GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Relationship between *RUNX3* methylation and hepatocellular carcinoma in Asian populations: a systematic review

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Genet. Mol. Res. 13 (3): 5182-5189 (2014)
Received May 9, 2013
Accepted October 5, 2013
Published July 7, 2014
DOI http://dx.doi.org/10.4238/2014.July.7.11

ABSTRACT. Runt-related transcription factor 3 (*RUNX3*) is a potential tumor suppressor that is frequently hypermethylated in hepatocellular carcinoma (HCC). The present meta-analysis of case-control studies was carried out to determine whether *RUNX3* hypermethylation is associated with HCC. The PubMed, Embase, and Chinese National Knowledge Infrastructure databases were searched for all relevant studies published between May 2000 and May 2012. A total of 11 studies were identified, and 8 studies involving 491 patients with HCC and 409 patients without tumors were found to satisfy the inclusion criteria for the meta-analysis. All tissue samples were from Asian populations. There was significant heterogeneity between the studies. Over the entire sample, the odds ratio (OR) of *RUNX3* promoter methylation was 18.5 [95% confidence interval

(CI), 11.6-29.6] for HCC tissues relative to control tissues. The ORs of *RUNX3* methylation were 16.6 (95%CI = 6.5-42.4) for tumor tissues relative to tumor-adjacent tissues in patients with HCC, 67.3 (95%CI = 13.0-348.5) for tumor tissues from patients with HCC relative to liver tissues from patients with non-neoplastic liver diseases, and 3.26 (95%CI = 1.54-6.90) for tissues from patients with hepatitis C virus (HCV)-related HCC relative to liver tissues from patients with HCC unrelated to HCV. There was no association between *RUNX3* methylation and age, gender, pathological stage, or hepatitis B virus infection in HCC tissues. Methylation of the *RUNX3* promoter strongly correlated with HCC in Asian populations, especially in individuals with HCV-related HCC, and may be a useful marker for HCC diagnosis in these populations.

Key words: *RUNX3*; Methylation; Meta-analysis; Asian; Hepatocellular carcinoma

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third most common cause of cancer death in the world, especially in Asia, and its incidence is increasing (Parkin et al., 2005; El-Serag and Rudolph, 2007). The main risk factors of HCC have been postulated to be chronic infection with hepatic viruses, such as hepatitis B virus (HBV) and hepatitis C virus (HCV), as well as exposure to aflatoxin B_1 (AFB_1) (Wang et al., 1996; El-Serag, 2012; Ha et al., 2012). However, the underlying molecular mechanisms contributing to hepatocarcinogenesis remain unclear.

The gene Runt-related transcription factor 3 (*RUNX3*) encodes a member of the Runt domain-containing family of transcription factors (Fan et al., 2011). *RUNX3* is an important component of the transforming growth factor-beta signaling pathway, and *RUNX3* may be one of the key tumor suppressor genes on 1p36. This chromosome region is frequently deleted in HCC (Moribe et al., 2009; Shitani et al., 2012), and decreased *RUNX3* expression has been linked to cell cycle deregulation, inhibition of apoptosis and enhancement of angiogenesis (Wu et al., 2012). Nevertheless, the possible relationship between decreased *RUNX3* expression and HCC has not been fully elucidated.

Reports suggest that aberrant DNA methylation of CpG islands is one of the most consistent epigenetic changes in human cancers (Levanon et al., 1994; Bae and Choi, 2004). In HCC, some genes in liver tissues are consistently found to be hypermethylated, suggesting that determination of the methylation status of specific genes may be useful for HCC diagnosis (Xiao and Liu, 2004; Shiraha et al., 2011; Wu et al., 2011). The promoter region of the *RUNX3* gene contains CpG islands that can be methylated, inactivating the gene. *RUNX3* methylation has been associated with numerous cancers, including cancers of the stomach (Lu et al., 2012), prostate (Mahapatra et al., 2012), breast (Subramaniam et al., 2009), colorectum (Zheng et al., 2011; Kang et al., 2012), bladder (Yan et al., 2012), and esophagus (Zheng et al., 2011). Some studies have also reported an association between *RUNX3* methylation and HCC (Park et al., 2005). In fact, the methylation status of *RUNX3* has already proven to be useful as a clonal marker and for characterizing tumors in HCC. Tan et al. (2007) suggested that serum hypermethylation of *RUNX3* is an HCC marker as sensitive as, and perhaps more sensitive than other tumor suppressor genes.

Despite these insights, some studies on *RUNX3* methylation and HCC have not examined cancerous tissue against healthy tissue, or they have not systematically examined HBV- and HCV-related HCC in the same population.

The present systematic review sought to take into account all relevant data to explore the association between *RUNX3* promoter methylation and HCC with a larger sample size, and to determine whether it could be an effective marker for HCC diagnosis.

MATERIAL AND METHODS

Study identification and selection

The PubMed, Embase, and Chinese National Knowledge Infrastructure databases were searched for the period from May 2000 to May 2012 using the following search string: ("liver cancer" or "hepatocellular carcinoma") and ("Runt-related transcription factor 3" or "RUNX3" or "AML2" or "CBFA3" or "PEBP") and ("methylation" or "hypermethylation"). Relevant studies were independently identified by two authors (X.L. and F.P.), who read the titles and abstracts.

To be included in the meta-analysis, studies had to satisfy the following inclusion criteria: 1) they had to be original research studies of the relationship between *RUNX3* hypermethylation and HCC; 2) HCC cases had to be diagnosed based on histopathological biopsy or elevated serum alpha-fetoprotein (AFP) and distinct changes visible by computed tomography (CT) and/or magnetic resonance imaging (MRI); 3) control subjects had to be free of cancer, such as healthy subjects or patients with chronic liver disease; 4) methylation status of *RUNX3* had to be determined by methylation-specific PCR (MSP) or quantitative MSP (QMSP). Review articles and duplicate publications were excluded.

Data extraction and synthesis

The following information was extracted from each study: name of the first author, year of publication, country, method for determining methylation status, source of control, rates of *RUNX3* hypermethylation in both case and control groups, study characteristics (sample source, gender and age distributions), and hepatitis virus infection status. The extracted data were used to perform meta-analyses.

Statistical methods

Data were analyzed using STATA (version 11.1, Stata Corporation, USA). Strength of association was expressed as pooled odds ratio (OR) with 95% confidence interval (CI). Pooled ORs were combined using Mantel-Haenszel methods (Mori et al., 2005). $P < 0.05$ was taken to indicate significant differences. All significance tests were two-sided.

Heterogeneity was considered to exist between studies if $P < 0.10$ and $I^2 > 25\%$. I^2 was used to determine how much heterogeneity was explained by subgroup differences (Nomoto et al., 2007). If there was no heterogeneity, logistic regression with a fixed-effects model was used to evaluate overall gene effects; otherwise, a random-effects model was used. Potential publication bias was tested using the Egger regression test and Begg test for funnel plot asymmetry (Wallenstein and Wittes, 1993). $P < 0.1$ indicated significant publication bias.

RESULTS

Search results

Eight articles (Egger et al., 1997; Higgins et al., 2003; Xiao and Liu, 2004; Kim et al., 2004; Park et al., 2005; Nishida et al., 2008; Hua et al., 2011; Shitani et al., 2012) met the inclusion criteria and reported data on *RUNX3* hypermethylation in 491 patients with HCC and 409 patients without tumors (Table 1). Four of these studies were conducted in Japan, and two in China and two in Korea. The control samples in these studies comprised samples from healthy individuals or patients with non-cancerous, non-liver disease. When patients had multiple HCC or repeatedly underwent surgical resection (Nishida et al., 2008), only data for tissue samples taken at the first resection were used.

The pooled OR for *RUNX3* methylation in HCC tissue relative to control tissue across all studies was 18.5 (95%CI = 11.6-29.6, z = 12.23, P = 0.007), indicating an increased likelihood of methylation in HCC tissue.

RUNX3 methylation in tumor tissue relative to tumor-adjacent tissue in patients with HCC

Seven studies (Higgins et al., 2003; Kim et al., 2004; Xiao and Liu, 2004; Park et al., 2005; Nishida et al., 2008; Hua et al., 2011; Shitani et al., 2012) examined 344 HCC tissue samples and 339 corresponding HCC-adjacent tissues. In total, 184 (52.4%) and 25 (7.2%) of those samples, respectively, showed *RUNX3* hypermethylation. This corresponds to a pooled OR for *RUNX3* hypermethylation of 16.6 (95%CI = 6.5-42.4, z = 5.87, P < 0.0001). However, these studies showed heterogeneity (P for heterogeneity = 0.025, I^2 = 58.5%; Figure 1), which was incorporated into the random-effects model. Funnel plot analysis and the Egger test did not show evidence of publication bias.

RUNX3 methylation in tissues from patients with HCC relative to liver tissue from patients with non-cancer liver disease and liver tissue from healthy individuals

Three studies (Egger et al., 1997; Higgins et al., 2003; Kim et al., 2004) examined a total of 157 tissue samples from patients with HCC, 40 from patients with HBV and 30 from healthy subjects. The pooled OR for *RUNX3* methylation across all these studies was 67.3 (95%CI = 13.0-348.5, z = 5.02, P < 0.0001), indicating increased likelihood of methylation in HCC tissue. Funnel plots did not show evidence of publication bias.

To determine the pooled OR for *RUNX3* methylation relative to completely healthy tissue, we performed risk analysis using the 157 tissue samples from patients with HCC and only the 30 tissue samples from healthy subjects. The pooled OR was 38.0 (95%CI = 5.2-275.4).

RUNX3 methylation in HBV- or HCV-related HCC

Four studies (Kim et al., 2004; Park et al., 2005; Nishida et al., 2008; Hua et al., 2011) reported sufficient information to evaluate the possible relationship between methylation of

CpG islands in the *RUNX3* promoter and HCC related to HBV or HCV infection. In total, 31 (42.5%) and 71 (76.3%) cases of *RUNX3* hypermethylation were observed, respectively, in tissue from patients with HBV-related HCC and tissue from patients with HCV-related HCC. This corresponds to an OR of 1.62 (95%CI = 0.71-3.71) for *RUNX3* methylation in hepatitis virus-positive HCC tissue relative to hepatitis virus-negative HCC tissue. The P value for heterogeneity was 0.046.

Table 1. Demographic data from the studies included in the meta-analysis.

Study and year	HCC tissue/ control tissue*	Patients	Controls	Methylation status method	Country	Mean or median age (range, year)	Gender (M:F)
Kim et al., 2004	Tumor/hbv	48	40	MSP	Korea	Not reported	Not reported
Xiao and Liu, 2004	Tumor/cap	62	62	MSP	China	48.6 (29-72)	52:10
Mori et al., 2005	Tumor/cap	41	41	MSP	Japan	Not reported	4:37
Park et al., 2005	Tumor/cap	73	73	MSP	Korea	51.6 (26-89)	60:13
Nomoto et al., 2007	Tumor/cap	19	19	MSP	Japan	59.3 (36-72)	16:3
Nishida et al., 2008	Tumor/cap+normal	176	99	MSP	Japan	(20-81)	70:27
Moribe et al., 2009	Tumor/cap+normal	25	20	MSP	Japan	65.9	33:15
Hua et al., 2011	Tumor/cap+normal	47	55	MSP	China	55 (27-78)	36:11

*hbv = hbv-infected liver tissue; cap = HCC-adjacent tissue; normal = normal liver tissue.

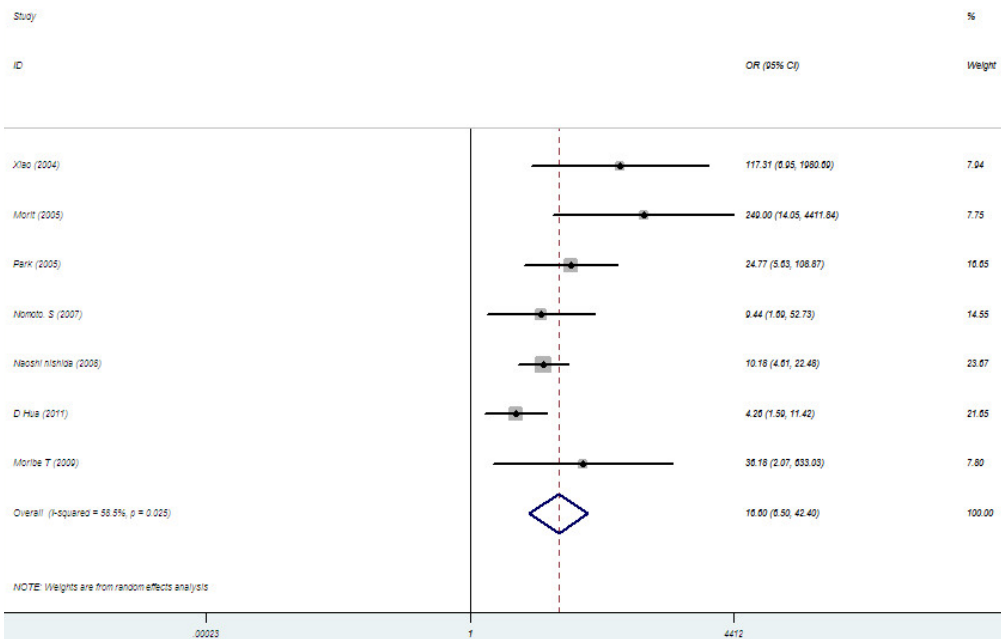

Figure 1. Forest plot of the meta-analysis for the association of RUNX3 hypermethylation with HCC.

When the study examining HCV-related HCC was excluded, the results showed that *RUNX3* methylation was not associated with HBV-related HCC (OR 0.69; 95%CI = 0.25-1.87; P for heterogeneity = 0.301; Table 2). In contrast, when the data from the HCV-related

HCC study were analyzed on their own, the odds ratio increased (OR 3.26; 95%CI = 1.54-6.90; P for heterogeneity = 0.198). Funnel plots did not show evidence of publication bias.

Table 2. *RUNX3* promoter methylation in relation to pathological stage, age, gender, and infection with hepatitis virus.

Clinicopathology	Status	Hypermethylation frequency (N = %)	OR (95%CI)	Heterogeneity test	Publication bias test
HBV infection	HBV+	31 (42.5)	0.69 (0.25-1.87)	I^2 = 16.70%, P = 0.301	P = 0.577
	HBV-	40 (66.7)			
HCV infection	HCV+	101 (70.1)	3.26 (1.54-6.90)	I^2 = 35.70%, P = 0.198	P = 0.071
	HCV-	56 (56.0)			
Pathological stage	stage III/IV	21 (50.0)	0.99 (0.44-2.23)	I^2 = 0.00%, P = 0.774	-
	stage I/II	40 (55.6)			
Age	≥60 years	32 (58.2)	0.80 (0.36-1.76)	I^2 = 53.00%, P = 0.119	P = 0.998
	<60 years	39 (50.0)			
Gender	female	18 (54.6)	1.30 (0.48-3.50)	I^2 = 0.00%, P = 0.587	P = 0.009
	male	53 (53.0)			

RUNX3 methylation in relation to age, gender and pathological stage of HCC patients

To identify relationships between methylation of CpG islands in the *RUNX3* promoter and the clinicopathology of HCC, we looked for associations between *RUNX3* promoter methylation and pathological stage, age, and gender of patients with HCC. Pooled ORs of *RUNX3* methylation were 1.30 (95%CI = 0.48-3.50) for females relative to males, 0.80 (95%CI = 0.36-1.76) for subjects aged ≥60 years relative to subjects aged <60 years, and 1.30 (95%CI = 0.48-3.50) for patients with stage III/IV HCC relative to patients with stage I/II (Table 2). Thus, no significant differences in *RUNX3* methylation were found between the subgroups. It was impossible to determine the P value for publication bias in the pathological stage subgroup, probably because of the limited sample size.

DISCUSSION

Methylation of the *RUNX3* promoter is one of the most common aberrant methylation events in cancer, leading to the suggestion that *RUNX3* methylation may be a useful biomarker for improving clinical management of HCC (Suzuki et al., 2005; Zhang et al., 2011). To assess this possibility with as much statistical power as possible, the present meta-analysis was carried out on eight studies involving 491 patients with HCC and 409 patients without HCC. Pooled ORs indicated that the risk of *RUNX3* hypermethylation was 16.6-fold higher in HCC tissue than HCC-adjacent tissue, and 67.3-fold higher in HCC tissue than normal liver tissue. Moreover, the risk of hypermethylation was 3.26-fold higher in HCV-related HCC tissue than non-HCV-related HCC tissue. Our results suggest that determining the status of *RUNX3* methylation may be a promising strategy for improving the diagnosis of HCC in Asian populations.

Our pooled analysis showed *RUNX3* methylation to be associated with HCV-related HCC but not with HBV-related HCC. While this may indicate that *RUNX3* inactivation plays a more important role in HCV-related HCC, our negative result with HBV-related HCC may be an artifact of small sample size. Future large-scale studies should address this question.

The present meta-analysis has several potential limitations. First, calculating pooled ORs relative to control samples of HCC-adjacent noncancerous tissue may be misleading. Such tissue is actually abnormal, since it frequently shows hepatitis, cirrhosis, and hyperplastic hepatocytes (Ha et al., 2012). Nevertheless, we confirmed the association between *RUNX3* methylation and HCC by examining 157 tissue samples from patients with HCC against 30 tissue samples from healthy subjects. Second, we conducted database searches only in PubMed, Embase and CNKI, so relatively few studies were initially identified and included in the final analysis. Third, the studies included used different MSP primer sequences, which might have created heterogeneity in the methylation results.

CONCLUSIONS

This meta-analysis of pooled data identified a strong association between methylation of the *RUNX3* promoter and HCC in Asians. HCV-infected HCC patients had higher *RUNX3* methylation rates than did non-HCV HCC patients. However, gender, age, pathological stage and HBV infection status showed no significant association with *RUNX3* methylation in HCC tissues.

Conflicts of interest

The authors declare conflict of interest.

ACKNOWLEDGMENTS

Research supported by the National Natural Science Foundation of China (#30960428) and the Natural Science Fund of Guangxi Province (#2012GXNSFAA053167).

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