RESEARCH COMMUNICATION

DNA Copy-number Loss on 1p36.1 Harboring RUNX3 with Promoter Hypermethylation and Associated Loss of RUNX3 Expression in Liver Fluke-associated Intrahepatic Cholangiocarcinoma

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Abstract

Runt-related transcription factor 3 (RUNX3) is a candidate tumor suppressor gene, localized on 1p36, involved in TGF-ß-Smads signaling. To assess its role in liver fluke-associated intrahepatic cholangiocarcinoma (ICC), the promoter methylation status was investigated in 53 ICCs by methylation-specific PCR, with determination of loss of 1p36.1 by microarray comparative genomic hybridization and RUNX3 protein expression by immunohistochemistry. Loss at 1p36.1 was found 41.5% of ICCs (22/53). In addition, DNA hypermethylation of the RUNX3 promoter was found in 49.1% (26/53) of cancers and 57.1% (4/7) of ICC cell lines. The protein was highly expressed in normal bile ducts but mostly decreased in ICCs, 67.9% (n=36) being negative for immunohistochemical staining. Promoter hypermethylation of RUNX3 was associated with reversible decrease or absence of RUNX3 protein expression (p<0.001), but this was not found to differ with the ICC subtype. In contrast, loss of 1p36.1 demonstrated a significant link (p=0.020). In conclusion, RUNX3 promoter hypermethylation and loss of 1p36.1 are causal mechanisms for loss of RUNX3 function in liver fluke-associated ICC carcinogenesis.

Key Words: RUNX3 - DNA hypermethylation - chromosome 1p36 - microarray comparative genomic hybridization

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Introduction

Intrahepatic cholangiocarcinoma (ICC) is a relatively rare malignancy arising in the epithelial bile ducts in the biliary tree, generally being much less common than the hepatocellular carcinoma (HCC) due to infection of hepatitis B and C virus. ICC is highly prevalent in Southeast Asia including Thailand, Lao People's Democratic Republic, Vietnam and Cambodia, where there is strongly epidemiological and experimental evidence of a role for infestation of liver fluke, Opisthorchis viverrini (OV), endemic in this region, as the major risk factor. At the present time, ICC is very difficult to diagnosis and is usually associated with a high mortality because of its late clinical presentation and the lack of effective non-surgical therapeutics (Khan et al., 2005). Therefore, the most patients with ICC were often diagnosed at advanced stages and poor prognosis to treatment. However, the pathogenesis of OV associated with ICC in carcinogenesis has been poorly understood. In particular, the molecular carcinogenesis links between ICC associated with Opisthorchiasis is still unclear.

The development and progression of liver fluke-associated ICC was multi-step process resulting in genetic material alterations and accumulated these affecting to genetic instability. The genetic instability is well known, a major mechanism to process cancer development and progression (Murnane, 2006). These associate a cancer-related genes controlling cell cycle regulation, DNA repairing, apoptosis, cell proliferation and differentiation, angiogenesis and metastasis. The recently reports of molecular carcinogenesis in ICC showed several mechanisms involving in genetic alterations such as oncogene activation of K-ras by point mutaion, amplification of c-myc, cyclin D1 and HER-2/neu (Ukita et al., 2002; Takahashi et al., 2007). Additionally, loss of activity in tumor suppressor gene (TSG) has mainly occurred via chromosomal deletion, loss of heterozygous (LOH) and mutation such as p53 mutation (Kiba et al. 1993; Wattanasirichaigoon et al., 1998; Tannapfel et al.,

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2000; Khan et al., 2005; Lui et al., 2006), E-cadherin mutation (Endo et al., 2001) and LOH on 1p36 (Limpaiboon et al., 2006). Especially, several studies on methylation modification on CpG islands in or near promoters of TSG and DNA repairing genes have been correlated with epigenetic alterations contributing to gene silence (Jones and Laird, 1999) and its occurred in TSG for loss of expression involving in carcinogenesis. However, a report of TSG silencing by alternative epigenetic pathway has been found in liver fluke associated with ICC (Limpaiboon et al., 2005) which is also seen in various solid tumors associated with tumorigenesis (Baylin and Ohm, 2006).

Runt-related transcription factor 3 (RUNX3), a member of the runt domain-containing family of transcription factors, plays an important role in both of normal development processes and carcinogenesis, due to tumor suppressor activity on the TGF-ß signaling pathway (Bae et al., 2004). RUNX3 has established a one of TSG in gastric cancer (Ito and Miyazono, 2003; Anglin and Passaniti, 2004; Bae and Choi, 2004). DNA promoter hypermethylation on RUNX3 gene has been reported in several of solids and hematopoietic malignancies by which is down regulated and correlated with tumor development and progression (Ito, 2004). In addition, RUNX3 is located on 1p36.1, which is commonly deleted in a variety of solid tumors, like HCCs, lung cancer, breast cancer, neuroblastomas, and colon cancer (Kim et al., 2004; Miyagawa et al., 2006). Recently, Limpaiboon et al (2006) reported on LOH on 1p36 and microsatellite alterations on the in liver fluke related ICC in which might be involved in tumorigenesis. Therefore, in this study we further elucidated promoter hypermethylation of RUNX3 gene and chromosomal loss at 1p36.1 in liver fluke-associated ICCs and assessed the relationship to loss of RUNX3 expression thought to contribute to ICC carcinogenesis.

Materials and Methods

Tissue samples, cell lines and DNA extraction

53 ICCs were diagnosed, including 38 mass forming (MFs) and 15 intraductal growing (IDs) lesions and their clinicopathological data were recorded. The specimens were collected at Srinagarind University Hospital at the Faculty of Medicine, Khon Kaen University, Thailandand stored immediately at -80°C until analysis. All patients gave informed consent approved by our ethics committee for use of all specimens.

In addition, 7 ICC cell lines were maintained in RPMI 1640 (KKU-M055, KKU-M139, KKU-M156, KKU-M213 and KKU-M214) or Ham F-10 (KKU-100 and KKU-OCA17) medium supplemented with 10% fetal bovine serum (FBS) and streptomycin-penicillin 100 U. In addition, we collected normal DNA from blood healthy donors. Aliquots of frozen tumors and cell lines (50 grams) were also extracted for genomic DNA using PUREGENE (Gentra System, Minneapolis, MN, USA) according to the manufacturer's instructions.

RNA extraction and RT-PCR

Total RNA (one µg) was isolated from 7 ICC cell lines (KKU-M055, KKU-M139, KKU-M156, KKU-M213, KKU-M214, KKU-100 and KKU-OCA17) by TRIzol (Invitrogen, Carlsbad, CA, USA). For synthesis complementary DNA (cDNA) was generated by reverse transcription system (Promega, Madison, WI, USA) according to the manufacturer's manual and then amplified with primer for RUNX3 and the PCR reaction as previously described (Wada et al., 2004), which amplify a 300 base pair (bp). GAPDH was used a house keeping gene, cDNA of GAPDH was amplified product size of 118 bp by using the sense primer 5'-TCATCAGCAATGCCTCCTGCA-3' and the anit-sense primer 5'- TGG GTG GCA GTG ATG GCA -3'. For GAPDH of PCR reaction was performed a thermal cycles as initial denaturing for 5 min at 94°C, then for 35 cycles of 30s at 94°C, 30s at 55°C, 30s at 72°C and final extension 7 min at 72°C. The PCR products were checked by electrophoresis on 2% agarose gel stained with ethidium bromide and visualized the product under UV illumination.

Microarray comparative genomic hybridization (aCGH)

The human bacterial artificial chromosomome (BAC) arrays were obtained from the UCSF Cancer Center Array Core (http://cc.ucsf.edu/microarray) and prepared as described previously (REF??). Human BAC array contain 2,440 BAC clones spotted in triplicate with an average resolution of 1.4 Mb.

For microarray hybridization procedure has been described REF??. In brief, the 1 µg of tumor DNA and 1 µg mached- sex reference DNA were diferntially fluorescently labeled with Cy3/Cy5 and added 100 µg of cot-1 DNA (Invitrogen, Carlsbad, CA, USA) to block repeated sequences in genome. After that the hybridization mixture will be hybridized to a BAC array. The array slides were mouthed with 0.3 µg/ml DAPI in 90% glycerol for taking a array image by CCD imaging system that acquire 16-bit 1,024 x 1,024 pixel DAPI, Cy3 and Cy5 images. Three single color intensity images were collected from each array using a charge coupled device camera.

aCGH data analysis

The image data was analyzed as described previously (ref) using UCSF SOPT and SPROC software, which were obtained to download at http://jainlab.ucsf.edu/Downloads.html. The SPOT software was used to analyze the spots image based on DAPI and identify each spot for analysis of Cy3/Cy5 ratios. The SPROC software was used to associate clone identities and a mapping the position of chromosomal regions with each spot according to UCSC human sequences draft of July 2003.

DNA copy-number changes were classified by log2 Cy3/Cy5 ratios when the normalized log2 Cy3/Cy5 ratio was higher than 0.225 and lower than -0.225, these indicating chromosomal copy-number gain and loss, respectively. In addition, DNA amplification of log2 Cy3/Cy5 ratios was higher than 0.8 while, log2 Cy3/Cy5 ratios of -0.7 or lower were classified as reflecting homozygous deletions.

Bisulfite modification

1 µg genomic DNA of 60 primary tumor of ICCs and

7 ICC cell lines as well as healthy donor leucocytes and non-cancerous liver tissue for unmethlylated DNA as a negative control were treated with sodium bisulfite using CpGenome Universial DNA Modification Kit (Chemicon International, Temecula, CA, USA) according to manufacturer's instruction. After desalting, desulfonation, bisulfite-treated DNA was eluted in TE buffer and aliquotted at -80 °C until use.

Methylation-specific PCR (MSP)

Bisulfite-treated DNA samples were analyzed the methylation status of RUNX3 gene by MSP. The primer pairs for unmethylated (U) were amplified sense (5'-GTGGGTGGTTGTTGGGTTAGT-3') and anti sense (5'-TCCTCAACCACCACTACCACA-3'), which amplify a 138 bp product while, methylated (M) were amplified by sense (5'-CGTCGGGTTAGCGAGGTTTC-3') and anti sense (5'-GCCGCTACCGCGAAAAACGA-3'), which amplify a 120 bp product. The primers designed and PCR amplification was the same as previously described (Mori T,2005). The DNA of placenta was treated with SssI methylase (New England Biolabs, Beverly, MA, USA) for positive control (M: methylated), DNA of normal liver and lymphocytes were used for negative control (U: unmethylated). The PCR products were loaded onto 2% agarose gel containing ethidium bromide and directly visualized under UV illumination.

Reactivation of RUNX3 expression by 5-Aza-2_-deoxycytidine (5-aza-dc) treatment

$1x10^5$ cells of methylated (KKU-100, M055 and M214), partial methyalted (M213) were seeded in 6 cm dish, growth for 24 hr before treated DNA methlyation inhibitor, 5-aza-dc (Sigma-Aldrich, St. Louis, MO, USA). The cell lines were cultured for 5 days with 5μM of 5-aza-dc medium changes everyday. After incubation, the cells were harvested for RNA extraction. RT-PCR was performed to evaluate the reactivation of RUNX3 expression as described above.

Immunohistochemistry (IHC)

The formalin-fixed paraffin-embedded of ICC tumors were cut the section with 5μm according to standard method. The sections were deparaffinized and rehydrate by routine techniques. Antigen retrieval of the tissues sections were performed in 10 mM sodium citrate (pH 6.0) for 10 min by high power microwave. After cooling for 20 min, the tissues sections were quenched with 3% H_2O_2 for 10 minutes and block a non-specific binding with 5% goat serum in phosphate buffer saline (PBS) for 20 min at room temperature in a humidified chamber. The sections were incubated for 1 hr at room temperature with rabbit polyclonal antibody against human RUNX3 (Activemotif, Carlsbad, CA, USA) in a 1:500 dilution. The secondary antibody (goat anti-rabbit-HRP) (ZYMED, San Francisco, CA, USA) was incubated the sections for 30 min (1:300 dilutions in PBS). The reaction products of peroxidate were visualized by 3, 3'-diaminobenzidine (DAB) (Sigma-Aldrich, St. Louis, MO, USA). Counterstaining was performed with Mayers hematoxylin for 5 minutes. Protein expression of RUNX3 was indicated a positive by a reddish-brown precipitate in cytoplasm and occasionally seen in nucleus (ref). The positive staining were divided into four group: Negative < 10% of tumor cells , +1 > 10-25% of tumor cells (weakly) , + 2 > 25-50 % of tumor cells (moderately) and +3 > 50 % of tumor cells (strongly).

Statistical analysis

The associations between clinicopathological factors and RUNX3 expression, methylation status were defined with two-tailed Chi square test. The statistical significant was considered as p-values less than 0.05. The TreeView software was used to graphically generate hierarchical clustering based on aCGH data and the program classified the regions of gain or loss and also amplification or deletion. TreeView is available online at http://rana.lbl.gov/EisenSoftware.htm.

Results

Frequent loss of DNA copy-number at 1p tel to 1p36 determined by high resolution aCGH

Using 2,440 human BAC clones screening genome-wide DNA copy-number changes in 53 ICCs revealed the whole genomic alteration with high resolution 1.4 Mb by which can detect micro deletion and/or narrow amplification. This high throughput tool overcame the limitation of other molecular cytogenetics as previously not defined in ICC. The heat map clustering of log2 ratio (Cy3/Cy5) was presented on the BAC clones changed at 1p36 in Figure 1. aCGH results on 1p starting at 1p tel to 1p36 that covered 25 BAC clones approximately spanning a size range 32,248 kb (starting at BAC clone of GS1-232B23 to CTD-2098K5). The DNA copy-number loss on 1p tel to 1p36 was counted as log2 ratio Cy3/Cy5 <-0.225. Resulting that the most frequent loss of DNA copy-number at 1p tel to 1p36 was occurred on BAC clone of RP11-57F20 at 1p36.1 nearly on RUNX3 gene in 22 cases (41.5%) (N=53) (Table 1) and this region was found to

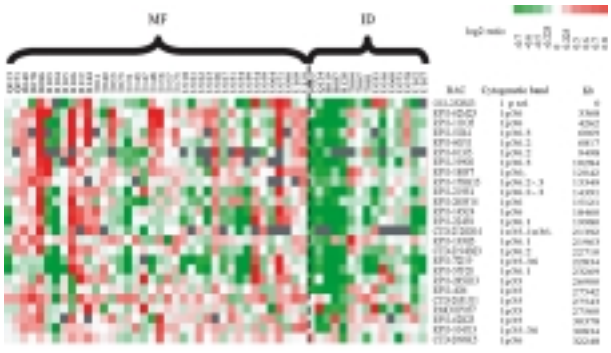

Figure 1. Heat Map Custering of BAC Clone Changes on Chromosome 1p tel to 1p36. The BAC clones gained (red) and lost (green) are observed individual tumors. The heat map of ICCs was clustered in two groups according to ICC subtypes [MF, and ID] and black loci are excluded. The DNA copy-number loss on 1p tel to 1p36 was individually counted as log2 ratio Cy3/Cy5 <-0.225. Loss on 1p36 was frequently occurred in ICCs. Loss 1p36.1 on RP11-57F20 at position 23,269 kb was found the most in all subtypes of ICC by which this locus is nearly RUNX3 gene

Table 1. Clinicopathological Parameters and RUNX3 Hypermethylation, Loss on 1p36.1 by aCGH and RUNX3 Protein Expression in ICCs

Tumor	ICC subtype	Methylation status	1p36.1	IHC
P010	ID	M	Loss	-
Q013	MF	P	Loss	+3
Q071	MF	U	No loss	-
Q124	ID	P	Loss	+1
R019	ID	M	Loss	-
R049	MF	M	No loss	-
R078	MF	M	No loss	-
R086	MF	M	No loss	-
R087	ID	U	Loss	-
R103	MF	P	Loss	+1
R104	MF	U	Loss	-
R105	MF	M	Loss	+2
R106	MF	U	No loss	-
R112	MF	M	No loss	-
R128	ID	U	Loss	-
R144	MF	M	No loss	-
T004	ID	M	No loss	-
T007	ID	M	No loss	-
T011	MF	P	No loss	+2
T040	MF	P	No loss	-
T053	MF	U	No loss	-
T073	MF	P	No loss	+2
T081	ID	U	Loss	-
T131	MF	M	Loss	-
T143	MF	P	No loss	+2
T147	MF	P	No loss	+3
T148	MF	M	Loss	-
T156	MF	M	No loss	-
T157	MF	M	No loss	-
T173	MF	M	No loss	-
U018	MF	M	Loss	-
U021	MF	M	No loss	-
U022	MF	M	No loss	-
U023	MF	M	Loss	-
U026	MF	M	Loss	-
U027	MF	P	No loss	+1
U031	MF	M	Loss	-
U034	MF	P	No loss	+2
U035	ID	P	Loss	+2
U039	MF	U	No loss	-
U054	MF	P	Loss	+1
U056	ID	M	Loss	-
U059	MF	M	No loss	-
U060	ID	P	No loss	-
U064	MF	P	No loss	+3
U071	MF	U	No loss	+1
U075	ID	U	Loss	+1
U078	ID	M	Loss	-
U090	MF	U	Loss	+3
U102	ID	P	Loss	+3
U107	ID	M	No loss	-
U109	MF	M	No loss	-
U110	MF	U	Loss	-

MF, Mass forming type; ID, Intraductal growing type; U, Unmethylated; P=15 (28.3%); M=26 (49.1%);Loss=24 (45.3%); No loss=29 (54.7%); Neg = 36 (67.9%)+1 = 6 (11.3%)+2 = 6 (11.3%)+3 = 5 (9.5%)

significant difference of ICC subtypes (p=0.020) (Table 2). In ICC cell lines, loss 1p36.1 occurred in KKU-100 (Table 3). However, loss on this region showed the most cases with hemizygous deletions while, homozygous deletion on RP11-57F20 at 1p36.1 represented only 2 samples as defined log2 ratio Cy3/Cy5 <-0.7.

Promoter hypermethylation of RUNX3 gene in ICCs and cell lines

To determine DNA methylation on CpG islands around the promoter of RUNX3 gene by MSP in ICCs and cell lines. The results are shown in Table 1 and Figure 2A. The alterations of DNA methylation on promoter can inactivate tumor suppressor gene (TSG) lead to silencing of gene expression (Jones and Laird, 1999). Suggesting that loss expression of RUNX3 via aberrations of DNA methylation in CpG islands on promoter of RUNX3 may play an important role in the development of ICC. 53 ICC were extracted genomic DNA and subsequentially treated with sodium bisulfite after that analyzed by MSP assay. Hypermethylation on promoter RUNX3 gene was commonly found with 49.1% (26/53) including 50.0% (19/38) in MFs, 53.3% (7/8) in IDs (Table 1). Moreover, 28.3% (15/53) exhibited partial methylation with unmethylated and methylated RUNX3 promoter regions. Interestingly, unmethylated was found 22.6% (12/53) consisting of 21.1% (8/38 in MFs), 26.7% (4/15 in IDs). DNA methylations in ICC cell lines, 57.1% (4/7) of ICC cell lines were found hypermethylation including KKU-100, M055, M156 and M214. In contrast, OCA-17, M139 and M213 exhibited partial methylation. An unmethylated status was not found in any of the ICC cell lines. There was no statistically significant association between methylation status and clinicopathological information (Table 2).

Reactivation of mRNA RUNX3 expression by 5'AZA in ICC cell lines

7 ICC cell lines were determined for mRNA RUNX3 expression by RT-PCR as shown in Figure 2B. 6 ICC cell lines consisting of OCA17, M055, M139, M156, M213 and M214 were slightly decreased mRNA of RUNX3 expression. In contrast, KKU-100 did not express. To confirm whether the silencing of RUNX3 gene in ICC cell lines resulting from DNA hypermethylation on promoter of RUNX3. 4 ICC cell lines (KKU100, M055, M214 were methylated, and M213 was partial methylated) were demonstrated reactivation of RUNX3 expression and treated with 5'AZA; DNA methyltransferase inhibitor that may be reactivated RUNX3 expression. RT-PCR product was evaluated the mRNA of RUNX3 expression after incubated with 5'AZA as shown in Figure 2C. The RT-PCR products were seen the intensity which was stronger in all ICC cell lines with treated by 5'AZA than when compared in untreated corresponding cell lines. This study was confirmed the inactivation of RUNX3 expression in ICC cell lines by DNA hypermethylation.

Loss of RUNX3 protein expression determining by IHC

RUNX3 protein was evaluated by IHC staining in 59 primary bile duct tumors that positive staining was seen reddish-brown precipitate in cytoplasm and occasionally found in nucleus. These results showed that RUNX3 was highly expressed in normal bile ducts but decreased or

Table 2. Associations of Clinical Features with Promoter Hypermethylation of RUNX3, Loss at 1p36.1 and RUNX3 Protein Expression

Clinical parameters	Category	n	Methylation U+P	Methylation M	p	Loss at 1p36.1 L	Loss at 1p36.1 N	p	RUNX3 protein -	RUNX3 protein +	p
Age (y)	<55	53	11 (41.8)	12 (52.2)	0.691	12 (52.2)	11 (47.8)	0.168	17 (73.9)	6 (24.0)	0.413
	>55		16 (53.3)	14 (46.7)		10 (33.3)	20 (66.7)		19 (63.3)	11 (36.7)	
Sex	Male	53	16 (50.0)	16 (50.0)	0.865	12 (37.5)	20 (62.5)	0.465	10 (47.6)	11 (52.4)	0.658
	Female		11 (52.4)	10 (47.6)		21 (65.6)	11 (34.4)		15 (71.4)	6 (28.6)	
Tumor size (cm3)	< 175	47	9 (47.4)	10 (52.6)	0.676	8 (42.1)	11 (57.9)	0.658	14 (73.7)	5 (26.3)	0.668
	≥ 175		15 (53.6)	13 (46.4)		10 (35.7)	18 (64.3)		19 (67.9)	9 (32.1)	
ICC subtypes	MF	53	19 (50.0)	19 (50.0)	0.827	12 (31.6)	26 (68.4)	0.020	25 (65.8)	13 (34.2)	0.596
	ID		8 (53.3)	7 (46.7)		10 (66.7)	5 (33.3)		11 (73.3)	4 (26.7)	
Staging	I-II	53	4 (80.0)	1 (20.0)	0.172	3 (60.0)	2 (40.0)	0.378	4 (80.0)	1 (20.0)	0.543
	IIIA-IV		23 (47.9)	25 (52.1)		19 (39.6)	29 (60.4)		32 (66.7)	16 (33.3)	
Grading	Well	53	20 (51.3)	19 (48.7)	0.997	17 (43.6)	22 (56.4)	0.866	28 (71.8)	11 (28.2)	0.484
	Moderate		4 (50.0)	4 (50.0)		3 (37.5)	5 (62.5)		4 (50.0)	4 (50.0)	
	Poorly		3 (50.0)	3 (50.0)		2 (33.3)	4 (66.7)		4 (66.7)	2 (33.3)	
Histological types	Papillary	53	7 (38.9)	11 (61.1)	0.208	10 (55.6)	8 (44.4)	0.137	15 (83.3)	3 (16.7)	0.085
	Non papillary		20 (57.1)	15 (42.9)		12 (34.3)	23 (65.7)		21 (60.0)	14 (40.0)	
Blood invasion	No	53	17 (45.9)	20 (54.1)	0.268	18 (48.6)	19 (51.4)	0.109	26 (70.3)	11 (29.7)	0.578
	Yes		10 (62.5)	6 (37.5)		4 (25.0)	12 (75.0)		10 (62.5)	6 (37.5)	
Lymphatic invasion	No	53	21 (52.5)	19 (47.5)	0.691	17 (42.5)	23 (57.5)	0.797	29 (72.5)	11 (27.5)	0.211
	Yes		6 (46.2)	7 (53.8)		5 (38.5)	8 (61.5)		7 (53.8)	6 (46.2)	
Metastasis	No	53	13 (61.9)	8 (38.1)	0.196	10 (47.6)	11 (52.4)	0.465	15 (71.4)	6 (28.6)	0.658
	Yes		14 (43.8)	18 (56.3)		12 (37.5)	20 (62.5)		21 (65.6)	11 (34.4)	

absent in bile duct cancer cells (Figure 3). Interestingly, mostly of ICCs displayed the negative to weakly staining (Table 1) results as 67.9% (36/53) negative, 11.3% (6/53) weakly, 11.3% (6/53) moderately and 9.5% (5/53) strongly positive. The hypermethylation on promoter of RUNX3 associated to decrease the RUNX3 protein (P<0.001) by IHC. However, loss of RUNX3 protein via promoter hypermethylation was not difference between subtypes of ICC.

Discussion

RUNX3 gene (RUNT-related transcription factor) is essential for normal tissue development and it involves

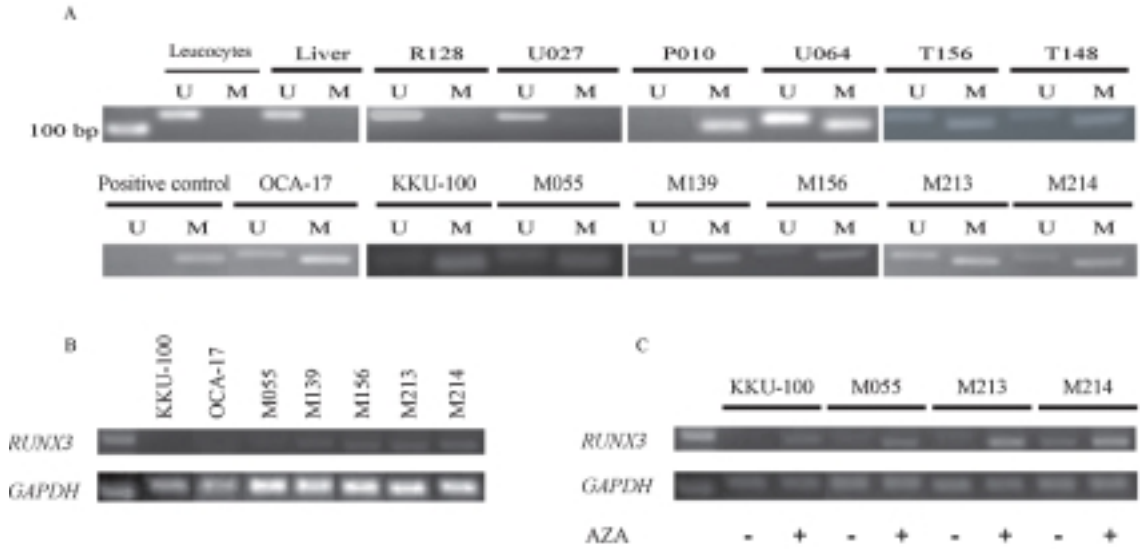

Figure 2. Blotting Results. (A) Methylation -specific PCR (MSP) determining DNA methylation status in ICCs and cell lines. Normal lymphocytes and liver tissue were used as negative control representing that one unmethylated (U) allele while, in placenta treated with SssI methylase as a positive control showed one allele of methylated (M). Unmethylated ICCs were demonstrated in R128 and U027 that represent only one unmethylated allele and methylated was demonstrated in P010, T156 and T148 and in cell lines KKU-100, M055, M156 and M214 showing that one methylated allele. U064 and cell lines OCA-17, M139 and M213 were heterogeneity with partial methylated representing in both of unmethylated and methylated allele. (B) mRNA of RUNX3 expression in ICCs and cell lines performing by RT-PCR and reactivation mRNA of RUNX3 expression treating with 5'AZA (C). Seven ICC cell lines were evaluated mRNA of RUNX3 gene with GAPDH as internal control. Six cell lines (OCA-17, M055, M139, M156, M123 and M214) were decreased mRNA expression and KKU-100 did not express. Reactivation of mRNA of RUNX3 expression were performed to confirm DNA methylation resulting to silencing of RUNX3 expression by treated with 5'AZA; inhibitor of DNA methyltransferase in cell lines (methylated; KKU-100, M055 and M214, partial methylated; M213). After cell lines were cultured with 5'AZA (5μM) that total RNA was extracted from control and treated cell lines then performed by RT-PCR. All cell lines were restored the mRNA of RUNX3 expression.

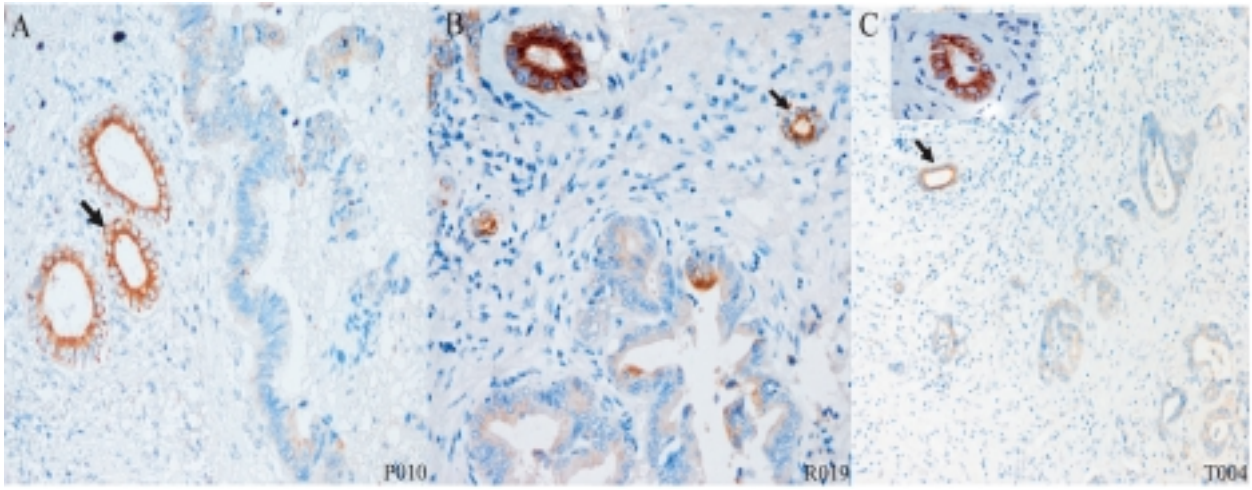

Figure 3. Immunohistochemistry (IHC) analysis of RUNX3 protein expression in primary ICCs. RUNX3 protein was strongly localized in cytoplasm of normal bile duct (arrows: normal bile duct) as shown in B, and C. RUNX3 protein negative in ICC cancer was demonstrated by which these negative results represent strong RUNX3 protein expression in normal bile duct (arrows) but not expressed in bile duct cancers. RUNX3 protein in normal bile ducts was expressed higher than cancer cells showed in P010 (A), R019 (B) and T004 (C).

Table 3. Methylation Status, mRNA Expression, Reactivation of RUNX3 and Loss at 1p36.1

Cell lines	RUNX3 expression	Methylation	Reactivation	1p36.1 status
KKU-100	-	M	Up	Loss
OCA-17	Down	P	ND	No loss
M055	Down	M	Up	No loss
M139	Down	P	ND	No loss
M156	Down	M	ND	No loss
M213	Down	P	Up	No loss
M214	Down	M	Up	No loss

ND, not determined; M, methylated; P, partial methylated

Table 4. Association of Methylation Status, Loss on 1p36.1 and RUNX3 Protein Expression

Status		RUNX3 protein expression		p
		Negative	Positive	
1p36.1	No loss	14 (63.6)	8 (36.4)	0.573
	Loss	22 (71.0)	9 (29.0)	
Methylation	No	11 (40.7)	16 (59.3)	<0.001
	Yes	25 (96.2)	1 (3.8)	

in carcinogenesis (Cameron et al., 2003; Ito et al., 2003; Anglin and Passaniti, 2004; Bae and Choi, 2004). RUNX3 has been established a tumor suppressor gene in gastric cancer (Coe et al., 2005; Or et al., 2005; Takada et al., 2005; Horsley et al., 2006; Hussenet et al., 2006; Saito et al., 2006; Lockwood et al., 2007; Cooke et al., 2008; Shann et al., 2008;Harada et al., 2009; Missiaglia et al., 2009). Studies in RUNX3-null mice showed that gastric epithelial displays hyperplasia and involves anti apoptosis (McMillan et al., 2003; Fukamachi, 2006), low sensitivity to TGF- ß, increasing cell proliferation and abnormal gastric epithelial differentiation (Fukamachi et al., 2004). RUNX3 plays an important role in TGF-ß signal (transforming growth factor-beta) inducing tumor suppressor pathways (Ito and Miyazono, 2003; Miyazono et al., 2003; 2004; Torquati et al., 2004; Suzuki et al., 2005). The role of RUNX3 in TGF-ß signaling pathway has been studied to interact with proteins of the Smad family of tumor suppressors including Smad 2, Smad 3 and Smad 4 by which TGF-ß signal can activate Smad 2 and Smad 3 then bind with RUNX3 protein (Ito and Miyazono, 2003; Miyazono et al., 2004; Miyazono et al., 2003; Park et al., 2003; Seo et al., 2009; Torquati et al., 2004). So that, loss of RUNX3 expression has directly associated TGF-ß signaling pathway. Recent studies have elucidated the role of RUNX3 in TGF-ß signaling in biliary tract cancer cell lines resulting that after restored RUNX3 expression results to upstream TGF-ß signaling by induced expression of p21 and FOXO3A and downregulated the Cyclin D1 lead to cell cycle G1 arrest (Hasegawa et al., 2007).

This first study analyzed the promoter hypermethylation on RUNX3 and loss on 1p36.1 in 60 primary bile duct tumors-associated liver fluke infection. Within the BAC clones on 1p tel to 1p36 were detected the most common loss on 1p36.1 on BAC clone of RP11-57F20 starting at the position at 23,269 Kb on 1p36.1 nearly on RUNX3 gene (position 25,098-25,164 Kb based on www.ensemble.org). Interestingly, loss on 1p36 has been often found in a wide range of human cancers where locus of RUNX3 gene (Wada et al., 2004; Xiao and Liu, 2004; Yanada et al., 2005; Hwang et al., 2007; Lefeuvre et al., 2009). Therefore, loss on chromosome 1p36.1 was interested to investigate RUNX3 possibly as a candidate tumor suppressor gene in ICC.

Loss on 1p36.1 in ID types was significantly associated with ICC subtypes (p=0.020). However, several candidate tumor suppressor genes are located in the neighborhood of the RUNX3 gene and these have been reported to be altered in solid tumors, such as RIZ1 (Huang, 1999; Fang et al., 2000; Tokumaru et al., 2003; Geli et al., 2005; Akahira et al., 2007; Piao et al., 2008), p73 (Rosenbluth and Pietenpol, 2008; Tomasini et al., 2008), UBE4B (Caren et al., 2005; Hosoda et al., 2005; Lefeuvre et al., 2009), RAP1GAP (Zhang et al., 2006a; 2006b;Tsygankova et al., 2007; Lefeuvre et al., 2009; Nellore et al., 2009; Zheng et al., 2009), ALPL (Muller et al., 1999), ZNF436 (Li et al., 2006) and RPL11 (Zhang et al., 2006). Furthermore, these candidate genes may be

involved the development of ICC. Importantly, loss on 1p36 in ICC is likely large size by which deleted region should be refined mapping.

The promoter hypermethylation of RUNX3 were frequently found in ICCs and loss on 1p36.1. Similarly, promoter hypermethylation on RUNX3 have reported in solid cancers with loss on 1p36 such as in HCC (Xiao and Liu, 2004), breast cancer (Hwang et al., 2007) and lung cancer (Yanada et al., 2005). Moreover, we found the association of hypermethylation of RUNX3 result to absent or decreased of RUNX3 protein ($p<0.001$). Taken together, our results indicate that promoter hypermethylation of RUNX3 and loss on 1p36 might be a causal mechanisms of loss function of RUNX3 in ICCs and cell lines. Additionally, recent studied in LOH analysis and microsatellite instability (MSI) on the chromosomal region of 1p36-pter in ICCs associated liver fluke infection revealed that 75.6% (68/90) represented LOH and MSI found in 37.8% (34/90) at one or more loci on 1p36-pter and associated with poor prognostic in MSI alterations (Limpaiboon et al., 2006). Unfortunately, mutation of RUNX3 gene has not been reported yet in ICCs and also not found in HCC (Xiao and Liu, 2004) but rare mutation in bladder cancer (Kim et al., 2005).

In summary, we successfully demonstrated common loss on 1p36 harboring RUNX3 as a potent candidate tumor suppressor gene in ICC by high resolution aCGH accompanied by methylation status analysis. Furthermore, transcriptional and protein expression analysis exhibited RUNX3 alteration with allelic loss, microsatellite alterations at chromosome 1p36 and epigenetic changes appeared to have a major role in the inactivation of RUNX3 functions which may be contributing to cholangiocarcinogenesis.

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References

Akahira J, Suzuki F, Suzuki T, et al (2007). Decreased expression of RIZ1 and its clinicopathological significance in epithelial ovarian carcinoma: correlation with epigenetic inactivation by aberrant DNA methylation. *Pathol Int*, **57**, 725-33.

Anglin I, Passaniti A (2004). Runx protein signaling in human cancers. *Cancer Treat Res*, **119**, 189-215.

Bae SC, Choi JK (2004). Tumor suppressor activity of RUNX3. *Oncogene*, **23**, 4336-40.

Baylin SB, Ohm JE (2006). Epigenetic gene silencing in cancer - a mechanism for early oncogenic pathway addiction? *Nat Rev Cancer*, **6**, 107-16.

Cameron ER, Blyth K, Hanlon L, et al (2003). The Runx genes as dominant oncogenes. *Blood Cells Mol Dis*, **30**, 194-200.

Caren H, Ejeskar K, Fransson S, et al (2005). A cluster of genes located in 1p36 are down-regulated in neuroblastomas with poor prognosis, but not due to CpG island methylation. *Mol Cancer*, **4**, 10.

Coe BP, Henderson LJ, Garnis C, et al (2005). High-resolution chromosome arm 5p array CGH analysis of small cell lung carcinoma cell lines. *Genes Chromosomes Cancer*, **42**, 308-13.

Cooke SL, Pole JC, Chin SF, et al (2008). High-resolution array CGH clarifies events occurring on 8p in carcinogenesis. *BMC Cancer*, **8**, 288.

Endo K, Ashida K, Miyake N, Terada T (2001). E-cadherin gene mutations in human intrahepatic cholangiocarcinoma. *J Pathol*, **193**, 310-7.

Fang W, Piao Z, Simon D, Sheu JC, Huang S (2000). Mapping of a minimal deleted region in human hepatocellular carcinoma to 1p36.13-p36.23 and mutational analysis of the RIZ (PRDM2) gene localized to the region. *Genes Chromosomes Cancer*, **28**, 269-275.

Fukamachi H (2006). Runx3 controls growth and differentiation of gastric epithelial cells in mammals. *Dev Growth Differ*, **48**, 1-13.

Fukamachi H, Ito K, Ito Y (2004). Runx3-/- gastric epithelial cells differentiate into intestinal type cells. *Biochem Biophys Res Commun*, **321**, 58-64.

Geli J, Nord B, Frisk T, et al (2005). Deletions and altered expression of the RIZ1 tumour suppressor gene in 1p36 in pheochromocytomas and abdominal paragangliomas. *Int J Oncol*, **26**, 1385-91.

Harada T, Chelala C, Crnogorac-Jurcevic T, Lemoine NR (2009). Genome-wide analysis of pancreatic cancer using microarray-based techniques. *Pancreatology*, **9**, 13-24.

Hasegawa K, Yazumi S, Wada M, et al (2007). Restoration of RUNX3 enhances transforming growth factor-beta-dependent p21 expression in a biliary tract cancer cell line. *Cancer Sci*, **98**, 838-43.

Horsley SW, Mackay A, Iravani M, et al (2006). Array CGH of fusion gene-positive leukemia-derived cell lines reveals cryptic regions of genomic gain and loss. *Genes Chromosomes Cancer*, 45, 554-64.

Hosoda M, Ozaki T, Miyazaki K, et al (2005). UFD2a mediates the proteasomal turnover of p73 without promoting p73 ubiquitination. *Oncogene*, **24**, 7156-69.

Huang S (1999). The retinoblastoma protein-interacting zinc finger gene RIZ in 1p36-linked cancers. *Front Biosci*, **4**, D528-32.

Hussenet T, Mallem N, Redon R, et al (2006). Overlapping 3q28 amplifications in the COMA cell line and undifferentiated primary sarcoma. *Cancer Genet Cytogenet*, **169**, 102-13.

Hwang KT, Han W, Bae JY, et al (2007). Downregulation of the RUNX3 gene by promoter hypermethylation and hemizygous deletion in breast cancer. *J Korean Med Sci*, **22 Suppl**, S24-31.

Ito Y (2004) Oncogenic potential of the RUNX gene family: 'overview'. *Oncogene*, **23**, 4198-208.

Ito Y, Miyazono K (2003). RUNX transcription factors as key targets of TGF-beta superfamily signaling. *Curr Opin Genet Dev*, **13**, 43-7.

Ito Y, Osato M, Ito K (2003). RUNX and cancer. *Ann Acad Med Singapore*, **32 (5 Suppl)**, S6-7.

Jones PA, Laird PW (1999). Cancer epigenetics comes of age. *Nat Genet*, **21**, 163-7.

Kiba T, Tsuda H, Pairojkul C, et al (1993). Mutations of the p53 tumor suppressor gene and the ras gene family in intrahepatic cholangiocellular carcinomas in Japan and Thailand. *Mol Carcinog*, **8**, 312-8.

Kim TY, Lee HJ, Hwang KS, et al (2004). Methylation of RUNX3 in various types of human cancers and premalignant stages of gastric carcinoma. *Lab Invest*, **84**, 479-84.

Kim WJ, Kim EJ, Jeong P, et al (2005). RUNX3 inactivation by point mutations and aberrant DNA methylation in bladder

tumors. *Cancer Res*, **65**, 9347-54.
Khan SA, Thomas HC, Davidson BR, Taylor-Robinson SD (2005). Cholangiocarcinoma. *Lancet*, **366**, 1303-14.
Lefeuvre M, Gunduz M, Nagatsuka H, Gunduz E, Al Sheikh Ali M, Beder L, et al (2009). Fine deletion analysis of 1p36 chromosomal region in oral squamous cell carcinomas. *J Oral Pathol Med*, **38**, 94-8.
Li Y, Du X, Li F, Deng Y, Yang Z, Wang Y, et al (2006). A novel zinc-finger protein ZNF436 suppresses transcriptional activities of AP-1 and SRE. *Mol Biol Rep*, **33**, 287-94.
Limpaiboon T, Khaenam P, Chinnasri P, et al (2005). Promoter hypermethylation is a major event of hMLH1 gene inactivation in liver fluke related cholangiocarcinoma. *Cancer Lett*, **217**, 213-9.
Limpaiboon T, Tapdara S, Jearanaikoon P, Sripa B, Bhudhisawasdi V (2006). Prognostic significance of microsatellite alterations at 1p36 in cholangiocarcinoma. *World J Gastroenterol*, **12**, 4377-82.
Liu XF, Zhang H, Zhu SG, et al (2006). Correlation of p53 gene mutation and expression of P53 protein in cholangiocarcinoma. *World J Gastroenterol*, **12**, 4706-9.
Lockwood WW, Coe BP, et al (2007). Whole genome tiling path array CGH analysis of segmental copy number alterations in cervical cancer cell lines. *Int J Cancer*, **120**, 436-43.
McMillan JR, Haftek M, Akiyama M, et al (2003). Alterations in desmosome size and number coincide with the loss of keratinocyte cohesion in skin with homozygous and heterozygous defects in the desmosomal protein plakophilin 1. *J Invest Dermatol*, **121**, 96-103.
Missiaglia E, Selfe J, Hamdi M, et al (2009). Genomic imbalances in rhabdomyosarcoma cell lines affect expression of genes frequently altered in primary tumors: an approach to identify candidate genes involved in tumor development. *Genes Chromosomes Cancer*, 48, 455-67.
Miyagawa K, Sakakura C, Nakashima S, et al (2006). Down-regulation of RUNX1, RUNX3 and CBFbeta in hepatocellular carcinomas in an early stage of hepatocarcinogenesis. *Anticancer Res*, **26**, 3633-43.
Miyazono K, Maeda S, Imamura T (2004). Coordinate regulation of cell growth and differentiation by TGF-beta superfamily and Runx proteins. *Oncogene*, **23**, 4232-4237.
Miyazono K, Suzuki H, Imamura, T (2003). Regulation of TGF-beta signaling and its roles in progression of tumors. *Cancer Sci*, **94**, 230-234.
Muller P, Henn W, Niedermayer I, et al (1999). Deletion of chromosome 1p and loss of expression of alkaline phosphatase indicate progression of meningiomas. *Clin Cancer Res*, **5**, 3569-77.
Murnane JP (2006). Telomeres and chromosome instability. *DNA Repair*, 5, 1082-92.
Nellore A, Paziana K, Ma C, et al (2009). Loss of Rap1GAP in papillary thyroid cancer. *J Clin Endocrinol Metab*, **94**, 1026-32.
Or YY, Hui AB, Tam KY, Huang DP, Lo KW (2005). Characterization of chromosome 3q and 12q amplicons in nasopharyngeal carcinoma cell lines. *Int J Oncol*, **26**, 49-56.
Park SR, Lee EK, Kim BC, Kim PH (2003). p300 cooperates with Smad3/4 and Runx3 in TGFbeta1-induced IgA isotype expression. *Eur J Immunol*, **33**, 3386-92.
Piao GH, Piao WH, He Y, et al (2008). Hyper-methylation of RIZ1 tumor suppressor gene is involved in the early tumorigenesis of hepatocellular carcinoma. *Histol Histopathol*, **23**, 1171-5.
Rosenbluth JM, Pietenpol JA (2008). The jury is in: p73 is a tumor suppressor after all. *Genes Dev*, **22**, 2591-5.
Saito S, Ghosh M, Morita K, et al (2006). The genetic differences between gallbladder and bile duct cancer cell lines. *Oncol Rep*, **16**, 949-56.
Seo GY, Park SR, Kim PH (2009). Analyses of TGF-beta1-inducible Ig germ-line gamma2b promoter activity: involvement of Smads and NF-kappaB. *Eur J Immunol*, **39**, 1157-66.
Shann YJ, Cheng C, Chiao CH, et al (2008). Genome-wide mapping and characterization of hypomethylated sites in human tissues and breast cancer cell lines. *Genome Res*, **18**, 791-801.
Suzuki M, Shigematsu H, Shames DS, et al (2005). DNA methylation-associated inactivation of TGFbeta-related genes DRM/Gremlin, RUNX3, and HPP1 in human cancers. Br J Cancer, 93, 1029-37.
Takada H, Imoto I, Tsuda H, et al (2005). Screening of DNA copy-number aberrations in gastric cancer cell lines by array-based comparative genomic hybridization. *Cancer Sci*, **96**, 100-10.
Takahashi Y, Kawate S, Watanabe M, et al (2007). Amplification of c-myc and cyclin D1 genes in primary and metastatic carcinomas of the liver. *Pathol Int*, **57**, 437-42.
Tannapfel A, Benicke M, Katalinic A, et al (2000). Frequency of p16(INK4A) alterations and K-ras mutations in intrahepatic cholangiocarcinoma of the liver. *Gut*, **47**, 721-7.
Tokumaru Y, Nomoto S, Jeronimo C, et al (2003). Biallelic inactivation of the RIZ1 gene in human gastric cancer. *Oncogene*, **22**, 6954-8.
Tomasini R, Tsuchihara K, Wilhelm M, et al (2008). TAp73 knockout shows genomic instability with infertility and tumor suppressor functions. *Genes Dev*, **22**, 2677-91.
Torquati A, O'Rear L, Longobardi L, et al (2004). RUNX3 inhibits cell proliferation and induces apoptosis by reinstating transforming growth factor beta responsiveness in esophageal adenocarcinoma cells. *Surgery*, **136**, 310-6.
Tsygankova OM, Prendergast GV, Puttaswamy K, et al (2007). Downregulation of Rap1GAP contributes to Ras transformation. *Mol Cell Biol*, **27**, 6647-58.
Ukita Y, Kato M, Terada T (2002). Gene amplification and mRNA and protein overexpression of c-erbB-2 (HER-2/neu) in human intrahepatic cholangiocarcinoma as detected by fluorescence in situ hybridization, in situ hybridization, and immunohistochemistry. *J Hepatol*, **36**, 780-5.
Wada M, Yazumi S, Takaishi S, et al (2004). Frequent loss of RUNX3 gene expression in human bile duct and pancreatic cancer cell lines. *Oncogene*, **23**, 2401-7.
Wattanasirichaigoon S, Tasanakhajorn U, Jesadapatarakul S (1998).The incidence of K-ras codon 12 mutations in cholangiocarcinoma detected by polymerase chain reaction technique. *J Med Assoc Thai*, **81**, 316-23.
Xiao W H, Liu WW (2004). Hemizygous deletion and hypermethylation of RUNX3 gene in hepatocellular carcinoma. *World J Gastroenterol*, **10**, 376-80.
Yanada M, Yaoi T, Shimada J, et al (2005). Frequent hemizygous deletion at 1p36 and hypermethylation downregulate RUNX3 expression in human lung cancer cell lines. *Oncol Rep*, **14**, 817-22.
Zhang F, Hamanaka RB, Bobrovnikova-Marjon E, et al (2006). Ribosomal stress couples the unfolded protein response to p53-dependent cell cycle arrest. *J Biol Chem*, **281**, 30036-45.
Zhang L, Chenwei L, Mahmood R, et al (2006). Identification of a putative tumor suppressor gene Rap1GAP in pancreatic cancer. *Cancer Res*, **66**, 898-906.
Zhang Z, Mitra RS, Henson BS, et al (2006). Rap1GAP inhibits tumor growth in oropharyngeal squamous cell carcinoma. *Am J Pathol*, **168**, 585-96.
Zheng H, Gao L, Feng Y, Yuan L, Zhao H, Cornelius LA (2009). Down-regulation of Rap1GAP via promoter hypermethylation promotes melanoma cell proliferation, survival, and migration. *Cancer Res*, **69**, 449-57.