9 (H3K9me2 or H3K9me3) and trimethylation of lysine 27 (H3K27me3) are marks of repression (Figure 1). The combination of chromatin immunoprecipitation (ChIP)-on-chip and miRNA microarray analyses in prostate cancer cells revealed that miRNA expression is positively correlated with H3K4me3 and inversely correlated with H3K27me3 in the miRNA promoter regions (Ke et al., 2009). Analysis of histone modification using ChIP-on-chip in acute lymphoblastic leukemia (ALL) revealed that the CpG islands of 13 miRNA genes are associated with high H3K9me2 and low H3K4me3, suggesting these miRNAs are epigenetically silenced in ALL (Roman-Gomez et al., 2009). Subsequent methylation analysis confirmed the hypermethylation of the selected miRNA genes, including the *miR-9* family, *miR-34* family and *miR-124* family genes. Hampering the identification of epigenetically dysregulated miRNA genes in cancer is the limited annotation of the primary transcripts of miRNA genes. Earlier studies have shown that H3K4me3 could be a useful mark with which to identify the active promoter regions of miRNA genes (Marson et al., 2008; Ozsolak et al.,

2008). In addition, we recently assessed genome-wide histone modification by performing deep sequencing (ChIP-seq) in CRC cells (Suzuki et al., 2011). Using the approach, we identified the putative promoter regions for 174 primary miRNA genes, among which 37 were predicted to be targets of epigenetic silencing in CRC.

The epigenetic silencing of a subset of tumor suppressive miRNAs was discovered through functional screening. Kozaki and colleagues tested the anti-proliferative effects of a panel of 327 synthetic miRNAs in oral and endometrial cancer cell lines (Tsuruta et al., 2011; Uesugi et al., 2011). Nearly 100 of the 327 miRNAs exerted growth suppressive effects, and approximately half of those were associated with CpG islands. By analyzing the DNA methylation and expression of the candidate miRNA genes, they identified *miR-218* and *miR-152* as targets of DNA methylation in oral and endometrial cancer, respectively.

3. miRNA genes epigenetically silenced in cancer

3.1. miR-124 *family*

Epigenetic silencing of *miR-124* family genes was first reported in CRC cells (Lujambio et al., 2007), and is now known to be methylated in various types of cancer. *miR-124* is thought to exert tumor suppressor effects by targeting *cyclin-dependent kinase 6* (*CDK6*), and epigenetic silencing of *miR-124* leads to CDK6 activation and Rb phosphorylation (Agirre et al., 2009; Lujambio et al., 2007). Within the human genome, three independent loci (*miR-124-1*, *miR-124-2* and *miR-124-3*) encode the identical mature *miR-124*, and all are associated with CpG islands, which may be targets of hypermethylation in cancer (Lujambio et al., 2007). In primary CRC tissues, methylation of *miR-124* family genes is observed in more than 70% of the cases (Lujambio et al., 2007). Methylation of *miR-124* is also reported in hematological malignancies, including approximately half of all cases of ALL (Agirre et al., 2009) and non-Hodgkin's lymphoma (Wong et al., 2011b). In ALL, moreover, *miR-124* methylation is associated with higher recurrence and mortality rates, and may be an independent prognostic factor for disease-free and overall survival (Agirre et al., 2009). *miR-124* family genes are also frequently methylated in the gastric mucosa of *Helicobacter pylori* (*H. pylori*)-positive healthy individuals, suggesting their methylation could be induced by chronic inflammation (Ando et al., 2009). Importantly, among *H. pylori*-negative individuals, *miR-124* genes were more highly methylated in the noncancerous gastric mucosae of gastric cancer patients than in those of healthy individuals, suggesting *miR-124* methylation may be involved in an epigenetic field defect. In addition, *miR-124* family genes are also frequently methylated in cervical cancer, and ectopic expression of *miR-124* inhibits cell proliferation and migration (Wilting et al., 2010). *miR-124* methylation is also acquired during human papilloma virus-mediated transformation, suggesting *miR-124* methylation may be a useful marker for detection of cervical cancer and high-grade precursors.

3.2. miR-34 *family*

Members of the *miR-34* gene family (*miR-34a*, *miR-34b* and *miR-34c*) are direct targets of p53, and their ectopic expression in cancer cells induces cell cycle arrest and apoptosis (Bommer et al., 2007; He et al., 2007). Within the human genome, *miR-34a* is located on chromosome 1p36, while *miR-34b* and *miR-34c* are co-transcribed from a single transcription unit on chromosome 11q23, and both are targets of CpG island hypermethylation in oral, esophageal, gastric, colorectal, pancreatic, breast, lung and renal cancer; malignant mesothelioma; and melanoma (Chen et al., 2012; Kozaki et al., 2008; Kubo et al., 2011; Lodygin et al., 2008; Mazar et al., 2011b; Suzuki et al., 2010; Toyota et al., 2008; Vogt et al., 2011; Wang et al., 2011b). Methylation of *miR-34b/c* has also been linked to cancer metastasis (Lujambio et al., 2008) and invasion (Watanabe et al., 2012). Similar to *miR-124*, methylation of *miR-34b/c* in the gastric mucosa is associated with *H. pylori* infection, and the noncancerous gastric mucosae of patients with multiple gastric cancers show higher levels of *miR-34b/c* methylation than those of patients with a single gastric cancer, indicating its involvement in an epigenetic field defect (Suzuki et al., 2010). In non-small cell lung cancer, methylation of *miR-34b/c* is associated with a high probability of recurrence and poor overall survival (Wang et al., 2011b). In addition, methylation-associated silencing of *miR-34c* was recently shown to promote self-renewal and epithelial–mesenchymal transition in breast tumor-initiating cells (Yu et al., 2012). These findings, as well as its contribution to the p53 network, strongly imply that *miR-34* family members act as tumor suppressors in cancer. Introduction of *miR-34b/c* into cancer cells leads to the downregulation of candidate target genes, including *MET*, *cyclin-dependent kinase 4* (*CDK4*), *cyclin E2* (*CCNE2*) and *MYC* (Lujambio et al., 2008; Toyota et al., 2008). Likewise, restoration of endogenous miRNA expression through demethylation also downregulates target genes, suggesting miRNAs could be important targets for epigenetic cancer therapy (Toyota et al., 2008).

3.3. miR-9 *family*

Methylation of the CpG island in the *miR-9-1* promoter was first reported in breast and pancreatic cancer (Lehmann et al., 2008; Omura et al., 2008). Shortly thereafter, methylation of *miR-9* family genes (*miR-9-1*, *miR-9-2* and *miR-9-3*) was also identified in several metastatic cancer cell lines (Lujambio et al., 2008). Consistent with that finding, methylation of *miR-9-1* is reportedly associated with lymph node metastasis in CRC (Bandres et al., 2009), and methylation of *miR-9-1* and *miR-9-3* is correlated with metastatic recurrence of renal cell carcinoma (Hildebrandt et al., 2010). All three *miR-9* family genes are simultaneously methylated in gastric cancer, and ectopic expression of *miR-9* inhibits proliferation, migration and invasion by gastric cancer cells (Tsai et al., 2011a). *miR-9* has been shown to target *fibroblast growth factor receptor 1* (*FGFR1*) and *CDK6* in ALL (Rodriguez-Otero et al., 2011) and *caudal-type homeobox 2* (*CDX2*) in gastric cancer cells (Rotkrua et al., 2011), suggesting a tumor suppressive function. Interestingly, xenoestrogen, which may increase ones risk of developing breast cancer, can induce methylation-associated silencing of *miR-9-3* in breast epithelial cells, indicating that methylation of this miRNA gene could be a hallmark of early breast cancer development (Hsu et al., 2009). In contrast to these findings, however, one recent study showed that *miR-9*

is activated by MYC and MYCN in breast cancer, and that *miR-9* promotes metastasis through downregulation of *E-cadherin* (*CDH1*) (Ma et al., 2010). These results are indicative of the functional complexity of miRNAs in cancer cells and suggest that miRNAs may exert opposite effects in different tissues or settings.

3.4. miR-200 *family and* miR-205

The *miR-200* gene family (*miR-200a*, *miR-200b*, *miR-200c*, *miR-141* and *miR-429*) and *miR-205* encode key regulators of epithelial–mesenchymal transition (EMT) that act by directly targeting *zinc finger E-box binding homeobox 1* (*ZEB1*) and ZEB2, which are transcriptional repressors that downregulate *CDH1* (Gregory et al., 2008; Korpal et al., 2008; Park et al., 2008). Within the human genome, the *miR-200* family genes are grouped into two polycistronic units, *miR-200b/200a/429* and *miR-200c/141*, located on chromosomes 1 and 12, respectively (Davalos et al., 2012). Expression of *miR-200c/141* is regulated by promoter CpG islands in normal mammary epithelial cells and fibroblasts, and their silencing is associated with aberrant methylation in breast and prostate cancer cells (Vrba et al., 2010). Methylation of *miR-200c/141* is tightly correlated with the invasive capacity of breast cancer cells, and induction of EMT by ectopic expression of Twist in immortalized human mammary epithelial cells is accompanied by increased methylation of *miR-200c/141* (Neves et al., 2010). Similarly, in non-small cell lung cancer, promoter methylation is associated with loss of *miR-200c* expression, which is in turn associated with poor differentiation, lymph node metastasis and weaker E-cadherin expression (Ceppi et al., 2010). Davalos et al. demonstrated that the upstream CpG islands of both units (*miR-200b/200a/429* and *miR-200c/141*) are unmethylated in cancer cells with epithelial features, but are both methylated and silenced in transformed cells with mesenchymal characteristics (Davalos et al., 2012). In bladder cancer, both units of the *miR-200* family and *miR-205* are coordinately silenced in association with promoter methylation (Wiklund et al., 2011). Epigenetic silencing of the *miR-200* family and *miR-205* was also observed in carcinogen-treated lung epithelial cells, suggesting that induction of EMT through miRNA dysregulation occurs early during lung carcinogenesis (Tellez et al., 2011).

3.5. *Other miRNA genes*

Reduced expression of *miR-1* is reported in many malignancies (Bueno et al., 2008; Nasser et al., 2008; Rao et al., 2010), and methylation of the upstream CpG island and silencing of *miR-1-1* have been reported in both hepatocellular carcinoma (HCC) (Datta et al., 2008) and CRC (Suzuki et al., 2011). Ectopic expression of *miR-1* in HCC cells inhibits cellular growth through suppression of its target genes, which include *MET*, *forkhead box P1* (*FOXP1*) and *histone deacetylase 4* (*HDAC4*) (Datta et al., 2008). In CRC cells, *miR-1* suppresses cellular proliferation, motility and invasion by targeting a number of genes, including *annexin A2* (*ANXA2*) and *brain-derived neurotrophic factor* (*BDNF*) (Suzuki et al., 2011), both of which are frequently overexpressed in cancer and are implicated in invasion and metastasis (Diaz et al., 2004; Douma et al., 2004; Emoto et al., 2001). Another recent study also provides evidence that expression of *miR-1* is downregulated in primary CRC, and that *miR-1* suppresses CRC cell proliferation and motility by targeting *MET* (Reid et al., 2012). Methylation of *miR-1-1* is observed in approximately 80% of primary CRC tissue samples tested and in 70% of colorectal adenoma tissue samples, suggesting that methylation of *miR-1-1* is an early event during colorectal tumorigenesis (Suzuki et al., 2011).

miR-125b is a brain-enriched miRNA and a good example of an miRNA that has opposite functions in different tumor types; that is, it can exert oncogenic or tumor suppressor effects, depending of the cellular context. Expression of miR-125b is upregulated in several types of malignancies, including prostate cancer, but it is downregulated in breast cancer, where it acts as a tumor suppressor by directly targeting *ETS1* (Zhang et al., 2011a). In breast cancer, the silencing of *miR-125b* is associated with methylation of the *miR-125b-1* promoter, and the weak expression of *miR-125b* correlates with a poor prognosis.

Methylation of *miR-129-2* has been reported in endometrial, esophageal, gastric, and colorectal cancer (Bandres et al., 2009; Chen et al., 2012; Huang et al., 2009; Shen et al., 2010). *miR-129-2* targets *SRY-related high-mobility group box 4* (*SOX4*), an oncogene frequently upregulated in malignancies, and an association between *miR-129-2* methylation and *SOX4* overexpression was found in both endometrial and gastric cancers (Huang et al., 2009; Shen et al., 2010). In endometrial cancer, *miR-129-2* methylation is associated with microsatellite instability, *MLH1* methylation and poor overall survival (Craig et al., 2011). Similarly, downregulated expression of *miR-129* is reportedly associated with poor clinicopathological features in gastric cancer (Tsai et al., 2011b).

Methylation of *miR-137* was first noted in oral cancer (Kozaki et al., 2008) and was subsequently reported in colon (Balaguer et al., 2010; Bandres et al., 2009) and gastric cancer (Chen et al., 2011). *miR-137* methylation is associated with a poorer survival rate among patients with head and neck squamous cell carcinoma (HNSCC) (Langevin et al., 2011), and was detected in oral rinses collected from HNSCC patients, suggesting its utility as a cancer biomarker (Langevin et al., 2010). Within cancer cells, *miR-137* targets *CDK6*, *lysine-specific demethylase 1* (*LSD1*) and *cell division cycle 42* (*CDC42*), indicating it to be a tumor suppressor (Balaguer et al., 2010; Kozaki et al., 2008; Liu et al., 2011b), while in normal cells, *miR-137* regulates neuronal differentiation by targeting *enhancer of zeste homolog 2* (*EZH2*) and *mindbomb 1* (*MIB1*) (Smrt et al., 2010; Szulwach et al., 2010).

miR-145 encodes a well-known tumor suppressor, expression of which is reduced in a colorectal (Michael et al., 2003), breast (Iorio et al., 2005), ovarian (Iorio et al., 2007) and prostate cancers (Zaman et al., 2010). In breast and colon cancer cells, expression of miR-145 is induced by p53, and miR-145 directly targets *MYC*, suggesting that p53 represses *MYC* through induction of *miR-145* expression. *miR-145* also suppresses cancer cell proliferation, invasion and metastasis by targeting *mucin 1* (*MUC1*), *insulin receptor substrate-1* (*IRS-1*) and *Fascin homolog 1* (FSCN1) (Fuse et al., 2011; Sachdeva and Mo, 2010; Shi et al., 2007). In prostate cancer, downregulation of *miR-145* expression is associated with an aggressive phenotype and a poor prognosis, and promoter methylation is associated with its silencing (Zaman et al., 2010). It was also recently

shown that both DNA methylation and *p53* mutation are major causes of diminished *miR-145* expression in prostate cancer (Suh et al., 2011).

Screening for epigenetically silenced miRNA genes in metastatic cancer cell lines revealed a set of genes that included *miR-148a* (Lujambio et al., 2008). Introduction of *miR-148a* into methylated cancer cells inhibited cell motility, growth and metastasis. *miR-148a* is methylated in colorectal, breast, lung, head and neck cancers, and is associated with lymph node metastasis (Lujambio et al., 2008). Methylation-induced silencing of *miR-148a* is also seen in pancreatic cancer and preneoplastic pancreatic lesions (pancreatic intraepithelial neoplasia; PanIN), suggesting it is an early event in pancreatic carcinogenesis (Hanoun et al., 2010).

Aberrant activation of the proto-oncogene *KIT* is observed in various malignancies, including acute myeloid leukemia (AML). Analysis of the 3′ UTR region of *KIT* suggested several miRNAs may serve as regulators; among those, methylation-induced silencing of *miR-193a* is causally related to *KIT* overexpression in AML (Gao et al., 2011). Interestingly, in HCC cells, *miR-193a* dictates chemoresistance to 5-fluorouracil by targeting *serine/arginine-rich splicing factor 2* (*SRSF2*) (Ma et al., 2012). Expression of *miR-193a* is regulated by DNA methylation, and 5-fluorouracil-sensitive HCC cells show promoter methylation and reduced expression of *miR-196a*, suggesting it could be an important prognostic indicator in HCC.

miR-203 encodes a candidate tumor suppressor and is epigenetically silenced in oral cancer (Kozaki et al., 2008), hematopoietic malignancies (Bueno et al., 2008; Wong et al., 2011a) and HCC (Furuta et al., 2010). *miR-203* directly controls *ABL1* expression, and it also targets the BCR-ABL1 translocation protein induced by Philadelphia chromosome in chronic myelogenous leukemia and B cell ALL in children (Bueno et al., 2008). The CpG island of *miR-203* is specifically methylated in Philadelphia-positive tumors, as compared to other hematopoietic malignancies, suggesting that epigenetic silencing of *miR-203* enhances the expression of the *BCR-ABL1* oncogene. Epigenetic silencing of *miR-203* has also been shown to activate *ABL1* in *H. pylori*-associated gastric B-cell lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) (Craig et al., 2011). In HCC cells, *miR-203* suppresses cellular growth and downregulates various target genes, including *ATP-binding cassette, subfamily E, member 1* (*ABCE1*) and *CDK6* (Furuta et al., 2010).

Dysregulated expression of *miR-375* is frequently observed in cancers; for example, it is downregulated in esophageal (Kong et al., 2012), gastric (Tsukamoto et al., 2010), and head and neck cancers (Hui et al., 2011), whereas it is overexpressed in breast cancer (de Souza Rocha Simonini et al., 2010; Giricz et al., 2012). Methylation-induced silencing of *miR-375* is reported in esophageal squamous cell carcinoma (Kong et al., 2012), HCC and melanoma (Mazar et al., 2011a). In the esophageal cancer cells, *miR-375* suppresses cell proliferation, motility, invasion and metastasis by targeting *insulin-like growth factor 1 receptor* (*IGF1R*) (Kong et al., 2012). In esophageal and gastric cancer, *miR-375* reportedly exerts its tumor suppressive function by downregulating *3-phosphoinositide-dependent protein kinase 1* (*PDK1*), which in turn reduces Akt phosphorylation (Li et al., 2011; Tsukamoto et al., 2010). Interestingly, however, *miR-375* exerts oncogenic effects by targeting *RAS dexamethasone-induced 1* (*RASD1*), and its overexpression is associated with promoter hypomethylation (see below) (de Souza Rocha Simonini et al., 2010).

4. CpG island shore methylation and miRNA dysregulation

Most of the studies on the epigenetic silencing of miRNA genes in cancer described above have focused on CpG island hypermethylation. By contrast, one recent study demonstrated that CpG island shore methylation is frequently associated with miRNA downregulation in bladder cancer. CpG island shores are regions located within 2 kb of CpG islands, and their methylation strongly affects gene expression (Irizarry et al., 2009). Microarray analysis of miRNA expression coupled with demethylating treatment in bladder cancer cells revealed a number of epigenetically silenced miRNA genes (Dudziec et al., 2011). Interestingly, several miRNA genes are more frequently hypermethylated in the CpG island shore region than the CpG island itself (*miR-9*, *miR-149*, *miR-210*, *miR-212*, *miR-328*, *miR-503*, *miR-1224*, *miR-1227* and *miR-1229*). Methylation of these genes is associated with tumor grade, stage and prognosis, and the reduced expression of the silenced miRNAs is apparent in urine specimens from bladder cancer patients, suggesting their utility as diagnostic biomarkers.

5. Hypomethylation of miRNA genes in cancer

Although epigenetic dysregulation leads to the silencing of many miRNAs in cancer, several are upregulated though epigenetic mechanisms. The CpG island of *let-7a-3* is heavily methylated in normal cells but is hypomethylated in lung adenocarcinoma, leading to its elevated expression (Brueckner et al., 2007). In lung cancer cells, *let-7a-3* exerts oncogenic effects through actions on several genes involved in cell proliferation, adhesion and differentiation. Another study showed that elevated expression of *miR-375* in estrogen receptor α (ERα)-positive breast cancer cells promotes tumor cell proliferation (de Souza Rocha Simonini et al., 2010). Similarly, overexpression of *miR-375* is caused by loss of repressive histone marks and DNA methylation, which leads to dissociation of the transcriptional repressor CTCF from the *miR-375* promoter, and the binding of ERα to its regulatory region. In addition, whereas expression of *miR-200a* and *miR-200b* is downregulated in many types of cancer, these genes are overexpressed in pancreatic cancer due to hypomethylation (Li et al., 2010). Moreover, the elevation of *miR-200a* and *miR-200b* in the serum of pancreatic cancer patients means they could potentially serve as diagnostic biomarkers. *miR-196* family genes (*miR-196a* and *miR-196b*) are located within the *HOX* gene cluster and are often overexpressed in tumors, which is indicative of their oncogenic role (Popovic et al., 2009). *miR-196b* is embedded within a CpG island, and its overexpression in gastric cancer is associated with its hypomethylation (Tsai et al., 2010). In HCC, expression of *miR-191* is frequently elevated due to hypomethylation of the gene locus (He et al., 2011). *miR-191* directly targets *tissue inhibitor of*

metalloproteinase 3 (*TIMP3*), and its elevated expression in HCC cells induces transition to mesenchymal-like cells.

6. Clinical implications of epigenetically silenced miRNAs

A number of studies have demonstrated that the miRNA expression profile is potentially useful for detecting cancers and predicting outcomes. In addition to the altered expression, epigenetically silenced miRNA genes also exhibit aberrant DNA methylation, which could also be a useful marker for cancer diagnosis. For example, as mentioned above, methylation of *miR-124* family genes is an independent prognostic factor for disease-free and overall survival (Agirre et al., 2009). A more comprehensive analysis of epigenetically silenced miRNA genes in ALL has identified methylation of 13 miRNA genes (*miR-9-1*, *miR-9-2*, *miR-9-3*, *miR-10b*, *miR-34b/c*, *miR124-1*, *miR-124-2*, *miR-124-3*, *miR-132/212*, *miR-196b*, *miR-203*) (Roman-Gomez et al., 2009). ALLs with at least one methylated miRNA genes, which account for 65% of all ALLs, show significantly poorer disease-free and overall survival than the unmethylated group, suggesting miRNA gene methylation is an important prognostic factor predictive of disease outcome. Similarly, methylation of multiple miRNA genes is associated with larger tumor size and poorer progression-free survival in non-small cell lung cancer (Kitano et al., 2011).

As mentioned above, *miR-34b/c* is one of the most frequently methylated miRNA genes among tumors. The CpG island of *miR-34b/c* is methylated in more than 90% of primary CRCs, and methylation was detected in 75% of fecal specimens from CRC patients and in 16% of specimens from high-grade dysplasia patients, suggesting *miR-34b/c* methylation could be a useful feces-based screening marker (Kalimutho et al., 2011). In addition, we recently reported that methylation of a panel of genes, including *miR-34b/c*, in mucosal wash fluid collected during colonoscopy could be a useful biomarker for predicting the invasiveness of CRCs (Kamimae et al., 2011).

Given that some epigenetically silenced miRNAs appear to have tumor suppressive potential, restoration of their expression may be an effective strategy for treating cancer. It has been demonstrated experimentally that re-expression of methylation-silenced miRNAs through treatment with a demethylating agent leads to downregulation of target oncogenes and suppression of tumor growth (Lujambio et al., 2007; Toyota et al., 2008). In addition, the antitumor effects of miRNA replacement using an oligonucleotide mimic or a miRNA expression vector has been tested in numerous studies (Henry et al., 2011). For example, restoration of *miR-34* family expression in pancreatic cancer cells led to a significant reduction in the fraction of CD44+/CD133+ tumor-initiating cells and inhibits tumor growth in vitro and in vivo (Ji et al., 2009). In mouse xenograft models of lung cancer, systemic delivery of a *miR-34a* mimic using a lipid-based delivery vehicle inhibited tumor growth (Wiggins et al., 2010). *miR-34a* also reportedly inhibits prostate cancer stem cells, and the therapeutic efficacy of its systemic delivery was confirmed in a mouse xenograft model (Liu et al., 2011a). Moreover, using a xenograft model of CRC, polyethylenimine-mediated delivery of *miR-145* and *miR-33a* was shown to downregulate expression of *MYC* and *ERK5*, and to suppress tumor growth (Ibrahim et al., 2011).

7. miRNA dysregulation and aberrant DNA methylation

In contrast to the observations described above, several lines of evidence support the idea that dysregulation of miRNA can lead to aberrant DNA methylation in cancer. For instance, using a CRC cell line with hypomorphic *DICER* (Cummins et al., 2006), we showed that DICER is required to maintain CpG island methylation of several gene promoters in cancer (Ting et al., 2008). To date, several miRNAs able to regulate *DNMT* genes are reportedly downregulated in cancer. The *miR-29* family (*miR-29a*, *miR-29b* and *miR-29c*), which is downregulated in lung cancer, directly targets DNMT3A and DNMT3B (Fabbri et al., 2007). Ectopic expression of *miR-29* family in lung cancer cells restores expression of methylation-silenced tumor suppressor genes, including *fragile histidine triad* (*FHIT*) and *WW domain containing oxidoreductase* (*WWOX*). In CRC, *miR-143* is frequently downregulated, and experimental evidence suggests that *miR-143* targets *DNMT3A* in CRC cells (Ng et al., 2009). In addition, downregulated expression of *miR-152* in HBV-related HCC correlates with increased expression of *DNMT1* (Huang et al., 2010). Forced expression of *miR-152* in liver cell lines reduces *DNMT1* expression and in turn global DNA methylation, whereas inhibition of *miR-152* causes global DNA hypermethylation and increased methylation of the *glutathione S-transferase pi 1* (*GSTP1*) and *CDH1* promoter regions. Similarly, in cholangiocarcinoma cells, *DNMT1* is targeted by *miR-148a* and *miR-152*, and their ectopic expression suppresses *DNMT1* and induces expression of the tumor suppressor genes *Ras association domain family* 1A (RASSF1A) and p16 (Braconi et al., 2010). Recently, *miR-342* was found to be downregulated in CRC cells, and restoration of its expression downregulated DNMT1 and reactivated expression of *ADAM metallopeptidase domain 23* (*ADAM23*), *histidine triad nucleotide binding protein 1* (HINT1), RASSF1A and *reversion-inducing-cysteine-rich protein with kazal motifs* (*RECK*) through demethylation of their promoter regions (Wang et al., 2011a). And in glioma, *miR-185* targets *DNMT1* to regulate the methylation of several gene promoters (Zhang et al., 2011b). Collectively, these results suggest that dysregulation of specific miRNAs may be causally related to aberrant methylation of promoter CpG islands.

8. Concluding remarks

In this review, we highlighted the relationship between epigenetic alteration and dysregulation of miRNAs in cancer. Aberrant DNA methylation and histone modification are major mechanisms underlying miRNA dysregulation in cancer, and methylation of a subset of miRNA genes may be useful biomarkers for detecting cancer and/or predicting clinical outcome. Moreover, replacement of silenced tumor-suppressive miRNAs in cancer cells could be a promising strategy for cancer treatment. We anticipate that further studies of the cancer epigenome and miRNAs will lead to the discovery of a variety of novel biomarkers and potential therapeutic targets.

Conflict of interest

All authors disclose no conflict of interest.

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RESEARCH ARTICLE

Genome-wide analysis of DNA methylation identifies novel cancer-related genes in hepatocellular carcinoma

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Abstract Aberrant DNA methylation has been implicated in the development of hepatocellular carcinoma (HCC). Our aim was to clarify its molecular mechanism and to identify useful biomarkers by screening for DNA methylation in HCC. Methylated CpG island amplification coupled with CpG island microarray (MCAM) analysis was carried out to screen for methylated genes in primary HCC specimens [hepatitis B virus (HBV)-positive, n=4; hepatitis C virus (HCV)-positive, n=5; HBV/HCV-negative, n=7]. Bisulfite pyrosequencing was used to analyze the methylation of selected genes and long interspersed nuclear element (LINE)-1 in HCC tissue (n=57) and noncancerous liver tissue (n=50) from HCC patients and in HCC cell lines (n=10). MCAM analysis identified 332, 342, and 259 genes that were methylated in HBV-positive, HCV-positive, and HBV/HCV-negative HCC tissues, respectively. Among these genes, methylation of *KLHL35*, *PAX5*, *PENK*, and *SPDYA* was significantly higher in HCC tissue than in noncancerous liver tissue, irrespective of the hepatitis virus status. LINE-1 hypomethylation was also prevalent in HCC and correlated positively with *KLHL35* and *SPDYA* methylation. Receiver operating characteristic curve analysis revealed that methylation of the four genes and LINE-1 strongly discriminated between HCC tissue and noncancerous liver tissue. Our data suggest that aberrant hyper- and hypomethylation may contribute to a common pathogenesis mechanism in HCC. Hypermethylation of *KLHL35*, *PAX*, *PENK*, and *SDPYA* and hypomethylation of LINE-1 could be useful biomarkers for the detection of HCC.

Keywords Hepatocellular carcinoma · DNA methylation · CpG island · LINE-1 · Biomarker

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common human malignancies, worldwide [1]. Chronic infection by hepatitis B virus (HBV) and hepatitis C virus (HCV) are well-documented risk factors for the development of HCC, while chronic alcoholism and various environmental factors, including aflatoxin B1, are also believed to be important risk

factors [2, 3]. The development and progression of HCC is often a complex, multistep process entailing the evolution of normal liver through chronic hepatitis and cirrhosis to HCC, but HCC can also arise in a noncirrhotic liver. In either case, the process is influenced by multiple genetic changes, including allelic deletions, chromosomal losses and gains, DNA rearrangements, and gene mutations [4]. In addition, a growing body of evidence suggests that epigenetic changes such as DNA methylation and histone modification also play crucial roles in hepatocarcinogenesis.

Two seemingly contradictory epigenetic events coexist in cancer: global hypomethylation, which is mainly observed in repetitive sequences throughout the genome, and regional hypermethylation, which is frequently associated with CpG islands within gene promoters [5]. Hypermethylation of CpG islands is a common feature of cancer and is associated with gene silencing. Although the classical two-hit theory posits that tumor suppressor genes are inactivated by gene mutation or deletion, it is now recognized that DNA hypermethylation is a third mechanism by which inactivation of tumor suppressor genes occurs, and that it plays a significant role in tumorigenesis. In contrast to the CpG islands, repetitive DNA elements are normally heavily methylated in somatic tissues. About 45 % of the human genome is composed of repetitive sequences, including long interspersed nuclear elements (LINEs) and short interspersed nuclear element [6], and studies have shown that methylation of such repetitive elements can serve as a surrogate for the global methylcytosine content [7]. In that regard, LINE-1 hypomethylation is known to occur during the development of various human malignancies, including HCC [8, 9].

HCC is generally diagnosed at an advanced stage of tumor progression, and a large fraction of HCC cases are fatal. Thus, a better understanding of the underlying molecular mechanisms and identification of genes critical for early detection of HCC and therapeutic intervention would be highly desirable. Although a number of hyper- or hypomethylated loci have been identified in HCC [10–12], only a few studies have been conducted to unravel the genome-wide methylation status [13–15]. In the present study, we carried out genome-wide CpG island methylation analysis in a set of primary HCC specimens, with and without hepatitis virus infection. We also evaluated the hypomethylation of LINE-1 and assessed its association with aberrant CpG island hypermethylation in HCC.

Materials and methods

Tissue samples and cell lines

A total of 57 primary HCC specimens (HBV-positive, n=21; HCV-positive, n=21; HBV/HCV-negative, n=15) were obtained through surgical resection or needle biopsy at Sapporo Medical University Hospital. Corresponding samples of noncancerous liver tissue were also obtained from 50 patients. HBV surface (HBs) antigen and anti-HCV antibody were measured serologically. An informed consent was obtained from all patients before collection of the specimens. The ten liver cancer cell lines (HT17, PLC/PRF/5, Li-7, huH-1, HuH-7, HepG2, Hep3B, HLE, HLF, and JHH-4) used have been described previously [11]. To analyze restoration of gene expression, cells were treated with 2.0 μM 5-aza-2′-deoxycytidine (5-aza-dC) (Sigma, St Louis, MO, USA) for 72 h, replacing the drug and medium every 24 h. Genomic DNA was extracted using the standard phenol–chloroform procedure. Total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) and then treated with a DNA-free kit (Ambion, Austin, TX, USA). Genomic DNA and total RNA from normal liver tissue from a healthy individual were purchased from BioChain (Hayward, CA, USA).

Methylated CpG island amplification coupled with CpG island microarray

Methylated CpG island amplification (MCA) was performed as described previously [13]. Briefly, 500 ng of genomic DNA was digested with the methylation-sensitive restriction endonuclease *Sma*I (New England Biolabs, Ipswich, MA, USA), after which it was digested with the methylation-insensitive restriction endonuclease *Xma*I. The adaptors were prepared by addition of the oligonucleotides RMCA12 (5′-CCGGGCAGAAAG-3′) and RMCA24 (5′-CCACCGCCATCCGAGCCTTTCTGC-3′). After ligation of the digested DNA to the adaptors, PCR amplification was carried out. Using a BioPrime Plus Array CGH Genomic Labeling System (Invitrogen), MCA amplicons from the HCC samples were labeled with Alexa Fluor 647, while amplicons from a normal liver sample was labeled with Alexa Fluor 555. The labeled MCA amplicons were then hybridized to a custom human CpG island microarray containing 15,134 probes covering 6,157 unique genes (G4497A; Agilent Technologies, Santa Clara, CA, USA) [16]. After washing, the array was scanned using an Agilent DNA Microarray Scanner (Agilent technologies), and the data were processed using Feature Extraction software ver. 10.7 (Agilent Technologies). The data were then analyzed using GeneSpring GX ver. 11 (Agilent Technologies).

Methylation-specific PCR

Genomic DNA (1 μg) was modified with sodium bisulfite using an EpiTect Bisulfite Kit (Qiagen, Hilden, Germany), and methylation-specific PCR (MSP) was performed as described previously [17]. Briefly, PCR was run in a 25-μl

volume containing 50 ng of bisulfite-treated DNA, 1× MSP buffer [67 mM Tris–HCl (pH 8.8), 16.6 mM $(NH4)_2SO_4$, 6.7 mM $MgCl_2$, and 10 mM 2-mercaptoethanol], 1.25 mM dNTP, 0.4 μM each primer, and 0.5 U of JumpStart REDTaq DNA Polymerase (Sigma). The PCR protocol for MSP entailed 5 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C; and a 7 min final extension at 72°C. Primer sequences and PCR product sizes are shown in Supplementary Table 1.

Bisulfite pyrosequencing analysis

Bisulfite pyrosequencing analysis was performed as described previously [17]. The PCR protocol entailed 5 min at 95°C; 45 cycles of 1 min at 95°C, 1 min at 60°C, and 1 min at 72°C; and a 7-min final extension at 72°C. PCR products were then bound to Streptavidin Sepharose beads HP (Amersham Biosciences, Piscataway, NJ); after which, the beads containing the immobilized PCR product were purified, washed, and denatured using a 0.2 M NaOH solution. After addition of 0.3 μM sequencing primer to the purified PCR product, pyrosequencing was carried out using a PSQ96MA system (Qiagen, Hilden, Germany) and Pyro Q-CpG software (Qiagen). Primer sequences and PCR product sizes are shown in Supplementary Table 1.

Quantitative RT-PCR

Single-stranded cDNA was prepared using SuperScript III reverse transcriptase (Invitrogen). Quantitative RT-PCR was carried out using TaqMan Gene Expression Assays (*KLHL35*, Hs00400533_m1; *PAX5*, Hs00172003_m1; *PENK*, Hs00175049_m1; *SPDYA*, Hs00736925_m1; *GAPDH*, Hs99999905_m1; Applied Biosystems, Foster City, CA, USA) and a 7500 Fast Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. SDS1.4 software (Applied Biosystems) was used for comparative delta Ct analysis, and *GAPDH* served as an endogenous control.

Statistical analysis

To compare differences in continuous variables between groups, *t* tests or ANOVA with post hoc Tukey's tests were performed. Fisher's exact test or chi-squared test was used for analysis of categorical data. Receiver operator characteristic (ROC) curves were constructed based on the levels of methylation. Values of $P<0.05$ (two-sided) were considered statistically significant. Statistical analyses were carried out using SPSS statistics 18 (IBM Corporation, Somers, NY, USA) and GraphPad Prism ver. 5.0.2 (GraphPad Software, La Jolla, CA, USA).

Results

Genome-wide CpG island methylation analysis in HCC

To screen for CpG island hypermethylation in HCC, we carried out methylated CpG island amplification coupled with CpG island microarray (MCAM) analysis using a set of HCC tissue specimens (HBV-positive, $n=4$; HCV-positive, $n=5$; HBV/HCV-negative, $n=7$). As in an earlier study in which the same array system was used, we utilized a signal ratio (Cy5/Cy3) of >2.0 as the criterion for a methylation-positive probe [13]. The average number of methylated probe sets in the HCC specimens was 566 (range 159–846). To assess the association between hepatitis virus infection and methylation status, we categorized the HCC specimens according to their viral status. The average numbers of methylated probe sets in HBV-positive, HCV-positive, and the HBV/HCV-negative HCC specimens were 574, 598, and 539, respectively, which did not significantly differ ($P=0.840$). Interestingly, however, the numbers of methylated probe sets were more varied among HBV/HCV-negative HCCs, which is indicative of their varied pathological backgrounds (Fig. 1a).

To identify commonly methylated genes in HCC, we selected genes that were methylated in at least two tumors in each group. Among the HBV-positive HCCs, 443 probe sets (corresponding to 332 unique genes) satisfied this criterion. Among the HCV-positive HCCs, 476 probe sets (342 unique genes) satisfied the criterion, and among the HBV/HCV-negative HCCs, 348 probe sets (259 unique genes) satisfied the criterion. Collectively, 714 probes (514 unique genes) were selected as commonly methylated genes. Of those, 137, 146, and 47 probe sets were methylated in only HBV-positive, HCV-positive, or HBV/HCV-negative HCC tissues, respectively (Fig. 1b). By contrast, a large number of genes were methylated in multiple categories, and 169 probe sets were methylated in all three groups (Fig. 1b). Consistent with the above results, unsupervised hierarchical clustering analysis demonstrated that some genes were methylated irrespective of the hepatitis virus status, and that HCV-positive HCCs exhibited the largest number of methylated genes (Fig. 1c, Supplementary Fig. 1). Gene ontology analysis of the commonly methylated genes revealed that genes related to "multicellular organismal process," "developmental process," and "system development" are significantly enriched among the methylated genes (Supplementary Table 2). In addition, pathway analysis suggested that some of the methylated genes are involved in differentiation and development (Supplementary Fig. 2).

Identification of novel genes methylated in HCC

Our MCAM analysis suggested that some genes were methylated in a hepatitis virus-specific manner, but a larger

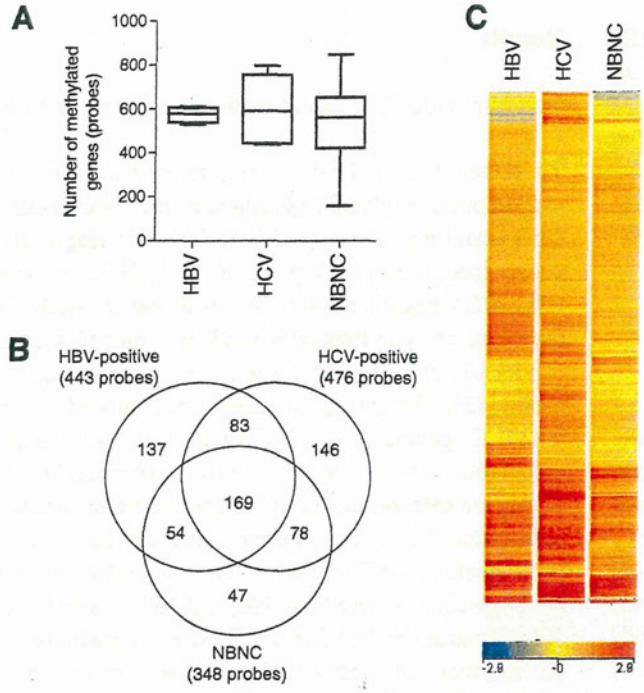


Fig. 1 Genome-wide analysis of CpG island methylation. **a** MCAM analysis was carried out using a series of HCC tissue specimens (HBV-positive, $n=4$; HCV-positive, $n=5$; HBV/HCV-negative, NBNC, $n=7$). MCAM data were categorized into three groups based on the hepatitis virus status, and the numbers of methylated genes in the respective categories are shown. **b** Venn diagram analysis of the methylated genes in the indicated categories. **c** Gene tree view of the MCAM analysis results. A set of 714 probes (514 unique genes) were selected as commonly methylated genes, after which, hierarchical clustering was performed. Each row represents a single probe

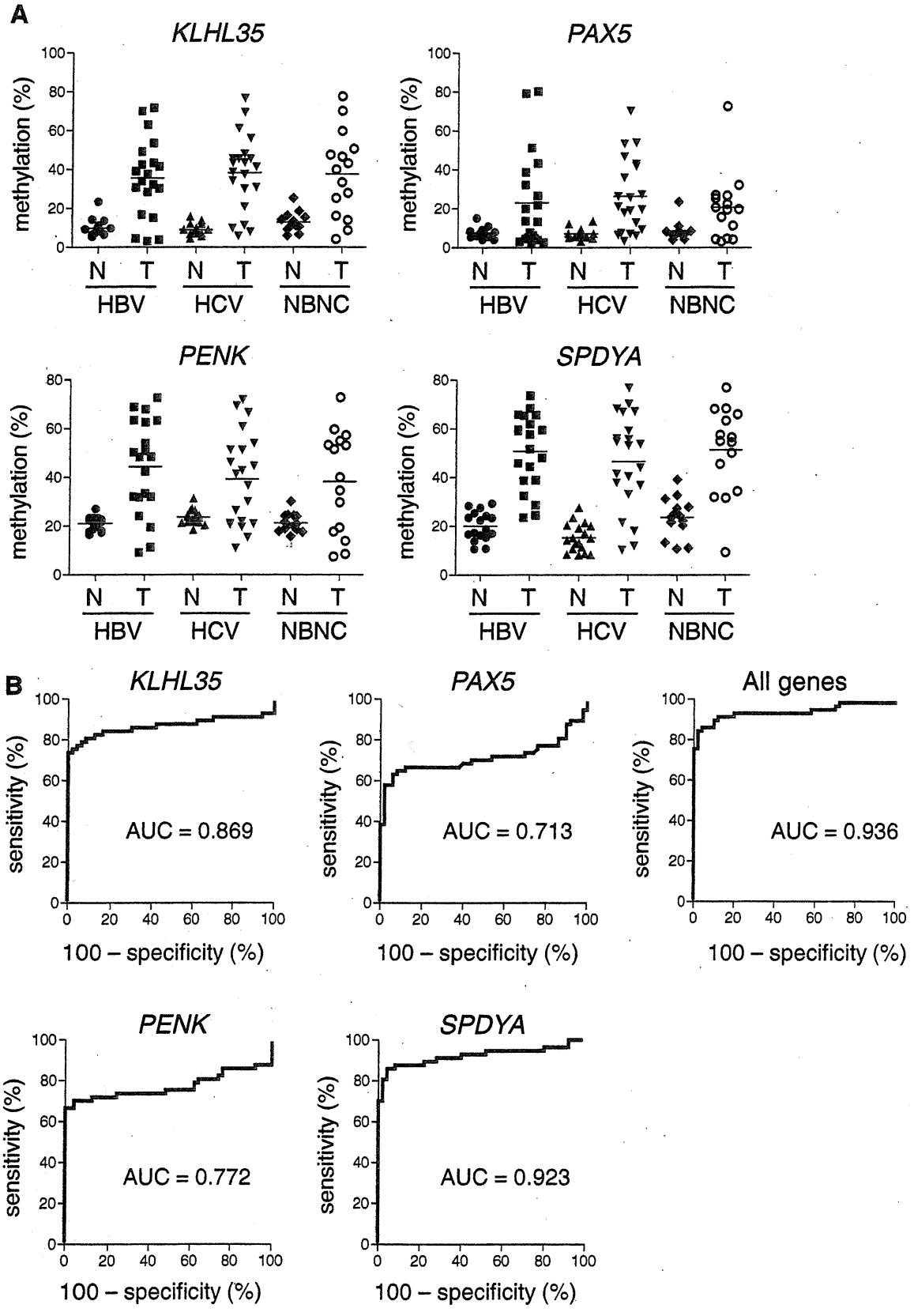
number were commonly methylated in HCC. Because recent studies have suggested that aberrant DNA methylation could be a useful diagnostic marker for HCC, we next aimed to identify novel genes frequently methylated in HCC. Among the genes commonly methylated irrespective of hepatitis virus status, we selected 14 (*KLHL35*, *PAX5*, *PENK*, *SPDYA*, *LTBP2*, *DLX1*, *PGBD1*, *WNT9A*, *ADRA1A*, *RHOBTB1*, *GDNF*, *WNT11*, *MLL*, and *PLEC1*) and carried out MSP to assess their methylation status in a series of HCC cell lines (Supplementary Fig. 3). We found that four (*KLHL35*, *PAX5*, *PENK*, and *SPDYA*) of the genes were frequently methylated in HCC cell lines, but showed only little or no methylation in normal liver tissue from a healthy individual (Supplementary Fig. 3). We therefore used quantitative bisulfite pyrosequencing to further analyze the methylation levels of these four genes (Supplementary Figs. 4 and 5).

To determine the extent to which these genes are aberrantly methylated in primary tumors, we analyzed a set of primary HCC specimens (HBV-positive, $n=21$; HCV-positive, $n=21$; HBV/HCV-negative, $n=15$) and corresponding noncancerous liver tissues from the same patients (HBV-positive, $n=18$; HCV-positive, $n=18$; HBV/HCV-negative, $n=14$). Bisulfite pyrosequencing analysis revealed the methylation levels of the four genes to be significantly higher in tumor tissues than in their noncancerous counterparts (*KLHL35*, 37.9 vs. 10.4 %, $P<0.001$; *PAX5*, 23.4 vs. 7.7 %, $P<0.001$; *PENK* 41.1 vs. 22.0 %, $P<0.001$; *SPDYA*, 49.7 vs. 19.3 %, $P<0.001$) (Supplementary Fig. 6). Moreover, these genes were frequently methylated in HCCs, irrespective of the hepatitis virus infection (*KLHL35*, HBV-positive, 37.5 vs. 9.9 %, $P<0.001$; HCV-positive, 38.3 vs. 9.0 %, $P<0.001$; HBV/HCV-negative, 37.7 vs. 13.0 %, $P<0.001$; *PAX5*, HBV-positive, 22.2 vs. 7.6 %, $P=0.014$; HCV-positive, 26.5 vs. 7.2 %, $P<0.001$; HBV/HCV-negative, 20.7 vs. 8.5 %, $P=0.017$; *PENK*, HBV-positive, 45.1 vs. 20.9 %, $P<0.001$; HCV-positive, 39.2 vs. 23.5 %, $P=0.001$; HBV/HCV-negative, 38.2 vs. 21.3 %, $P=0.006$; *SPDYA*, HBV-positive, 51.5 vs. 19.8 %, $P<0.001$; HCV-positive, 46.6 vs. 15.3 %, $P<0.001$; HBV/HCV-negative, 51.3 vs. 23.7 %, $P<0.001$) (Fig. 2a). The association between the methylation of each gene and the clinicopathological features are shown in Table 1. Methylation of *KLHL35* and *PAX5* was correlated with greater age, and *SPDYA* methylation was moderately correlated with higher PIVKA-II levels, but we found no other significant correlations (Table 1). We also generated an ROC curve and observed that methylation of the four genes discriminated strongly between tumor tissues and noncancerous liver tissue, suggesting that methylation of these genes could be a useful tumor marker (Fig. 2b). The most discriminating cutoffs for *KLHL35*, *PAX5*, *PENK*, and *SPDYA* were 14.8 % (sensitivity, 82.5 %; specificity, 88.0 %), 12.5 % (sensitivity, 63.2 %; specificity, 94.0 %), 28.4 % (sensitivity, 70.2 %; specificity, 96.0 %), and 30.3 % (sensitivity, 86.0 %; specificity, 94.0 %), respectively.

Analysis of *KLHL35*, *PAX5*, *PENK*, and *SPYDA* methylation and expression

We next tested whether methylation of *KLHL35*, *PAX5*, *PENK*, and *SPYDA* was associated with their silencing in HCC. Bisulfite pyrosequencing analysis revealed that the degree to which these genes were methylated varied among the HCC cell lines, but it was always much higher than in normal liver tissue from a healthy individual (Fig. 3a). Quantitative RT-PCR analysis confirmed an inverse relationship between methylation and expression of *KLHL35*

Fig. 2 Quantitative methylation analysis of the genes identified by MCAM. **a** Summary of the bisulfite pyrosequencing analysis of *KLHL35*, *PAX5*, *PENK*, and *SPDYA* in tumor tissue (*T*) and noncancerous liver tissue (*N*) from HBV-positive, HCV-positive, and HBV/HCB-negative (NBNC) HCC patients. **b** ROC curve analysis of the methylation of the indicated genes. The area under the ROC curve (*AUC*) for each site conveys its utility (in terms of sensitivity and specificity) for distinguishing between HCC tissue and corresponding noncancerous liver tissue from the same HCC patients



and *PAX5* in the cell lines and normal liver tissue (Fig. 3b), whereas methylation of *PENK* and *SPDYA* did not correlate significantly with their expression levels. The expression of *PENK* was undetectable in seven HCC cell lines and in

Table 1 Association between clinicopathological features and DNA methylation in HCC

	N	*KLHL35* methylation			*PAX5* methylation			*PENK* methylation			*SPDYA* methylation			LINE-1 methylation		
		Mean	SD	*P* value	Mean	SD	*P* value	Mean	SD	*P* value	Mean	SD	*P* value	Mean	SD	*P* value
Age																
≤63	24	30.3	17.7	0.003	18.3	17.1	0.026	41.7	19.3	0.583	51.2	17.9	0.945	49.4	14.8	0.571
>64	23	47.2	18.5		32.3	24.1		44.8	19.6		50.9	15.0		47.2	10.6	
Sex																
M	39	37.5	20.3		22.6	20.9		40.2	19.6		50.1	18.1		47.8	13.3	
F	18	37.2	22.0	0.953	25.2	19.9	0.652	43.1	19.6	0.602	48.7	16.7	0.771	51.5	12.0	0.318
Virus																
HBV	21	35.6	20.3		23.1	24.5		44.3	19.4		50.7	15.4		50.4	13.9	
HCV	21	38.3	19.5		26.5	19.0		39.2	18.8		46.6	19.6		50.2	12.2	
NBNC	15	36.1	22.2	0.900	20.7	17.2	0.698	38.2	21.0	0.603	51.3	18.0	0.668	44.7	12.9	0.359
Child-Pugh																
A	44	39.2	20.0		25.5	22.3		43.4	19.6		51.4	15.6		48.6	12.4	
B	3	29.7	18.2	0.426	19.9	11.8	0.672	41.0	17.1	0.842	45.3	29.5	0.536	44.6	20.6	0.609
PIVKA-II (mAU/ml)																
≤21	16	40.0	19.5		24.0	25.7		42.1	19.1		53.5	14.1		48.0	11.5	
22–66	16	35.8	11.8		23.4	14.2		44.6	14.0		42.9	15.9		52.9	10.7	
>67	15	40.1	26.9	0.795	28.3	24.8	0.802	42.8	24.9	0.933	57.1	16.6	0.039	43.8	15.1	0.136
AFP (ng/ml)																
≤7.4	16	39.3	19.3		25.8	24.1		41.4	19.0		49.4	17.0		47.4	9.2	
7.5–55.0	16	44.9	20.2		31.1	23.2		51.5	15.6		55.0	16.7		50.6	13.4	
>55.1	15	31.1	18.7	0.150	18.1	16.3	0.256	36.3	21.0	0.078	48.7	15.8	0.509	46.9	15.7	0.695
Cirrhosis																
0	27	35.3	23.4		22.2	22.2		40.0	22.1		51.8	18.2		47.7	14.7	
1	24	40.7	16.5	0.353	25.7	20.3	0.559	44.2	17.8	0.467	50.5	15.8	0.795	49.2	11.3	0.687
Vascular invasion																
0	42	38.3	18.5		24.0	21.0		43.9	19.5		52.3	15.1		48.2	12.8	
1	9	35.7	28.9	0.353	23.1	23.3	0.559	33.1	21.5	0.467	46.1	24.2	0.795	49.3	15.1	0.687
TNM stage																
1	6	29.5	15.4		15.0	9.8		50.6	12.1		43.3	20.8		58.4	11.8	
2	20	37.7	20.4		24.6	20.3		44.7	19.3		53.7	11.8		47.5	13.2	
3	13	45.4	14.3		24.4	23.4		43.0	19.3		55.5	13.8		44.8	10.6	
4	6	32.4	28.6	0.335	30.9	28.4	0.639	29.4	23.3	0.262	41.6	25.5	0.181	47.5	16.1	0.200
Multiple cancer																
0	33	38.3	22.1		26.2	23.9		43.9	20.8		51.1	18.5		48.8	14.2	
1	13	38.6	14.3	0.964	22.4	16.9	0.609	41.7	16.4	0.732	50.5	10.7	0.911	48.3	8.5	0.916

NBNC HBV/HCV-negative

normal liver tissue, irrespective of the methylation status (Fig. 3b). Conversely, although *SPDYA* was highly methylated in a majority of HCC cell lines, its expression was detectable in all cells, and most of the HCC lines exhibited greater *SPDYA* expression than did normal liver tissue (Fig. 3b). The above results suggest that *KLHL35* and *PAX5* are epigenetically silenced in HCC cells. Consistent with that idea, treating methylated cell lines with a DNA methyltransferase inhibitor, 5-aza-dC, restored the expression of *KLHL35* and *PAX5* (Fig. 3c). On the other hand, the expression of *PENK* and *SPDYA* does not appear to be affected by methylation.

Analysis of LINE-1 methylation and its association with gene hypermethylation

It was previously reported that LINE-1 is frequently hypomethylated in HCC, though most of those studies focused on HBV-positive tumors. Similarly, by using the bisulfite

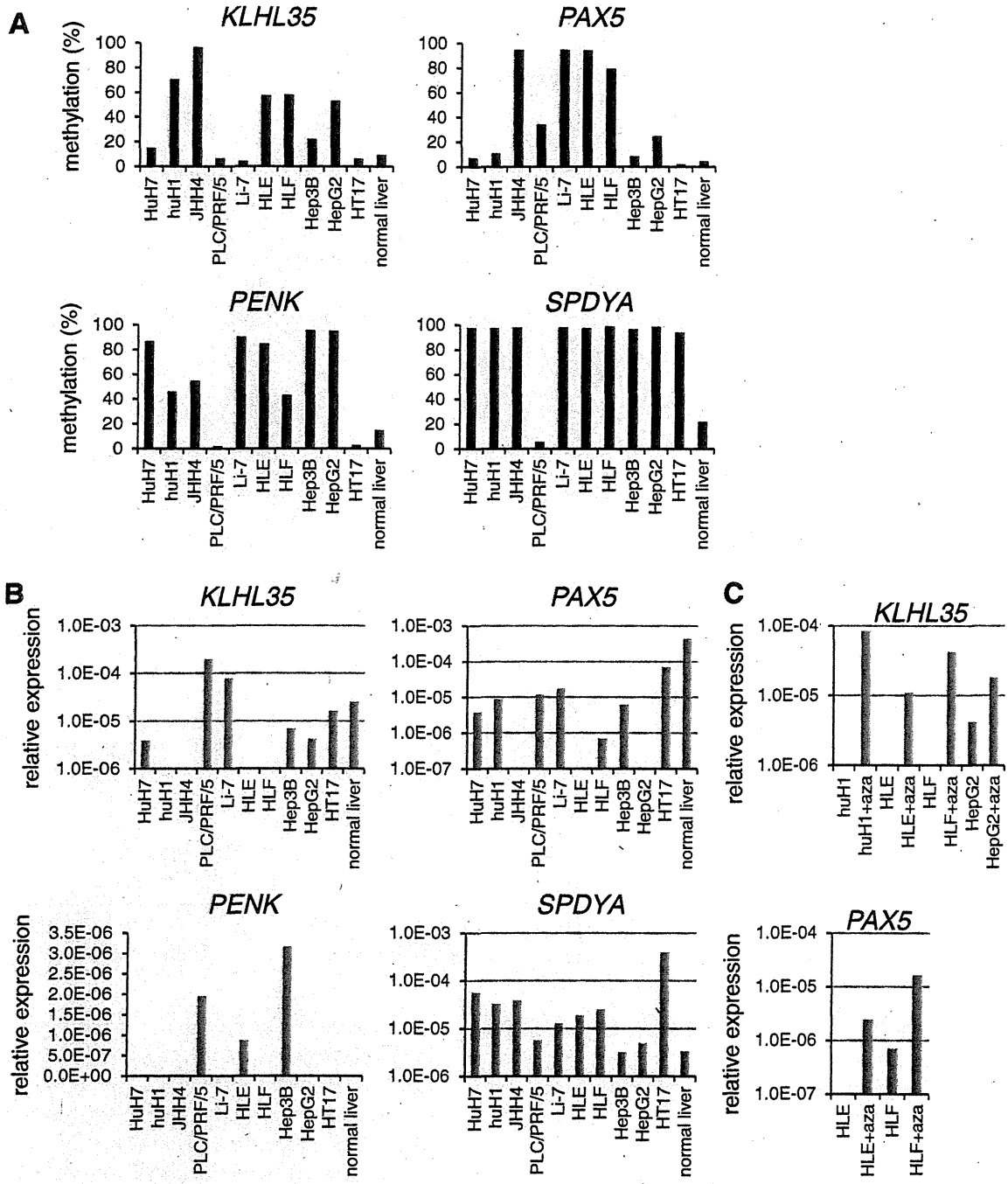


Fig. 3 Analysis of the methylation and expression of the indicated genes in HCC cell lines. **a** Bisulfite pyrosequencing of *KLHL35*, *PAX5*, *PENK*, and *SPDYA* in HCC cell lines and normal liver tissue from a healthy individual. **b** Quantitative RT-PCR of the four genes in HCC cell lines and normal liver tissue. **c** Quantitative RT-PCR of *KLHL35* and *PAX5* in HCC cell lines, with and without 5-aza-dC (*aza*) treatment

pyrosequencing, we found that levels of LINE-1 methylation were significantly lower in tumor tissues than in their noncancerous counterparts (48.5 vs. 66.8 %, $P<0.001$). LINE-1 hypomethylation was prevalent, regardless of the tumor's hepatitis virus status, but the average methylation level was lowest in the HBV/HCV-negative tumors (HBV-positive, 50.8 vs. 66.3 %, $P<0.001$; HCV-positive, 48.9 vs. 67.4 %, $P<0.001$; HBV/HCV-negative, 44.7 vs. 66.6 %, $P<0.001$; Fig. 4a). The ROC curve analysis revealed that LINE-1 methylation discriminated strongly between HCC tissue and noncancerous liver tissue (Fig. 4b), though no significant correlation was found between the levels of LINE-1 methylation and the clinicopathological characteristics of the samples (Table 1). Finally, we tested whether LINE-1 hypomethylation is linked to gene hypermethylation. We found an inverse relationship between the level of

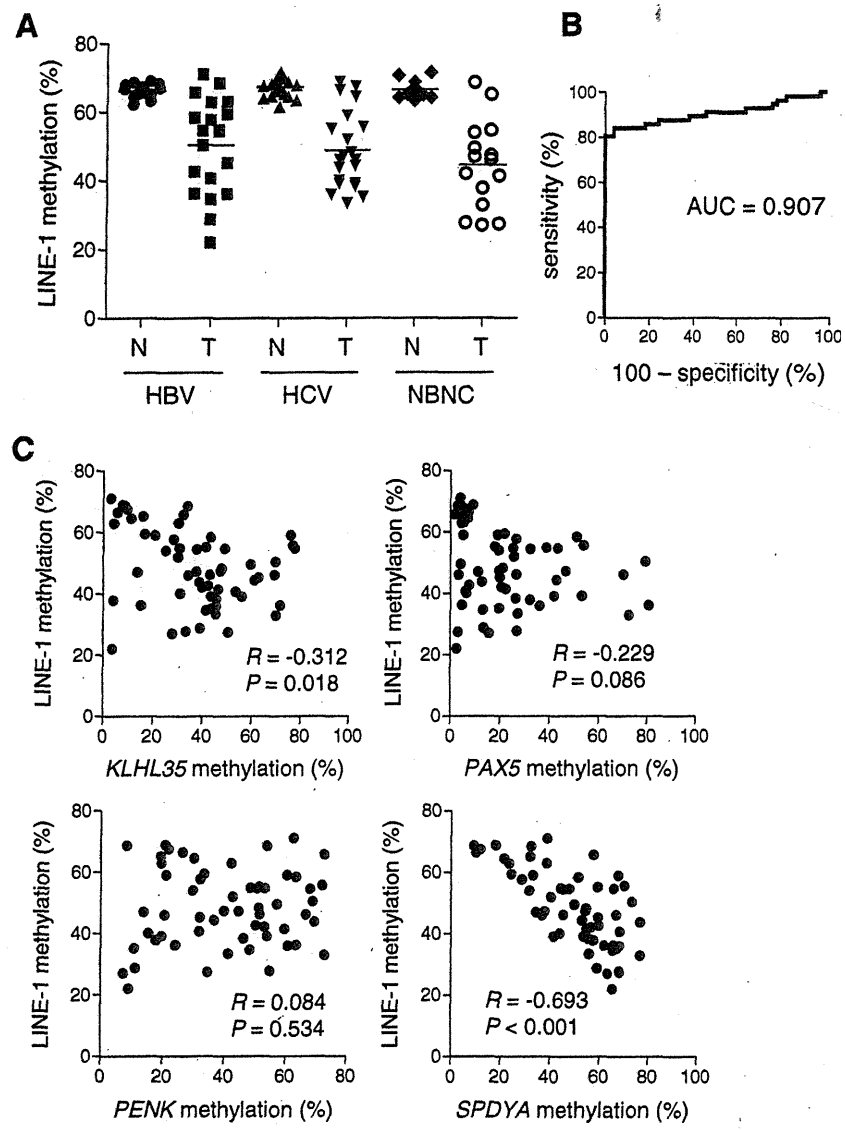


Fig. 4 Analysis of LINE-1 methylation and its association with CpG island hypermethylation in HCC. **a** Summary of bisulfite pyrosequencing analysis of LINE-1 in tumor tissue (*T*) and corresponding noncancerous liver tissue (*N*) from HBV-positive, HCV-positive, and HBV/HCB-negative (*NBNC*) HCC patients. **b** ROC curve analysis of the utility of LINE-1 methylation for distinguishing between HCC tissue and corresponding noncancerous liver tissue from the same HCC patients. **c** Correlation between the level of LINE-1 methylation and methylation of the indicated genes in HCC tissues. The Pearson correlation coefficients and *P* values are shown

LINE-1 methylation and levels of *KLHL35* and *SPDYA* methylation. On the other hand, we found no significant correlation between the LINE-1 hypomethylation and *PAX5* or *PENK* methylation (Fig. 4c).

Discussion

In the present study, we carried out high-throughput CpG island methylation profiling in a set of primary HCC tissues with and without hepatitis virus infection. MCAM analysis enabled us to evaluate the methylation status of more than 6,000 gene promoters with high specificity and sensitivity [13]. Consistent with earlier studies that showed methylation to be more abundant in the HCV-positive HCCs than in the HBV-positive or hepatitis virus-negative HCCs [15, 18], we observed the highest number of methylated genes in HCV-positive HCC tissue. However, we also noted that a large number of genes were commonly methylated among HCCs, irrespective of the hepatitis virus status, indicating that aberrant methylation of multiple genes may be involved in a common mechanism underlying hepatocarcinogenesis. Moreover, studies have also shown that aberrant methylation detected in tissues or blood samples could be a useful biomarker for early detection of HCC [19, 20]. We therefore validated the methylation status of 14 genes and identified four genes that were frequently methylated in HCC tissues but showed little or no methylation in surrounding noncancerous tissues. The high-tumor specificity suggests that methylation of these genes may not occur at precancerous stages, such as chronic hepatitis or liver cirrhosis; instead, they may be acquired during malignant transformation.

The paired box 5 (*PAX5*) gene is a member of the paired box-containing family of transcription factors, which are involved in the control of organ development and tissue differentiation [21]. PAX5 is also known to be a B cell-

specific activator protein that plays an essential role during B cell differentiation, neural development, and spermatogenesis. Methylation of the CpG island of *PAX5* was first discovered in breast cancer cells using the MCA technique [22]. Subsequently, methylation and downregulation of *PAX5* were found in lymphoid neoplasms [23]. In addition, while we are preparing the present manuscript, methylation of *PAX5* was reported in HCC and gastric cancer [24, 25]. Restoration of *PAX5* expression in HCC cells induced growth arrest and apoptosis through upregulation of various target genes, including p53, p21, and Fas ligand, suggesting that the PAX5 acts as a tumor suppressor [24].

The involvement of the kelch-like 35 (*KLHL35*) gene in cancer had not been reported until recently, when a genome-wide analysis of DNA methylation in renal cell carcinoma identified frequent hypermethylation of nine genes, including *KHLH35* [26]. Although the function of the gene product remains unknown, RNAi-induced knockdown of *KHLH35* in HEK293 cells promoted anchorage-independent growth, indicating its possible role in tumorigenesis [26].

The proenkephalin (*PENK*) gene encodes preproenkephalin, a precursor protein that is proteolytically cleaved to produce the endogenous opioid peptides met- and leu-enkephalin. Methylation of the CpG island of *PENK* was first identified in pancreatic cancer cells using the MCA technique [27]. Downregulated expression of *PENK* has also been reported in prostate cancer, suggesting its possible involvement in cancer development [28], and *PENK* methylation was recently identified in lung cancer, bladder cancer, and meningioma [29–31]. Although its functional role in cancer is not fully understood, a recent study showed that in response to cellular stress, PENK physically associates with p53 and RelA (p65) and regulates stress-induced apoptosis [32].

The *SPDYA* encodes Spy1, also known as Speedy, an atypical CDK activator known to promote cell survival, prevent apoptosis, and inhibit checkpoint activation in response to DNA damage [33]. The expression of *SPDYA* is upregulated in breast cancer [34], and its overexpression in a mouse model has been shown to accelerate mammary tumorigenesis [35]. Moreover, a recent study showed overexpression of *SYPDA* in HCC and its association with poor prognosis [36]. These results strongly suggest its involvement in oncogenesis. In the present study, we also observed that most of the HCC cell lines tested exhibited greater expression of *SPDYA* than normal liver tissue, regardless of the methylation status. Among the three transcription variants of *SPDYA* annotated in the NCBI Reference Sequence database, transcription start sites of variants 1 and 3 are located within the CpG island, while that of variant 2 are located approximately 5 kb downstream of the CpG island. Thus, the *SPDYA* transcript in HCC cells may be derived from the downstream transcription start site.

By analyzing the LINE-1 methylation levels, we and others have shown that global hypomethylation is a commonly observed feature of HCC [8, 9, 37]. Earlier studies have suggested that the association between global methylation and hepatitis status may be attributable to hepatitis B virus X protein, which can induce aberrant methylation of specific genes and global hypomethylation [38]. By contrast, we found in the present study that LINE-1 hypomethylation is prevalent among HCC tissues, regardless of the hepatitis virus infection, which suggests that global hypomethylation is involved in a common mechanism underlying hepatocarcinogenesis. It has been shown that the timing of global hypomethylation differs among tumor types. For example, hypomethylation is often observed during the early stages of colorectal and gastric carcinogenesis. By contrast, LINE-1 hypomethylation appears to be tumor-specific in HCC; it is rarely found in precancerous lesions such as chronic hepatitis or liver cirrhosis [8, 9]. A recent study showed that global hypomethylation is associated with a poorer prognosis in HCC patients [39]. In addition, the levels of serum LINE-1 hypomethylation in HCC patients reportedly correlate with serum HBs antigen status, large tumor size, and advanced tumor stage [40]. This suggests that hypomethylation may not occur at precancerous stages, and that LINE-1 methylation could be a useful biomarker with which to identify HCC and predict its clinical outcome.

The relationship between LINE-1 hypomethylation and CpG island hypermethylation in cancer is controversial. In one study, LINE-1 methylation levels were reduced in HCCs with the CpG island methylator phenotype, indicating a positive correlation between global hypomethylation and CpG island hypermethylation [9]. Another study showed that LINE-1 hypomethylation was positively correlated with hypermethylation of only a few genes (*p16*, *CACNA1G*, and *CDKN1C*), while methylation of a large number of genes showed inverse or no correlation with LINE-1 hypomethylation [12]. In the present study, we found that methylation of *KLHL35* and *SPYDA* correlates positively with LINE-1 hypomethylation, whereas levels of *PAX5* or *PENK* methylation are independent of LINE-1 methylation. These results suggest that the association between CpG island methylation and global hypomethylation may be site specific, and that hypomethylation of LINE-1 is a more generalized phenomenon than hypermethylation of CpG islands in HCC.

In summary, by screening targets of DNA methylation in HCC, we identified four frequently methylated genes. These genes are methylated in a cancer-specific manner and could be useful molecular markers for diagnosing HCC. In addition, we observed prevalent LINE-1 hypomethylation in HCC, irrespective of hepatitis virus infection. Identification of aberrant methylation in HCC may provide valuable information that not only contributes to our understanding of the pathogenesis