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Genetic polymorphism of *NFKB1* and *NFKBIA* genes and liver cancer risk: a nested case-control study in Shanghai, China

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ABSTRACT

Objectives: Genetic variations of NF-κB signaling pathway were found to be associated with inflammatory diseases and several malignancies. However, little is known about NF-κB pathway gene polymorphisms and susceptibility of liver cancer. The aim of this study was to investigate whether genetic variants of NFKB1 and NFKBIA were associated with risk of liver cancer in a Chinese population.

Design: The study was designed as a nested case-control study within two prospective cohorts (the Shanghai Women's Health Study, SWHS, 1996–2000 and the Shanghai Men's Health Study, SMHS, 2002–2006).

Settings: This population-based study was conducted in urban Shanghai, China.

Participants: A total of 218 incident liver cancer cases diagnosed through December 31, 2011 and 436 healthy controls matched by sex, age at baseline (±2 years) and date (±30 days) of sample collection were included in the study.

Primary and secondary outcome measures: Genetic polymorphisms of *NFKB1* and *NFKBIA* were determined by TaqMan SNP genotyping assay blindly. ORs and its 95% CIs were estimated by unconditional logistic regression model to measure the association between selected SNPs and the risk of liver cancer.

Results: After adjusted for potential confounding factors, rs28362491 ins/del or del/del genotypes were associated with higher risk of liver cancer with an adjusted OR of 1.50(95%CI: 1.02-2.49). rs230496 AG and GG genotypes were also noted with higher risk of liver cancer with an adjusted OR of 1.49 (95%CI: 1.01-2.21). Haplotype analysis indicated that carriers of the *NFKB1* GA and AA (rs230525-rs230530) haplotypes had higher risk of liver cancer under additive model. No assiciation was observed between *NFKBIA* variants and risk of live cancer.

Conclusions: Our results suggest that genetic variants of *NFKB1* influence liver cancer susceptibility in Chinese population, although replication in other studies is needed.

Article summary-strengths and limitations of this study

- This study was the first population-based study to evaluate the polymorphic variants of NF-κB and risk of liver cancer.
- Only incident cases from two prospective cohorts were included in the study which ruled out the possibility of recall and selection bias.
- The limitations of the study include relatively small sample size, unmeasured HBV infection, HCV infection and aflatoxin exposure. However, we did take into

consideration of the participants' history of hepatitis and liver cirrhosis, and the presents of HCV infection and aflatoxin are very low in the study population.

INTRODUCTION

Liver cancer is a common disorder worldwide which ranks the 5th and 7th most common cancer among men and women. It was estimated that more than 80% liver cancers occur in developing countries and about 54% occur in China[1]. Among the main risk factors for liver cancer, chronic infections of hepatitis B virus (HBV) and hepatitis C virus (HCV) are the most important in humans, accounting for more than 70% of liver cancer cases worldwide[2-4]. Liver cirrhosis, heavy alcohol consumption, exposure to aflatoxin B1, and diabetes also account for part of liver cancer occurrence[2-4].

Chronic inflammation has been widely accepted to play an important role in hepatocarcinogenesis. Most of the known risk factors of liver cancer such as HBV, HCV infection and alcohol drinking can cause persistent inflammatory reaction of the liver and promote cancer development[5, 6]. However, the molecular and cellular mechanisms linking inflammation and liver cancer remain unclear. Recent findings have suggested that NF-κB may play a crucial role in bridging the actions of growth factors and chronic inflammation to hepatic oncogenesis[7-10].

NF-κB, a collection of dimeric transcription factors, was originally identified as a nuclear factor specific to B cells bound to the B site of the κ-light chain gene enhancer[11] and presents in all cell types[12]. It is a major transcription regulator of the immune response, cell adhesion, differentiation, proliferation, and apoptosis[13]. NF-κB dimers are formed by seven distinct proteins: NF-κB1 (p105 and p50), NF-κB2 (p100 and p52), RelA (p65), RelB and c-Rel, of which NF-κB p50/RelA is the most common dimer form[9]. In the resting cell, most NF-κB dimers are inactivated in the cytoplasm by binding to specific inhabitors-IκB family, of which IκBα is the most common one. In the classical activation pathway, IκB is phosphorylated and degraded by IκB kinase complex, and then NF-κB dimers are released and translocated to the nucleus where they coordinate the transcriptional activation of target genes[14]. Several genetic variations of NF-κB signaling pathway have been reported to be associated with cancer risks such as breast[15], prostate[16], stomach[17], colorectum[18] and mouth[19]. However, little is known about role of genetic polymorphisms NF-κB genes and susceptibility of liver cancer.

In a population based case-control study nested in two prospective cohorts of the Shanghai Women's and Men's Health Studies, we investigated the relationships between genetic variants of *NFKB1* and *NFKBIA*, two key genes involved in classic signaling pathway of NF-κB, and the risk of LIVER CANCER among Chinese men and women.

MATERIAL AND METHODS

Study population

Participants of this study came from the Shanghai Women's Health Study (SWHS) and Shanghai Men's Health Study (SMHS). The design and methods used in these two studies have been described in detail elsewhere [20-23]. Briefly, the SWHS enrolled 74,941 women aged 40-74 years between March 1, 1997 and May 31, 2000, with a response rate of 92.7%. SMHS enrolled 61,491 men aged 40-74 years without history of cancer at recruitment from April 1, 2002 to June 30, 2006, with a response rate of 74.1%. Both studies were approved by the relevant Institutional Review Boards for human research in China and the United States and a written informed consent was obtained from all participants.

In-person interview was conducted by trained interviewers using a structured questionnaire at baseline to obtain information on demographics, lifestyle, dietary habits, medical history and other characteristics. Anthropometric measurements, including current weight, height and circumferences of the waist and hips, were also measured. Of the eligible participants, 56,831 (75.8%) of the SWHS and 46,332 (75.3%) of the SMHS provided a 10-ml blood sample at baseline. The samples were drawn into an EDTA Vacutainer tube and then kept in a portable styrofoam box with ice packs (at approximately 0-4°C) and processed within 6 hours for long-term storage at -70°C. A bio-specimen collection form was completed for each participant at the time of sample procurement which included the date and time of collection, time of last meal, and date of last menstruation, intake of selected foods, smoking, as well as use of any medications over the previous 24 hours and during the previous week.

Cohort follow-up and outcome ascertainment

Both cohorts were followed for occurrence of cancer and other chronic diseases by active in-person surveys conducted every 2-3 years as well as annual record linkage to the databases of the population-based Shanghai Cancer Registry, Shanghai Vital Statistics Registry, and Shanghai Resident Registry. For the SWHS, four rounds

of in-person follow-ups were completed and the response rates for the first (2000-2002), second (2002-2004), third (2004-2007), and fourth (2008-2011) follow-up surveys were 99.8%, 98.7%, 96.7%, and 92.0%, respectively. For the SMHS, two rounds of follow-up surveys have completed. The response rates for the first (2004-2008) and second (2008-2011) follow-up surveys were 97.6% and 93.6%, respectively. For cohort members who developed liver cancer during the follow-up, medical chart were reviewed by a panel of oncologists to verify the diagnosis. Liver cancer data through December 31, 2011 was used for the present study.

Included in this nested case-control study are 218 incident liver cancer cases and 436 matched controls who had donated blood sample. Liver cancer cases were defined as having an International Classification of Disease, Ninth Revision (ICD-9), codes of 155.0 (primary malignant neoplasms), 155.1(malignant neoplasms of the intrahepatic bile ducts), or 155.2 (unspecified malignant neoplasms of the liver)[24]. Two control subjects were randomly selected from the cohorts who donated a blood sample at baseline and matched to each case for sex, age at baseline (±2 years) and date (±30 days) of sample collection. All controls were free of any cancer at the time of cancer diagnosis for the corresponding case.

Genotyping

Single-nucleotide polymorphisms (SNPs) were selected based on both TagSNP and their putative functional significance. Tagging SNPs were selected by searching the Han Chinese data from the Hapmap project[25]. The following criteria were used to identify tagging SNPs: (i) SNPs located in the genes or within the 5-kb flanking region, (ii) a minor allele frequency $\geqslant 0.05$, and (iii) other unselected single-nucleotide polymorphisms could be captured by one of the tagging SNPs with a linkage disequilibrium of $r^2 \geqslant 0.90$. A total of 8 SNPs were selected for genotyping which were rs28362491, rs230530, rs230525, rs230496 for *NFKB1* and rs3138053, rs3138055, rs2273650, rs696 for *NFKBIA* (table 1). Genomic DNA was extracted from buffycoat using Promega DNA Extraction Kit according to the manufacturer's instructions (Promega Corporation, Madison, WI, USA). Genotyping were performed by the TaqMan assay, using the ABIPRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA), in 384-wellformat, with dual fluorescent reporter probes VIC and FAM. The quality and potential misclassification of the genotyping results were assessed by evaluating 5% of duplicate DNA samples that were randomly selected from the whole samples. There replicates were 100%

concordant. All serum samples were tested blindly and were identified only by an unique identification number blinded with case-control status.

Table 1. Descriptions of Genetic Polymorphisms of the *NFKB1* and *NFKBIA* genes under investigation

Gene	Assay ID	Sequence	Location
NFKB1	rs28362491	CTCCGTGCTGCCTGCGTTCCCCGACC[-/ATTG]ATTGGGCC CGGCAGGCGCTTCCTGG	5'-near gene
	rs230530	TTTTTAGCACCAAACATCTTAATTT[A/G]CATTCAAATAAA TGAGAACCACCAT	intron
	rs230525	TACGGGAAAAGTGATTCTTGTTTAC[A/G]GAGCCCTCTTT CACAGTTTCATGTT	intron
	rs230496	TGTCTGGATTTGCTTGAGACAGCCC[A/G]GTTTGCCCCTG ACCTAATTGTTTAT	intron
NFKBIA	rs3138053	ATTCGTTTATGCTATCTGACCTACA[C/T]TGTGCTCCCGCA GAAAAAGGATCGT	5'-near gene
	rs3138055	AATCAACGGGATGACAGAATGACAA[C/T]GGAGAGGTCT CCAACCACAGGCCAA	3'-near gene
	rs2273650	AACAATACATTATGTACACCATTTA[C/T]AGGAGGGTAAC ACAAACCTTGACAG	3'-UTR
	rs696	CCTACCACAATAAGACGTTTTGGGC[C/T]AGGCAGTGTGC AGTGTGGATATAAG	3'-UTR

Statistical analysis

Subjects with both survey data and genotyping results were included in the final analysis. Means and percentages of selected characteristics for cases and controls were calculated. The distributions of selected characteristics were compared between cases and controls by either student's *t*-test (continuous variables) or χ^2 test (categorical variables). Odds ratio and its 95% confidence interval were estimated by unconditional logistic regression model to measure the association between selected SNPs and the risk of primary liver cancer. In the multivariable analysis, potential confounding factors were adjusted for, which include age (continuous variable); education level (four categories: elementary school or less, middle school, high school, and college or above); history of hepatitis (yes or no); family history of liver cancer (yes or no); and history of other chronic liver diseases or cirrhosis (yes or no). Statistical analyses were carried out using the SAS software package (version9.2; SAS Institute, Cary, NC). Tests for trend were performed by entering categorical variables as continuous variables in the regression model. All P values were calculated by two-sided tests and were considered statistically significant if P was less than 0.05.

Hardy–Weinberg equilibrium and Linkage disequilibrium were accessed with HaploView version 4.0[26]. Associations between haplotypes and the risk of liver cancer

were evaluated with HAPSTAT version 3.0 using the most common haplotype as the referent category, assumming an additive model[27].

RESULTS

Selected baseline characteristics of study participants were presented in table 2. The average ages of cases and control were 59.65 and 59.48. Compared with controls, liver cancer cases were more likely to have a lower education level, a history of hepatitis, a family history of liver cancer in first degree relatives, and history of chronic liver diseases or cirrhosis. Besides, male liver cancer cases were more probably to have lower body mass index, and be a non-regular exerciser compared to controls, although the difference were at borderline significance. Whereas in women, cases were more likely to have a history of type 2 diabetes than controls. No differences were observed in family income, smoking, drinking habits, waist to hip ratio, and family history of other cancers between the two groups.

The associations of *NFKB1* SNPs with liver cancer risk were summarized in table 3. The genotypes of rs28362491, rs230530 and rs230525 showed no deviation from Hardy-Weinberg equilibrium in controls except for rs230496. After adjusted for potential confounding factors, rs28362191 ins/del or del/del genotypes were associated with higher risk of liver cancer with an OR of 1.50(95%CI: 1.02-2.49). rs230496 AG and GG genotypes were also noted with higher risk of liver cancer with an adjusted OR of 1.49 (95%CI: 1.01-2.21). Carriers of rs230525 AG or GG genotypes had about 30% percent increased risk of liver cancer, but the risk was not insignificant. No association was found between rs230530 and liver cancer risk.

Table 4 presents the distribution of *NFKBIA* SNPs in cases and controls. The genotypes of rs3138055, rs696 and rs2273650 showed no deviation from Hardy-Weinberg equilibrium in controls but for rs3138053. Generally, all the four SNPs showed no relationship with liver cancer.

We further analyzed the haplotypes of these SNPs with risk of liver cancer (table 5). For *NFKB1* gene, two SNPs (rs230525, rs230530) demonstrated strong linkage disequilibrium (D' =1.0, r^2=0.58). Compared to men carrying rs230525-rs230530 AG haplotype, those with GA or AA haplotypes were at increased risk of liver cancer with ORs of 1.46(95%CI: 1.05-2.03) and 1.81(95%CI: 1.15-2.86), respectively. For *NFKBIA*, rs3138053 and rs2273650 were in linkage disequilibrium (D' =0.97, r^2=0.31) but none of the haplotypes was significantly associated with liver cancer.

Table 2. Distribution of selected characteristics in the study cases and controls[†]

Characteristics	All subjects			Male			Female		
	Cases (*N*=218)	Controls (*N*=436)	*P*	Cases (*N*=131)	Controls (*N*=393)	*P*	Cases (*N*=87)	Controls (*N*=174)	*P*
Age at interview, Mean±SD,	59.65±9.55	59.48±9.53	0.835	60.05±9.93	59.86±9.95	0.858	59.06±8.98	58.93±8.86	0.914
Education level (%)									
Elementary school or less	63(29.17)	120(27.59)		18(13.95)	35(13.41)		45(51.72)	85(48.85)	
Middle school	70(32.41)	151(34.71)		54(41.86)	104(39.85)		16(18.39)	47(27.01)	
High school	62(28.70)	92(21.15)		41(31.78)	61(23.37)		21(21.14)	31(17.82)	
College or above	21(9.72)	72(16.55)	0.036	16(12.40)	61(23.37)	0.053	5(5.75)	11(6.32)	0.380
Family income (%)[†]									
Low	50(22.94)	95(21.84)		17(12.98)	37(14.12)		33(37.93)	58(33.53)	
Medium	112(51.38)	211(48.51)		76(58.02)	130(49.62)		36(41.38)	81(46.82)	
High	56(25.69)	129(29.66)	0.569	38(29.01)	95(36.25)	0.271	18(20.69)	34(19.65)	0.694
Ever smoked (%)	93(42.66)	174(39.91)	0.50	90(68.70)	163(62.21)	0.205	3(3.45)	11(6.32)	0.331
Ever drank alcohol (%)	45(20.64)	98(22.48)	0.593	42(32.06)	97(37.02)	0.332	3(3.45)	1(0.57)	0.109
Body mass index ,kg/m^2, Mean±SD	23.80±3.64	24.16±3.33	0.209	23.16±3.25	23.77±2.89	0.06	24.76±4.00	24.74±3.83	0.97
WHR, Mean±SD	0.87±0.07	0.87±0.07	0.995	0.90±0.06	0.90±0.06	0.379	0.82±0.05	0.83±0.06	0.231
Regular physical activity (%)	95(43.58)	211(48.39)	0.245	49(37.40)	124(47.33)	0.062	46(52.87)	87(50.00)	0.662
physical activity, MET-hours/week	82.16±47.67	83.57±43.43	0.696	66.86±40.33	68.00±34.61	0.78	105.1±48.84	107.0±44.90	0.752
History of hepatitis (%)	74(33.94)	25(5.73)	<0.001	57(43.51)	16(6.11)	<0.001	17(19.54)	9(5.17)	<0.001
Family history of cancer (%)	69(31.65)	117(26.83)	0.198	41(31.30)	70(26.72)	0.342	28(32.18)	47(27.01)	0.384
Family history of liver cancer (%)	28(12.84)	18(4.13)	<0.001	20(15.27)	10(3.82)	<0.001	8(9.20)	8(4.60)	0.144
History of type 2 diabetes (%)	26(11.93)	36(8.26)	0.131	14(10.69)	25(9.54)	0.72	12(13.79)	11(6.32)	0.045
History of chronic liver disease or	35(16.06)	11(2.52)	<0.001	26(19.82)	10(3.82)	<0.001	9(10.34)	1(0.57)	<0.001

* Missing data was excluded from the analysis

[†] Family income level (low income for <5000 yuan/year in the SWHS and <12 000 yuan/year in the SMHS; medium income for 5000 to <10 000 yuan/year in the SWHS and 12 000 to <24 000 yuan/year in the SMHS; and high income for >10 000 yuan/year in the SWHS and >24 000 yuan/year in the SMHS)

Table 3. *NFKB1* genetic polymorphisms with the risk of primary liver cancer

SNPs	Cases	Controls	P for χ^2	OR*	95%CI	OR†	95%CI	OR‡	95%CI
rs28362491									
ins/ins	69	174		1.00	-	1.00	-	1.00	-
ins/del	102	166		1.55	1.07-2.25	1.55	1.07-2.25	1.64	1.09-2.49
del/del	40	79	0.069	1.28	0.80-2.05	1.28	0.80-2.05	1.21	0.71-2.04
P for trend				0.148		0.148		0.246	
ins/del or del/del	142	245	0.031	1.46	1.03-2.07	1.46	1.03-2.07	1.50	1.02-2.21
rs230496									
AA	65	167		1.00	-	1.00	-	1.00	-
AG	101	173		1.50	1.03-2.19	1.50	1.03-2.19	1.64	1.08-2.50
GG	47	92	0.106	1.32	0.84-2.07	1.31	0.84-2.07	1.24	0.74-2.05
P for trend				0.152		0.152		0.259	
AG or GG	148	265	0.041	1.44	1.01-2.04	1.44	1.01-2.04	1.49	1.01-2.21
rs230525									
AA	80	189		1.00	-	1.00	-	1.00	-
AG	102	180		1.34	0.94-1.92	1.35	0.94-1.92	1.42	0.95-2.11
GG	32	64	0.277	1.18	0.72-1.95	1.18	0.72-1.95	1.08	0.62-1.89
P for trend				0.268		0.258		0.402	
AG or GG	134	244	0.127	1.30	0.93-1.82	1.30	0.93-1.82	1.33	0.91-1.93
rs230530									
AA	64	116		1.00	-	1.00	-	1.00	-
AG	99	181		0.99	0.67-1.46	0.99	0.67-1.47	1.03	0.67-1.59
GG	49	130	0.152	0.68	0.44-1.07	0.68	0.44-1.07	0.70	0.42-1.15
P for trend				0.1		0.1		0.169	
AG or GG	148	311	0.426	0.86	0.60-1.24	0.86	0.60-1.24	0.89	0.60-1.34

*Adjusted for age

† Adjusted for age, sex.

‡ Adjusted for age, sex, education level, family history of liver cancer, history of hepatitis, and chronic liver diseases or cirrhosis.

Table 4. *NFKBIA* genetic polymorphisms with the risk of primary liver cancer

SNPs	Cases	Controls	P	OR*		OR†		OR‡	
rs3138053									
AA	174	342		1.00	-	1.00	-	1.00	-
AG	21	51		0.81	0.47-1.39	0.80	0.47-1.39	0.92	0.51-1.64
GG	19	40	0.736	0.93	0.53-1.66	0.94	0.53-1.68	0.98	0.51-1.89
P for trend				0.618		0.611		0.861	
AG or GG	40	91	0.487	0.86	0.57-1.31	0.86	0.57-1.31	0.94	0.60-1.49
rs3138055									
CC	63	129		1.00	-	1.00	-	1.00	-
CT	109	220		1.02	0.70-1.49	1.02	0.70-1.49	1.17	0.76-1.80
TT	42	84	0.995	1.02	0.63-1.65	1.02	0.63-1.65	1.26	0.74-2.14
P for trend				0.923		0.923		0.379	
CT or TT	151	304	0.926	1.02	0.71-1.46	1.02	0.71-1.46	1.20	0.80-1.79
rs696									
CC	66	150		1.00	-	1.00	-	1.00	-
CT	115	202		1.30	0.90-1.88	1.30	0.90-1.88	1.41	0.93-2.13
TT	33	78	0.268	0.96	0.58-1.58	0.96	0.58-1.58	1.12	0.64-1.94
P for trend				0.822		0.823		0.459	
CT or TT	148	280	0.304	1.20	0.85-1.71	1.20	0.85-1.71	1.33	0.89-1.97
rs2273650									
CC	108	331		1.00	-	1.00	-	1.00	-
CT	85	176		0.99	0.70-1.40	0.99	0.70-1.40	0.88	0.60-1.30
TT	20	37	0.934	1.11	0.61-1.99	1.11	0.61-2.00	0.92	0.47-1.79
P for trend				0.831		0.830		0.596	
CT or TT	105	213	0.959	1.01	0.73-1.41	1.01	0.73-1.41	0.89	0.62-1.28

*Adjusted for age

† Adjusted for age, sex.

‡ Adjusted for age, sex, education level, family history of liver cancer, history of hepatitis, and chronic liver diseases or cirrhosis.

Table 5. ORs and 95%CIs for liver cancer in relation to *NFKB1*/*NFKBIA* haplotypes

	All subjects*			Female†			Male†		
	Cases (%)	Controls (%)	OR	Cases (%)	Controls (%)	OR	Cases (%)	Controls (%)	OR
NFKB1(rs230525-rs230530)				n=87	n=174		n=129	n=260	
AG	46.74	51.85	ref	52.70	49.13	ref	42.69	53.53	ref
GA	38.79	35.57	1.21(0.94-1.56)	36.05	36.71	0.92(0.62-1.36)	40.55	34.88	1.46(1.05-2.03)
AA	14.47	12.58	1.28(0.89-1.82)	11.25	14.16	0.74(0.41-1.33)	16.76	11.59	1.81(1.15-2.86)
NFKBIA(rs3138055-rs2273650)									
TC	44.78	44.30	ref	47.67	43.28	ref	43.15	45.14	ref
CT	29.15	28.33	1.02(0.77-1.34)	31.51	30.46	0.94(0.61-1.44)	27.79	27.12	1.07(0.75-1.55)
CC	25.76	26.90	0.95(0.72-1.27)	20.81	26.26	0.72(0.45-1.15)	28.50	26.95	1.11(0.77-1.60)

* Adjusted for age and sex.

† Adjusted for age

DISCUSSION

In this nested case-control study, we found that the variants of rs28362491 and rs230496 of *NFKB1* gene might be associated with risk of primary liver cancer. After adjusting for possible confounders, rs28362491 deletion allelle and rs230496 AG or GG genotypes were found to increase the risk of liver cancer. In addition, haplotype analysis indicated that carriers of the *NFKB1* GA and AA (rs230525-rs230530) haplotypes had higher risk of liver cancer under additive model, although this association was only observed in man. These findings suggested that variants of NF-κB signaling pathway may play a role in liver cancer susceptibility.

NFKB1 gene was mapped on chromosome 4q23-q24 and composed of 24 exons[28]. This gene encodes for two proteins, p105 is a none-DNA binding protein and is activated to p50, a DNA binding protein by proteasome-mediated degradation. Several genetic polymorphisms were defined in *NFKB1* and researches have been focused on a common polymorphism of -94 del/ins (rs28362491) in the promoter region. Recent studies showed that genetic polymorphism of rs28362491 was associated with a number of cancer risks including sporadic breast cancer[15], prostate cancer[16], gastric cancer[17], colorectal cancer[18], and oral cancer[19], but little is known about its relationship with liver cancer. Cao and his colleagues conducted a case-control study of 202 HCC cases of HBV carrier and 404 healthy controls without HBV infection. Results showed that after adjusting for age and gender, -94 ins/del and ins/ins genotypes might increase the risk of HCC, with ORs of 1.60 (95%CI:1.01-2.53) and 3.01 (95%CI:1.87-4.85), respectively[29]. A report from Taiwan also found ins allele more previlent in HCC patients (OR=2.23,95%CI:1.32-3.77)[30]. In our study, we found that ins/del and del/del genotypes were more prevalent in liver cancer cases than controls. It was observed that the association of rs28362491 polymorphism with cancer susceptibility varied with cancer site and study populations. Ins allele was reported to increase the risk of oral cancer[31], melanoma[32], prostate cancer[16], gastric cancer[17], nasopharyngeal carcinoma[33] and cervical cancer[34]. Two studies in European found del allele might increase the risk of colorectal cancer[35, 36], while in Chinese population, none or even reverse association were obtained [35, 37]. The difference of polymorphisms may probably result from interactions or combined effects with none genetic risk factors. Well-designed studies with larger sample size are needed to validate these findings.

To our knowledge, this is the first report on the variants of rs230496, rs230525 and rs230530 with liver cancer susceptibility. A study in European American descent found

rs230530 polymorphism associated with alcohol dependence, and the evidence came primarily from those individuals who met criteria for alcoholism earlier[38]. As alcohol is one of the major risk factors of liver cancer, rs230530 might play a role in alcohol associated liver cancer. Unfortunately, subject to the limitation of relatively small samplesize, we were not able to explore this issue.

NFKBIA gene, which encodes IκBα, the inhibitor of *NFKB1*, was mapped to 14q13 with six exons spanning approximately 3.5kb[39]. As a major component of IκB family, the dysfunction or down regulation of IκBα will lead to over activation of NF-κB. Epidemiological studies on *NFKBIA* were relatively rare. A 2758G/A polymorphysim (rs696) in 3' untranstated region might regulate the expression of IκBα and thus affect the activation of NF-κB. Sun et al. found the frequency of AG genotype was increased in Chinese patients ≥50 years of age (OR=3.06, 95% CI:1.55-6.02) with colonrectal cancer[40]. Another study on breast cancer fail to obtain a significant association[15]. There was no previous report on rs696 and risk of liver cancer.

Of the four SNPs of *NFKBIA* gene evaluated, we did not observed an significant association. In previous studies, rs3138053 variant was found to be associated with hepatocellular carcinoma in a Chinese mainland population[29] but not Taiwanese[30].

There are several strengths of our study. This study was based on two well-designed prospective cohort studies. To the best of our knowledge, it was the first population based study to evaluate the polymorphic variants of NF-κB and risk of liver cancer. All study participants were ethnic Chinese and residents of Shanghai with similar genetic background, which minimized the potential confounding of ethnics. Only incident cases were included which ruled out the possibility of recall and selection bias. Liver cancer cases were carefully verified with multiple approaches which minimized the disease misclassification. Also, we controlled potential confounding variables in the analysis. The limitations of our study should also be noted. Firstly, we focused on only two genes involved in canonical pathway of NF-κB, other regulatory genes in NF-κB signaling pathway may also contribute to the pathogenesis of liver cancer. Secondly, we did not test for HBV infection, HCV infection or aflatoxin exposure, so we cannot rule out the possible confounding although the presents of HCV infection and aflatoxin are very low in the study population[41], but we did take into consideration of the participants' history of hepatitis and liver cirrhosis. Finally, due to the relatively small sample size, the frequencies of some homozygous variants were low in subgroups therefore reduced the statistical power and limited us from evaluating the

joint effects in stratified analysis. Replication in other studies is needed.

In summary, in this nested case-control study, we provided additional evidence for a role of NF-κB SNPs and haplotypes in the etiology of liver cancer. Studies in larger, varied polulations are warranted to confirm these findings. Furthermore, functional studies are required in order to explore the underling mechanisms.

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Contributors YBX obtained the funding, developed the research design, drafted the manuscript, interpreted the results, and also had primary responsibility for the final content; W Zheng and XOS designed, directed and obtained funding for the parent cohorts, and contributed to the revisions and interpretation of the results; JG obtained part of funding, drafted the manuscript, analyzed the data and interpreted the results; JG and HLX conducted experiments; All authors critically reviewed and approval manuscript.

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Competing interests None. The funding sponsor had no role in the study design, data collection, statistical analysis and result interpretation, as well as in the writing of the report and the decision to submit for publication. The corresponding author had full access to all data in the study and final responsibility for the decision to submit for publication.

Study approval Institutional review board.

Ethics approval Vanderbilt University IRB and Shanghai Cancer Institute IRB.

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References:

1. Ferlay J, Shin HR, Bray F, *et al*. GLOBOCAN 2008 v2.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10 [Internet]. Lyon, France: International Agency for Research on Cancer, 2010. Cited 28 Sep 2013. Available from URL: http://globocan.iarc.fr.

2. Gao J, Xie L, Yang WS, *et al*. Risk factors of hepatocellular carcinoma-current status and perspectives. *Asian Pac J Cancer Prev* 2012; 13: 743-52.

3. Schottenfeld D, Fraumeni JF. Cancer epidemiology and prevention. 3rd ed. Oxford: Oxford University Press, 2006.

4. Boyle P, Levin B. World cancer report 2008. Lyon: International Agency for Research on Cancer, 2008.

5. Seitz HK, Stickel F. Molecular mechanisms of alcohol-mediated carcinogenesis. *Nat Rev Cancer* 2007; 7: 599-612.

6. Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002; 420: 860-7.

7. Arsura M, Cavin LG. Nuclear factor-kappaB and liver carcinogenesis. *Cancer Lett* 2005; 229: 157-69.

8. Pikarsky E, Porat RM, Stein I, *et al*. NF-kappaB functions as a tumour promoter in inflammation-associated cancer. *Nature* 2004; 431: 461-6.

9. He G, Karin M. NF-kappaB and STAT3 - key players in liver inflammation and cancer. *Cell Res* 2011; 21: 159-68.

10. Berasain C, Castillo J, Perugorria MJ, *et al*. Inflammation and liver cancer: new molecular links. *Ann N Y Acad Sci* 2009; 1155: 206-21.

11. Sen R, Baltimore D. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell* 1986; 46: 705-16.

12. Barnes PJ, Karin M. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* 1997; 336: 1066-71.

13. Baldwin AJ. Series introduction: the transcription factor NF-kappaB and human disease. *J Clin Invest* 2001; 107: 3-6.

14. Karin M. Nuclear factor-kappaB in cancer development and progression. *Nature* 2006; 441: 431-6.

15. Curran JE, Weinstein SR, Griffiths LR. Polymorphic variants of NFKB1 and its inhibitory protein NFKBIA, and their involvement in sporadic breast cancer. *Cancer Lett* 2002; 188: 103-7.

16. Zhang P, Wei Q, Li X, *et al*. A functional insertion/deletion polymorphism in the promoter region of the NFKB1 gene increases susceptibility for prostate cancer. *Cancer Genet Cytogenet* 2009; 191: 73-7.

17. Lo SS, Chen JH, Wu CW, *et al*. Functional polymorphism of NFKB1 promoter may correlate to

the susceptibility of gastric cancer in aged patients. *Surgery* 2009; 145: 280-5.

18. Yu Y, Liu H, Jin M, *et al*. The joint association of REST and NFKB1 polymorphisms on the risk of colorectal cancer. *Ann Hum Genet* 2012; 76: 269-76.

19. Lin CW, Hsieh YS, Hsin CH, *et al*. Effects of NFKB1 and NFKBIA gene polymorphisms on susceptibility to environmental factors and the clinicopathologic development of oral cancer. *PLoS One* 2012; 7: e35078.

20. Shu XO, Yang G, Jin F, *et al*. Validity and reproducibility of the food frequency questionnaire used in the Shanghai Women's Health Study. *Eur J Clin Nutr* 2004; 58: 17-23.

21. Zheng W, Chow WH, Yang G, *et al*. The Shanghai Women's Health Study: rationale, study design, and baseline characteristics. *Am J Epidemiol* 2005; 162: 1123-31.

22. Villegas R, Yang G, Liu D, *et al*. Validity and reproducibility of the food-frequency questionnaire used in the Shanghai men's health study. *Br J Nutr* 2007; 97: 993-1000.

23. Cai H, Zheng W, Xiang YB, *et al*. Dietary patterns and their correlates among middle-aged and elderly Chinese men: a report from the Shanghai Men's Health Study. *Br J Nutr* 2007; 98: 1006-13.

24. World Health Organization. International Classification of Diseases, ninth revision (ICD-9). Geneva, Switzerland: World Health Organization, 1977.

25. de Bakker PI, McVean G, Sabeti PC, *et al*. A high-resolution HLA and SNP haplotype map for disease association studies in the extended human MHC. *Nat Genet* 2006; 38: 1166-72.

26. Barrett JC, Fry B, Maller J, *et al*. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005; 21: 263-5.

27. Lin DY, Zeng D, Millikan R. Maximum likelihood estimation of haplotype effects and haplotype-environment interactions in association studies. *Genet Epidemiol* 2005; 29: 299-312.

28. Mathew S, Murty VV, Dalla-Favera R, *et al*. Chromosomal localization of genes encoding the transcription factors, c-rel, NF-kappa Bp50, NF-kappa Bp65, and lyt-10 by fluorescence in situ hybridization. *Oncogene* 1993; 8: 191-3.

29. He Y, Zhang H, Yin J, *et al*. IkappaBalpha gene promoter polymorphisms are associated with hepatocarcinogenesis in patients infected with hepatitis B virus genotype C. *Carcinogenesis* 2009; 30: 1916-22.

30. Cheng CW, Su JL, Lin CW, *et al*. Effects of NFKB1 and NFKBIA gene polymorphisms on hepatocellular carcinoma susceptibility and clinicopathological features. *PLoS One* 2013; 8: e56130.

31. Lin SC, Liu CJ, Yeh WI, *et al*. Functional polymorphism in NFKB1 promoter is related to the risks of oral squamous cell carcinoma occurring on older male areca (betel) chewers. *Cancer Lett* 2006; 243: 47-54.

32. Bu H, Rosdahl I, Sun XF, *et al*. Importance of polymorphisms in NF-kappaB1 and NF-kappaBIalpha genes for melanoma risk, clinicopathological features and tumor progression in Swedish melanoma patients. *J Cancer Res Clin Oncol* 2007; 133: 859-66.

33. Zhou B, Rao L, Li Y, *et al*. A functional insertion/deletion polymorphism in the promoter region of NFKB1 gene increases susceptibility for nasopharyngeal carcinoma. *Cancer Lett* 2009; 275: 72-6.

34. Zhou B, Qie M, Wang Y, *et al*. Relationship between NFKB1 -94 insertion/deletion ATTG polymorphism and susceptibility of cervical squamous cell carcinoma risk. *Ann Oncol* 2010; 21: 506-11.

35. Lewander A, Butchi AK, Gao J, *et al*. Polymorphism in the promoter region of the NFKB1 gene increases the risk of sporadic colorectal cancer in Swedish but not in Chinese populations. *Scand J Gastroenterol* 2007; 42: 1332-8.

36. Andersen V, Christensen J, Overvad K, *et al*. Polymorphisms in NFkB, PXR, LXR and risk of colorectal cancer in a prospective study of Danes. *Bmc Cancer* 2010; 10: 484.

37. Song S, Chen D, Lu J, *et al*. NFkappaB1 and NFkappaBIA polymorphisms are associated with increased risk for sporadic colorectal cancer in a southern Chinese population. *PLoS One* 2011; 6: e21726.

38. Edenberg HJ, Xuei X, Wetherill LF, *et al*. Association of NFKB1, which encodes a subunit of the transcription factor NF-kappaB, with alcohol dependence. *Hum Mol Genet* 2008; 17: 963-70.

39. Le Beau MM, Ito C, Cogswell P, *et al*. Chromosomal localization of the genes encoding the p50/p105 subunits of NF-kappa B (NFKB2) and the I kappa B/MAD-3 (NFKBI) inhibitor of NF-kappa B to 4q24 and 14q13, respectively. *Genomics* 1992; 14: 529-31.

40. Gao J, Pfeifer D, He LJ, *et al*. Association of NFKBIA polymorphism with colorectal cancer risk and prognosis in Swedish and Chinese populations. Scand J Gastroenterol. 2007; 42: 345-50.

41. Yuan JM, Ross RK, Stanczyk FZ, *et al*. A cohort study of serum testosterone and hepatocellular carcinoma in Shanghai, China. *Int J Cancer* 1995; 63: 491-3.

STROBE Statement—Checklist of items that should be included in reports of ***cohort studies***

	Item No	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1-3
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2, 3
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3
Objectives	3	State specific objectives, including any prespecified hypotheses	4
Methods			
Study design	4	Present key elements of study design early in the paper	4, 5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	4, 5
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	4, 5
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	5
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	4-6
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	4-6
Bias	9	Describe any efforts to address potential sources of bias	4-6
Study size	10	Explain how the study size was arrived at	5
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	6
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	6
		(*b*) Describe any methods used to examine subgroups and interactions	N/A
		(*c*) Explain how missing data were addressed	9
		(*d*) If applicable, explain how loss to follow-up was addressed	4-5
		(*e*) Describe any sensitivity analyses	N/A
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	5, 9
		(b) Give reasons for non-participation at each stage	4-5
		(c) Consider use of a flow diagram	N/A
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	8, 9
		(b) Indicate number of participants with missing data for each variable of interest	9-12
		(c) Summarise follow-up time (eg, average and total amount)	4-5
Outcome data	15*	Report numbers of outcome events or summary measures over time	5

Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	6,7, 9-12
		(*b*) Report category boundaries when continuous variables were categorized	9
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	N/A
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	12
Discussion			
Key results	18	Summarise key results with reference to study objectives	13-15
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	14-15
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	15
Generalisability	21	Discuss the generalisability (external validity) of the study results	13, 14
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	15

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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Genetic polymorphism of NFKB1 and NFKBIA genes and liver cancer risk: a nested case-control study in Shanghai, China

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Genetic polymorphism of *NFKB1* and *NFKBIA* genes and liver cancer risk: a nested case-control study in Shanghai, China

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ABSTRACT

Objectives: Genetic variations of NF-κB signaling pathway were found to be associated with inflammatory diseases and several malignancies. However, little is known about NF-κB pathway gene polymorphisms and susceptibility of liver cancer. The aim of this study was to investigate whether genetic variants of NFKB1 and NFKBIA were associated with risk of liver cancer in a Chinese population.

Design: The study was designed as a nested case-control study within two prospective cohorts (the Shanghai Women's Health Study, SWHS, 1996–2000 and the Shanghai Men's Health Study, SMHS, 2002–2006).

Settings: This population-based study was conducted in urban Shanghai, China.

Participants: A total of 217 incident liver cancer cases diagnosed through December 31, 2009 and 427 healthy controls matched by sex, age at baseline (±2 years) and date (±30 days) of sample collection were included in the study.

Primary and secondary outcome measures: Genetic polymorphisms of *NFKB1* and *NFKBIA* were determined by TaqMan SNP genotyping assay blindly. OR and its 95% CIs were estimated by unconditional logistic regression model to measure the association between selected SNPs and the risk of liver cancer.

Results: After adjusted for potential confounding factors, rs28362491 ins/del or del/del genotypes were associated with higher risk of liver cancer with an adjusted OR of 1.54(95%CI: 1.04-2.28). rs230496 AG and GG genotypes were also noted with higher risk of liver cancer with an adjusted OR of 1.53 (95%CI: 1.03-2.26). Haplotype analysis indicated that carriers of the *NFKB1* GA and AA (rs230525-rs230530) haplotypes had higher risk of liver cancer under additive model. No association was observed between *NFKBIA* variants and risk of live cancer.

Conclusions: Our results suggest that genetic variants of *NFKB1* influence liver cancer susceptibility in Chinese population, although replication in other studies is needed.

Article summary-strengths and limitations of this study

- This study was the first population-based study to evaluate the polymorphic variants of NF-κB and risk of liver cancer.
- Only incident cases from two prospective cohorts were included in the study which ruled out the possibility of recall and selection bias.
- The limitations of the study include relatively small sample size, unmeasured HBV infection, HCV infection and aflatoxin exposure. However, we did take into

consideration of the participants' history of hepatitis and liver cirrhosis, and the presents of HCV infection and aflatoxin are very low in the study population.

INTRODUCTION

Liver cancer is a common disorder worldwide which ranks the 5th and 7th most common cancer among men and women. It was estimated that more than 80% liver cancers occur in developing countries and about 54% occur in China[1]. Among the main risk factors for liver cancer, chronic infections of hepatitis B virus (HBV) and hepatitis C virus (HCV) are the most important in humans, accounting for more than 70% of liver cancer cases worldwide[2-4]. Liver cirrhosis, heavy alcohol consumption, exposure to aflatoxin, and diabetes also account for part of liver cancer occurrence[2-4].

Chronic inflammation has been widely accepted to play an important role in hepatocarcinogenesis. Most of the known risk factors of liver cancer such as HBV, HCV infection and alcohol drinking can cause persistent inflammatory reaction of the liver and promote cancer development[5, 6]. However, the molecular and cellular mechanisms linking inflammation and liver cancer remain unclear. Recent findings have suggested that NF-κB may play a crucial role in bridging the actions of growth factors and chronic inflammation to hepatic oncogenesis[7-10].

NF-κB, a collection of dimeric transcription factors, was originally identified as a nuclear factor bound to the enhancer of the immunoglobulin κ-light chain gene [11] specific to B cells and presents in all cell types[12]. It is a major transcription regulator of the immune response, cell adhesion, differentiation, proliferation, and apoptosis[13]. NF-κB dimers are formed by seven distinct proteins: NF-κB1 (p105 and p50), NF-κB2 (p100 and p52), RelA (p65), RelB and c-Rel, of which NF-κB p50/RelA is the most common dimer form[9]. In the resting cell, most NF-κB dimers are inactivated in the cytoplasm by binding to specific inhabitors-IκB family, of which IκBα is the most common one. In the classical activation pathway, IκB is phosphorylated and degraded by IκB kinase complex, and then NF-κB dimers are released and translocate to the nucleus where they coordinate the transcriptional activation of target genes[14]. Several genetic variations of NF-κB signaling pathway have been reported to be associated with cancer risks such as breast[15], prostate[16], stomach[17], colorectum[18] and mouth[19]. However, little is known about role of genetic polymorphisms of NF-κB genes and susceptibility of liver cancer.

In a population based case-control study nested in two prospective cohorts of the

Shanghai Women's and Men's Health Studies, we investigated the relationships between genetic variants of *NFKB1* and *NFKBIA*, two key genes involved in classic signaling pathway of NF-κB, and the risk of liver cancer among Chinese men and women.

MATERIAL AND METHODS

Study population

Participants of this study came from the Shanghai Women's Health Study (SWHS) and Shanghai Men's Health Study (SMHS). The design and methods used in these two studies have been described in detail elsewhere [20-23]. Briefly, the SWHS enrolled 74,941 women aged 40-74 years between March 1, 1997 and May 31, 2000, with a response rate of 92.7%. SMHS enrolled 61,491 men aged 40-74 years without history of cancer at recruitment from April 1, 2002 to June 30, 2006, with a response rate of 74.1%. Both studies were approved by the relevant Institutional Review Boards for human research in China and the United States and a written informed consent was obtained from all participants.

In-person interview was conducted by trained interviewers using a structured questionnaire at baseline to obtain information on demographics, lifestyle, dietary habits, medical history and other characteristics. Anthropometric measurements, including current weight, height and circumferences of the waist and hips, were also measured. Of the eligible participants, 56,831 (75.8%) of the SWHS and 46,332 (75.3%) of the SMHS provided a 10-ml blood sample at baseline. The samples were drawn into an EDTA Vacutainer tube and then kept in a portable styrofoam box with ice packs (at approximately 0-4°C) and processed within 6 hours for long-term storage at -70°C. A bio-specimen collection form was completed for each participant at the time of sample procurement which included the date and time of collection, time of last meal, and date of last menstruation, intake of selected foods, smoking, as well as use of any medications over the previous 24 hours and during the previous week.

Cohort follow-up and outcome ascertainment

Both cohorts were followed for occurrence of cancer and other chronic diseases by active in-person surveys conducted every 2-3 years as well as annual record linkage to the databases of the population-based Shanghai Cancer Registry, Shanghai Vital Statistics Registry, and Shanghai Resident Registry. For the SWHS, four rounds of in-person follow-ups were completed and the response rates for the first

(2000-2002), second (2002-2004), third (2004-2007), and fourth (2008-2011) follow-up surveys were 99.8%, 98.7%, 96.7%, and 92.0%, respectively. For the SMHS, two rounds of follow-up surveys have completed. The response rates for the first (2004-2008) and second (2008-2011) follow-up surveys were 97.6% and 93.6%, respectively. For cohort members who developed liver cancer during the follow-up, medical chart were reviewed by a panel of oncologists to verify the diagnosis. Liver cancer data through December 31, 2009 was used for the present study.

Included in this nested case-control study are 217 incident liver cancer cases and 427 matched controls who had donated blood sample. Liver cancer cases were defined as having an International Classification of Disease, Ninth Revision (ICD-9), codes of 155.0 (primary malignant neoplasms), 155.1(malignant neoplasms of the intrahepatic bile ducts), or 155.2 (unspecified malignant neoplasms of the liver)[24]. Two control subjects were randomly selected from the cohorts who donated a blood sample at baseline and matched to each case for sex, age at baseline (±2 years) and date (±30 days) of sample collection. All controls were free of any cancer at the time of cancer diagnosis for the corresponding case.

Genotyping

Single-nucleotide polymorphisms (SNPs) were selected based on both TagSNP and their putative functional significance. Tagging SNPs were selected by searching the Han Chinese data from the Hapmap project[25]. The following criteria were used to identify tagging SNPs: (i) SNPs located in the genes or within the 5-kb flanking region, (ii) a minor allele frequency $\geqslant 0.05$, and (iii) other unselected single-nucleotide polymorphisms could be captured by one of the tagging SNPs with a linkage disequilibrium of $r^2 \geqslant 0.90$. A total of 8 SNPs were selected for genotyping which were rs28362491, rs230530, rs230525, rs230496 for *NFKB1* and rs3138053, rs3138055, rs2273650, rs696 for *NFKBIA* (table 1). Genomic DNA was extracted from buffycoat using Promega DNA Extraction Kit according to the manufacturer's instructions (Promega Corporation, Madison, WI, USA). Genotyping were performed by the TaqMan assay, using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA), in 384-wellformat, with dual fluorescent reporter probes VIC and FAM. The quality and potential misclassification of the genotyping results were assessed by evaluating 5% of duplicate DNA samples that were randomly selected from the whole samples. There replicates were 100% concordant. All serum samples were tested blindly and were identified only by an

unique identification number blinded with case-control status.

Table 1. Descriptions of Genetic Polymorphisms of the *NFKB1* and *NFKBIA* genes under investigation

Gene	Assay ID	Sequence	Location
NFKB1	rs28362491	CTCCGTGCTGCCTGCGTTCCCCGACC[-/ATTG]ATTGGGCC CGGCAGGCGCTTCCTGG	5'-near gene
	rs230530	TTTTTAGCACCAAACATCTTAATTT[A/G]CATTCAAATAAA TGAGAACCACCAT	intron
	rs230525	TACGGGAAAAGTGATTCTTGTTTAC[A/G]GAGCCCTCTTT CACAGTTTCATGTT	intron
	rs230496	TGTCTGGATTTGCTTGAGACAGCCC[A/G]GTTTGCCCCTG ACCTAATTGTTTAT	intron
NFKBIA	rs3138053	ATTCGTTTATGCTATCTGACCTACA[C/T]TGTGCTCCCGCA GAAAAAGGATCGT	5'-near gene
	rs3138055	AATCAACGGGATGACAGAATGACAA[C/T]GGAGAGGTCT CCAACCACAGGCCAA	3'-near gene
	rs2273650	AACAATACATTATGTACACCATTTA[C/T]AGGAGGGTAAC ACAAACCTTGACAG	3'-UTR
	rs696	CCTACCACAATAAGACGTTTTGGGC[C/T]AGGCAGTGTGC AGTGTGGATATAAG	3'-UTR

Statistical analysis

Subjects with both survey data and genotyping results were included in the final analysis. Means and percentages of selected characteristics for cases and controls were calculated. The distributions of selected characteristics were compared between cases and controls by either student's *t*-test (continuous variables) or χ^2 test (categorical variables). Odds ratio and its 95% confidence interval were estimated by unconditional logistic regression model to measure the association between selected SNPs and the risk of primary liver cancer. In the multivariable analysis, potential confounding factors were adjusted for, which include age (continuous variable); education level (four categories: elementary school or less, middle school, high school, and college or above); history of hepatitis (yes or no); family history of liver cancer (yes or no); and history of other chronic liver diseases or cirrhosis (yes or no). Statistical analyses were carried out using the SAS software package (version9.2; SAS Institute, Cary, NC). Tests for trend were performed by entering categorical variables as continuous variables in the regression model. All P values were calculated by two-sided tests and were considered statistically significant if P was less than 0.05.

Hardy–Weinberg equilibrium and Linkage disequilibrium were accessed with HaploView version 4.0[26]. Associations between haplotypes and the risk of liver cancer were evaluated with HAPSTAT version 3.0 using the most common haplotype as the

referent category, assumming an additive model[27].

RESULTS

Selected baseline characteristics of study participants were presented in table 2. The average ages of cases and control were 59.61 and 59.47. Compared with controls, liver cancer cases were more likely to have a lower education level, a history of hepatitis, a family history of liver cancer in first degree relatives, and history of chronic liver diseases or cirrhosis. Besides, male liver cancer cases were more probably to have lower body mass index, and be a non-regular exerciser compared to controls, although the difference were at borderline significance. Whereas in women, cases were more likely to have a history of type 2 diabetes than controls. No differences were observed in family income, smoking, drinking habits, waist to hip ratio, and family history of other cancers between the two groups.

The associations of *NFKB1* SNPs with liver cancer risk were summarized in table 3. The genotypes of rs28362491, rs230530 and rs230525 showed no deviation from Hardy-Weinberg equilibrium in controls except for rs230496. After adjusted for potential confounding factors, rs28362191 ins/del or del/del genotypes were associated with higher risk of liver cancer with an OR of 1.54(95%CI: 1.04-2.28). rs230496 AG and GG genotypes were also noted with higher risk of liver cancer with an adjusted OR of 1.53 (95%CI: 1.03-2.26). Carriers of rs230525 AG or GG genotypes had about 30% percent increased risk of liver cancer, but the risk was not insignificant. No association was found between rs230530 and liver cancer risk.

Table 4 presents the distribution of *NFKBIA* SNPs in cases and controls. The genotypes of rs3138055, rs696 and rs2273650 showed no deviation from Hardy-Weinberg equilibrium in controls but for rs3138053. Generally, all the four SNPs showed no relationship with liver cancer.

We further analyzed the haplotypes of these SNPs with risk of liver cancer (table 5). For *NFKB1* gene, two SNPs (rs230525, rs230530) demonstrated strong linkage disequilibrium (D' =1.0, r^2=0.59). Compared to men carrying rs230525-rs230530 AG haplotype, those with GA or AA haplotypes were at increased risk of liver cancer with ORs of 1.46(95%CI: 1.05-2.03) and 1.81(95%CI: 1.15-2.86), respectively. For *NFKBIA*, rs3138053 and rs2273650 were in linkage disequilibrium (D' =0.97, r^2=0.31) but none of the haplotypes was significantly associated with liver cancer.

Table 2. Distribution of selected characteristics in the study cases and controls[†]

Characteristics	All subjects			Male			Female		
	Cases (*N*=217)	Controls (*N*=427)	*P*	Cases (*N*=131)	Controls (*N*=262)	*P*	Cases (*N*=86)	Controls (*N*=165)	*P*
Age at interview, Mean±SD,	59.61±9.56	59.47±9.55	0.853	60.05±9.93	59.86±9.95	0.858	58.95±8.98	58.85±8.87	0.928
Education level (%)									
Elementary school or less	63(29.30)	115(27.00)		18(13.95)	35(13.41)		45(52.33)	80(48.48)	
Middle school	69(32.09)	148(37.74)		54(41.86)	104(39.85)		15(17.44)	44(26.67)	
High school	62(28.84)	91(21.36)		41(31.78)	61(23.37)		21(24.42)	30(18.18)	
College or above	21(9.77)	72(16.90)	0.031	16(12.40)	61(23.37)	0.053	5(5.81)	11(6.67)	0.341
Family income (%)[†]									
Low	50(23.04)	90(21.13)		17(12.98)	37(14.12)		33(38.37)	53(32.32)	
Medium	112(51.61)	208(48.83)		76(58.02)	130(49.62)		36(41.86)	78(47.56)	
High	55(25.35)	128(30.05)	0.454	38(29.01)	95(36.26)	0.271	17(19.77)	33(20.12)	0.606
Ever smoked (%)	93(42.86)	173(40.52)	0.569	90(68.70)	163(62.21)	0.206	3(3.49)	10(6.06)	0.384
Ever drank alcohol (%)	45(20.74)	98(22.95)	0.523	42(32.06)	97(37.02)	0.333	3(3.49)	1(0.61)	0.084
Body mass index ,kg/m^2, Mean±SD	23.79±3.65	24.16±3.31	0.198	23.16±3.25	23.77±2.89	0.06	24.75±4.02	24.78±3.80	0.961
WHR, Mean±SD	0.87±0.07	0.87±0.07	0.936	0.90±0.06	0.90±0.06	0.379	0.82±0.05	0.83±0.06	0.261
Regular physical activity (%)	94(43.32)	207(48.48)	0.215	49(37.40)	124(47.33)	0.062	45(52.33)	83(50.30)	0.761
physical activity, MET-hours/week	81.58±47.12	83.71±43.59	0.570	66.86±40.33	68.00±34.61	0.78	104.00±48.09	108.60±44.83	0.450
History of hepatitis (%)	74(34.10)	25(5.85)	<0.001	57(43.51)	16(6.11)	<0.001	17(19.77)	9(9.45)	<0.001
Family history of cancer (%)	69(31.80)	116(27.17)	0.220	41(31.30)	70(26.72)	0.342	28(32.56)	46(27.88)	0.441
Family history of liver cancer (%)	28(12.90)	18(4.22)	<0.001	20(15.27)	10(3.82)	<0.001	8(9.30)	8(4.85)	0.171
History of type 2 diabetes (%)	25(11.52)	35(8.20)	0.171	14(10.69)	25(9.54)	0.72	11(12.79)	10(6.06)	0.068
History of chronic liver disease or	35(16.13)	11(2.58)	<0.001	26(19.85)	10(3.82)	<0.001	9(10.47)	1(0.61)	<0.001

* Missing data was excluded from the analysis

[†] Family income level (low income for <5000 yuan/year in the SWHS and <12 000 yuan/year in the SMHS; medium income for 5000 to <10 000 yuan/year in the SWHS and 12 000 to <24 000 yuan/year in the SMHS; and high income for >10 000 yuan/year in the SWHS and >24 000 yuan/year in the SMHS)

Table 3. *NFKB1* genetic polymorphisms with the risk of primary liver cancer

SNPs	Cases	Controls	P for χ^2	OR*	95%CI	OR†	95%CI	OR‡	95%CI
rs28362491									
ins/ins	68	171		1.00	-	1.00	-	1.00	-
ins/del	102	160		1.60	1.10-2.33	1.60	1.10-2.33	1.71	1.13-2.60
del/del	40	79	0.047	1.27	0.79-2.05	1.27	0.79-2.04	1.21	0.71-2.05
P for trend				0.144		0.146		0.233	
ins/del or del/del	142	239	0.023	1.50	1.05-2.12	1.49	1.05-2.12	1.54	1.04-2.28
rs230496									
AA	64	164		1.00	-	1.00	-	1.00	-
AG	101	169		1.53	1.05-2.24	1.53	1.05-2.24	1.68	1.10-2.58
GG	47	91	0.087	1.33	0.84-2.09	1.32	0.84-2.09	1.25	0.75-2.09
P for trend				0.141		0.143		0.235	
AG or GG	148	260	0.041	1.46	1.03-2.08	1.46	1.03-2.08	1.53	1.03-2.26
rs230525									
AA	79	186		1.00	-	1.00	-	1.00	-
AG	102	175		1.38	0.96-1.97	1.38	0.96-1.98	1.46	0.98-2.18
GG	32	63	0.224	1.20	0.73-1.98	1.20	0.73-1.97	1.11	0.63-1.94
P for trend				0.236		0.236		0.347	
AG or GG	134	238	0.100	1.33	0.95-1.87	1.33	0.95-1.87	1.36	0.94-1.99
rs230530									
AA	64	114		1.00	-	1.00	-	1.00	-
AG	99	175		1.01	0.68-1.49	1.01	0.68-1.49	1.05	0.68-1.62
GG	48	129	0.102	0.66	0.42-1.04	0.66	0.42-1.04	0.67	0.40-1.12
P for trend				0.079		0.078		0.132	
AG or GG	147	304	0.423	0.86	0.60-1.24	0.86	0.60-1.24	0.89	0.59-1.34

*Adjusted for age

† Adjusted for age, sex.

‡ Adjusted for age, sex, education level, family history of liver cancer, history of hepatitis, and chronic liver diseases or cirrhosis.

Table 4. *NFKBIA* genetic polymorphisms with the risk of primary liver cancer

SNPs	Cases	Controls	P	OR*		OR†		OR‡	
rs3138053									
AA	173	336		1.00	-	1.00	-	1.00	-
AG	21	48		0.85	0.49-1.47	0.84	0.48-1.45	0.97	0.54-1.74
GG	19	40	0.823	0.92	0.52-1.64	0.94	0.52-1.68	0.98	0.51-1.88
P for trend				0.638		0.653		0.920	
AG or GG	40	88	0.556	0.88	0.58-1.34	0.88	0.58-1.34	0.97	0.61-1.54
rs3138055									
CC	62	128		1.00	-	1.00	-	1.00	-
CT	109	215		1.05	0.72-1.54	1.05	0.72-1.54	1.22	0.79-1.87
TT	42	81	0.956	1.07	0.66-1.73	1.07	0.66-1.73	1.33	0.78-2.27
P for trend				0.772		0.771		0.276	
CT or TT	151	296	0.778	1.06	0.74-1.51	1.06	0.74-1.52	1.25	0.83-1.88
rs696									
CC	65	149		1.00	-	1.00	-	1.00	-
CT	115	196		1.35	0.93-1.96	1.35	0.93-1.96	1.47	0.97-2.23
TT	33	76	0.210	0.99	0.60-1.64	0.99	0.60-1.64	1.17	0.67-2.03
P for trend				0.694		0.695		0.360	
CT or TT	148	272	0.218	1.25	0.88-1.78	1.25	0.88-1.78	1.38	0.93-2.06
rs2273650									
CC	108	215		1.00	-	1.00	-	1.00	-
CT	84	173		0.97	0.68-1.37	0.97	0.68-1.37	0.86	0.58-1.26
TT	20	37	0.938	1.08	0.60-1.95	1.07	0.59-1.94	0.89	0.45-1.73
P for trend				0.937		0.945		0.493	
CT or TT	104	210	0.933	0.99	0.71-1.38	0.99	0.71-1.37	0.86	0.60-1.24

*Adjusted for age

† Adjusted for age, sex.

‡ Adjusted for age, sex, education level, family history of liver cancer, history of hepatitis, and chronic liver diseases or cirrhosis.

Table 5. ORs and 95%CIs for liver cancer in relation to *NFKB1*/*NFKBIA* haplotypes

	All subjects*			Female†			Male†		
	Cases (%)	Controls (%)	OR	Cases (%)	Controls (%)	OR	Cases (%)	Controls (%)	OR
NFKB1(rs230525-rs230530)	n=215	n=425		n=86	n=165		n=129	n=260	
AG	46.49	52.01	ref	52.14	49.39	ref	42.69	53.53	ref
GA	38.973	35.50	1.23(0.95-1.58)	36.47	36.59	0.94(0.63-1.41)	40.55	34.88	1.46(1.05-2.03)
AA	14.54	12.49	1.30(0.91-1.86)	11.39	14.02	0.77(0.42-1.39)	16.76	11.59	1.81(1.15-2.86)
NFKBIA(rs3138055-rs2273650)									
TC	45.00	43.94	ref	48.24	42.31	ref	43.15	45.14	ref
CT	29.05	28.58	1.00(0.75-1.31)	31.30	31.21	0.88(0.57-1.35)	27.79	27.12	1.07(0.75-1.55)
CC	25.65	27.00	0.93(0.70-1.24)	20.47	26.48	0.68(0.42-1.10)	28.50	26.95	1.11(0.77-1.60)

* Adjusted for age and sex.

† Adjusted for age

DISCUSSION

In this nested case-control study, we found that the variants of rs28362491 and rs230496 of *NFKB1* gene might be associated with risk of primary liver cancer. After adjusting for possible confounders, rs28362491 deletion allelle and rs230496 AG or GG genotypes were found to increase the risk of liver cancer. In addition, haplotype analysis indicated that carriers of the *NFKB1* GA and AA (rs230525-rs230530) haplotypes had higher risk of liver cancer under additive model, although this association was only observed in man. These findings suggested that variants of NF-κB signaling pathway may play a role in liver cancer susceptibility.

NFKB1 gene was mapped on chromosome 4q23-q24 and composed of 24 exons[28]. This gene encodes for two proteins-p105 and p50. p105 is a none-DNA binding protein and is activated to p50, a DNA binding protein by proteasome-mediated degradation. Several genetic polymorphisms were defined in *NFKB1* and researches have been focused on a common polymorphism of -94 del/ins (rs28362491) in the promoter region. Recent studies showed that genetic polymorphism of rs28362491 was associated with a number of cancer risks including sporadic breast cancer[15], prostate cancer[16], gastric cancer[17], colorectal cancer[18], and oral cancer[19], but little is known about its relationship with liver cancer. He and his colleagues conducted a case-control study of 202 HCC cases of HBV carrier and 404 healthy controls without HBV infection. Results showed that after adjusting for age and gender, -94 ins/del and ins/ins genotypes might increase the risk of HCC, with ORs of 1.60 (95%CI:1.01-2.53) and 3.01 (95%CI:1.87-4.85), respectively[29]. A report from Taiwan also found ins allele more previlent in HCC patients (OR=2.23,95%CI:1.32-3.77)[30]. In our study, we found that ins/del and del/del genotypes were more prevalent in liver cancer cases than controls. It was observed that the association of rs28362491 polymorphism with cancer susceptibility varied with cancer site and study populations. Ins allele was reported to increase the risk of oral cancer[31], melanoma[32], prostate cancer[16], gastric cancer[17], nasopharyngeal carcinoma[33] and cervical cancer[34]. Two studies in European found del allele might increase the risk of colorectal cancer[35, 36], while in Chinese population, none or even reverse association were obtained [35, 37]. The difference of polymorphisms may probably result from interactions or combined effects with none genetic risk factors. Well-designed studies with larger sample size are needed to validate these findings.

To our knowledge, this is the first report on the variants of rs230496, rs230525 and rs230530 with liver cancer susceptibility. A study in European American descent found

rs230530 polymorphism associated with alcohol dependence, and the evidence came primarily from those individuals who met criteria for alcoholism earlier[38]. As alcohol is one of the major risk factors of liver cancer, rs230530 might play a role in alcohol associated liver cancer. Unfortunately, subject to the limitation of relatively small samplesize, we were not able to explore this issue. In addition, although the functions of intronic SNPs are still abscure, studies have indicated that they can affect either local DNA or RNA secondary structure, thereby regulating gene expression[39,40]

NFKBIA gene, which encodes IκBα, the inhibitor of *NFKB1*, was mapped to 14q13 with six exons spanning approximately 3.5kb[41]. As a major component of IκB family, the dysfunction or down regulation of IκBα will lead to over activation of NF-κB. Epidemiological studies on *NFKBIA* were relatively rare. A 2758G/A polymorphysim (rs696) in 3' untranstated region might regulate the expression of IκBα and thus affect the activation of NF-κB. Sun et al. found the frequency of AG genotype was increased in Chinese patients ≥50 years of age (OR=3.06, 95% CI:1.55-6.02) with colorectal cancer[42]. Another study on breast cancer fail to obtain a significant association[15]. There was no previous report on rs696 and risk of liver cancer.

Of the four SNPs of *NFKBIA* gene evaluated, we did not observed an significant association. In previous studies, rs3138053 variant was found to be associated with hepatocellular carcinoma in a Chinese mainland population[29] but not Taiwanese[30].

There are several strengths of our study. This study was based on two well-designed prospective cohort studies. To the best of our knowledge, it was the first population based study to evaluate the polymorphic variants of NF-κB and risk of liver cancer. All study participants were ethnic Chinese and residents of Shanghai with similar genetic background, which minimized the potential confounding of ethnics. Only incident cases were included which ruled out the possibility of recall and selection bias. Liver cancer cases were carefully verified with multiple approaches which minimized the disease misclassification. Also, we controlled potential confounding variables in the analysis. The limitations of our study should also be noted. Firstly, we focused on only two genes involved in canonical pathway of NF-κB, other regulatory genes in NF-κB signaling pathway may also contribute to the pathogenesis of liver cancer. Secondly, we did not test for HBV infection, HCV infection or aflatoxin exposure, so we cannot rule out the possible confoundings although the presents of HCV infection and aflatoxin are very low in the study population[43], but we did take into consideration of the participants' history of hepatitis and liver cirrhosis. Finally, due to

the relatively small sample size, the frequencies of some homozygous variants were low in subgroups therefore reduced the statistical power and limited us from evaluating the joint effects in stratified analysis. Replication in other studies is needed.

In summary, in this nested case-control study, we provided additional evidence for a role of NF-κB SNPs and haplotypes in the etiology of liver cancer. Studies in larger, varied polulations are warranted to confirm these findings. Furthermore, functional studies are required in order to explore the underling mechanisms.

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References:

1. Ferlay J, Shin HR, Bray F, *et al*. GLOBOCAN 2008 v2.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10 [Internet]. Lyon, France: International Agency for Research on Cancer, 2010. Cited 28 Sep 2013. Available from URL: http://globocan.iarc.fr.

2. Gao J, Xie L, Yang WS, *et al*. Risk factors of hepatocellular carcinoma-current status and perspectives. *Asian Pac J Cancer Prev* 2012; 13: 743-52.

3. Schottenfeld D, Fraumeni JF. Cancer epidemiology and prevention. 3rd ed. Oxford: Oxford University Press, 2006.

4. Boyle P, Levin B. World cancer report 2008. Lyon: International Agency for Research on Cancer, 2008.

5. Seitz HK, Stickel F. Molecular mechanisms of alcohol-mediated carcinogenesis. *Nat Rev Cancer* 2007; 7: 599-612.

6. Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002; 420: 860-7.

7. Arsura M, Cavin LG. Nuclear factor-kappaB and liver carcinogenesis. *Cancer Lett* 2005; 229: 157-69.

8. Pikarsky E, Porat RM, Stein I, *et al*. NF-kappaB functions as a tumour promoter in inflammation-associated cancer. *Nature* 2004; 431: 461-6.

9. He G, Karin M. NF-kappaB and STAT3 - key players in liver inflammation and cancer. *Cell Res* 2011; 21: 159-68.

10. Berasain C, Castillo J, Perugorria MJ, *et al*. Inflammation and liver cancer: new molecular links. *Ann N Y Acad Sci* 2009; 1155: 206-21.

11. Sen R, Baltimore D. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell* 1986; 46: 705-16.

12. Barnes PJ, Karin M. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* 1997; 336: 1066-71.

13. Baldwin AJ. Series introduction: the transcription factor NF-kappaB and human disease. *J Clin Invest* 2001; 107: 3-6.

14. Karin M. Nuclear factor-kappaB in cancer development and progression. *Nature* 2006; 441: 431-6.

15. Curran JE, Weinstein SR, Griffiths LR. Polymorphic variants of NFKB1 and its inhibitory protein NFKBIA, and their involvement in sporadic breast cancer. *Cancer Lett* 2002; 188: 103-7.

16. Zhang P, Wei Q, Li X, *et al*. A functional insertion/deletion polymorphism in the promoter region of the NFKB1 gene increases susceptibility for prostate cancer. *Cancer Genet Cytogenet* 2009; 191: 73-7.

17. Lo SS, Chen JH, Wu CW, *et al*. Functional polymorphism of NFKB1 promoter may correlate to the susceptibility of gastric cancer in aged patients. *Surgery* 2009; 145: 280-5.

18. Yu Y, Liu H, Jin M, *et al*. The joint association of REST and NFKB1 polymorphisms on the risk of colorectal cancer. *Ann Hum Genet* 2012; 76: 269-76.

19. Lin CW, Hsieh YS, Hsin CH, *et al*. Effects of NFKB1 and NFKBIA gene polymorphisms on susceptibility to environmental factors and the clinicopathologic development of oral cancer. *PLoS*

One 2012; 7: e35078.

20. Shu XO, Yang G, Jin F, *et al*. Validity and reproducibility of the food frequency questionnaire used in the Shanghai Women's Health Study. *Eur J Clin Nutr* 2004; 58: 17-23.

21. Zheng W, Chow WH, Yang G, *et al*. The Shanghai Women's Health Study: rationale, study design, and baseline characteristics. *Am J Epidemiol* 2005; 162: 1123-31.

22. Villegas R, Yang G, Liu D, *et al*. Validity and reproducibility of the food-frequency questionnaire used in the Shanghai men's health study. *Br J Nutr* 2007; 97: 993-1000.

23. Cai H, Zheng W, Xiang YB, *et al*. Dietary patterns and their correlates among middle-aged and elderly Chinese men: a report from the Shanghai Men's Health Study. *Br J Nutr* 2007; 98: 1006-13.

24. World Health Organization. International Classification of Diseases, ninth revision (ICD-9). Geneva, Switzerland: World Health Organization, 1977.

25. de Bakker PI, McVean G, Sabeti PC, *et al*. A high-resolution HLA and SNP haplotype map for disease association studies in the extended human MHC. *Nat Genet* 2006; 38: 1166-72.

26. Barrett JC, Fry B, Maller J, *et al*. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005; 21: 263-5.

27. Lin DY, Zeng D, Millikan R. Maximum likelihood estimation of haplotype effects and haplotype-environment interactions in association studies. *Genet Epidemiol* 2005; 29: 299-312.

28. Mathew S, Murty VV, Dalla-Favera R, *et al*. Chromosomal localization of genes encoding the transcription factors, c-rel, NF-kappa Bp50, NF-kappa Bp65, and lyt-10 by fluorescence in situ hybridization. *Oncogene* 1993; 8: 191-3.

29. He Y, Zhang H, Yin J, *et al*. IkappaBalpha gene promoter polymorphisms are associated with hepatocarcinogenesis in patients infected with hepatitis B virus genotype C. *Carcinogenesis* 2009; 30: 1916-22.

30. Cheng CW, Su JL, Lin CW, *et al*. Effects of NFKB1 and NFKBIA gene polymorphisms on hepatocellular carcinoma susceptibility and clinicopathological features. *PLoS One* 2013; 8: e56130.

31. Lin SC, Liu CJ, Yeh WI, *et al*. Functional polymorphism in NFKB1 promoter is related to the risks of oral squamous cell carcinoma occurring on older male areca (betel) chewers. *Cancer Lett* 2006; 243: 47-54.

32. Bu H, Rosdahl I, Sun XF, *et al*. Importance of polymorphisms in NF-kappaB1 and NF-kappaBIalpha genes for melanoma risk, clinicopathological features and tumor progression in Swedish melanoma patients. *J Cancer Res Clin Oncol* 2007; 133: 859-66.

33. Zhou B, Rao L, Li Y, *et al*. A functional insertion/deletion polymorphism in the promoter region of NFKB1 gene increases susceptibility for nasopharyngeal carcinoma. *Cancer Lett* 2009; 275: 72-6.

34. Zhou B, Qie M, Wang Y, *et al*. Relationship between NFKB1 -94 insertion/deletion ATTG polymorphism and susceptibility of cervical squamous cell carcinoma risk. *Ann Oncol* 2010; 21: 506-11.

35. Lewander A, Butchi AK, Gao J, *et al*. Polymorphism in the promoter region of the NFKB1 gene increases the risk of sporadic colorectal cancer in Swedish but not in Chinese populations. *Scand J Gastroenterol* 2007; 42: 1332-8.

36. Andersen V, Christensen J, Overvad K, *et al*. Polymorphisms in NFkB, PXR, LXR and risk of colorectal cancer in a prospective study of Danes. *Bmc Cancer* 2010; 10: 484.

37. Song S, Chen D, Lu J, *et al*. NFkappaB1 and NFkappaBIA polymorphisms are associated with increased risk for sporadic colorectal cancer in a southern Chinese population. *PLoS One* 2011; 6: e21726.

38. Edenberg HJ, Xuei X, Wetherill LF, *et al*. Association of NFKB1, which encodes a subunit of the transcription factor NF-kappaB, with alcohol dependence. *Hum Mol Genet* 2008; 17: 963-70.

39. Lu L, Risch E, Deng Q, *et al*. An insulin-like growth factor-II intronic variant affects local DNA conformation and ovarian cancer survival. *Carcinogenesis* 2013;34:2024-30.

40. Lu L, Katsaros D, Mayne ST, *et al*. Functional study of risk loci of stem cell-associated gene lin-28B and associations with disease survival outcomes in epithelial ovarian cancer. *Carcinogenesis* 2012;33:2119-25.

41. Le Beau MM, Ito C, Cogswell P, *et al*. Chromosomal localization of the genes encoding the p50/p105 subunits of NF-kappa B (NFKB2) and the I kappa B/MAD-3 (NFKBI) inhibitor of NF-kappa B to 4q24 and 14q13, respectively. *Genomics* 1992; 14: 529-31.

42. Gao J, Pfeifer D, He LJ, *et al*. Association of NFKBIA polymorphism with colorectal cancer risk and prognosis in Swedish and Chinese populations. Scand J Gastroenterol. 2007; 42: 345-50.

43. Yuan JM, Ross RK, Stanczyk FZ, *et al*. A cohort study of serum testosterone and hepatocellular carcinoma in Shanghai, China. *Int J Cancer* 1995; 63: 491-3.

Genetic polymorphism of *NFKB1* and *NFKBIA* genes and liver cancer risk: a nested case-control study in Shanghai, China

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ABSTRACT

Objectives: Genetic variations of NF-κB signaling pathway were found to be associated with inflammatory diseases and several malignancies. However, little is known about NF-κB pathway gene polymorphisms and susceptibility of liver cancer. The aim of this study was to investigate whether genetic variants of NFKB1 and NFKBIA were associated with risk of liver cancer in a Chinese population.

Design: The study was designed as a nested case-control study within two prospective cohorts (the Shanghai Women's Health Study, SWHS, 1996–2000 and the Shanghai Men's Health Study, SMHS, 2002–2006).

Settings: This population-based study was conducted in urban Shanghai, China.

Participants: A total of ~~218~~ 217 incident liver cancer cases diagnosed through December 31, ~~2011~~ 2009 and ~~436~~ 427 healthy controls matched by sex, age at baseline (±2 years) and date (±30 days) of sample collection were included in the study.

Primary and secondary outcome measures: Genetic polymorphisms of *NFKB1* and *NFKBIA* were determined by TaqMan SNP genotyping assay blindly. OR~~s~~ and its 95% CIs were estimated by unconditional logistic regression model to measure the association between selected SNPs and the risk of liver cancer.

Results: After adjusted for potential confounding factors, rs28362491 ins/del or del/del genotypes were associated with higher risk of liver cancer with an adjusted OR of 1.~~50~~54(95%CI: 1.~~02~~04-2.~~49~~28). rs230496 AG and GG genotypes were also noted with higher risk of liver cancer with an adjusted OR of 1.~~49~~ 53 (95%CI: 1.~~01~~03-2.~~21~~26). Haplotype analysis indicated that carriers of the *NFKB1* GA and AA (rs230525-rs230530) haplotypes had higher risk of liver cancer under additive model. No ~~assiciation~~ association was observed between *NFKBIA* variants and risk of live cancer.

Conclusions: Our results suggest that genetic variants of *NFKB1* influence liver cancer susceptibility in Chinese population, although replication in other studies is needed.

Article summary-strengths and limitations of this study

- This study was the first population-based study to evaluate the polymorphic variants of NF-κB and risk of liver cancer.
- Only incident cases from two prospective cohorts were included in the study which ruled out the possibility of recall and selection bias.

- The limitations of the study include relatively small sample size, unmeasured HBV infection, HCV infection and aflatoxin exposure. However, we did take into consideration of the participants' history of hepatitis and liver cirrhosis, and the presents of HCV infection and aflatoxin are very low in the study population.

INTRODUCTION

Liver cancer is a common disorder worldwide which ranks the 5th and 7th most common cancer among men and women. It was estimated that more than 80% liver cancers occur in developing countries and about 54% occur in China[1]. Among the main risk factors for liver cancer, chronic infections of hepatitis B virus (HBV) and hepatitis C virus (HCV) are the most important in humans, accounting for more than 70% of liver cancer cases worldwide[2-4]. Liver cirrhosis, heavy alcohol consumption, exposure to aflatoxin ~~B1~~, and diabetes also account for part of liver cancer occurrence[2-4].

Chronic inflammation has been widely accepted to play an important role in hepatocarcinogenesis. Most of the known risk factors of liver cancer such as HBV, HCV infection and alcohol drinking can cause persistent inflammatory reaction of the liver and promote cancer development[5, 6]. However, the molecular and cellular mechanisms linking inflammation and liver cancer remain unclear. Recent findings have suggested that NF-κB may play a crucial role in bridging the actions of growth factors and chronic inflammation to hepatic oncogenesis[7-10].

NF-κB, a collection of dimeric transcription factors, was originally identified as a nuclear factor ~~specific to B cells~~ bound to the ~~B site~~ enhancer of the immunoglobulin κ-light chain gene ~~enhancer~~[11] specific to B cells and presents in all cell types[12]. It is a major transcription regulator of the immune response, cell adhesion, differentiation, proliferation, and apoptosis[13]. NF-κB dimers are formed by seven distinct proteins: NF-κB1 (p105 and p50), NF-κB2 (p100 and p52), RelA (p65), RelB and c-Rel, of which NF-κB p50/RelA is the most common dimer form[9]. In the resting cell, most NF-κB dimers are inactivated in the cytoplasm by binding to specific inhabitors-IκB family, of which IκBα is the most common one. In the classical activation pathway, IκB is phosphorylated and degraded by IκB kinase complex, and then NF-κB dimers are released and ~~translocated~~translocate to the nucleus where they coordinate the transcriptional activation of target genes[14]. Several genetic variations of NF-κB signaling pathway have been reported to be associated with cancer risks such

as breast[15], prostate[16], stomach[17], colorectum[18] and mouth[19]. However, little is known about role of genetic polymorphisms of NF-κB genes and susceptibility of liver cancer.

In a population based case-control study nested in two prospective cohorts of the Shanghai Women's and Men's Health Studies, we investigated the relationships between genetic variants of *NFKB1* and *NFKBIA*, two key genes involved in classic signaling pathway of NF-κB, and the risk of ~~LIVER CANCER~~ liver cancer among Chinese men and women.

MATERIAL AND METHODS

Study population

Participants of this study came from the Shanghai Women's Health Study (SWHS) and Shanghai Men's Health Study (SMHS). The design and methods used in these two studies have been described in detail elsewhere [20-23]. Briefly, the SWHS enrolled 74,941 women aged 40-74 years between March 1, 1997 and May 31, 2000, with a response rate of 92.7%. SMHS enrolled 61,491 men aged 40-74 years without history of cancer at recruitment from April 1, 2002 to June 30, 2006, with a response rate of 74.1%. Both studies were approved by the relevant Institutional Review Boards for human research in China and the United States and a written informed consent was obtained from all participants.

In-person interview was conducted by trained interviewers using a structured questionnaire at baseline to obtain information on demographics, lifestyle, dietary habits, medical history and other characteristics. Anthropometric measurements, including current weight, height and circumferences of the waist and hips, were also measured. Of the eligible participants, 56,831 (75.8%) of the SWHS and 46,332 (75.3%) of the SMHS provided a 10-ml blood sample at baseline. The samples were drawn into an EDTA Vacutainer tube and then kept in a portable styrofoam box with ice packs (at approximately 0-4°C) and processed within 6 hours for long-term storage at -70°C. A bio-specimen collection form was completed for each participant at the time of sample procurement which included the date and time of collection, time of last meal, and date of last menstruation, intake of selected foods, smoking, as well as use of any medications over the previous 24 hours and during the previous week.

Cohort follow-up and outcome ascertainment

Both cohorts were followed for occurrence of cancer and other chronic diseases

by active in-person surveys conducted every 2-3 years as well as annual record linkage to the databases of the population-based Shanghai Cancer Registry, Shanghai Vital Statistics Registry, and Shanghai Resident Registry. For the SWHS, four rounds of in-person follow-ups were completed and the response rates for the first (2000-2002), second (2002-2004), third (2004-2007), and fourth (2008-2011) follow-up surveys were 99.8%, 98.7%, 96.7%, and 92.0%, respectively. For the SMHS, two rounds of follow-up surveys have completed. The response rates for the first (2004-2008) and second (2008-2011) follow-up surveys were 97.6% and 93.6%, respectively. For cohort members who developed liver cancer during the follow-up, medical chart were reviewed by a panel of oncologists to verify the diagnosis. Liver cancer data through December 31, ~~2011~~ 2009 was used for the present study.

Included in this nested case-control study are ~~218~~ 217 incident liver cancer cases and ~~436~~ 427 matched controls who had donated blood sample. Liver cancer cases were defined as having an International Classification of Disease, Ninth Revision (ICD-9), codes of 155.0 (primary malignant neoplasms), 155.1(malignant neoplasms of the intrahepatic bile ducts), or 155.2 (unspecified malignant neoplasms of the liver)[24]. Two control subjects were randomly selected from the cohorts who donated a blood sample at baseline and matched to each case for sex, age at baseline (±2 years) and date (±30 days) of sample collection. All controls were free of any cancer at the time of cancer diagnosis for the corresponding case.

Genotyping

Single-nucleotide polymorphisms (SNPs) were selected based on both TagSNP and their putative functional significance. Tagging SNPs were selected by searching the Han Chinese data from the Hapmap project[25]. The following criteria were used to identify tagging SNPs: (i) SNPs located in the genes or within the 5-kb flanking region, (ii) a minor allele frequency $\geqslant 0.05$, and (iii) other unselected single-nucleotide polymorphisms could be captured by one of the tagging SNPs with a linkage disequilibrium of $r^2 \geqslant 0.90$. A total of 8 SNPs were selected for genotyping which were rs28362491, rs230530, rs230525, rs230496 for *NFKB1* and rs3138053, rs3138055, rs2273650, rs696 for *NFKBIA* (table 1). Genomic DNA was extracted from buffycoat using Promega DNA Extraction Kit according to the manufacturer's instructions (Promega Corporation, Madison, WI, USA). Genotyping were performed by the TaqMan assay, using the ABI_PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA), in 384-wellformat, with dual

fluorescent reporter probes VIC and FAM. The quality and potential misclassification of the genotyping results were assessed by evaluating 5% of duplicate DNA samples that were randomly selected from the whole samples. There replicates were 100% concordant. All serum samples were tested blindly and were identified only by an unique identification number blinded with case-control status.

Table 1. Descriptions of Genetic Polymorphisms of the *NFKB1* and *NFKBIA* genes under investigation

Gene	Assay ID	Sequence	Location
NFKB1	rs28362491	CTCCGTGCTGCCTGCGTTCCCCGACC[-/ATTG]ATTGGGCCCGGCAGGCGCTTCCTGG	5'-near gene
	rs230530	TTTTTAGCACCAAACATCTTAATTT[A/G]CATTCAAATAAATGAGAACCACCAT	intron
	rs230525	TACGGGAAAAGTGATTCTTGTTTAC[A/G]GAGCCCTCTTTCACAGTTTCATGTT	intron
	rs230496	TGTCTGGATTTGCTTGAGACAGCCC[A/G]GTTTGCCCCTGACCTAATTGTTTAT	intron
NFKBIA	rs3138053	ATTCGTTTATGCTATCTGACCTACA[C/T]TGTGCTCCCGCAGAAAAAGGATCGT	5'-near gene
	rs3138055	AATCAACGGGATGACAGAATGACAA[C/T]GGAGAGGTCTCCAACCACAGGCCAA	3'-near gene
	rs2273650	AACAATACATTATGTACACCATTTA[C/T]AGGAGGGTAACACAAACCTTGACAG	3'-UTR
	rs696	CCTACCACAATAAGACGTTTTGGGC[C/T]AGGCAGTGTGCAGTGTGGATATAAG	3'-UTR

Statistical analysis

Subjects with both survey data and genotyping results were included in the final analysis. Means and percentages of selected characteristics for cases and controls were calculated. The distributions of selected characteristics were compared between cases and controls by either student's *t*-test (continuous variables) or χ^2 test (categorical variables). Odds ratio and its 95% confidence interval were estimated by unconditional logistic regression model to measure the association between selected SNPs and the risk of primary liver cancer. In the multivariable analysis, potential confounding factors were adjusted for, which include age (continuous variable); education level (four categories: elementary school or less, middle school, high school, and college or above); history of hepatitis (yes or no); family history of liver cancer (yes or no); and history of other chronic liver diseases or cirrhosis (yes or no). Statistical analyses were carried out using the SAS software package (version9.2; SAS Institute, Cary, NC). Tests for trend were performed by entering categorical variables as continuous variables in the regression model. All P values were calculated

by two-sided tests and were considered statistically significant if P was less than 0.05.

Hardy–Weinberg equilibrium and Linkage disequilibrium were accessed with HaploView version 4.0[26]. Associations between haplotypes and the risk of liver cancer were evaluated with HAPSTAT version 3.0 using the most common haplotype as the referent category, assumming an additive model[27].

RESULTS

Selected baseline characteristics of study participants were presented in table 2. The average ages of cases and control were 59.~~65~~ 61 and 59.~~48~~47. Compared with controls, liver cancer cases were more likely to have a lower education level, a history of hepatitis, a family history of liver cancer in first degree relatives, and history of chronic liver diseases or cirrhosis. Besides, male liver cancer cases were more probably to have lower body mass index, and be a non-regular exerciser compared to controls, although the difference were at borderline significance. Whereas in women, cases were more likely to have a history of type 2 diabetes than controls. No differences were observed in family income, smoking, drinking habits, waist to hip ratio, and family history of other cancers between the two groups.

The associations of *NFKB1* SNPs with liver cancer risk were summarized in table 3. The genotypes of rs28362491, rs230530 and rs230525 showed no deviation from Hardy-Weinberg equilibrium in controls except for rs230496. After adjusted for potential confounding factors, rs28362191 ins/del or del/del genotypes were associated with higher risk of liver cancer with an OR of 1.~~50~~54(95%CI: 1.~~02~~04-2.~~49~~28). rs230496 AG and GG genotypes were also noted with higher risk of liver cancer with an adjusted OR of 1.~~49~~ 53 (95%CI: 1.~~01~~03-2.~~21~~26). Carriers of rs230525 AG or GG genotypes had about 30% percent increased risk of liver cancer, but the risk was not insignificant. No association was found between rs230530 and liver cancer risk.

Table 4 presents the distribution of *NFKBIA* SNPs in cases and controls. The genotypes of rs3138055, rs696 and rs2273650 showed no deviation from Hardy-Weinberg equilibrium in controls but for rs3138053. Generally, all the four SNPs showed no relationship with liver cancer.

We further analyzed the haplotypes of these SNPs with risk of liver cancer (table 5). For *NFKB1* gene, two SNPs (rs230525, rs230530) demonstrated strong linkage disequilibrium (D' =1.0, r^2=0.~~58~~59). Compared to men carrying rs230525-rs230530 AG haplotype, those with GA or AA haplotypes were at increased risk of liver cancer with ORs of 1.46(95%CI: 1.05-2.03) and 1.81(95%CI: 1.15-2.86), respectively. For *NFKBIA*, rs3138053 and rs2273650 were in linkage disequilibrium (D' =0.97, r^2=0.31) but none of the haplotypes was significantly associated with liver cancer.

Table 2. Distribution of selected characteristics in the study cases and controls[†]

Characteristics	All subjects			Male			Female		
	Cases	Controls	*P*[*]	Cases	Controls	*P*[*]	Cases	Controls	*P*[*]
Age at interview, Mean±SD,	59.61±9.5659.6	59.47±9.5559.48±	0.8530.	60.05±9.9360.0	59.86±9.9559.86±9.	0.8580.	58.95±8.98	58.85±8.8758.93±	0.9280.
Education level (%)									
Elementary school or less	63(29.30)63(29.	115(27.00)120(27		18(13.95)18(13.	35(13.41)35(13.41)		45(52.33)45(5	80(48.48)85(48.8	
Middle school	69(32.09)70(32.	148(37.74)151(34		54(41.86)54(41.	104(39.85)104(39.8		15(17.44)16(1	44(26.67)47(27.0	
High school	62(28.84)62(28.	91(21.36)92(21.1		41(31.78)41(31.	61(23.37)61(23.37)		21(24.42)21(2	30(18.18)31(17.8	
College or above	21(9.77)21(9.72	72(16.90)72(16.5	0.0310.	16(12.40)16(12.	61(23.37)61(23.37)	0.0530.	5(5.81)5(5.75)	11(6.67)11(6.32)	0.3410.
Family income (%)[†]									
Low	50(23.04)50(22.	90(21.13)95(21.8		17(12.98)	37(14.12) 37(14.12)		33(38.37)33(3	53(32.32)58(33.5	
Medium	112(51.61)112(	208(48.83)211(48		76(58.02)	130(49.62)		36(41.86)36(4	78(47.56)81(46.8	
High	55(25.35)56(25.	128(30.05)129(29	0.4540.	38(29.01)	95(36.26) 95(36.25)	0.2710.	17(19.77)18(2	33(20.12)34(19.6	0.6060.
Ever smoked (%)	93(42.86)93(42.	173(40.52)174(39	0.5690.	90(68.70)90(68.	163(62.21)163(62.2	0.2060.	3(3.49)3(3.45)	10(6.06)11(6.32)	0.3840.
Ever drank alcohol (%)	45(20.74)45(20.	98(22.95)98(22.4	0.5230.	42(32.06)42(32.	97(37.02)97(37.02)	0.3330.	3(3.49)3(3.45)	1(0.61)1(0.57)	0.0840.
Body mass index ,kg/m^2, Mean±SD	23.79±3.6523.8	24.16±3.3124.16±	0.1980.	23.16±3.2523.1	23.77±2.8923.77±2.	0.0600	24.75±4.0224.	24.78±3.8024.74±	0.9610.
WHR, Mean±SD	0.87±0.070.87±	0.87±0.070.87±0.	0.9360.	0.90±0.060.90±	0.90±0.060.90±0.06	0.3790.	0.82±0.050.82	0.83±0.060.83±0.	0.2610.
Regular physical activity (%)	94(43.32)95(43.	207(48.48)211(48	0.2150.	49(37.40)49(37.	124(47.33)124(47.3	0.0620.	45(52.33)46(5	83(50.30)87(50.0	0.7610.
physical activity, MET-hours/week	81.58±47.1282.	83.71±43.5983.57	0.5700.	66.86±40.3366.	68.00±34.6168.00±	0.7807	104.00±48.09	108.60±44.83107.	0.4500.
History of hepatitis (%)	74(34.10)74(33.	25(5.85)25(5.73)	<0.001	57(43.51)57(43.	16(6.11)16(6.11)	<0.001	17(19.77)17(1	9(9.45)9(5.17)	<0.001
Family history of cancer (%)	69(31.80)69(31.	116(27.17)117(26	0.2200.	41(31.30)41(31.	70(26.72)70(26.72)	0.3420.	28(32.56)28(3	46(27.88)47(27.0	0.4410.
Family history of liver cancer (%)	28(12.90)28(12.	18(4.22)18(4.13)	<0.001	20(15.27)20(15.	10(3.82)10(3.82)	<0.001	8(9.30)8(9.20)	8(4.85)8(4.60)	0.1710.
History of type 2 diabetes (%)	25(11.52)26(11.	35(8.20)36(8.26)	0.1710.	14(10.69)14(10.	25(9.54)25(9.54)	0.7207	11(12.79)12(1	10(6.06)11(6.32)	0.0680.
History of chronic liver disease or	35(16.13)35(16.	11(2.58)11(2.52)	<0.001	26(19.85)26(19.	10(3.82)10(3.82)	<0.001	9(10.47)9(10.	1(0.61)1(0.57)	<0.001

* Missing data was excluded from the analysis

† Family income level (low income for <5000 yuan/year in the SWHS and <12 000 yuan/year in the SMHS; medium income for 5000 to <10 000 yuan/year in the SWHS and 12 000 to <24 000 yuan/year in the SMHS; and high income for >10 000 yuan/year in the SWHS and >24 000 yuan/year in the SMHS)

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Table 3. *NFKB1* genetic polymorphisms with the risk of primary liver cancer

SNPs	Cases	Controls	P for χ^2	OR*	95%CI	OR†	95%CI	OR‡	95%CI
rs28362491									
ins/ins	68	171		1.00	1.00	--	1.00	1.00	--	1.00	1.00	--
ins/del	102	160		1.60	1.55	1.10-2.33	1.07-2.25	1.60	1.55	1.10-2.33	1.07-2.25	1.71	1.64	1.13-2.60	1.09-2.49
del/del	40	79	0.047	0.069	1.27	1.28	0.79-2.05	0.80-2.05	1.27	1.28	0.79-2.04	0.80-2.05	1.21	1.21	0.71-2.05	0.71-2.04
P for trend				0.144	0.148			0.146	0.148			0.233	0.246		
ins/del or del/del	142	239	0.023	0.031	1.50	1.46	1.05-2.12	1.03-2.07	1.49	1.46	1.05-2.12	1.03-2.07	1.54	1.50	1.04-2.28	1.02-2.21
rs230496									
AA	64	164		1.00	1.00	--	1.00	1.00	--	1.00	1.00	--
AG	101	169		1.53	1.50	1.05-2.24	1.03-2.19	1.53	1.50	1.05-2.24	1.03-2.19	1.68	1.64	1.10-2.58	1.08-2.50
GG	47	91	0.087	0.106	1.33	1.32	0.84-2.09	0.84-2.07	1.32	1.31	0.84-2.09	0.84-2.07	1.25	1.24	0.75-2.09	0.74-2.05
P for trend				0.141	0.152			0.143	0.152			0.235	0.259		
AG or GG	148	260	0.041	0.041	1.46	1.44	1.03-2.08	1.01-2.04	1.46	1.44	1.03-2.08	1.01-2.04	1.53	1.49	1.03-2.26	1.01-2.21
rs230525									
AA	79	186		1.00	1.00	--	1.00	1.00	--	1.00	1.00	--
AG	102	175		1.38	1.34	0.96-1.97	0.94-1.92	1.38	1.35	0.96-1.98	0.94-1.92	1.46	1.42	0.98-2.18	0.95-2.11
GG	32	63	0.224	0.277	1.20	1.18	0.73-1.98	0.72-1.95	1.20	1.18	0.73-1.97	0.72-1.95	1.11	1.08	0.63-1.94	0.62-1.89

P for trend				0.236	0.2		0.236	0.2	
					68			58	
									0.347	0.
									402	
AG or GG	134	134	238	244	0.100	0.12	1.33	1.30	0.95-1.87	0.	1.33	1.30	0.95-1.87	0.	1.36	1.3	0.94-1.99	0.
					7				93-1.82				93-1.82		3		91-1.93	
rs230530																		
AA	64	64	114	116			1.00	1.00	--		1.00	1.00	--		1.00	1.0	--	
															0			
AG	99	99	175	181			1.01	0.99	0.68-1.49	0.	1.01	0.99	0.68-1.49	0.	1.05	1.0	0.68-1.62	0.
									67-1.46				67-1.47		3		67-1.59	
GG	48	49	129	130	0.102	0.15	0.66	0.68	0.42-1.04	0.	0.66	0.68	0.42-1.04	0.	0.67	0.7	0.40-1.12	0.
					2				44-1.07				44-1.07		0		42-1.15	
P for trend							0.079	0.1			0.078	0.1			0.132	0.		
															169			
AG or GG	147	148	304	311	0.423	0.42	0.86	0.86	0.60-1.24	0.	0.86	0.86	0.60-1.24	0.	0.89	0.8	0.59-1.34	0.
					6				60-1.24				60-1.24		9		60-1.34	

*Adjusted for age

† Adjusted for age, sex.

‡ Adjusted for age, sex, education level, family history of liver cancer, history of hepatitis, and chronic liver diseases or cirrhosis.

Table 4. *NFKBIA* genetic polymorphisms with the risk of primary liver cancer

SNPs	Cases	Controls	P	OR*		OR†		OR‡	
rs3138053									
AA	173	174	336	342	1.00	1.0	--	1.00	1.0	--	1.00	1.0	--
					0			0			0		
AG	21	21	48	51	0.85	0.8	0.49-1.47	0.	0.84	0.8	0.48-1.45	0.	0.97	0.9	0.54-1.74	0.
					1		47-1.39		0		47-1.39		2		51-1.64	
GG	19	19	40	40	0.823	0.7	0.92	0.9	0.52-1.64	0.	0.94	0.9	0.52-1.68	0.	0.98	0.9	0.51-1.88	0.
					36		3		53-1.66		4		53-1.68		8		51-1.89	
P for trend							0.638	0.			0.653	0.			0.920	0.		
							618				611				861			
AG or GG	40	40	88	91	0.556	0.4	0.88	0.8	0.58-1.34	0.	0.88	0.8	0.58-1.34	0.	0.97	0.9	0.61-1.54	0.
					87		6		57-1.31		6		57-1.31		4		60-1.49	

rs3138055										
CC	6263	128129		1.001.00	--	1.001.00	--	1.001.00	--	
CT	109109	215220		1.051.02	0.72-1.540.70-1.49	1.051.02	0.72-1.540.70-1.49	1.221.17	0.79-1.870.76-1.80	
TT	4242	8184	0.9560.995	1.071.02	0.66-1.730.63-1.65	1.071.02	0.66-1.730.63-1.65	1.331.26	0.78-2.270.74-2.14	
P for trend				0.7720.923		0.7710.923		0.2760.379		
CT or TT	151151	296304	0.7780.926	1.061.02	0.74-1.510.71-1.46	1.061.02	0.74-1.520.71-1.46	1.251.20	0.83-1.880.80-1.79	
rs696										
CC	6566	149150		1.001.00	--	1.001.00	--	1.001.00	--	
CT	115115	196202		1.351.30	0.93-1.960.90-1.88	1.351.30	0.93-1.960.90-1.88	1.471.41	0.97-2.230.93-2.13	
TT	3333	7678	0.2100.268	0.990.96	0.60-1.640.58-1.58	0.990.96	0.60-1.640.58-1.58	1.171.12	0.67-2.030.64-1.94	
P for trend				0.6940.822		0.6950.823		0.3600.459		
CT or TT	148148	272280	0.2180.304	1.251.20	0.88-1.780.85-1.71	1.251.20	0.88-1.780.85-1.71	1.381.33	0.93-2.060.89-1.97	
rs2273650										
CC	108108	215331		1.001.00	--	1.001.00	--	1.001.00	--	
CT	8485	173176		0.970.99	0.68-1.370.70-1.40	0.970.99	0.68-1.370.70-1.40	0.860.88	0.58-1.260.60-1.30	
TT	2020	3737	0.9380.934	1.081.11	0.60-1.950.61-1.99	1.071.11	0.59-1.940.61-2.00	0.890.92	0.45-1.730.47-1.79	
P for trend				0.9370.831		0.9450.830		0.4930.596		

CT or TT	104105	210213	0.9330.9 59	0.991.0 1	0.71-1.380. 73-1.41	0.991.0 1	0.71-1.370. 73-1.41	0.860.8 9	0.60-1.240. 62-1.28

*Adjusted for age

† Adjusted for age, sex.

‡ Adjusted for age, sex, education level, family history of liver cancer, history of hepatitis, and chronic liver diseases or cirrhosis.

Table 5. ORs and 95%CIs for liver cancer in relation to *NFKB1*/*NFKBIA* haplotypes

	All subjects*			Female†			Male†		
	Cases (%)	Controls (%)	OR	Cases (%)	Controls (%)	OR	Cases (%)	Controls (%)	OR
NFKB1(rs230525-rs230530)	n=215	n=425		n=86n=87	n=165n=174		n=129n=129	n=260n=260	
AG	46.4946.74	52.0151.85	ref/ref	52.1452.70	49.3949.13	ref/ref	42.6942.69	53.5353.53	ref/ref
GA	38.9738.79	35.5035.57	1.23(0.95-1.58)1.21(0.94-1.56)	36.4736.05	36.5936.71	0.94(0.63-1.41)0.92(0.62-1.36)	40.5540.55	34.8834.88	1.46(1.05-2.03)1.46(1.05-2.03)
AA	14.5414.47	12.4912.58	1.30(0.91-1.86)1.28(0.89-1.82)	11.3911.25	14.0214.16	0.77(0.42-1.39)0.74(0.41-1.33)	16.7616.76	11.5911.59	1.81(1.15-2.86)1.81(1.15-2.86)
NFKBIA(rs3138055-rs2273650)									
TC	45.0044.78	43.9444.30	ref/ref	48.2447.67	42.3143.28	ref/ref	43.1543.15	45.1445.14	ref/ref
CT	29.0529.15	28.5828.33	1.00(0.75-1.31)1.02(0.77-1.34)	31.3031.51	31.2130.46	0.88(0.57-1.35)0.94(0.61-1.44)	27.7927.79	27.1227.12	1.07(0.75-1.55)1.07(0.75-1.55)
CC	25.6525.76	27.0026.90	0.93(0.70-1.24)0.95(0.72-1.27)	20.4720.81	26.4826.26	0.68(0.42-1.10)0.72(0.45-1.15)	28.5028.50	26.9526.95	1.11(0.77-1.60)1.11(0.77-1.60)

* Adjusted for age and sex.

† Adjusted for age

DISCUSSION

In this nested case-control study, we found that the variants of rs28362491 and rs230496 of *NFKB1* gene might be associated with risk of primary liver cancer. After adjusting for possible confounders, rs28362491 deletion allelle and rs230496 AG or GG genotypes were found to increase the risk of liver cancer. In addition, haplotype analysis indicated that carriers of the *NFKB1* GA and AA (rs230525-rs230530) haplotypes had higher risk of liver cancer under additive model, although this association was only observed in man. These findings suggested that variants of NF-κB signaling pathway may play a role in liver cancer susceptibility.

NFKB1 gene was mapped on chromosome 4q23-q24 and composed of 24 exons[28]. This gene encodes for two proteins-p105 and p50-, p105 is a none-DNA binding protein and is activated to p50, a DNA binding protein by proteasome-mediated degradation. Several genetic polymorphisms were defined in *NFKB1* and researches have been focused on a common polymorphism of -94 del/ins (rs28362491) in the promoter region. Recent studies showed that genetic polymorphism of rs28362491 was associated with a number of cancer risks including sporadic breast cancer[15], prostate cancer[16], gastric cancer[17], colorectal cancer[18], and oral cancer[19], but little is known about its relationship with liver cancer. ~~Cao~~ He and his colleagues conducted a case-control study of 202 HCC cases of HBV carrier and 404 healthy controls without HBV infection. Results showed that after adjusting for age and gender, -94 ins/del and ins/ins genotypes might increase the risk of HCC, with ORs of 1.60 (95%CI:1.01-2.53) and 3.01 (95%CI:1.87-4.85), respectively[29]. A report from Taiwan also found ins allele more previlent in HCC patients (OR=2.23,95%CI:1.32-3.77)[30]. In our study, we found that ins/del and del/del genotypes were more prevalent in liver cancer cases than controls. It was observed that the association of rs28362491 polymorphism with cancer susceptibility varied with cancer site and study populations. Ins allele was reported to increase the risk of oral cancer[31], melanoma[32], prostate cancer[16], gastric cancer[17], nasopharyngeal carcinoma[33] and cervical cancer[34]. Two studies in European found del allele might increase the risk of colorectal cancer[35, 36], while in Chinese population, none or even reverse association were obtained [35, 37]. The difference of polymorphisms may probably result from interactions or combined effects with none genetic risk factors. Well-designed studies with larger sample size are needed to validate these findings.

To our knowledge, this is the first report on the variants of rs230496, rs230525 and rs230530 with liver cancer susceptibility. A study in European American descent found

rs230530 polymorphism associated with alcohol dependence, and the evidence came primarily from those individuals who met criteria for alcoholism earlier[38]. As alcohol is one of the major risk factors of liver cancer, rs230530 might play a role in alcohol associated liver cancer. Unfortunately, subject to the limitation of relatively small samplesize, we were not able to explore this issue. In addition, although the functions of intronic SNPs are still abscure, studies have indicated that they can affect either local DNA or RNA secondary structure, thereby regulating gene expression[39,40]

NFKBIA gene, which encodes IκBα, the inhibitor of *NFKB1*, was mapped to 14q13 with six exons spanning approximately 3.5kb[41~~39~~]. As a major component of IκB family, the dysfunction or down regulation of IκBα will lead to over activation of NF-κB. Epidemiological studies on *NFKBIA* were relatively rare. A 2758G/A polymorphysim (rs696) in 3' untranstated region might regulate the expression of IκBα and thus affect the activation of NF-κB. Sun et al. found the frequency of AG genotype was increased in Chinese patients ≥50 years of age (OR=3.06, 95% CI:1.55-6.02) with colo~~n~~rectal cancer[42~~40~~]. Another study on breast cancer fail to obtain a significant association[15]. There was no previous report on rs696 and risk of liver cancer.

Of the four SNPs of *NFKBIA* gene evaluated, we did not observed an significant association. In previous studies, rs3138053 variant was found to be associated with hepatocellular carcinoma in a Chinese mainland population[29] but not Taiwanese[30].

There are several strengths of our study. This study was based on two well-designed prospective cohort studies. To the best of our knowledge, it was the first population based study to evaluate the polymorphic variants of NF-κB and risk of liver cancer. All study participants were ethnic Chinese and residents of Shanghai with similar genetic background, which minimized the potential confounding of ethnics. Only incident cases were included which ruled out the possibility of recall and selection bias. Liver cancer cases were carefully verified with multiple approaches which minimized the disease misclassification. Also, we controlled potential confounding variables in the analysis. The limitations of our study should also be noted. Firstly, we focused on only two genes involved in canonical pathway of NF-κB, other regulatory genes in NF-κB signaling pathway may also contribute to the pathogenesis of liver cancer. Secondly, we did not test for HBV infection, HCV infection or aflatoxin exposure, so we cannot rule out the possible confoundings although the presents of HCV infection and aflatoxin are very low in the study population[43~~41~~], but we did take into consideration of the participants' history of hepatitis and liver cirrhosis. Finally, due

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to the relatively small sample size, the frequencies of some homozygous variants were low in subgroups therefore reduced the statistical power and limited us from evaluating the joint effects in stratified analysis. Replication in other studies is needed.

In summary, in this nested case-control study, we provided additional evidence for a role of NF-κB SNPs and haplotypes in the etiology of liver cancer. Studies in larger, varied polulations are warranted to confirm these findings. Furthermore, functional studies are required in order to explore the underling mechanisms.

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Contributors YBX obtained the funding, developed the research design, drafted the manuscript, interpreted the results, and also had primary responsibility for the final content; W Zheng and XOS designed, directed and obtained funding for the parent cohorts, and contributed to the revisions and interpretation of the results; JG obtained part of funding, drafted the manuscript, analyzed the data and interpreted the results; JG and HLX conducted experiments; All authors critically reviewed and approval manuscript.

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Competing interests None. The funding sponsor had no role in the study design, data collection, statistical analysis and result interpretation, as well as in the writing of the report and the decision to submit for publication. The corresponding author had full access to all data in the study and final responsibility for the decision to submit for publication.

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Ethics approval Vanderbilt University IRB and Shanghai Cancer Institute IRB.

Provenance and peer review Not commissioned; externally peer reviewed.

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References:

1. Ferlay J, Shin HR, Bray F, *et al*. GLOBOCAN 2008 v2.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10 [Internet]. Lyon, France: International Agency for Research on Cancer, 2010. Cited 28 Sep 2013. Available from URL: http://globocan.iarc.fr.

2. Gao J, Xie L, Yang WS, *et al*. Risk factors of hepatocellular carcinoma-current status and perspectives. *Asian Pac J Cancer Prev* 2012; 13: 743-52.

3. Schottenfeld D, Fraumeni JF. Cancer epidemiology and prevention. 3rd ed. Oxford: Oxford University Press, 2006.

4. Boyle P, Levin B. World cancer report 2008. Lyon: International Agency for Research on Cancer, 2008.

5. Seitz HK, Stickel F. Molecular mechanisms of alcohol-mediated carcinogenesis. *Nat Rev Cancer* 2007; 7: 599-612.

6. Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002; 420: 860-7.

7. Arsura M, Cavin LG. Nuclear factor-kappaB and liver carcinogenesis. *Cancer Lett* 2005; 229: 157-69.

8. Pikarsky E, Porat RM, Stein I, *et al*. NF-kappaB functions as a tumour promoter in inflammation-associated cancer. *Nature* 2004; 431: 461-6.

9. He G, Karin M. NF-kappaB and STAT3 - key players in liver inflammation and cancer. *Cell Res* 2011; 21: 159-68.

10. Berasain C, Castillo J, Perugorria MJ, *et al*. Inflammation and liver cancer: new molecular links. *Ann N Y Acad Sci* 2009; 1155: 206-21.

11. Sen R, Baltimore D. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell* 1986; 46: 705-16.

12. Barnes PJ, Karin M. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* 1997; 336: 1066-71.

13. Baldwin AJ. Series introduction: the transcription factor NF-kappaB and human disease. *J Clin Invest* 2001; 107: 3-6.

14. Karin M. Nuclear factor-kappaB in cancer development and progression. *Nature* 2006; 441: 431-6.

15. Curran JE, Weinstein SR, Griffiths LR. Polymorphic variants of NFKB1 and its inhibitory protein NFKBIA, and their involvement in sporadic breast cancer. *Cancer Lett* 2002; 188: 103-7.

16. Zhang P, Wei Q, Li X, *et al*. A functional insertion/deletion polymorphism in the promoter region of the NFKB1 gene increases susceptibility for prostate cancer. *Cancer Genet Cytogenet* 2009; 191:

73-7.

17. Lo SS, Chen JH, Wu CW, *et al*. Functional polymorphism of NFKB1 promoter may correlate to the susceptibility of gastric cancer in aged patients. *Surgery* 2009; 145: 280-5.

18. Yu Y, Liu H, Jin M, *et al*. The joint association of REST and NFKB1 polymorphisms on the risk of colorectal cancer. *Ann Hum Genet* 2012; 76: 269-76.

19. Lin CW, Hsieh YS, Hsin CH, *et al*. Effects of NFKB1 and NFKBIA gene polymorphisms on susceptibility to environmental factors and the clinicopathologic development of oral cancer. *PLoS One* 2012; 7: e35078.

20. Shu XO, Yang G, Jin F, *et al*. Validity and reproducibility of the food frequency questionnaire used in the Shanghai Women's Health Study. *Eur J Clin Nutr* 2004; 58: 17-23.

21. Zheng W, Chow WH, Yang G, *et al*. The Shanghai Women's Health Study: rationale, study design, and baseline characteristics. *Am J Epidemiol* 2005; 162: 1123-31.

22. Villegas R, Yang G, Liu D, *et al*. Validity and reproducibility of the food-frequency questionnaire used in the Shanghai men's health study. *Br J Nutr* 2007; 97: 993-1000.

23. Cai H, Zheng W, Xiang YB, *et al*. Dietary patterns and their correlates among middle-aged and elderly Chinese men: a report from the Shanghai Men's Health Study. *Br J Nutr* 2007; 98: 1006-13.

24. World Health Organization. International Classification of Diseases, ninth revision (ICD-9). Geneva, Switzerland: World Health Organization, 1977.

25. de Bakker PI, McVean G, Sabeti PC, *et al*. A high-resolution HLA and SNP haplotype map for disease association studies in the extended human MHC. *Nat Genet* 2006; 38: 1166-72.

26. Barrett JC, Fry B, Maller J, *et al*. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005; 21: 263-5.

27. Lin DY, Zeng D, Millikan R. Maximum likelihood estimation of haplotype effects and haplotype-environment interactions in association studies. *Genet Epidemiol* 2005; 29: 299-312.

28. Mathew S, Murty VV, Dalla-Favera R, *et al*. Chromosomal localization of genes encoding the transcription factors, c-rel, NF-kappa Bp50, NF-kappa Bp65, and lyt-10 by fluorescence in situ hybridization. *Oncogene* 1993; 8: 191-3.

29. He Y, Zhang H, Yin J, *et al*. IkappaBalpha gene promoter polymorphisms are associated with hepatocarcinogenesis in patients infected with hepatitis B virus genotype C. *Carcinogenesis* 2009; 30: 1916-22.

30. Cheng CW, Su JL, Lin CW, *et al*. Effects of NFKB1 and NFKBIA gene polymorphisms on hepatocellular carcinoma susceptibility and clinicopathological features. *PLoS One* 2013; 8: e56130.

31. Lin SC, Liu CJ, Yeh WI, *et al*. Functional polymorphism in NFKB1 promoter is related to the risks of oral squamous cell carcinoma occurring on older male areca (betel) chewers. *Cancer Lett* 2006; 243: 47-54.

32. Bu H, Rosdahl I, Sun XF, *et al*. Importance of polymorphisms in NF-kappaB1 and NF-kappaBIalpha genes for melanoma risk, clinicopathological features and tumor progression in Swedish melanoma patients. *J Cancer Res Clin Oncol* 2007; 133: 859-66.

33. Zhou B, Rao L, Li Y, *et al*. A functional insertion/deletion polymorphism in the promoter region of NFKB1 gene increases susceptibility for nasopharyngeal carcinoma. *Cancer Lett* 2009; 275: 72-6.

34. Zhou B, Qie M, Wang Y, *et al*. Relationship between NFKB1 -94 insertion/deletion ATTG

polymorphism and susceptibility of cervical squamous cell carcinoma risk. *Ann Oncol* 2010; 21: 506-11.

35. Lewander A, Butchi AK, Gao J, *et al*. Polymorphism in the promoter region of the NFKB1 gene increases the risk of sporadic colorectal cancer in Swedish but not in Chinese populations. *Scand J Gastroenterol* 2007; 42: 1332-8.

36. Andersen V, Christensen J, Overvad K, *et al*. Polymorphisms in NFkB, PXR, LXR and risk of colorectal cancer in a prospective study of Danes. *Bmc Cancer* 2010; 10: 484.

37. Song S, Chen D, Lu J, *et al*. NFkappaB1 and NFkappaBIA polymorphisms are associated with increased risk for sporadic colorectal cancer in a southern Chinese population. *PLoS One* 2011; 6: e21726.

38. Edenberg HJ, Xuei X, Wetherill LF, *et al*. Association of NFKB1, which encodes a subunit of the transcription factor NF-kappaB, with alcohol dependence. *Hum Mol Genet* 2008; 17: 963-70.

39. Lu L, Risch E, Deng Q, *et al*. An insulin-like growth factor-II intronic variant affects local DNA conformation and ovarian cancer survival. *Carcinogenesis* 2013;34:2024-30.

40. Lu L, Katsaros D, Mayne ST, *et al*. Functional study of risk loci of stem cell-associated gene lin-28B and associations with disease survival outcomes in epithelial ovarian cancer. *Carcinogenesis* 2012;33:2119-25.

~~39~~41. Le Beau MM, Ito C, Cogswell P, *et al*. Chromosomal localization of the genes encoding the p50/p105 subunits of NF-kappa B (NFKB2) and the I kappa B/MAD-3 (NFKBI) inhibitor of NF-kappa B to 4q24 and 14q13, respectively. *Genomics* 1992; 14: 529-31.

~~40~~42. Gao J, Pfeifer D, He LJ, *et al*. Association of NFKBIA polymorphism with colorectal cancer risk and prognosis in Swedish and Chinese populations. Scand J Gastroenterol. 2007; 42: 345-50.

~~41~~43. Yuan JM, Ross RK, Stanczyk FZ, *et al*. A cohort study of serum testosterone and hepatocellular carcinoma in Shanghai, China. *Int J Cancer* 1995; 63: 491-3.

STROBE Statement—Checklist of items that should be included in reports of ***cohort studies***

	Item No	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1-3
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2, 3
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3
Objectives	3	State specific objectives, including any prespecified hypotheses	4
Methods			
Study design	4	Present key elements of study design early in the paper	4, 5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	4, 5
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	4, 5
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	5
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	4-6
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	4-6
Bias	9	Describe any efforts to address potential sources of bias	4-6
Study size	10	Explain how the study size was arrived at	5
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	6
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	6
		(*b*) Describe any methods used to examine subgroups and interactions	N/A
		(*c*) Explain how missing data were addressed	9
		(*d*) If applicable, explain how loss to follow-up was addressed	4-5
		(*e*) Describe any sensitivity analyses	N/A
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	5, 9
		(b) Give reasons for non-participation at each stage	4-5
		(c) Consider use of a flow diagram	N/A
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	8, 9
		(b) Indicate number of participants with missing data for each variable of interest	9-12
		(c) Summarise follow-up time (eg, average and total amount)	4-5
Outcome data	15*	Report numbers of outcome events or summary measures over time	5

Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	6,7, 9-12
		(*b*) Report category boundaries when continuous variables were categorized	9
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	N/A
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	12
Discussion			
Key results	18	Summarise key results with reference to study objectives	13-15
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	14-15
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	15
Generalisability	21	Discuss the generalisability (external validity) of the study results	13, 14
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	15

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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Genetic polymorphism of NFKB1 and NFKBIA genes and liver cancer risk: a nested case-control study in Shanghai, China

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Genetic polymorphism of *NFKB1* and *NFKBIA* genes and liver cancer risk: a nested case-control study in Shanghai, China

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Tables: 5
Figures: 0
Supplemental tables: 0

ABSTRACT

Objectives: Genetic variations of NF-κB signaling pathway were found to be associated with inflammatory diseases and several malignancies. However, little is known about NF-κB pathway gene polymorphisms and susceptibility of liver cancer. The aim of this study was to investigate whether genetic variants of NFKB1 and NFKBIA were associated with risk of liver cancer in a Chinese population.

Design: The study was designed as a nested case-control study within two prospective cohorts (the Shanghai Women's Health Study, SWHS, 1996–2000 and the Shanghai Men's Health Study, SMHS, 2002–2006).

Settings: This population-based study was conducted in urban Shanghai, China.

Participants: A total of 217 incident liver cancer cases diagnosed through December 31, 2009 and 427 healthy controls matched by sex, age at baseline (±2 years) and date (±30 days) of sample collection were included in the study.

Primary and secondary outcome measures: Genetic polymorphisms of *NFKB1* and *NFKBIA* were determined by TaqMan SNP genotyping assay blindly. OR and its 95% CIs were estimated by unconditional logistic regression model to measure the association between selected SNPs and the risk of liver cancer.

Results: After adjusted for potential confounding factors, rs28362491 ins/del or del/del genotypes were associated with higher risk of liver cancer with an adjusted OR of 1.54(95%CI: 1.04-2.28). rs230496 AG and GG genotypes were also noted with higher risk of liver cancer with an adjusted OR of 1.53 (95%CI: 1.03-2.26). Haplotype analysis indicated that carriers of the *NFKB1* GA and AA (rs230525-rs230530) haplotypes had higher risk of liver cancer under additive model. No association was observed between *NFKBIA* variants and risk of live cancer.

Conclusions: Our results suggest that genetic variants of *NFKB1* influence liver cancer susceptibility in Chinese population, although replication in other studies is needed.

Article summary-strengths and limitations of this study

- This study was the first population-based study to evaluate the polymorphic variants of NF-κB and risk of liver cancer.
- Only incident cases from two prospective cohorts were included in the study which ruled out the possibility of recall and selection bias.
- The limitations of the study include relatively small sample size, unmeasured HBV infection, HCV infection and aflatoxin exposure. However, we did take into

consideration of the participants' history of hepatitis and liver cirrhosis, and the presents of HCV infection and aflatoxin are very low in the study population.

INTRODUCTION

Liver cancer is a common disorder worldwide which ranks the 5th and 7th most common cancer among men and women. It was estimated that more than 80% liver cancers occur in developing countries and about 54% occur in China[1]. Among the main risk factors for liver cancer, chronic infections of hepatitis B virus (HBV) and hepatitis C virus (HCV) are the most important in humans, accounting for more than 70% of liver cancer cases worldwide[2-4]. Liver cirrhosis, heavy alcohol consumption, exposure to aflatoxin, and diabetes also account for part of liver cancer occurrence[2-4].

Chronic inflammation has been widely accepted to play an important role in hepatocarcinogenesis. Most of the known risk factors of liver cancer such as HBV, HCV infection and alcohol drinking can cause persistent inflammatory reaction of the liver and promote cancer development[5, 6]. However, the molecular and cellular mechanisms linking inflammation and liver cancer remain unclear. Recent findings have suggested that NF-κB may play a crucial role in bridging the actions of growth factors and chronic inflammation to hepatic oncogenesis[7-10].

NF-κB, a collection of dimeric transcription factors, was originally identified as a nuclear factor bound to the enhancer of the immunoglobulin κ-light chain gene [11] specific to B cells and presents in all cell types[12]. It is a major transcription regulator of the immune response, cell adhesion, differentiation, proliferation, and apoptosis[13]. NF-κB dimers are formed by seven distinct proteins: NF-κB1 (p105 and p50), NF-κB2 (p100 and p52), RelA (p65), RelB and c-Rel, of which NF-κB p50/RelA is the most common dimer form[9]. In the resting cell, most NF-κB dimers are inactivated in the cytoplasm by binding to specific inhabitors-IκB family, of which IκBα is the most common one. In the classical activation pathway, IκB is phosphorylated and degraded by IκB kinase complex, and then NF-κB dimers are released and translocate to the nucleus where they coordinate the transcriptional activation of target genes[14]. Several genetic variations of NF-κB signaling pathway have been reported to be associated with cancer risks such as breast[15], prostate[16], stomach[17], colorectum[18] and mouth[19]. However, little is known about role of genetic polymorphisms of NF-κB genes and susceptibility of liver cancer.

In a population based case-control study nested in two prospective cohorts of the

Shanghai Women's and Men's Health Studies, we investigated the relationships between genetic variants of *NFKB1* and *NFKBIA*, two key genes involved in classic signaling pathway of NF-κB, and the risk of liver cancer among Chinese men and women.

MATERIAL AND METHODS

Study population

Participants of this study came from the Shanghai Women's Health Study (SWHS) and Shanghai Men's Health Study (SMHS). The design and methods used in these two studies have been described in detail elsewhere [20-23]. Briefly, the SWHS enrolled 74,941 women aged 40-74 years between March 1, 1997 and May 31, 2000, with a response rate of 92.7%. SMHS enrolled 61,491 men aged 40-74 years without history of cancer at recruitment from April 1, 2002 to June 30, 2006, with a response rate of 74.1%. Both studies were approved by the relevant Institutional Review Boards for human research in China and the United States and a written informed consent was obtained from all participants.

In-person interview was conducted by trained interviewers using a structured questionnaire at baseline to obtain information on demographics, lifestyle, dietary habits, medical history and other characteristics. Anthropometric measurements, including current weight, height and circumferences of the waist and hips, were also measured. Of the eligible participants, 56,831 (75.8%) of the SWHS and 46,332 (75.3%) of the SMHS provided a 10-ml blood sample at baseline. The samples were drawn into an EDTA Vacutainer tube and then kept in a portable styrofoam box with ice packs (at approximately 0-4°C) and processed within 6 hours for long-term storage at -70°C. A bio-specimen collection form was completed for each participant at the time of sample procurement which included the date and time of collection, time of last meal, and date of last menstruation, intake of selected foods, smoking, as well as use of any medications over the previous 24 hours and during the previous week.

Cohort follow-up and outcome ascertainment

Both cohorts were followed for occurrence of cancer and other chronic diseases by active in-person surveys conducted every 2-3 years as well as annual record linkage to the databases of the population-based Shanghai Cancer Registry, Shanghai Vital Statistics Registry, and Shanghai Resident Registry. For the SWHS, four rounds of in-person follow-ups were completed and the response rates for the first

(2000-2002), second (2002-2004), third (2004-2007), and fourth (2008-2011) follow-up surveys were 99.8%, 98.7%, 96.7%, and 92.0%, respectively. For the SMHS, two rounds of follow-up surveys have completed. The response rates for the first (2004-2008) and second (2008-2011) follow-up surveys were 97.6% and 93.6%, respectively. For cohort members who developed liver cancer during the follow-up, medical chart were reviewed by a panel of oncologists to verify the diagnosis. Liver cancer data through December 31, 2009 was used for the present study.

Included in this nested case-control study are 217 incident liver cancer cases and 427 matched controls who had donated blood sample. Liver cancer cases were defined as having an International Classification of Disease, Ninth Revision (ICD-9), codes of 155.0 (primary malignant neoplasms), 155.1(malignant neoplasms of the intrahepatic bile ducts), or 155.2 (unspecified malignant neoplasms of the liver)[24]. Two control subjects were randomly selected from the cohorts who donated a blood sample at baseline and matched to each case for sex, age at baseline (±2 years) and date (±30 days) of sample collection. All controls were free of any cancer at the time of cancer diagnosis for the corresponding case.

Genotyping

Single-nucleotide polymorphisms (SNPs) were selected based on both TagSNP and their putative functional significance. Tagging SNPs were selected by searching the Han Chinese data from the Hapmap project[25]. The following criteria were used to identify tagging SNPs: (i) SNPs located in the genes or within the 5-kb flanking region, (ii) a minor allele frequency $\geqslant 0.05$, and (iii) other unselected single-nucleotide polymorphisms could be captured by one of the tagging SNPs with a linkage disequilibrium of $r^2 \geqslant 0.90$. A total of 8 SNPs were selected for genotyping which were rs28362491, rs230530, rs230525, rs230496 for *NFKB1* and rs3138053, rs3138055, rs2273650, rs696 for *NFKBIA* (table 1). Genomic DNA was extracted from buffycoat using Promega DNA Extraction Kit according to the manufacturer's instructions (Promega Corporation, Madison, WI, USA). Genotyping were performed by the TaqMan assay, using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA), in 384-wellformat, with dual fluorescent reporter probes VIC and FAM. rs28362491 was genotyped using custom-designed probes and primers. The primer sequences were: 5'-GCCTCCGTGCTGCCT-3'(forward primer), 3'-AGGGAAGCCCCCAGGAA -5'(reverse primer). The probe sequences were: 5'-TTCCCCGACCATTGG-3' (del),

5'-CCGACCATTGATTGG-3' (ins). Other SNPs were genotyped using pre-designed assays (Applied biosystems). The quality and potential misclassification of the genotyping results were assessed by evaluating 5% of duplicate DNA samples that were randomly selected from the whole samples. There replicates were 100% concordant. All serum samples were tested blindly and were identified only by an unique identification number blinded with case-control status.

Table 1. Descriptions of Genetic Polymorphisms of the *NFKB1* and *NFKBIA* genes under investigation

Gene	Assay ID	Sequence	Location
NFKB1	rs28362491	CTCCGTGCTGCCTGCGTTCCCCGACC[-/ATTG]ATTGGGCC CGGCAGGCGCTTCCTGG	5'-near gene
	rs230530	TTTTTAGCACCAAACATCTTAATTT[A/G]CATTCAAATAAA TGAGAACCACCAT	intron
	rs230525	TACGGGAAAAGTGATTCTTGTTTAC[A/G]GAGCCCTCTTT CACAGTTTCATGTT	intron
	rs230496	TGTCTGGATTTGCTTGAGACAGCCC[A/G]GTTTGCCCCTG ACCTAATTGTTTAT	intron
NFKBIA	rs3138053	ATTCGTTTATGCTATCTGACCTACA[C/T]TGTGCTCCCGCA GAAAAAGGATCGT	5'-near gene
	rs3138055	AATCAACGGGATGACAGAATGACAA[C/T]GGAGAGGTCT CCAACCACAGGCCAA	3'-near gene
	rs2273650	AACAATACATTATGTACACCATTTA[C/T]AGGAGGGTAAC ACAAACCTTGACAG	3'-UTR
	rs696	CCTACCACAATAAGACGTTTTGGGC[C/T]AGGCAGTGTGC AGTGTGGATATAAG	3'-UTR

Statistical analysis

Subjects with both survey data and genotyping results were included in the final analysis. Means and percentages of selected characteristics for cases and controls were calculated. The distributions of selected characteristics were compared between cases and controls by either student's *t*-test (continuous variables) or χ^2 test (categorical variables). Odds ratio and its 95% confidence interval were estimated by unconditional logistic regression model to measure the association between selected SNPs and the risk of primary liver cancer. In the multivariable analysis, potential confounding factors were adjusted for, which include age (continuous variable); education level (four categories: elementary school or less, middle school, high school, and college or above); history of hepatitis (yes or no); family history of liver cancer (yes or no); and history of other chronic liver diseases or cirrhosis (yes or no). Statistical analyses were carried out using the SAS software package (version9.2; SAS Institute, Cary, NC). Tests for trend were performed by entering categorical

variables as continuous variables in the regression model. All P values were calculated by two-sided tests and were considered statistically significant if P was less than 0.05.

Hardy–Weinberg equilibrium and Linkage disequilibrium were accessed with HaploView version 4.0[26]. Associations between haplotypes and the risk of liver cancer were evaluated with HAPSTAT version 3.0 using the most common haplotype as the referent category, assumming an additive model[27].

RESULTS

Selected baseline characteristics of study participants were presented in table 2. The average ages of cases and control were 59.61 and 59.47. Compared with controls, liver cancer cases were more likely to have a lower education level, a history of hepatitis, a family history of liver cancer in first degree relatives, and history of chronic liver diseases or cirrhosis. Besides, male liver cancer cases were more probably to have lower body mass index, and be a non-regular exerciser compared to controls, although the difference were at borderline significance. Whereas in women, cases were more likely to have a history of type 2 diabetes than controls. No differences were observed in family income, smoking, drinking habits, waist to hip ratio, and family history of other cancers between the two groups.

The associations of *NFKB1* SNPs with liver cancer risk were summarized in table 3. The genotypes of rs28362491, rs230530 and rs230525 showed no deviation from Hardy-Weinberg equilibrium in controls except for rs230496. After adjusted for potential confounding factors, rs28362191 ins/del or del/del genotypes were associated with higher risk of liver cancer with an OR of 1.54(95%CI: 1.04-2.28). rs230496 AG and GG genotypes were also noted with higher risk of liver cancer with an adjusted OR of 1.53 (95%CI: 1.03-2.26). Carriers of rs230525 AG or GG genotypes had about 30% percent increased risk of liver cancer, but the risk was not insignificant. No association was found between rs230530 and liver cancer risk.

Table 4 presents the distribution of *NFKBIA* SNPs in cases and controls. The genotypes of rs3138055, rs696 and rs2273650 showed no deviation from Hardy-Weinberg equilibrium in controls but for rs3138053. Generally, all the four SNPs showed no relationship with liver cancer.

We further analyzed the haplotypes of these SNPs with risk of liver cancer (table 5). For *NFKB1* gene, two SNPs (rs230525, rs230530) demonstrated strong linkage disequilibrium (D' =1.0, r^2=0.59). Compared to men carrying rs230525-rs230530 AG haplotype, those with GA or AA haplotypes were at increased risk of liver cancer with ORs of 1.46(95%CI: 1.05-2.03) and 1.81(95%CI: 1.15-2.86) in significance, respectively. For *NFKBIA*, rs3138053 and rs2273650 were in linkage disequilibrium (D' =0.97, r^2=0.31) but none of the haplotypes was significantly associated with liver cancer.

Table 2. Distribution of selected characteristics in the study cases and controls[†]

Characteristics	All subjects			Male			Female		
	Cases (*N*=217)	Controls (*N*=427)	*P*	Cases (*N*=131)	Controls (*N*=262)	*P*	Cases (*N*=86)	Controls (*N*=165)	*P*
Age at interview, Mean±SD,	59.61±9.56	59.47±9.55	0.853	60.05±9.93	59.86±9.95	0.858	58.95±8.98	58.85±8.87	0.928
Education level (%)									
Elementary school or less	63(29.30)	115(27.00)		18(13.95)	35(13.41)		45(52.33)	80(48.48)	
Middle school	69(32.09)	148(37.74)		54(41.86)	104(39.85)		15(17.44)	44(26.67)	
High school	62(28.84)	91(21.36)		41(31.78)	61(23.37)		21(24.42)	30(18.18)	
College or above	21(9.77)	72(16.90)	0.031	16(12.40)	61(23.37)	0.053	5(5.81)	11(6.67)	0.341
Family income (%)[†]									
Low	50(23.04)	90(21.13)		17(12.98)	37(14.12)		33(38.37)	53(32.32)	
Medium	112(51.61)	208(48.83)		76(58.02)	130(49.62)		36(41.86)	78(47.56)	
High	55(25.35)	128(30.05)	0.454	38(29.01)	95(36.26)	0.271	17(19.77)	33(20.12)	0.606
Ever smoked (%)	93(42.86)	173(40.52)	0.569	90(68.70)	163(62.21)	0.206	3(3.49)	10(6.06)	0.384
Ever drank alcohol (%)	45(20.74)	98(22.95)	0.523	42(32.06)	97(37.02)	0.333	3(3.49)	1(0.61)	0.084
Body mass index ,kg/m^2, Mean±SD	23.79±3.65	24.16±3.31	0.198	23.16±3.25	23.77±2.89	0.06	24.75±4.02	24.78±3.80	0.961
WHR, Mean±SD	0.87±0.07	0.87±0.07	0.936	0.90±0.06	0.90±0.06	0.379	0.82±0.05	0.83±0.06	0.261
Regular physical activity (%)	94(43.32)	207(48.48)	0.215	49(37.40)	124(47.33)	0.062	45(52.33)	83(50.30)	0.761
physical activity, MET-hours/week	81.58±47.12	83.71±43.59	0.570	66.86±40.33	68.00±34.61	0.78	104.00±48.09	108.60±44.83	0.450
History of hepatitis (%)	74(34.10)	25(5.85)	<0.001	57(43.51)	16(6.11)	<0.001	17(19.77)	9(9.45)	<0.001
Family history of cancer (%)	69(31.80)	116(27.17)	0.220	41(31.30)	70(26.72)	0.342	28(32.56)	46(27.88)	0.441
Family history of liver cancer (%)	28(12.90)	18(4.22)	<0.001	20(15.27)	10(3.82)	<0.001	8(9.30)	8(4.85)	0.171
History of type 2 diabetes (%)	25(11.52)	35(8.20)	0.171	14(10.69)	25(9.54)	0.72	11(12.79)	10(6.06)	0.068
History of chronic liver disease or	35(16.13)	11(2.58)	<0.001	26(19.85)	10(3.82)	<0.001	9(10.47)	1(0.61)	<0.001

* Missing data was excluded from the analysis

[†] Family income level (low income for <5000 yuan/year in the SWHS and <12 000 yuan/year in the SMHS; medium income for 5000 to <10 000 yuan/year in the SWHS and 12 000 to <24 000 yuan/year in the SMHS; and high income for >10 000 yuan/year in the SWHS and >24 000 yuan/year in the SMHS)

Table 3. *NFKB1* genetic polymorphisms with the risk of primary liver cancer

SNPs	Cases	Controls	P for χ^2	OR*	95%CI	OR†	95%CI	OR‡	95%CI
rs28362491									
ins/ins	68	171		1.00	-	1.00	-	1.00	-
ins/del	102	160		1.60	1.10-2.33	1.60	1.10-2.33	1.71	1.13-2.60
del/del	40	79	0.047	1.27	0.79-2.05	1.27	0.79-2.04	1.21	0.71-2.05
P for trend				0.144		0.146		0.233	
ins/del or del/del	142	239	0.023	1.50	1.05-2.12	1.49	1.05-2.12	1.54	1.04-2.28
rs230496									
AA	64	164		1.00	-	1.00	-	1.00	-
AG	101	169		1.53	1.05-2.24	1.53	1.05-2.24	1.68	1.10-2.58
GG	47	91	0.087	1.33	0.84-2.09	1.32	0.84-2.09	1.25	0.75-2.09
P for trend				0.141		0.143		0.235	
AG or GG	148	260	0.041	1.46	1.03-2.08	1.46	1.03-2.08	1.53	1.03-2.26
rs230525									
AA	79	186		1.00	-	1.00	-	1.00	-
AG	102	175		1.38	0.96-1.97	1.38	0.96-1.98	1.46	0.98-2.18
GG	32	63	0.224	1.20	0.73-1.98	1.20	0.73-1.97	1.11	0.63-1.94
P for trend				0.236		0.236		0.347	
AG or GG	134	238	0.100	1.33	0.95-1.87	1.33	0.95-1.87	1.36	0.94-1.99
rs230530									
AA	64	114		1.00	-	1.00	-	1.00	-
AG	99	175		1.01	0.68-1.49	1.01	0.68-1.49	1.05	0.68-1.62
GG	48	129	0.102	0.66	0.42-1.04	0.66	0.42-1.04	0.67	0.40-1.12
P for trend				0.079		0.078		0.132	
AG or GG	147	304	0.423	0.86	0.60-1.24	0.86	0.60-1.24	0.89	0.59-1.34

*Adjusted for age

† Adjusted for age, sex.

‡ Adjusted for age, sex, education level, family history of liver cancer, history of hepatitis, and chronic liver diseases or cirrhosis.

Table 4. *NFKBIA* genetic polymorphisms with the risk of primary liver cancer

SNPs	Cases	Controls	P	OR*		OR†		OR‡	
rs3138053									
AA	173	336		1.00	-	1.00	-	1.00	-
AG	21	48		0.85	0.49-1.47	0.84	0.48-1.45	0.97	0.54-1.74
GG	19	40	0.823	0.92	0.52-1.64	0.94	0.52-1.68	0.98	0.51-1.88
P for trend				0.638		0.653		0.920	
AG or GG	40	88	0.556	0.88	0.58-1.34	0.88	0.58-1.34	0.97	0.61-1.54
rs3138055									
CC	62	128		1.00	-	1.00	-	1.00	-
CT	109	215		1.05	0.72-1.54	1.05	0.72-1.54	1.22	0.79-1.87
TT	42	81	0.956	1.07	0.66-1.73	1.07	0.66-1.73	1.33	0.78-2.27
P for trend				0.772		0.771		0.276	
CT or TT	151	296	0.778	1.06	0.74-1.51	1.06	0.74-1.52	1.25	0.83-1.88
rs696									
CC	65	149		1.00	-	1.00	-	1.00	-
CT	115	196		1.35	0.93-1.96	1.35	0.93-1.96	1.47	0.97-2.23
TT	33	76	0.210	0.99	0.60-1.64	0.99	0.60-1.64	1.17	0.67-2.03
P for trend				0.694		0.695		0.360	
CT or TT	148	272	0.218	1.25	0.88-1.78	1.25	0.88-1.78	1.38	0.93-2.06
rs2273650									
CC	108	215		1.00	-	1.00	-	1.00	-
CT	84	173		0.97	0.68-1.37	0.97	0.68-1.37	0.86	0.58-1.26
TT	20	37	0.938	1.08	0.60-1.95	1.07	0.59-1.94	0.89	0.45-1.73
P for trend				0.937		0.945		0.493	
CT or TT	104	210	0.933	0.99	0.71-1.38	0.99	0.71-1.37	0.86	0.60-1.24

*Adjusted for age

† Adjusted for age, sex.

‡ Adjusted for age, sex, education level, family history of liver cancer, history of hepatitis, and chronic liver diseases or cirrhosis.

Table 5. ORs and 95%CIs for liver cancer in relation to *NFKB1*/*NFKBIA* haplotypes

	All subjects*			Female†			Male†		
	Cases (%)	Controls (%)	OR	Cases (%)	Controls (%)	OR	Cases (%)	Controls (%)	OR
NFKB1(rs230525-rs230530)	n=215	n=425		n=86	n=165		n=129	n=260	
AG	46.49	52.01	ref	52.14	49.39	ref	42.69	53.53	ref
GA	38.973	35.50	1.23(0.95-1.58)	36.47	36.59	0.94(0.63-1.41)	40.55	34.88	1.46(1.05-2.03)
AA	14.54	12.49	1.30(0.91-1.86)	11.39	14.02	0.77(0.42-1.39)	16.76	11.59	1.81(1.15-2.86)
NFKBIA(rs3138055-rs2273650)									
TC	45.00	43.94	ref	48.24	42.31	ref	43.15	45.14	ref
CT	29.05	28.58	1.00(0.75-1.31)	31.30	31.21	0.88(0.57-1.35)	27.79	27.12	1.07(0.75-1.55)
CC	25.65	27.00	0.93(0.70-1.24)	20.47	26.48	0.68(0.42-1.10)	28.50	26.95	1.11(0.77-1.60)

* Adjusted for age and sex.

† Adjusted for age

DISCUSSION

In this nested case-control study, we found that the variants of rs28362491 and rs230496 of *NFKB1* gene might be associated with risk of primary liver cancer. After adjusting for possible confounders, rs28362491 deletion allelle and rs230496 AG or GG genotypes were found to increase the risk of liver cancer. In addition, haplotype analysis indicated that carriers of the *NFKB1* GA and AA (rs230525-rs230530) haplotypes had higher risk of liver cancer under additive model, although this association was only observed in man. These findings suggested that variants of NF-κB signaling pathway may play a role in liver cancer susceptibility.

NFKB1 gene was mapped on chromosome 4q23-q24 and composed of 24 exons[28]. This gene encodes p105 which is a none-DNA binding protein. As an inactive precursor, it was activated to p50, a DNA binding protein by proteasome-mediated degradation. Several genetic polymorphisms were defined in *NFKB1* and researches have been focused on a common polymorphism of -94 del/ins (rs28362491) in the promoter region. Recent studies showed that genetic polymorphism of rs28362491 was associated with a number of cancer risks including sporadic breast cancer[15], prostate cancer[16], gastric cancer[17], colorectal cancer[18], and oral cancer[19], but little is known about its relationship with liver cancer. He and his colleagues conducted a case-control study of 202 HCC cases of HBV carrier and 404 healthy controls without HBV infection. Results showed that after adjusting for age and gender, -94 ins/del and ins/ins genotypes might increase the risk of HCC, with ORs of 1.60 (95%CI:1.01-2.53) and 3.01 (95%CI:1.87-4.85), respectively[29]. A report from Taiwan also found ins allele more previlent in HCC patients (OR=2.23,95%CI:1.32-3.77)[30]. In our study, we found that ins/del and del/del genotypes were more prevalent in liver cancer cases than controls. It was observed that the association of rs28362491 polymorphism with cancer susceptibility varied with cancer site and study populations. Ins allele was reported to increase the risk of oral cancer[31], melanoma[32], prostate cancer[16], gastric cancer[17], nasopharyngeal carcinoma[33] and cervical cancer[34]. Two studies in European found del allele might increase the risk of colorectal cancer[35, 36], while in Chinese population, none or even reverse association were obtained [35, 37]. The difference of polymorphisms may probably result from interactions or combined effects with none genetic risk factors. Well-designed studies with larger sample size are needed to validate these findings.

To our knowledge, this is the first report on the variants of rs230496, rs230525 and rs230530 with liver cancer susceptibility. A study in European American descent found

rs230530 polymorphism associated with alcohol dependence, and the evidence came primarily from those individuals who met criteria for alcoholism earlier[38]. As alcohol is one of the major risk factors of liver cancer, rs230530 might play a role in alcohol associated liver cancer. Unfortunately, subject to the limitation of relatively small samplesize, we were not able to explore this issue. In addition, although the functions of intronic SNPs are still abscure, studies have indicated that they can affect either local DNA or RNA secondary structure, thereby regulating gene expression[39,40]

NFKBIA gene, which encodes IκBα, the inhibitor of *NFKB1*, was mapped to 14q13 with six exons spanning approximately 3.5kb[41]. As a major component of IκB family, the dysfunction or down regulation of IκBα will lead to over activation of NF-κB. Epidemiological studies on *NFKBIA* were relatively rare. A 2758G/A polymorphysim (rs696) in 3' untranstated region might regulate the expression of IκBα and thus affect the activation of NF-κB. Sun et al. found the frequency of AG genotype was increased in Chinese patients ≥50 years of age (OR=3.06, 95% CI:1.55-6.02) with colorectal cancer[42]. Another study on breast cancer fail to obtain a significant association[15]. There was no previous report on rs696 and risk of liver cancer.

Of the four SNPs of *NFKBIA* gene evaluated, we did not observed an significant association. In previous studies, rs3138053 variant was found to be associated with hepatocellular carcinoma in a Chinese mainland population[29] but not Taiwanese[30].

There are several strengths of our study. This study was based on two well-designed prospective cohort studies. To the best of our knowledge, it was the first population based study to evaluate the polymorphic variants of NF-κB and risk of liver cancer. All study participants were ethnic Chinese and residents of Shanghai with similar genetic background, which minimized the potential confounding of ethnics. Only incident cases were included which ruled out the possibility of recall and selection bias. Liver cancer cases were carefully verified with multiple approaches which minimized the disease misclassification. Also, we controlled potential confounding variables in the analysis. The limitations of our study should also be noted. Firstly, we focused on only two genes involved in canonical pathway of NF-κB, other regulatory genes in NF-κB signaling pathway may also contribute to the pathogenesis of liver cancer. Secondly, we did not test for HBV infection, HCV infection or aflatoxin exposure, so we cannot rule out the possible confoundings although the presents of HCV infection and aflatoxin are very low in the study population[43], but we did take into consideration of the participants' history of hepatitis and liver cirrhosis. Finally, due to

the relatively small sample size, the frequencies of some homozygous variants were low in subgroups therefore reduced the statistical power and limited us from evaluating the joint effects in stratified analysis. Replication in other studies is needed.

In summary, in this nested case-control study, we provided additional evidence for a role of NF-κB SNPs and haplotypes in the etiology of liver cancer. Studies in larger, varied polulations are warranted to confirm these findings. Furthermore, functional studies are required in order to explore the underling mechanisms.

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References:

1. Ferlay J, Shin HR, Bray F, *et al*. GLOBOCAN 2008 v2.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10 [Internet]. Lyon, France: International Agency for Research on Cancer, 2010. Cited 28 Sep 2013. Available from URL: http://globocan.iarc.fr.

2. Gao J, Xie L, Yang WS, *et al*. Risk factors of hepatocellular carcinoma-current status and perspectives. *Asian Pac J Cancer Prev* 2012; 13: 743-52.

3. Schottenfeld D, Fraumeni JF. Cancer epidemiology and prevention. 3rd ed. Oxford: Oxford University Press, 2006.

4. Boyle P, Levin B. World cancer report 2008. Lyon: International Agency for Research on Cancer, 2008.

5. Seitz HK, Stickel F. Molecular mechanisms of alcohol-mediated carcinogenesis. *Nat Rev Cancer* 2007; 7: 599-612.

6. Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002; 420: 860-7.

7. Arsura M, Cavin LG. Nuclear factor-kappaB and liver carcinogenesis. *Cancer Lett* 2005; 229: 157-69.

8. Pikarsky E, Porat RM, Stein I, *et al*. NF-kappaB functions as a tumour promoter in inflammation-associated cancer. *Nature* 2004; 431: 461-6.

9. He G, Karin M. NF-kappaB and STAT3 - key players in liver inflammation and cancer. *Cell Res* 2011; 21: 159-68.

10. Berasain C, Castillo J, Perugorria MJ, *et al*. Inflammation and liver cancer: new molecular links. *Ann N Y Acad Sci* 2009; 1155: 206-21.

11. Sen R, Baltimore D. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell* 1986; 46: 705-16.

12. Barnes PJ, Karin M. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* 1997; 336: 1066-71.

13. Baldwin AJ. Series introduction: the transcription factor NF-kappaB and human disease. *J Clin Invest* 2001; 107: 3-6.

14. Karin M. Nuclear factor-kappaB in cancer development and progression. *Nature* 2006; 441: 431-6.

15. Curran JE, Weinstein SR, Griffiths LR. Polymorphic variants of NFKB1 and its inhibitory protein NFKBIA, and their involvement in sporadic breast cancer. *Cancer Lett* 2002; 188: 103-7.

16. Zhang P, Wei Q, Li X, *et al*. A functional insertion/deletion polymorphism in the promoter region of the NFKB1 gene increases susceptibility for prostate cancer. *Cancer Genet Cytogenet* 2009; 191:

73-7.

17. Lo SS, Chen JH, Wu CW, *et al*. Functional polymorphism of NFKB1 promoter may correlate to the susceptibility of gastric cancer in aged patients. *Surgery* 2009; 145: 280-5.

18. Yu Y, Liu H, Jin M, *et al*. The joint association of REST and NFKB1 polymorphisms on the risk of colorectal cancer. *Ann Hum Genet* 2012; 76: 269-76.

19. Lin CW, Hsieh YS, Hsin CH, *et al*. Effects of NFKB1 and NFKBIA gene polymorphisms on susceptibility to environmental factors and the clinicopathologic development of oral cancer. *PLoS One* 2012; 7: e35078.

20. Shu XO, Yang G, Jin F, *et al*. Validity and reproducibility of the food frequency questionnaire used in the Shanghai Women's Health Study. *Eur J Clin Nutr* 2004; 58: 17-23.

21. Zheng W, Chow WH, Yang G, *et al*. The Shanghai Women's Health Study: rationale, study design, and baseline characteristics. *Am J Epidemiol* 2005; 162: 1123-31.

22. Villegas R, Yang G, Liu D, *et al*. Validity and reproducibility of the food-frequency questionnaire used in the Shanghai men's health study. *Br J Nutr* 2007; 97: 993-1000.

23. Cai H, Zheng W, Xiang YB, *et al*. Dietary patterns and their correlates among middle-aged and elderly Chinese men: a report from the Shanghai Men's Health Study. *Br J Nutr* 2007; 98: 1006-13.

24. World Health Organization. International Classification of Diseases, ninth revision (ICD-9). Geneva, Switzerland: World Health Organization, 1977.

25. de Bakker PI, McVean G, Sabeti PC, *et al*. A high-resolution HLA and SNP haplotype map for disease association studies in the extended human MHC. *Nat Genet* 2006; 38: 1166-72.

26. Barrett JC, Fry B, Maller J, *et al*. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005; 21: 263-5.

27. Lin DY, Zeng D, Millikan R. Maximum likelihood estimation of haplotype effects and haplotype-environment interactions in association studies. *Genet Epidemiol* 2005; 29: 299-312.

28. Mathew S, Murty VV, Dalla-Favera R, *et al*. Chromosomal localization of genes encoding the transcription factors, c-rel, NF-kappa Bp50, NF-kappa Bp65, and lyt-10 by fluorescence in situ hybridization. *Oncogene* 1993; 8: 191-3.

29. He Y, Zhang H, Yin J, *et al*. IkappaBalpha gene promoter polymorphisms are associated with hepatocarcinogenesis in patients infected with hepatitis B virus genotype C. *Carcinogenesis* 2009; 30: 1916-22.

30. Cheng CW, Su JL, Lin CW, *et al*. Effects of NFKB1 and NFKBIA gene polymorphisms on hepatocellular carcinoma susceptibility and clinicopathological features. *PLoS One* 2013; 8: e56130.

31. Lin SC, Liu CJ, Yeh WI, *et al*. Functional polymorphism in NFKB1 promoter is related to the risks of oral squamous cell carcinoma occurring on older male areca (betel) chewers. *Cancer Lett* 2006; 243: 47-54.

32. Bu H, Rosdahl I, Sun XF, *et al*. Importance of polymorphisms in NF-kappaB1 and NF-kappaBIalpha genes for melanoma risk, clinicopathological features and tumor progression in Swedish melanoma patients. *J Cancer Res Clin Oncol* 2007; 133: 859-66.

33. Zhou B, Rao L, Li Y, *et al*. A functional insertion/deletion polymorphism in the promoter region of NFKB1 gene increases susceptibility for nasopharyngeal carcinoma. *Cancer Lett* 2009; 275: 72-6.

34. Zhou B, Qie M, Wang Y, *et al*. Relationship between NFKB1 -94 insertion/deletion ATTG

polymorphism and susceptibility of cervical squamous cell carcinoma risk. *Ann Oncol* 2010; 21: 506-11.

35. Lewander A, Butchi AK, Gao J, *et al*. Polymorphism in the promoter region of the NFKB1 gene increases the risk of sporadic colorectal cancer in Swedish but not in Chinese populations. *Scand J Gastroenterol* 2007; 42: 1332-8.

36. Andersen V, Christensen J, Overvad K, *et al*. Polymorphisms in NFkB, PXR, LXR and risk of colorectal cancer in a prospective study of Danes. *Bmc Cancer* 2010; 10: 484.

37. Song S, Chen D, Lu J, *et al*. NFkappaB1 and NFkappaBIA polymorphisms are associated with increased risk for sporadic colorectal cancer in a southern Chinese population. *PLoS One* 2011; 6: e21726.

38. Edenberg HJ, Xuei X, Wetherill LF, *et al*. Association of NFKB1, which encodes a subunit of the transcription factor NF-kappaB, with alcohol dependence. *Hum Mol Genet* 2008; 17: 963-70.

39. Lu L, Risch E, Deng Q, *et al*. An insulin-like growth factor-II intronic variant affects local DNA conformation and ovarian cancer survival. *Carcinogenesis* 2013;34:2024-30.

40. Lu L, Katsaros D, Mayne ST, *et al*. Functional study of risk loci of stem cell-associated gene lin-28B and associations with disease survival outcomes in epithelial ovarian cancer. *Carcinogenesis* 2012;33:2119-25.

41. Le Beau MM, Ito C, Cogswell P, *et al*. Chromosomal localization of the genes encoding the p50/p105 subunits of NF-kappa B (NFKB2) and the I kappa B/MAD-3 (NFKBI) inhibitor of NF-kappa B to 4q24 and 14q13, respectively. *Genomics* 1992; 14: 529-31.

42. Gao J, Pfeifer D, He LJ, *et al*. Association of NFKBIA polymorphism with colorectal cancer risk and prognosis in Swedish and Chinese populations. Scand J Gastroenterol. 2007; 42: 345-50.

43. Yuan JM, Ross RK, Stanczyk FZ, *et al*. A cohort study of serum testosterone and hepatocellular carcinoma in Shanghai, China. *Int J Cancer* 1995; 63: 491-3.

Genetic polymorphism of *NFKB1* and *NFKBIA* genes and liver cancer risk: a nested case-control study in Shanghai, China

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ABSTRACT

Objectives: Genetic variations of NF-κB signaling pathway were found to be associated with inflammatory diseases and several malignancies. However, little is known about NF-κB pathway gene polymorphisms and susceptibility of liver cancer. The aim of this study was to investigate whether genetic variants of NFKB1 and NFKBIA were associated with risk of liver cancer in a Chinese population.

Design: The study was designed as a nested case-control study within two prospective cohorts (the Shanghai Women's Health Study, SWHS, 1996–2000 and the Shanghai Men's Health Study, SMHS, 2002–2006).

Settings: This population-based study was conducted in urban Shanghai, China.

Participants: A total of 217 incident liver cancer cases diagnosed through December 31, 2009 and 427 healthy controls matched by sex, age at baseline (±2 years) and date (±30 days) of sample collection were included in the study.

Primary and secondary outcome measures: Genetic polymorphisms of *NFKB1* and *NFKBIA* were determined by TaqMan SNP genotyping assay blindly. OR and its 95% CIs were estimated by unconditional logistic regression model to measure the association between selected SNPs and the risk of liver cancer.

Results: After adjusted for potential confounding factors, rs28362491 ins/del or del/del genotypes were associated with higher risk of liver cancer with an adjusted OR of 1.54(95%CI: 1.04-2.28). rs230496 AG and GG genotypes were also noted with higher risk of liver cancer with an adjusted OR of 1.53 (95%CI: 1.03-2.26). Haplotype analysis indicated that carriers of the *NFKB1* GA and AA (rs230525-rs230530) haplotypes had higher risk of liver cancer under additive model. No association was observed between *NFKBIA* variants and risk of live cancer.

Conclusions: Our results suggest that genetic variants of *NFKB1* influence liver cancer susceptibility in Chinese population, although replication in other studies is needed.

Article summary-strengths and limitations of this study

- This study was the first population-based study to evaluate the polymorphic variants of NF-κB and risk of liver cancer.
- Only incident cases from two prospective cohorts were included in the study which ruled out the possibility of recall and selection bias.
- The limitations of the study include relatively small sample size, unmeasured HBV infection, HCV infection and aflatoxin exposure. However, we did take into

consideration of the participants' history of hepatitis and liver cirrhosis, and the presents of HCV infection and aflatoxin are very low in the study population.

INTRODUCTION

Liver cancer is a common disorder worldwide which ranks the 5th and 7th most common cancer among men and women. It was estimated that more than 80% liver cancers occur in developing countries and about 54% occur in China[1]. Among the main risk factors for liver cancer, chronic infections of hepatitis B virus (HBV) and hepatitis C virus (HCV) are the most important in humans, accounting for more than 70% of liver cancer cases worldwide[2-4]. Liver cirrhosis, heavy alcohol consumption, exposure to aflatoxin, and diabetes also account for part of liver cancer occurrence[2-4].

Chronic inflammation has been widely accepted to play an important role in hepatocarcinogenesis. Most of the known risk factors of liver cancer such as HBV, HCV infection and alcohol drinking can cause persistent inflammatory reaction of the liver and promote cancer development[5, 6]. However, the molecular and cellular mechanisms linking inflammation and liver cancer remain unclear. Recent findings have suggested that NF-κB may play a crucial role in bridging the actions of growth factors and chronic inflammation to hepatic oncogenesis[7-10].

NF-κB, a collection of dimeric transcription factors, was originally identified as a nuclear factor bound to the enhancer of the immunoglobulin κ-light chain gene [11] specific to B cells and presents in all cell types[12]. It is a major transcription regulator of the immune response, cell adhesion, differentiation, proliferation, and apoptosis[13]. NF-κB dimers are formed by seven distinct proteins: NF-κB1 (p105 and p50), NF-κB2 (p100 and p52), RelA (p65), RelB and c-Rel, of which NF-κB p50/RelA is the most common dimer form[9]. In the resting cell, most NF-κB dimers are inactivated in the cytoplasm by binding to specific inhabitors-IκB family, of which IκBα is the most common one. In the classical activation pathway, IκB is phosphorylated and degraded by IκB kinase complex, and then NF-κB dimers are released and translocate to the nucleus where they coordinate the transcriptional activation of target genes[14]. Several genetic variations of NF-κB signaling pathway have been reported to be associated with cancer risks such as breast[15], prostate[16], stomach[17], colorectum[18] and mouth[19]. However, little is known about role of genetic polymorphisms of NF-κB genes and susceptibility of liver cancer.

In a population based case-control study nested in two prospective cohorts of the

Shanghai Women's and Men's Health Studies, we investigated the relationships between genetic variants of *NFKB1* and *NFKBIA*, two key genes involved in classic signaling pathway of NF-κB, and the risk of liver cancer among Chinese men and women.

MATERIAL AND METHODS

Study population

Participants of this study came from the Shanghai Women's Health Study (SWHS) and Shanghai Men's Health Study (SMHS). The design and methods used in these two studies have been described in detail elsewhere [20-23]. Briefly, the SWHS enrolled 74,941 women aged 40-74 years between March 1, 1997 and May 31, 2000, with a response rate of 92.7%. SMHS enrolled 61,491 men aged 40-74 years without history of cancer at recruitment from April 1, 2002 to June 30, 2006, with a response rate of 74.1%. Both studies were approved by the relevant Institutional Review Boards for human research in China and the United States and a written informed consent was obtained from all participants.

In-person interview was conducted by trained interviewers using a structured questionnaire at baseline to obtain information on demographics, lifestyle, dietary habits, medical history and other characteristics. Anthropometric measurements, including current weight, height and circumferences of the waist and hips, were also measured. Of the eligible participants, 56,831 (75.8%) of the SWHS and 46,332 (75.3%) of the SMHS provided a 10-ml blood sample at baseline. The samples were drawn into an EDTA Vacutainer tube and then kept in a portable styrofoam box with ice packs (at approximately 0-4°C) and processed within 6 hours for long-term storage at -70°C. A bio-specimen collection form was completed for each participant at the time of sample procurement which included the date and time of collection, time of last meal, and date of last menstruation, intake of selected foods, smoking, as well as use of any medications over the previous 24 hours and during the previous week.

Cohort follow-up and outcome ascertainment

Both cohorts were followed for occurrence of cancer and other chronic diseases by active in-person surveys conducted every 2-3 years as well as annual record linkage to the databases of the population-based Shanghai Cancer Registry, Shanghai Vital Statistics Registry, and Shanghai Resident Registry. For the SWHS, four rounds of in-person follow-ups were completed and the response rates for the first

(2000-2002), second (2002-2004), third (2004-2007), and fourth (2008-2011) follow-up surveys were 99.8%, 98.7%, 96.7%, and 92.0%, respectively. For the SMHS, two rounds of follow-up surveys have completed. The response rates for the first (2004-2008) and second (2008-2011) follow-up surveys were 97.6% and 93.6%, respectively. For cohort members who developed liver cancer during the follow-up, medical chart were reviewed by a panel of oncologists to verify the diagnosis. Liver cancer data through December 31, 2009 was used for the present study.

Included in this nested case-control study are 217 incident liver cancer cases and 427 matched controls who had donated blood sample. Liver cancer cases were defined as having an International Classification of Disease, Ninth Revision (ICD-9), codes of 155.0 (primary malignant neoplasms), 155.1(malignant neoplasms of the intrahepatic bile ducts), or 155.2 (unspecified malignant neoplasms of the liver)[24]. Two control subjects were randomly selected from the cohorts who donated a blood sample at baseline and matched to each case for sex, age at baseline (±2 years) and date (±30 days) of sample collection. All controls were free of any cancer at the time of cancer diagnosis for the corresponding case.

Genotyping

Single-nucleotide polymorphisms (SNPs) were selected based on both TagSNP and their putative functional significance. Tagging SNPs were selected by searching the Han Chinese data from the Hapmap project[25]. The following criteria were used to identify tagging SNPs: (i) SNPs located in the genes or within the 5-kb flanking region, (ii) a minor allele frequency $\geqslant 0.05$, and (iii) other unselected single-nucleotide polymorphisms could be captured by one of the tagging SNPs with a linkage disequilibrium of $r^2 \geqslant 0.90$. A total of 8 SNPs were selected for genotyping which were rs28362491, rs230530, rs230525, rs230496 for *NFKB1* and rs3138053, rs3138055, rs2273650, rs696 for *NFKBIA* (table 1). Genomic DNA was extracted from buffycoat using Promega DNA Extraction Kit according to the manufacturer's instructions (Promega Corporation, Madison, WI, USA). Genotyping were performed by the TaqMan assay, using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA), in 384-wellformat, with dual fluorescent reporter probes VIC and FAM.– rs28362491 was genotyped using custom-designed probes and primers. The primer sequences were: 5'-GCCTCCGTGCTGCCT-3'(forward primer), 3'-AGGGAAGCCCCCAGGAA-5'(reverse primer). The probe sequences were: 5'-TTCCCCGACCATTGG-3' (del),

5'-CCGACCATTGATTGG-3' (ins). Other SNPs were genotyped using pre-designed assays (Applied biosystems). The quality and potential misclassification of the genotyping results were assessed by evaluating 5% of duplicate DNA samples that were randomly selected from the whole samples. There replicates were 100% concordant. All serum samples were tested blindly and were identified only by an unique identification number blinded with case-control status.

Table 1. Descriptions of Genetic Polymorphisms of the *NFKB1* and *NFKBIA* genes under investigation

Gene	Assay ID	Sequence	Location
NFKB1	rs28362491	CTCCGTGCTGCCTGCGTTCCCCGACC[-/ATTG]ATTGGGCC CGGCAGGCGCTTCCTGG	5'-near gene
	rs230530	TTTTTAGCACCAAACATCTTAATTT[A/G]CATTCAAATAAA TGAGAACCACCAT	intron
	rs230525	TACGGGAAAAGTGATTCTTGTTTAC[A/G]GAGCCCTCTTT CACAGTTTCATGTT	intron
	rs230496	TGTCTGGATTTGCTTGAGACAGCCC[A/G]GTTTGCCCCTG ACCTAATTGTTTAT	intron
NFKBIA	rs3138053	ATTCGTTTATGCTATCTGACCTACA[C/T]TGTGCTCCCGCA GAAAAAGGATCGT	5'-near gene
	rs3138055	AATCAACGGGATGACAGAATGACAA[C/T]GGAGAGGTCT CCAACCACAGGCCAA	3'-near gene
	rs2273650	AACAATACATTATGTACACCATTTA[C/T]AGGAGGGTAAC ACAAACCTTGACAG	3'-UTR
	rs696	CCTACCACAATAAGACGTTTTGGGC[C/T]AGGCAGTGTGC AGTGTGGATATAAG	3'-UTR

Statistical analysis

Subjects with both survey data and genotyping results were included in the final analysis. Means and percentages of selected characteristics for cases and controls were calculated. The distributions of selected characteristics were compared between cases and controls by either student's *t*-test (continuous variables) or χ^2 test (categorical variables). Odds ratio and its 95% confidence interval were estimated by unconditional logistic regression model to measure the association between selected SNPs and the risk of primary liver cancer. In the multivariable analysis, potential confounding factors were adjusted for, which include age (continuous variable); education level (four categories: elementary school or less, middle school, high school, and college or above); history of hepatitis (yes or no); family history of liver cancer (yes or no); and history of other chronic liver diseases or cirrhosis (yes or no). Statistical analyses were carried out using the SAS software package (version9.2; SAS Institute, Cary, NC). Tests for trend were performed by entering categorical

variables as continuous variables in the regression model. All P values were calculated by two-sided tests and were considered statistically significant if P was less than 0.05.

Hardy–Weinberg equilibrium and Linkage disequilibrium were accessed with HaploView version 4.0[26]. Associations between haplotypes and the risk of liver cancer were evaluated with HAPSTAT version 3.0 using the most common haplotype as the referent category, assumming an additive model[27].

RESULTS

Selected baseline characteristics of study participants were presented in table 2. The average ages of cases and control were 59.61 and 59.47. Compared with controls, liver cancer cases were more likely to have a lower education level, a history of hepatitis, a family history of liver cancer in first degree relatives, and history of chronic liver diseases or cirrhosis. Besides, male liver cancer cases were more probably to have lower body mass index, and be a non-regular exerciser compared to controls, although the difference were at borderline significance. Whereas in women, cases were more likely to have a history of type 2 diabetes than controls. No differences were observed in family income, smoking, drinking habits, waist to hip ratio, and family history of other cancers between the two groups.

The associations of *NFKB1* SNPs with liver cancer risk were summarized in table 3. The genotypes of rs28362491, rs230530 and rs230525 showed no deviation from Hardy-Weinberg equilibrium in controls except for rs230496. After adjusted for potential confounding factors, rs28362191 ins/del or del/del genotypes were associated with higher risk of liver cancer with an OR of 1.54(95%CI: 1.04-2.28). rs230496 AG and GG genotypes were also noted with higher risk of liver cancer with an adjusted OR of 1.53 (95%CI: 1.03-2.26). Carriers of rs230525 AG or GG genotypes had about 30% percent increased risk of liver cancer, but the risk was not insignificant. No association was found between rs230530 and liver cancer risk.

Table 4 presents the distribution of *NFKBIA* SNPs in cases and controls. The genotypes of rs3138055, rs696 and rs2273650 showed no deviation from Hardy-Weinberg equilibrium in controls but for rs3138053. Generally, all the four SNPs showed no relationship with liver cancer.

We further analyzed the haplotypes of these SNPs with risk of liver cancer (table 5). For *NFKB1* gene, two SNPs (rs230525, rs230530) demonstrated strong linkage disequilibrium (D' =1.0, r^2=0.59). Compared to men carrying rs230525-rs230530 AG haplotype, those with GA or AA haplotypes were at increased risk of liver cancer with ORs of 1.46(95%CI: 1.05-2.03) and 1.81(95%CI: 1.15-2.86) in significance, respectively. For *NFKBIA*, rs3138053 and rs2273650 were in linkage disequilibrium (D' =0.97, r^2=0.31) but none of the haplotypes was significantly associated with liver cancer.

Table 2. Distribution of selected characteristics in the study cases and controls[†]

Characteristics	All subjects			Male			Female		
	Cases (*N*=217)	Controls (*N*=427)	*P*	Cases (*N*=131)	Controls (*N*=262)	*P*	Cases (*N*=86)	Controls (*N*=165)	*P*
Age at interview, Mean±SD,	59.61±9.56	59.47±9.55	0.853	60.05±9.93	59.86±9.95	0.858	58.95±8.98	58.85±8.87	0.928
Education level (%)									
Elementary school or less	63(29.30)	115(27.00)		18(13.95)	35(13.41)		45(52.33)	80(48.48)	
Middle school	69(32.09)	148(37.74)		54(41.86)	104(39.85)		15(17.44)	44(26.67)	
High school	62(28.84)	91(21.36)		41(31.78)	61(23.37)		21(24.42)	30(18.18)	
College or above	21(9.77)	72(16.90)	0.031	16(12.40)	61(23.37)	0.053	5(5.81)	11(6.67)	0.341
Family income (%)[†]									
Low	50(23.04)	90(21.13)		17(12.98)	37(14.12)		33(38.37)	53(32.32)	
Medium	112(51.61)	208(48.83)		76(58.02)	130(49.62)		36(41.86)	78(47.56)	
High	55(25.35)	128(30.05)	0.454	38(29.01)	95(36.26)	0.271	17(19.77)	33(20.12)	0.606
Ever smoked (%)	93(42.86)	173(40.52)	0.569	90(68.70)	163(62.21)	0.206	3(3.49)	10(6.06)	0.384
Ever drank alcohol (%)	45(20.74)	98(22.95)	0.523	42(32.06)	97(37.02)	0.333	3(3.49)	1(0.61)	0.084
Body mass index ,kg/m^2, Mean±SD	23.79±3.65	24.16±3.31	0.198	23.16±3.25	23.77±2.89	0.06	24.75±4.02	24.78±3.80	0.961
WHR, Mean±SD	0.87±0.07	0.87±0.07	0.936	0.90±0.06	0.90±0.06	0.379	0.82±0.05	0.83±0.06	0.261
Regular physical activity (%)	94(43.32)	207(48.48)	0.215	49(37.40)	124(47.33)	0.062	45(52.33)	83(50.30)	0.761
physical activity, MET-hours/week	81.58±47.12	83.71±43.59	0.570	66.86±40.33	68.00±34.61	0.78	104.00±48.09	108.60±44.83	0.450
History of hepatitis (%)	74(34.10)	25(5.85)	<0.001	57(43.51)	16(6.11)	<0.001	17(19.77)	9(9.45)	<0.001
Family history of cancer (%)	69(31.80)	116(27.17)	0.220	41(31.30)	70(26.72)	0.342	28(32.56)	46(27.88)	0.441
Family history of liver cancer (%)	28(12.90)	18(4.22)	<0.001	20(15.27)	10(3.82)	<0.001	8(9.30)	8(4.85)	0.171
History of type 2 diabetes (%)	25(11.52)	35(8.20)	0.171	14(10.69)	25(9.54)	0.72	11(12.79)	10(6.06)	0.068
History of chronic liver disease or	35(16.13)	11(2.58)	<0.001	26(19.85)	10(3.82)	<0.001	9(10.47)	1(0.61)	<0.001

* Missing data was excluded from the analysis

[†] Family income level (low income for <5000 yuan/year in the SWHS and <12 000 yuan/year in the SMHS; medium income for 5000 to <10 000 yuan/year in the SWHS and 12 000 to <24 000 yuan/year in the SMHS; and high income for >10 000 yuan/year in the SWHS and >24 000 yuan/year in the SMHS)

Table 3. *NFKB1* genetic polymorphisms with the risk of primary liver cancer

SNPs	Cases	Controls	P for χ^2	OR*	95%CI	OR†	95%CI	OR‡	95%CI
rs28362491									
ins/ins	68	171		1.00	-	1.00	-	1.00	-
ins/del	102	160		1.60	1.10-2.33	1.60	1.10-2.33	1.71	1.13-2.60
del/del	40	79	0.047	1.27	0.79-2.05	1.27	0.79-2.04	1.21	0.71-2.05
P for trend				0.144		0.146		0.233	
ins/del or del/del	142	239	0.023	1.50	1.05-2.12	1.49	1.05-2.12	1.54	1.04-2.28
rs230496									
AA	64	164		1.00	-	1.00	-	1.00	-
AG	101	169		1.53	1.05-2.24	1.53	1.05-2.24	1.68	1.10-2.58
GG	47	91	0.087	1.33	0.84-2.09	1.32	0.84-2.09	1.25	0.75-2.09
P for trend				0.141		0.143		0.235	
AG or GG	148	260	0.041	1.46	1.03-2.08	1.46	1.03-2.08	1.53	1.03-2.26
rs230525									
AA	79	186		1.00	-	1.00	-	1.00	-
AG	102	175		1.38	0.96-1.97	1.38	0.96-1.98	1.46	0.98-2.18
GG	32	63	0.224	1.20	0.73-1.98	1.20	0.73-1.97	1.11	0.63-1.94
P for trend				0.236		0.236		0.347	
AG or GG	134	238	0.100	1.33	0.95-1.87	1.33	0.95-1.87	1.36	0.94-1.99
rs230530									
AA	64	114		1.00	-	1.00	-	1.00	-
AG	99	175		1.01	0.68-1.49	1.01	0.68-1.49	1.05	0.68-1.62
GG	48	129	0.102	0.66	0.42-1.04	0.66	0.42-1.04	0.67	0.40-1.12
P for trend				0.079		0.078		0.132	
AG or GG	147	304	0.423	0.86	0.60-1.24	0.86	0.60-1.24	0.89	0.59-1.34

*Adjusted for age

† Adjusted for age, sex.

‡ Adjusted for age, sex, education level, family history of liver cancer, history of hepatitis, and chronic liver diseases or cirrhosis.

Table 4. *NFKBIA* genetic polymorphisms with the risk of primary liver cancer

SNPs	Cases	Controls	P	OR*		OR†		OR‡	
rs3138053									
AA	173	336		1.00	-	1.00	-	1.00	-
AG	21	48		0.85	0.49-1.47	0.84	0.48-1.45	0.97	0.54-1.74
GG	19	40	0.823	0.92	0.52-1.64	0.94	0.52-1.68	0.98	0.51-1.88
P for trend				0.638		0.653		0.920	
AG or GG	40	88	0.556	0.88	0.58-1.34	0.88	0.58-1.34	0.97	0.61-1.54
rs3138055									
CC	62	128		1.00	-	1.00	-	1.00	-
CT	109	215		1.05	0.72-1.54	1.05	0.72-1.54	1.22	0.79-1.87
TT	42	81	0.956	1.07	0.66-1.73	1.07	0.66-1.73	1.33	0.78-2.27
P for trend				0.772		0.771		0.276	
CT or TT	151	296	0.778	1.06	0.74-1.51	1.06	0.74-1.52	1.25	0.83-1.88
rs696									
CC	65	149		1.00	-	1.00	-	1.00	-
CT	115	196		1.35	0.93-1.96	1.35	0.93-1.96	1.47	0.97-2.23
TT	33	76	0.210	0.99	0.60-1.64	0.99	0.60-1.64	1.17	0.67-2.03
P for trend				0.694		0.695		0.360	
CT or TT	148	272	0.218	1.25	0.88-1.78	1.25	0.88-1.78	1.38	0.93-2.06
rs2273650									
CC	108	215		1.00	-	1.00	-	1.00	-
CT	84	173		0.97	0.68-1.37	0.97	0.68-1.37	0.86	0.58-1.26
TT	20	37	0.938	1.08	0.60-1.95	1.07	0.59-1.94	0.89	0.45-1.73
P for trend				0.937		0.945		0.493	
CT or TT	104	210	0.933	0.99	0.71-1.38	0.99	0.71-1.37	0.86	0.60-1.24

*Adjusted for age

† Adjusted for age, sex.

‡ Adjusted for age, sex, education level, family history of liver cancer, history of hepatitis, and chronic liver diseases or cirrhosis.

Table 5. ORs and 95%CIs for liver cancer in relation to *NFKB1*/*NFKBIA* haplotypes

	All subjects*			Female†			Male†		
	Cases (%)	Controls (%)	OR	Cases (%)	Controls (%)	OR	Cases (%)	Controls (%)	OR
NFKB1(rs230525-rs230530)	n=215	n=425		n=86	n=165		n=129	n=260	
AG	46.49	52.01	ref	52.14	49.39	ref	42.69	53.53	ref
GA	38.973	35.50	1.23(0.95-1.58)	36.47	36.59	0.94(0.63-1.41)	40.55	34.88	1.46(1.05-2.03)
AA	14.54	12.49	1.30(0.91-1.86)	11.39	14.02	0.77(0.42-1.39)	16.76	11.59	1.81(1.15-2.86)
NFKBIA(rs3138055-rs2273650)									
TC	45.00	43.94	ref	48.24	42.31	ref	43.15	45.14	ref
CT	29.05	28.58	1.00(0.75-1.31)	31.30	31.21	0.88(0.57-1.35)	27.79	27.12	1.07(0.75-1.55)
CC	25.65	27.00	0.93(0.70-1.24)	20.47	26.48	0.68(0.42-1.10)	28.50	26.95	1.11(0.77-1.60)

* Adjusted for age and sex.

† Adjusted for age

DISCUSSION

In this nested case-control study, we found that the variants of rs28362491 and rs230496 of *NFKB1* gene might be associated with risk of primary liver cancer. After adjusting for possible confounders, rs28362491 deletion allelle and rs230496 AG or GG genotypes were found to increase the risk of liver cancer. In addition, haplotype analysis indicated that carriers of the *NFKB1* GA and AA (rs230525-rs230530) haplotypes had higher risk of liver cancer under additive model, although this association was only observed in man. These findings suggested that variants of NF-κB signaling pathway may play a role in liver cancer susceptibility.

NFKB1 gene was mapped on chromosome 4q23-q24 and composed of 24 exons[28]. This gene encodes ~~for two proteins~~ p105 ~~and p50. p105~~ which is a none-DNA binding protein. As an inactive precursor, ~~and is~~it was activated to p50, a DNA binding protein by proteasome-mediated degradation. Several genetic polymorphisms were defined in *NFKB1* and researches have been focused on a common polymorphism of -94 del/ins (rs28362491) in the promoter region. Recent studies showed that genetic polymorphism of rs28362491 was associated with a number of cancer risks including sporadic breast cancer[15], prostate cancer[16], gastric cancer[17], colorectal cancer[18], and oral cancer[19], but little is known about its relationship with liver cancer. He and his colleagues conducted a case-control study of 202 HCC cases of HBV carrier and 404 healthy controls without HBV infection. Results showed that after adjusting for age and gender, -94 ins/del and ins/ins genotypes might increase the risk of HCC, with ORs of 1.60 (95%CI:1.01-2.53) and 3.01 (95%CI:1.87-4.85), respectively[29]. A report from Taiwan also found ins allele more previlent in HCC patients (OR=2.23,95%CI:1.32-3.77)[30]. In our study, we found that ins/del and del/del genotypes were more prevalent in liver cancer cases than controls. It was observed that the association of rs28362491 polymorphism with cancer susceptibility varied with cancer site and study populations. Ins allele was reported to increase the risk of oral cancer[31], melanoma[32], prostate cancer[16], gastric cancer[17], nasopharyngeal carcinoma[33] and cervical cancer[34]. Two studies in European found del allele might increase the risk of colorectal cancer[35, 36], while in Chinese population, none or even reverse association were obtained [35, 37]. The difference of polymorphisms may probably result from interactions or combined effects with none genetic risk factors. Well-designed studies with larger sample size are needed to validate these findings.

To our knowledge, this is the first report on the variants of rs230496, rs230525 and rs230530 with liver cancer susceptibility. A study in European American descent found

rs230530 polymorphism associated with alcohol dependence, and the evidence came primarily from those individuals who met criteria for alcoholism earlier[38]. As alcohol is one of the major risk factors of liver cancer, rs230530 might play a role in alcohol associated liver cancer. Unfortunately, subject to the limitation of relatively small samplesize, we were not able to explore this issue. In addition, although the functions of intronic SNPs are still abscure, studies have indicated that they can affect either local DNA or RNA secondary structure, thereby regulating gene expression[39,40]

NFKBIA gene, which encodes IκBα, the inhibitor of *NFKB1*, was mapped to 14q13 with six exons spanning approximately 3.5kb[41]. As a major component of IκB family, the dysfunction or down regulation of IκBα will lead to over activation of NF-κB. Epidemiological studies on *NFKBIA* were relatively rare. A 2758G/A polymorphysim (rs696) in 3' untranstated region might regulate the expression of IκBα and thus affect the activation of NF-κB. Sun et al. found the frequency of AG genotype was increased in Chinese patients ≥50 years of age (OR=3.06, 95% CI:1.55-6.02) with colorectal cancer[42]. Another study on breast cancer fail to obtain a significant association[15]. There was no previous report on rs696 and risk of liver cancer.

Of the four SNPs of *NFKBIA* gene evaluated, we did not observed an significant association. In previous studies, rs3138053 variant was found to be associated with hepatocellular carcinoma in a Chinese mainland population[29] but not Taiwanese[30].

There are several strengths of our study. This study was based on two well-designed prospective cohort studies. To the best of our knowledge, it was the first population based study to evaluate the polymorphic variants of NF-κB and risk of liver cancer. All study participants were ethnic Chinese and residents of Shanghai with similar genetic background, which minimized the potential confounding of ethnics. Only incident cases were included which ruled out the possibility of recall and selection bias. Liver cancer cases were carefully verified with multiple approaches which minimized the disease misclassification. Also, we controlled potential confounding variables in the analysis. The limitations of our study should also be noted. Firstly, we focused on only two genes involved in canonical pathway of NF-κB, other regulatory genes in NF-κB signaling pathway may also contribute to the pathogenesis of liver cancer. Secondly, we did not test for HBV infection, HCV infection or aflatoxin exposure, so we cannot rule out the possible confoundings although the presents of HCV infection and aflatoxin are very low in the study population[43], but we did take into consideration of the participants' history of hepatitis and liver cirrhosis. Finally, due to

the relatively small sample size, the frequencies of some homozygous variants were low in subgroups therefore reduced the statistical power and limited us from evaluating the joint effects in stratified analysis. Replication in other studies is needed.

In summary, in this nested case-control study, we provided additional evidence for a role of NF-κB SNPs and haplotypes in the etiology of liver cancer. Studies in larger, varied polulations are warranted to confirm these findings. Furthermore, functional studies are required in order to explore the underling mechanisms.

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Contributors YBX obtained the funding, developed the research design, drafted the manuscript, interpreted the results, and also had primary responsibility for the final content; W Zheng and XOS designed, directed and obtained funding for the parent cohorts, and contributed to the revisions and interpretation of the results; JG obtained part of funding, drafted the manuscript, analyzed the data and interpreted the results; JG and HLX conducted experiments; All authors critically reviewed and approval manuscript.

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Competing interests None. The funding sponsor had no role in the study design, data collection, statistical analysis and result interpretation, as well as in the writing of the report and the decision to submit for publication. The corresponding author had full access to all data in the study and final responsibility for the decision to submit for publication.

Study approval Institutional review board.

Ethics approval Vanderbilt University IRB and Shanghai Cancer Institute IRB.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement No additional data are available.

References:

1. Ferlay J, Shin HR, Bray F, *et al*. GLOBOCAN 2008 v2.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10 [Internet]. Lyon, France: International Agency for Research on Cancer, 2010. Cited 28 Sep 2013. Available from URL: http://globocan.iarc.fr.

2. Gao J, Xie L, Yang WS, *et al*. Risk factors of hepatocellular carcinoma-current status and perspectives. *Asian Pac J Cancer Prev* 2012; 13: 743-52.

3. Schottenfeld D, Fraumeni JF. Cancer epidemiology and prevention. 3rd ed. Oxford: Oxford University Press, 2006.

4. Boyle P, Levin B. World cancer report 2008. Lyon: International Agency for Research on Cancer, 2008.

5. Seitz HK, Stickel F. Molecular mechanisms of alcohol-mediated carcinogenesis. *Nat Rev Cancer* 2007; 7: 599-612.

6. Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002; 420: 860-7.

7. Arsura M, Cavin LG. Nuclear factor-kappaB and liver carcinogenesis. *Cancer Lett* 2005; 229: 157-69.

8. Pikarsky E, Porat RM, Stein I, *et al*. NF-kappaB functions as a tumour promoter in inflammation-associated cancer. *Nature* 2004; 431: 461-6.

9. He G, Karin M. NF-kappaB and STAT3 - key players in liver inflammation and cancer. *Cell Res* 2011; 21: 159-68.

10. Berasain C, Castillo J, Perugorria MJ, *et al*. Inflammation and liver cancer: new molecular links. *Ann N Y Acad Sci* 2009; 1155: 206-21.

11. Sen R, Baltimore D. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell* 1986; 46: 705-16.

12. Barnes PJ, Karin M. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* 1997; 336: 1066-71.

13. Baldwin AJ. Series introduction: the transcription factor NF-kappaB and human disease. *J Clin Invest* 2001; 107: 3-6.

14. Karin M. Nuclear factor-kappaB in cancer development and progression. *Nature* 2006; 441: 431-6.

15. Curran JE, Weinstein SR, Griffiths LR. Polymorphic variants of NFKB1 and its inhibitory protein NFKBIA, and their involvement in sporadic breast cancer. *Cancer Lett* 2002; 188: 103-7.

16. Zhang P, Wei Q, Li X, *et al*. A functional insertion/deletion polymorphism in the promoter region of the NFKB1 gene increases susceptibility for prostate cancer. *Cancer Genet Cytogenet* 2009; 191:

73-7.

17. Lo SS, Chen JH, Wu CW, *et al*. Functional polymorphism of NFKB1 promoter may correlate to the susceptibility of gastric cancer in aged patients. *Surgery* 2009; 145: 280-5.

18. Yu Y, Liu H, Jin M, *et al*. The joint association of REST and NFKB1 polymorphisms on the risk of colorectal cancer. *Ann Hum Genet* 2012; 76: 269-76.

19. Lin CW, Hsieh YS, Hsin CH, *et al*. Effects of NFKB1 and NFKBIA gene polymorphisms on susceptibility to environmental factors and the clinicopathologic development of oral cancer. *PLoS One* 2012; 7: e35078.

20. Shu XO, Yang G, Jin F, *et al*. Validity and reproducibility of the food frequency questionnaire used in the Shanghai Women's Health Study. *Eur J Clin Nutr* 2004; 58: 17-23.

21. Zheng W, Chow WH, Yang G, *et al*. The Shanghai Women's Health Study: rationale, study design, and baseline characteristics. *Am J Epidemiol* 2005; 162: 1123-31.

22. Villegas R, Yang G, Liu D, *et al*. Validity and reproducibility of the food-frequency questionnaire used in the Shanghai men's health study. *Br J Nutr* 2007; 97: 993-1000.

23. Cai H, Zheng W, Xiang YB, *et al*. Dietary patterns and their correlates among middle-aged and elderly Chinese men: a report from the Shanghai Men's Health Study. *Br J Nutr* 2007; 98: 1006-13.

24. World Health Organization. International Classification of Diseases, ninth revision (ICD-9). Geneva, Switzerland: World Health Organization, 1977.

25. de Bakker PI, McVean G, Sabeti PC, *et al*. A high-resolution HLA and SNP haplotype map for disease association studies in the extended human MHC. *Nat Genet* 2006; 38: 1166-72.

26. Barrett JC, Fry B, Maller J, *et al*. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005; 21: 263-5.

27. Lin DY, Zeng D, Millikan R. Maximum likelihood estimation of haplotype effects and haplotype-environment interactions in association studies. *Genet Epidemiol* 2005; 29: 299-312.

28. Mathew S, Murty VV, Dalla-Favera R, *et al*. Chromosomal localization of genes encoding the transcription factors, c-rel, NF-kappa Bp50, NF-kappa Bp65, and lyt-10 by fluorescence in situ hybridization. *Oncogene* 1993; 8: 191-3.

29. He Y, Zhang H, Yin J, *et al*. IkappaBalpha gene promoter polymorphisms are associated with hepatocarcinogenesis in patients infected with hepatitis B virus genotype C. *Carcinogenesis* 2009; 30: 1916-22.

30. Cheng CW, Su JL, Lin CW, *et al*. Effects of NFKB1 and NFKBIA gene polymorphisms on hepatocellular carcinoma susceptibility and clinicopathological features. *PLoS One* 2013; 8: e56130.

31. Lin SC, Liu CJ, Yeh WI, *et al*. Functional polymorphism in NFKB1 promoter is related to the risks of oral squamous cell carcinoma occurring on older male areca (betel) chewers. *Cancer Lett* 2006; 243: 47-54.

32. Bu H, Rosdahl I, Sun XF, *et al*. Importance of polymorphisms in NF-kappaB1 and NF-kappaBIalpha genes for melanoma risk, clinicopathological features and tumor progression in Swedish melanoma patients. *J Cancer Res Clin Oncol* 2007; 133: 859-66.

33. Zhou B, Rao L, Li Y, *et al*. A functional insertion/deletion polymorphism in the promoter region of NFKB1 gene increases susceptibility for nasopharyngeal carcinoma. *Cancer Lett* 2009; 275: 72-6.

34. Zhou B, Qie M, Wang Y, *et al*. Relationship between NFKB1 -94 insertion/deletion ATTG

polymorphism and susceptibility of cervical squamous cell carcinoma risk. *Ann Oncol* 2010; 21: 506-11.

35. Lewander A, Butchi AK, Gao J, *et al*. Polymorphism in the promoter region of the NFKB1 gene increases the risk of sporadic colorectal cancer in Swedish but not in Chinese populations. *Scand J Gastroenterol* 2007; 42: 1332-8.

36. Andersen V, Christensen J, Overvad K, *et al*. Polymorphisms in NFkB, PXR, LXR and risk of colorectal cancer in a prospective study of Danes. *Bmc Cancer* 2010; 10: 484.

37. Song S, Chen D, Lu J, *et al*. NFkappaB1 and NFkappaBIA polymorphisms are associated with increased risk for sporadic colorectal cancer in a southern Chinese population. *PLoS One* 2011; 6: e21726.

38. Edenberg HJ, Xuei X, Wetherill LF, *et al*. Association of NFKB1, which encodes a subunit of the transcription factor NF-kappaB, with alcohol dependence. *Hum Mol Genet* 2008; 17: 963-70.

39. Lu L, Risch E, Deng Q, *et al*. An insulin-like growth factor-II intronic variant affects local DNA conformation and ovarian cancer survival. *Carcinogenesis* 2013;34:2024-30.

40. Lu L, Katsaros D, Mayne ST, *et al*. Functional study of risk loci of stem cell-associated gene lin-28B and associations with disease survival outcomes in epithelial ovarian cancer. *Carcinogenesis* 2012;33:2119-25.

41. Le Beau MM, Ito C, Cogswell P, *et al*. Chromosomal localization of the genes encoding the p50/p105 subunits of NF-kappa B (NFKB2) and the I kappa B/MAD-3 (NFKBI) inhibitor of NF-kappa B to 4q24 and 14q13, respectively. *Genomics* 1992; 14: 529-31.

42. Gao J, Pfeifer D, He LJ, *et al*. Association of NFKBIA polymorphism with colorectal cancer risk and prognosis in Swedish and Chinese populations. Scand J Gastroenterol. 2007; 42: 345-50.

43. Yuan JM, Ross RK, Stanczyk FZ, *et al*. A cohort study of serum testosterone and hepatocellular carcinoma in Shanghai, China. *Int J Cancer* 1995; 63: 491-3.

STROBE Statement—Checklist of items that should be included in reports of ***cohort studies***

	Item No	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1-3
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2, 3
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3
Objectives	3	State specific objectives, including any prespecified hypotheses	4
Methods			
Study design	4	Present key elements of study design early in the paper	4, 5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	4, 5
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	4, 5
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	5
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	4-6
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	4-6
Bias	9	Describe any efforts to address potential sources of bias	4-6
Study size	10	Explain how the study size was arrived at	5
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	6
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	6
		(*b*) Describe any methods used to examine subgroups and interactions	N/A
		(*c*) Explain how missing data were addressed	9
		(*d*) If applicable, explain how loss to follow-up was addressed	4-5
		(*e*) Describe any sensitivity analyses	N/A
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	5, 9
		(b) Give reasons for non-participation at each stage	4-5
		(c) Consider use of a flow diagram	N/A
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	8, 9
		(b) Indicate number of participants with missing data for each variable of interest	9-12
		(c) Summarise follow-up time (eg, average and total amount)	4-5
Outcome data	15*	Report numbers of outcome events or summary measures over time	5

Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	6,7, 9-12
		(*b*) Report category boundaries when continuous variables were categorized	9
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	N/A
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	12
Discussion			
Key results	18	Summarise key results with reference to study objectives	13-15
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	14-15
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	15
Generalisability	21	Discuss the generalisability (external validity) of the study results	13, 14
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	15

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.