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RUNX3 promoter methylation correlation with pathogenesis of hepatocellular carcinoma in Asians

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ABSTRACT. The aim of this study was to elucidate the role of *RUNX3* promoter methylation in the pathogenesis of hepatocellular carcinoma (HCC) among Asians. For this purpose, we performed a comprehensive search of Chinese and English language scientific literature databases using stringent selection criteria; ultimately, we identified relevant studies that specifically assessed the correlation between *RUNX3* promoter methylation and HCC. All data was retrieved and analyzed by two independent investigators using the STATA software (version 12.0). Initially, 132 studies (103 in Chinese, 29 in English) were retrieved; 122 were eliminated through a stepwise filtering process. Finally, 10 studies conducted in Asian populations (5 Chinese, 4 Japanese, 1 Korean) fulfilled all the inclusion criteria of our meta-analysis. The studies included 588 HCC patients (641 cancer tissues; 593 adjacent normal

tissues) and 184 healthy controls. We observed that *RUNX3* promoter methylation was significantly higher in cancer tissues than in adjacent normal tissues (RR = 6.35, 95%CI = 3.62-11.14, P < 0.001) and normal control tissues (RR = 17.31, 95%CI = 7.08-42.34, P < 0.001). *RUNX3* promoter methylation status did not differ significantly between patients with different TNM stages (RR = 0.88, 95%CI = 0.70-1.10, P = 0.269) and histological grades (RR = 0.86, 95%CI = 0.65-1.14, P = 0.304), suggesting that *RUNX3* promoter methylation is linked to the origin of HCC but not to its progression from non-metastatic to metastatic stages. This in turn indicated that *RUNX3* could be an early diagnostic marker distinguishing benign from malignant hepatocellular carcinoma.

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

INTRODUCTION

Liver cancer is the third leading cause of cancer mortality. Liver cancer is responsible for the death of approximately 700,000 people annually worldwide, with an alarming increase in mortality rates over the past 20 years in the United States alone (Schwabe and Wang, 2011). Hepatocellular carcinoma (HCC) ranks fifth and seventh among cancers affecting males and females, respectively; over half a million new cases of HCC are reported worldwide every year (Mittal and El-Serag, 2013). The leading risk factors for HCC are infection with hepatitis B virus (HBV; >50%), hepatitis C virus (HCV; 44-66 and 80% of HCC patients in Italy and Japan, respectively), and non-alcoholic fatty liver disease (NAFLD), the last being the leading factor for chronic liver diseases in the United States. Additional factors that contribute to the origin and progression of HCC include aflatoxins, alcohol and coffee consumption, and a host of genetic factors, such as abnormal tumor necrosis factor (TNF) signaling (Huxley et al., 2009; El-Serag, 2011). In recent years, HBV vaccination, antiviral treatment, and HCC surveillance have been aggressively pursued as strategies for the prevention, management, and early treatment of HCC. Despite considerable efforts, the incidence of HCC is increasing at an alarming rate, and post-diagnostic 5-year survival rates continue to be very low (5%) in these patients. Therefore, there is an urgent need for effective treatment methods to address the public health crisis (Zhang, 2012). HCC is known to be accompanied by multiple epigenetic and genetic alterations (Hua et al., 2011). Previous studies have demonstrated that epigenetic modifications, especially hypermethylations, are a common occurrence in HCC patients, and indicated that the methylation status of critical genes might reveal pathways involved in the origin and progression of HCC. Therefore, these can be important early diagnostic markers for better disease management (Shiraha et al., 2011).

Runt-related transcription factor 3 (*RUNX3*) belongs to the runt domain family of transcription factors that regulate the expression of a number of genes involved in lineage commitment and cell proliferation during embryonic development and in adults (Lu et al., 2012). *RUNX3* is also a vital downstream target of the transforming growth factor beta (TGF-β) superfamily, and is linked to signal-dependent regulation of cyclin-dependent kinase inhibitor 1A (P21), cyclin-dependent kinase inhibitor 1B (P27), tumor protein 53 (p53), AT motif-

binding factor 1 (ATBF1), caspase 3, and notch 1 (Notch), the dysregulation of which leads to the development and progression of cancers (Chen et al., 2010; Gao et al., 2010; Yamada et al., 2010; Shio et al., 2011). *RUNX3* functions as a tumor suppressor in various cancers such as lung adenocarcinoma, esophageal adenocarcinoma, colorectal cancer, oral squamous cell carcinoma, breast cancer, gastric cancer, adenoid cystic carcinoma, and HCC (Gao et al., 2009; Soong et al., 2009; Subramaniam et al., 2009a,b; Ge et al., 2011; Li et al., 2011; Shiraha et al., 2011). Consistent with this, decreased *RUNX3* expression has been correlated with inhibition of apoptosis, cell cycle dysregulation, and increased angiogenesis (Wu et al., 2012). Previous studies have reported a lack of *RUNX3* expression in a majority of HCC cases (Li and Jiang, 2011; Shiraha et al., 2011; Shiraha et al., 2013). However, the biological function of *RUNX3* and its definite link to HCC tumorigenesis remains unclear. In order to address this knowledge gap, we conducted a meta-analysis to understand the correlation between *RUNX3* and HCC.

MATERIAL AND METHODS

Data sources and keywords

To identify all studies containing data involving a correlation between *RUNX3* and HCC, we performed a comprehensive search of the PubMed, EBSCO, Ovid, Springerlink, Wiley, Web of Science, Wanfang database, China National Knowledge Infrastructure (CNKI), and VIP databases (last updated search on September 30, 2014), utilizing the following combination of keywords: “hepatocellular carcinoma” or “liver neoplasms” or “primary hepatic carcinoma” or “metastatic hepatic carcinoma” or “secondary hepatic carcinoma” or “HCC” and “*RUNX3*” or “methylation”.

Selection criteria

All studies reporting the association between *RUNX3* and HCC in human samples were collected and the relative risk (RR) and 95% confidence intervals (CI) were estimated. The studies were selected based on the following inclusion criteria: 1) clinical cohort studies (research design) investigating the correlation between *RUNX3* promoter methylation and the pathogenesis of HCC; 2) HCC diagnosis by histopathology; and 3) the articles should provide complete information regarding *RUNX3* promoter methylation. Articles were excluded from this meta-analysis based on the following criteria: 1) incomplete data and 2) duplicate articles. The study with the largest sample size, or the latest publication, was included in case of studies published by the same author.

Data extraction and quality assessment

To reduce bias and increase confidence, data was independently extracted from the selected studies by two investigators based on the selection criteria; a consensus was reached on all extracted data by discussion and reexamination. The following information was extracted from the studies: surname of the first author, year of publication, country of origin, ethnicity, language, disease, sample source, age, gender, case load, number of individuals in the control group, detection method and research design, promoter methylation status, TNM

stage, and histological type. All investigators reached a consensus regarding the completeness of data during the final round of selection. Quality assessment was performed by more than two independent investigators using the critical appraisal skill program (CASP; http://www.casp-uk.net/). The questions included among the CASP criteria were: (CASP01) did the study address a clearly focused issue? (CASP02) was the cohort recruited in an acceptable way? (CASP03) was the exposure accurately measured to minimize bias? (CASP04) was the outcome accurately measured to minimize bias? (CASP05) (a) Have the authors identified all relevant confounding factors? (b) Have they taken into account the confounding factors in the design and/or analysis? (CASP06) (a) Was the follow up of subjects complete? (b) Was the follow up of subjects long enough? (CASP07) What are the results of this study? (CASP08) How precise are the results? (CASP09) Do you believe the results? (CASP10) Can the results be applied to the local population? (CASP11) Do the results of this study fit with other available evidence? (CASP12) What are the implications of this study for practice?

Statistical analysis

The RR with 95%CI was calculated using the random effects model; these values were used to investigate the correlation between *RUNX3* promoter methylation and the pathogenesis of HCC. The Z test was used to assess the pooled effect size. Quantitative evidence was provided for all selected articles using a random-effects model in case of the presence of heterogeneity among the included studies; if not, a fixed-effects model was used. Heterogeneity of the included studies was evaluated utilizing Cochran's Q-statistic; P values < 0.05 were considered statistically significant (Jackson et al., 2012). The I^2 test was used to assess the potential of heterogeneity among studies (0%, no heterogeneity; 100%, maximal heterogeneity) (Zintzaras and Ioannidis, 2005); in case of heterogeneity, a sensitivity analysis was performed by removing a single study at a time. A Begger's funnel plot and an Egger test helped evaluate the publication bias (Peters et al., 2006). All information was input by two investigators working independently, and analyzed using STATA software (version 12.0; Stata Corp, College Station, TX, USA); both investigators reached an identical conclusion.

RESULTS

Included studies

The selected studies were published between 2004 and 2012. A total of 132 articles were initially retrieved by an electronic database search (N = 129), followed by a manual search (N = 3). A total of 31 papers were retained for further scrutiny after removing duplicates (N = 34), letters, reviews or meta-analyses (N = 3), studies without human samples (N = 19), and studies unrelated to the research topic (N = 45). Sixteen studies were excluded because they were not cohort studies (N = 5), and did not pertain to *RUNX3* promoter methylation (N = 6) or HCC (N = 5). During the final selection step, 5 of the remaining 15 studies were rejected because of insufficient information. Ultimately, 10 studies, all conducted in Asian populations, matched all the inclusion criteria; these studies included 588 HCC patients (641 cancer tissue samples and 593 adjacent normal tissue samples) and a 184 healthy controls (Xiao and Liu, 2004; Mori et al., 2005; Park et al., 2005; Nomoto et al., 2007; Nishida et al., 2008; Moribe

et al., 2009; Zhang et al., 2009; Hua et al., 2011; Jiang et al., 2011; Li and Jiang, 2012). All study subjects were of Asian descent; 5 studies were conducted in a Chinese population, 4 in Japanese, and 1 in a Korean population. *RUNX3* promoter methylation was detected in tissue samples (68-225) using methylation-specific polymerase chain reaction (MSP) and bisulfite sequencing polymerase chain reaction (BSP). Baseline characteristics of the enrolled studies are summarized in Table 1.

Table 1. Baseline characteristics and methodological quality of all included studies.

First author	Country	Patients	Number			Gender (M/F)	Age (years)	Control	Method
			Tumor	Adjacent	Normal				
Xiao and Liu (2004)	China	62	62	62	0	52/10	48.6 (29-72)	Adjacent	MSP
Mori et al. (2005)	Japan	41	41	41	0	37/4	NR	Adjacent	MSP
Park et al. (2005)	Korea	73	73	73	0	60/13	51.6 (26-89)	Adjacent	MSP
Nomoto et al. (2007)	Japan	19	74	51	17	16/3	36-72	Adjacent + Normal	MSP
Nishida et al. (2008)	Japan	79	77	77	22	59/20	59.8 (20-81)	Adjacent + Normal	MSP
Moribe et al. (2009)	Japan	45	45	20	3	32/13	66.25 ± 7.75	Adjacent + Normal	MSP
Zhang et al. (2009)	China	52	52	52	0	43/9	NR	Adjacent	MSP+
Hua et al. (2011)	China	47	47	47	47	36/11	55 (27-78)	Adjacent + Normal	BSP
Jiang et al. (2011)	China	75	75	75	75	51/24	57.6 ± 5.7	Adjacent + Normal	MSP
Li and Jiang (2012)	China	95	95	95	20	65/30	56.8 ± 4.3	Adjacent + Normal	MSP

M = male; F = female; MSP = methylation-specific polymerase chain reaction; BSP = bisulfite sequencing polymerase chain reaction.

Correlation between *RUNX3* promoter methylation and pathogenesis of HCC in Asians

A strong correlation was observed between *RUNX3* promoter methylation and the pathogenesis of HCC in our meta-analysis. A random effect model was used to identify heterogeneity between tumor tissues and adjacent normal tissues (I^2 = 62.3%, P_h = 0.004). No heterogeneity was observed among tumor tissues and normal control tissues (I^2 = 40.7%, P_h = 0.134); therefore, the fixed effect model was applied. We observed that the *RUNX3* promoter methylation was significantly higher in cancer tissues than in adjacent normal tissues and normal control tissues (Figure 1; cancer tissues *vs* adjacent normal tissues: RR = 6.35, 95%CI = 3.62-11.14, P < 0.001; cancer tissues *vs* normal control tissues, RR = 17.31, 95%CI = 7.08-42.34, P < 0.001).

Correlation between *RUNX3* promoter methylation and pathological characteristics of HCC in Asians

Five studies documented the correlation between *RUNX3* promoter methylation and the pathological characteristics of HCC. These studies were separately tested for heterogeneity; the fixed effects model was used as no heterogeneity was observed among the studies (TNM stage: I^2 =0.0%, P_h = 0.622; histological grade: I^2 = 0.0%, P_h = 0.468). The outcomes of this meta-analysis (Figure 1) suggested that the *RUNX3* promoter methylation status in cancer tissues was not correlated with the TNM stages and histological grades (TNM stages I-II *vs* TNM stages III-IV: RR = 0.88, 95%CI = 0.70-1.10, P = 0.269; high/moderate histological grade *vs* low histological grade: RR = 0.86, 95%CI = 0.65-1.14, P = 0.304).

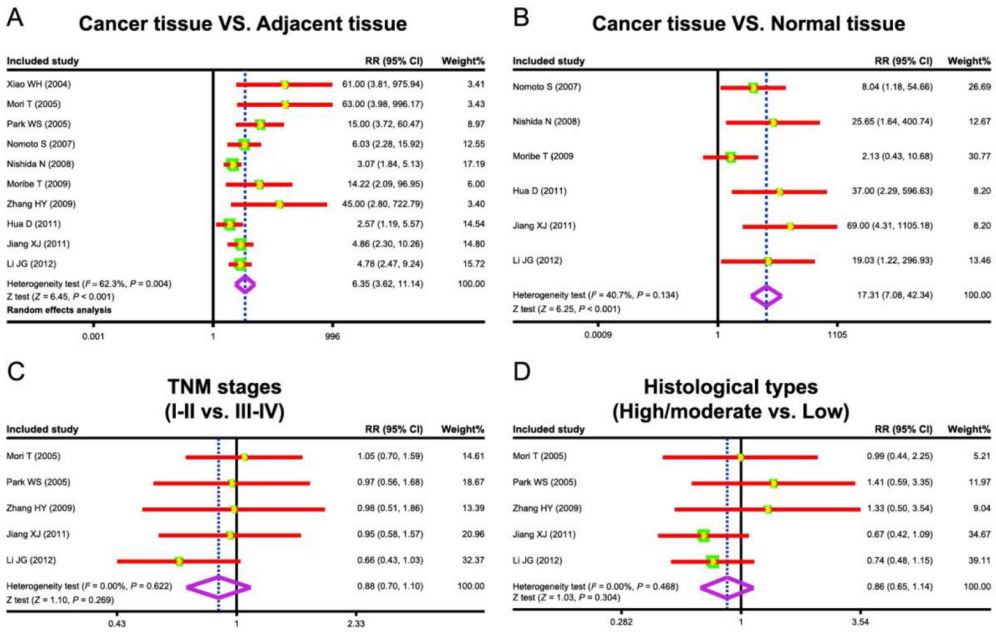

Figure 1. Forest plots for the correlation between *RUNX3* promoter methylation and hepatocellular carcinoma (HCC).

Sensitivity analysis and publication bias

The sensitivity analysis showed that no single study influenced the pooled RR of the correlation between *RUNX3* promoter methylation and the pathogenesis of HCC (Figure 2). The funnel plots were asymmetric, except for the plots detailing the TNM stages, indicating the presence of a publication bias. Furthermore, the Egger test confirmed the presence of a publication bias, possibly owing to the small sample sizes of the selected studies (cancer tissues *vs* adjacent normal tissues: $P < 0.001$; cancer tissues *vs* normal control tissues: $P = 0.004$; histological types, $P = 0.020$), excluding the research on TNM stages ($P = 0.729$) (Figure 3).

DISCUSSION

Our meta-analysis revealed an important link between *RUNX3* promoter methylation and HCC tumorigenesis, suggesting that *RUNX3* might be a critical early biomarker for HCC in Asians, which might also predict the course of the disease. Primary liver cancer includes HCC, hepatic angiosarcoma, and cholangiocarcinoma. HCC accounts for 85-90% of all primary liver cancers and is the third leading cause of cancer mortality worldwide; in China, the incidence of HCC is >50% (Liang et al., 2010). HCC is highly metastatic has a low 5-year survival rate; therefore, there is an urgent need for early diagnosis, estimation of disease progression, and effective treatment for HCC (McGivern and Lemon, 2011; Welzel et al., 2011). HCC is

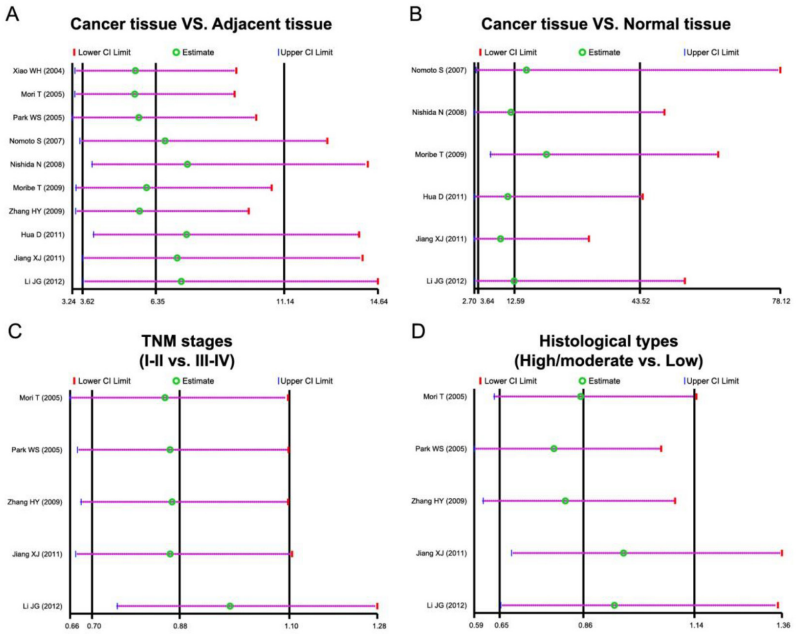

Figure 2. Sensitivity analysis of the correlation between *RUNX3* promoter methylation and hepatocellular carcinoma (HCC).

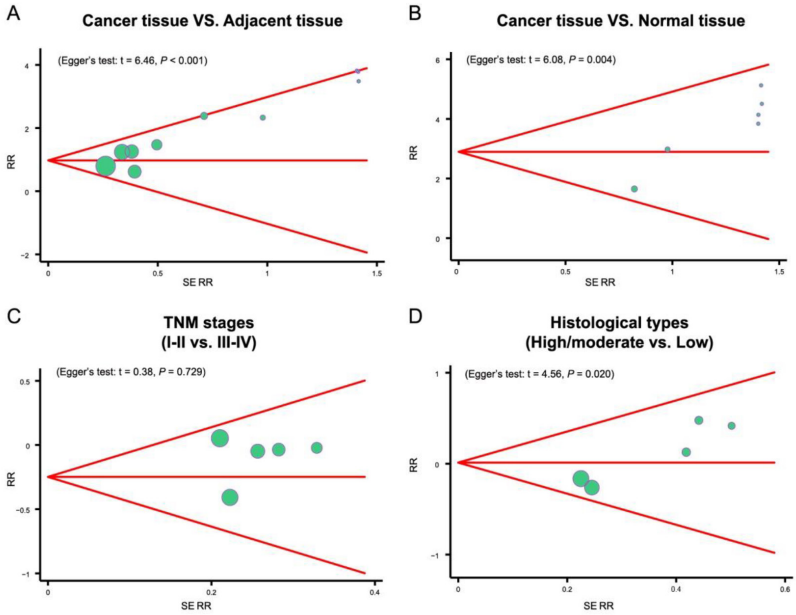

Figure 3. Funnel plot identifying publication bias.

common in patients infected with chronic HBV or HCV and hepatocyte cell death, due to host immune surveillance or intrinsic cytopathic effects of HCV or HBV. This induces long-lasting compensatory liver repair and regeneration mechanisms, eventually leading to serious liver fibrosis or cirrhosis (Zhang, 2012; Mittal and El-Serag, 2013).

RUNX3 is a vital downstream target of the transforming growth factor beta (TGF-β) superfamily and regulates several genes that are dysregulated in cancers, including cyclin-dependent kinase inhibitor 1A (*P21*), cyclin-dependent kinase inhibitor 1B (*P27*), tumor protein 53 (*p53*), AT motif-binding factor 1 (*ATBF1*), *caspase 3*, and notch 1 (*Notch*) (Chen et al., 2010; Gao et al., 2010; Yamada et al., 2010; Shio et al., 2011). *RUNX3* is believed to function as a tumor suppressor; evidence of this has been obtained from a variety of cancers, including lung adenocarcinoma, esophageal adenocarcinoma, colorectal cancer, oral squamous cell carcinoma, breast cancer, gastric cancer, adenoid cystic carcinoma, and HCC (Gao et al., 2009; Soong et al., 2009; Subramaniam et al., 2009a,b; Ge et al., 2011; Li et al., 2011; Shiraha et al., 2011). CpG islands (CGIs), clusters of CpGs in GC-rich 5' regions (Han et al., 2008), are prime targets of methylation. Growing evidence indicate that methylation changes in promoter-associated CGIs induce transcriptional silencing and disruption of gene function. In particular, abnormal hypermethylation in CGIs within the promoter region of tumor suppressor genes results in loss of tumor suppressor function of the gene, and might lead to tumorigenesis (Han and Zhao, 2009). The methylation status of *RUNX3* appears to be a sensitive marker, and is related to pathways that prominently drive the course of HCC (Li and Jiang, 2011; Shiraha et al., 2011; Lu et al., 2014). For example, Hua et al. (2011) observed that aberrant promoter methylation of *RUNX3* might cause liver carcinogenesis through the TGF-β and Notch signaling pathways (Nishida et al., 2008; Gao et al., 2010).

In this meta-analysis, *RUNX3* promoter methylation was found to be significantly higher in cancer tissues than in adjacent normal tissues and normal control tissues, indicating that *RUNX3* might be a biomarker for the pathogenesis of HCC in Asians. On the other hand, an analysis of the pathological characteristics of HCC revealed no statistical correlation between *RUNX3* promoter methylation and the TNM stage or the histological grade of tumors.

This study has some noteworthy limitations: firstly, although the studies were selected by a comprehensive search of English and Chinese language databases, the information retrieved could still be incomplete; secondly, the sample size is very small, with only 2 of the 10 included studies having large sample sizes ($N \geq 200$), indicating that our results should be considered with caution; thirdly, although the studies were published between 2004 and 2012, very few were published recently. Therefore, our results must be validated by further studies using recent data and larger sample sizes.

In conclusion, we observed a strong correlation between *RUNX3* promoter methylation and the pathogenesis of HCC in Asians. Unexpectedly, *RUNX3* methylation status was not associated with the tumor stage (TNM and histology), suggesting that *RUNX3* could be a valuable predicator for HCC development and a useful index for differentiating between benign and malignant tumors during an early tumor stage.

Conflicts of interest

The authors declare no conflict of interest.

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