Am J Cancer Res 2015;5(1):396-410
www.ajcr.us /ISSN:2156-6976/ajcr0003378

Original Article

Epidermal growth factor receptor pathway polymorphisms and the prognosis of hepatocellular carcinoma

Wenjia Wang[1,2,3,4*], Xiao-Pin Ma[1*], Zhuqing Shi[1,2,3,4*], Pengyin Zhang[1,2,3,4], Dong-Lin Ding[1], Hui-Xing Huang[1], Hexi Ge Saiyin[1], Tao-Yang Chen[5], Pei-Xin Lu[5], Neng-Jin Wang[5], Hongjie Yu[1,2,3,4], Jielin Sun[6], S Lilly Zheng[6], Long Yu[1,7], Jianfeng Xu[1,2,3,4,8,9], De-Ke Jiang[1,2,3,4,6]

[1]State Key Laboratory of Genetic Engineering, Collaborative Innovation Center for Genetics and Development, School of Life Sciences, Fudan University, Shanghai, China; [2]Ministry of Education Key Laboratory of Contemporary Anthropology, School of Life Sciences, Fudan University, Shanghai, China; [3]Center for Genetic Epidemiology, School of Life Sciences, Fudan University, Shanghai, China; [4]Center for Genetic Translational Medicine and Prevention, School of Public Health, Fudan University, Shanghai, China; [5]Qidong Liver Cancer Institute, Qidong People's Hospital, Qidong, Jiangsu, China; [6]Center for Cancer Genomics, Wake Forest University School of Medicine, Winston-Salem, North Carolina, USA; [7]Institute of Biomedical Science, Fudan University, Shanghai, China; [8]Fudan Institute of Urology, Huashan Hospital, Fudan University, Shanghai, China; [9]Center for Genomic Cancer Research, North Shore University Health System, Evanston, IL, USA. [*]Equal contributors.

Received October 25, 2014; Accepted November 20, 2014; Epub December 15, 2014; Published January 1, 2015

Abstract: The EGFR signaling pathway is important in the control of vital processes in the carcinogenesis of hepatocellular carcinoma (HCC), including cell survival, cell cycle progression, tumor invasion and angiogenesis. In the current study, we aim to assess if genetic variants in the genes of the EGFR signaling pathway are associated with the prognosis of HCC. We genotyped 36 single nucleotide polymorphisms (SNP) in four core genes (EGF, EGFR, VEGF, and VEGFR2) by using DNA from blood samples of 363 HCC patients with surgical resection. The associations between genotypes and overall survival (OS) and disease-free survival (DFS) were estimated using the Kaplan-Meier method. Hazard ratios (HRs) and 95% confident intervals (CIs) were estimated for the multivariate survival analyses by Cox proportional hazards regression models, adjusting for age, gender, family history, HBsAg and AFP. We found that five SNPs in the VEGFR2 gene were significantly associated with clinical outcomes of HCC patients. Among them, four SNPs (rs7692791, rs2305948, rs13109660, rs6838752) were associated with OS (p=0.035, 0.038, 0.029 and 0.028, respectively), and two SNPs (rs7692791 and rs2034965) were associated with DFS (p=0.039 and 0.017, respectively). Particularly, rs7692791 TT genotype was associated with both reduced OS (p=0.037) and DFS (p=0.043). However, only one SNP rs2034965 with the AA genotype was shown to be an independent effect on DFS (p=0.009) in the multivariate analysis. None of the other 31 polymorphisms or 9 haplotypes attained from the four genes was significantly associated with OS or DFS. Our results illustrated the potential use of VEGFR2 polymorphisms as prognostic markers for HCC patients.

Keywords: Hepatocellular carcinoma, survival, EGF, EGFR, VEGF, VEGFR2, genetic polymorphisms

Introduction

Hepatocellular carcinoma (HCC) is diagnosed in more than half a million people worldwide every year, and it is the third leading cause of cancer-related deaths [1]. About half of these cases and deaths are from China, mainly because chronic hepatitis B carriers account for 10% of its population [2]. In 2008, estimated 748,300 new liver cancer cases and 695,900 cancer-related deaths occurred worldwide, making the incidence and mortality rates almost equal [1].

Multiple clinical factors, including large tumor size, positive portal vein thrombosis, increased serum AFP, and advanced TNM stage are involved in poor survival of HCC patients [3]. Although these factors can be used to predict prognosis, recurrence is observed in 77-100%

of the patients within 5 years and the 5-year overall survival (OS) rate remains poor, at around 50% [4, 5]. Therefore more useful predictive markers are required to identify high-risk patients, thus establishing more appropriate cancer management strategies and improving better clinical outcomes of HCC.

The epidermal growth factor receptor (EGFR) is a tyrosine kinase transmembrane receptor in the ErB family of receptors expressed on the surface of epithelial cells. EGFR regulates important processes in carcinogenesis, including cell survival, cell cycle progression, tumor invasion and angiogenesis [6]. Ligands including epidermal growth factor (EGF) bind to EGFR and they activate signal transduction pathways that upregulate transcription factors leading to proliferation and differentiation of epidermal and epithelial tissue [7]. A few studies have suggested that genetic variants in the EGF and EGFR gene are associated with EGFR amplifications and contribute to cancer risk and prognosis [8-14].

Activation of the EGFR pathway also leads to the up-regulation of the ligand vascular endothelial growth factor (VEGF) and its receptor (VEGFR2) on endothelial cells, thus stimulating angiogenesis and vascular permeability. VEGF is considered to be a key mediator of both physiological and pathological angiogenesis. Angiogenesis, a process involving the growth of new blood vessels, is an important step in the development of cancer and plays a critical role in the primary tumor growth, invasiveness, and metastasis [15]. Several studies have reported the association between VEGF expression and worse prognosis in various cancers [16-19]. As for HCC, results from several reports have indicated that VEGF plays an important role in the development of HCC [20-22], and elevated serum level of VEGF is considered as an independent marker of HCC survival [23, 24]. Furthermore, genetic variability of VEGF and VEGFR2 may affect the risk and outcome of various kinds of cancers regulated by angiogenesis [25], and polymorphisms in the VEGF gene have the predictive value on the risk of HCC patients [26, 27].

These findings indicate that genetic variations of EGFR and its ligand EGF, VEGF and its ligand VEGFR2 probably affect HCC prognosis, but to our knowledge, the influence of genetic polymorphisms in the EGFR signaling pathway on the clinical outcomes of HCC patients have not been investigated extensively [28, 29]. Therefore, the goal of the present study was to determine whether inherited variations in four core genes of the EGFR signaling pathway (EGF, EGFR, VEGF and VEGFR2) modified HCC survival.

Materials and methods

Patients and samples collection

A total of 363 Han Chinese patients newly diagnosed with HCC and receiving surgical resection of HCC tumor were recruited by the Qidong Liver Cancer Institute in Qidong, Jiangsu province, China from April 1996 to September 2009. The clinical outcomes of HCC were recorded until October 2014, with a median follow-up time of 53.0 months (range 2-110 months). The diagnosis of HCC was based on histopathological examination and the National Comprehensive Cancer Network (NCCN) clinical practice guidelines in oncology. All tumors were proven to be HCC by two pathologists. All patients had no other cancers as determined by initial screening examination and were followed up prospectively every 3 months from the time of enrollment by personal or family contacts until death or last time of follow-up.

There were no recruitment restrictions on age, gender and tumor stage. 5 ml whole blood for each subject was extracted. Clinical information was collected at the time the blood specimens were collected from medical records with patients' consent. The histologic grade of tumor differentiation was assigned by the Edmondson grading system. The clinical typing of tumors was determined according to the TNM classification system of International Union Against Cancer (edition 6). The study endpoints were overall survival (OS), and disease-free survival (DFS). OS was calculated from the date of pathologic diagnosis/recruitment to death regardless of the cause or the end of available follow-up. DFS was defined as the time from pathologic diagnosis/recruitment to disease recurrence, metastasis, disease specific death or last follow-up.

This study was approved by the Department of Scientific Research of Fudan University and the Qidong Liver Cancer Institute, and a written informed consent with a signature was obtained from each patient before enrollment.

Table 1. SNPs selected in 4 EGFR pathway genes for analysis

Gene	SNP	Allelic change	MAF in Chinese	MAF in this study
EGF	rs3756261	A/G	0.239	0.228
EGF	rs11568835	A/G	0.068	0.127
EGF	rs4444903	A/G	0.367	0.450
EGF	rs11568943	A/T	0.244	0.219
EGF	rs2237051	A/G	0.378	0.291
EGF	rs11569017	A/G	0.256	0.229
EGF	rs3733625	A/G	0.239	0.204
EGFR	rs6965469	C/T	0.167	0.167
EGFR	rs4947492	A/G	0.419	0.351
EGFR	rs2227983	A/G	0.397	0.478
EGFR	rs11977388	A/C	0.464	0.370
EGFR	rs2293347	G/A	0.222	0.301
EGFR	rs884225	G/A	0.467	0.480
VEGF	rs699947	C/A	0.278	0.266
VEGF	rs833061	C/T	0.278	0.288
VEGF	rs2010963	G/C	0.333	0.470
VEGF	rs1413711	C/T	0.278	0.255
VEGF	rs833070	A/G	0.192	0.242
VEGF	rs3025000	C/T	0.309	0.445
VEGF	rs3025033	A/G	0.178	0.216
VEGF	rs3025035	C/T	0.200	0.156
VEGF	rs3025039	C/T	0.178	0.208
VEGF	rs10434	A/G	0.189	0.212
VEGFR2	rs2071559	C/T	0.300	0.335
VEGFR2	rs7667298	C/T	0.284	0.337
VEGFR2	rs1531290	A/G	0.233	0.273
VEGFR2	rs6837735	C/T	0.362	0.411
VEGFR2	rs7692791	T/C	0.411	0.398
VEGFR2	rs2305948	C/T	0.156	0.141
VEGFR2	rs2034965	A/G	0.178	0.221
VEGFR2	rs1870377	A/T	0.467	0.459
VEGFR2	rs13109660	G/A	0.278	0.297
VEGFR2	rs6838752	C/T	0.438	0.467
VEGFR2	rs17709898	A/G	0.389	0.303
VEGFR2	rs1531289	G/A	0.200	0.213
VEGFR2	rs7691507	T/C	0.156	0.182

MAF: Minor allele frequency.

SNP selection

To select all the potential functional SNPs of EGF, EGFR, VEGF, and VEGFR2, we utilized the International HapMap Project database (http://hapmap.ncbi.nlm.nih.gov/) [30], and dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) [31] to search for candidate variants in the promoter region, all exons including intron–exon boundaries and the 3'-untranslated region (3'-UTR). We identified 20 potential functional polymorphisms (**Table 1**). We selected tagging SNPs from 5-kb flanking and within the gene regions of four genes by using the tagger algorithm [32]. 21 tagging SNPs were identified with a cut-off value of r^2<0.8 and a minor allele frequency greater than 0.1 in the Chinese population, based on data from the HapMap Project (http://hapmap.ncbi.nlm.nih.gov/) [32]. In addition, functional SNPs and SNPs previously reported to be associated with cancer were also included. Finally, a total of 36 SNPs, including haplotype-tagging SNPs and potential functional SNPs, were selected for genotyping (**Table 1**).

DNA extraction, genotyping, and haplotypes reconstruction

Genomic DNA was extracted from blood samples using the QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany). Genotyping was performed with Sequenom MassARRAY iPLEX platform by use of allele-specific MALDI-TOF mass spectrometry assay. Polymerase chain reaction (PCR) and extension primers for these 36 SNPs were designed using the MassARRAY Assay Design 3.0 software (Sequenom). PCR and extension reactions were performed according to the manufacturer's instructions, and extension product sizes were determined by mass spectrometry using the Sequenom iPLEX system. Duplicate test samples and two water samples (PCR negative controls) that were blinded to the technician were included in each 96-well plate. Genotyping quality was examined by a detailed QC procedure consisting of >95% successful call rate, duplicate calling of genotypes, internal positive control samples.

The linkage disequilibrium (LD) status among SNPs was measured with Lewontin D and r^2 by using the Haploview software package (http://www.broad.mit.edu/mpg/haploview). LD blocks were inferred from the definition proposed by Gabriel and colleagues [30]. Probable haplo-

Table 2. Correlations between clinicopathologic features and prognosis of HCC patients

Characteristics	No. of patients	No. of events	Median survival time (95% CI)	Log-rank p	
				OS	DFS
Total	363	229	34.0 (27.4-40.6)		
Age (year)				0.157	0.281
≤50	187	123	30.0 (23.7-36.3)		
<50	176	106	42.0 (32.5-51.5)		
Gender				0.806	0.993
Female	63	40	38.0 (18.6-57.4)		
Male	300	189	33.0 (25.9-40.1)		
Family history				0.834	0.866
Absent	263	163	35.0 (29.6-40.4)		
Present	81	54	39.0 (21.1-56.9)		
Unknown	19	12			
HBsAg				0.996	0.939
Negative	58	38	20.0 (0.000-41.4)		
Positive	304	190	36.0 (29.5-42.5)		
Unknown	1	1			
AFP				0.009	0.007
Negative	142	79	51.0 (34.0-68.0)		
Positive	215	144	28.0 (21.5-34.5)		
Unknown	6	6			
Tumor size (cm)				0.029	0.046
≤5	145	86	46.0 (35.6-56.4)		
>5	153	92	24.0 (14.7-33.4)		
Unknown	65	51			
Differentiation				0.011	0.011
I+II	167	89	46.0 (33.36-58.64)		
III+IV	128	89	30.0 (22.52-37.48)		
Unknown	68	51			
Tumor capsule				0.001	0.001
Absent	144	98	26.0 (14.4-37.6)		
Present	146	77	47.0 (30.4-63.6)		
Unknown	73	54			
Cirrhosis				0.114	0.044
Absent	93	50	40.0 (13.3-66.7)		
Present	199	126	33.0 (26.9-39.1)		
Unknown	71	53			
Venous invasion				0.041	0.078
Absent	199	119	39.0 (30.0-48.0)		
Present	77	48	23.0 (16.0-30.0)		
Unknown	87	62			
HB history				0.684	0.604
Absent	148	92	35.0 (27.1-42.9)		
Present	113	65	30.0 (20.3-39.7)		
Unknown	102	72			
Tumor number				0.491	0.442
Solitary	210	123	37.0 (26.3-42.7)		
Multiple	65	41	31.0 (19.2-42.8)		
Unknown	88	65			
pTNM stage				0.001	<0.001
I	70	29	57.0 (33.2-80.8)		
II	152	97	31.0 (23.3-39.9)		
III	24	19	19.0 (0.000-44.3)		
IV	12	11	6.0 (0.000-17.9)		
Unknown	105	73			

CI: Confidence interval; OS: Overall survival; DFS: Disease-free survival.

Table 3. Influence of SNPs in 4 EGFR pathway genes on prognosis of HCC patients

Gene	SNP	Log-rank p for OS			Log-rank p for DFS		
		General	Dominant	Recessive	General	Dominant	Recessive
EGF	rs3756261	0.653	0.446	0.772	0.494	0.261	0.951
EGF	rs11568835	0.966	0.865	0.87	0.852	0.914	0.571
EGF	rs4444903	0.437	-	-	0.23	-	-
EGF	rs11568943	0.621	0.636	0.496	0.512	0.372	0.652
EGF	rs2237051	0.327	0.955	0.172	0.401	0.52	0.18
EGF	rs11569017	0.713	0.716	0.537	0.699	0.493	0.759
EGF	rs3733625	0.294	0.536	0.215	0.258	0.317	0.304
EGFR	rs6965469	0.969	0.831	0.935	0.883	0.817	0.631
EGFR	rs4947492	0.776	0.484	0.925	0.995	0.977	0.937
EGFR	rs2227983	0.493	0.332	0.795	0.69	0.642	0.62
EGFR	rs11977388	0.602	0.586	0.573	0.737	0.976	0.464
EGFR	rs2293347	0.371	0.176	0.975	0.356	0.152	0.793
EGFR	rs884225	0.435	0.631	0.198	0.345	0.611	0.793
VEGF	rs699947	0.169	0.885	0.085	0.182	0.838	0.097
VEGF	rs833061	0.277	0.622	0.109	0.296	0.736	0.123
VEGF	rs2010963	0.373	0.989	0.19	0.274	0.728	0.174
VEGF	rs1413711	0.231	0.716	0.09	0.251	0.794	0.103
VEGF	rs833070	0.23	0.937	0.114	0.271	0.911	0.139
VEGF	rs3025000	0.436	0.795	0.274	0.362	0.6	0.286
VEGF	rs3025033	0.896	0.657	0.99	0.865	0.677	0.845
VEGF	rs3025035	0.291	0.75	0.16	0.344	0.819	0.182
VEGF	rs3025039	0.891	0.923	0.673	0.815	0.844	0.602
VEGF	rs10434	0.267	0.206	0.55	0.452	0.382	0.538
VEGFR2	rs2071559	0.437	0.352	0.584	0.282	0.331	0.369
VEGFR2	rs7667298	0.33	0.245	0.607	0.214	0.236	0.386
VEGFR2	rs1531290	0.134	0.361	0.137	0.289	0.451	0.261
VEGFR2	rs6837735	0.788	0.51	0.994	0.679	0.406	0.972
VEGFR2	rs7692791	0.101	0.035	0.338	0.112	0.039	0.346
VEGFR2	rs2305948	0.038	0.025	0.132	0.163	0.078	0.331
VEGFR2	rs2034965	0.103	0.091	0.082	0.017	0.027	0.019
VEGFR2	rs1870377	0.113	0.34	0.163	0.141	0.134	0.498
VEGFR2	rs13109660	0.092	0.46	0.029	0.222	0.554	0.083
VEGFR2	rs6838752	0.028	0.263	0.066	0.063	0.123	0.272
VEGFR2	rs17709898	0.811	0.519	0.865	0.572	0.291	0.691
VEGFR2	rs1531289	0.384	0.341	0.219	0.118	0.164	0.066
VEGFR2	rs7691507	0.751	0.452	0.794	0.259	0.24	0.171

OS: Overall survival; DFS: Disease-free survival.

types and their frequencies were calculated on the basis of a Bayesian algorithm [33] using PHASE software (ver 2.1.1, Seattle, WA, USA).

Statistical analysis

Associations of genotypes and haplotypes with OS and DFS were estimated using the Kaplan-Meier method, and statistical significance was determined using the log-rank test. The most significant test among the 3 genetic models (general, dominant, and recessive) was used to determine the statistical significance of each SNP. The SNPs or haplotypes with a raw *p*-value <0.05 in the univariable analysis were included in the multivariate analysis to evaluate their effects on the clinical outcomes. Hazard ratios (HRs) and 95% confident intervals (CIs) were

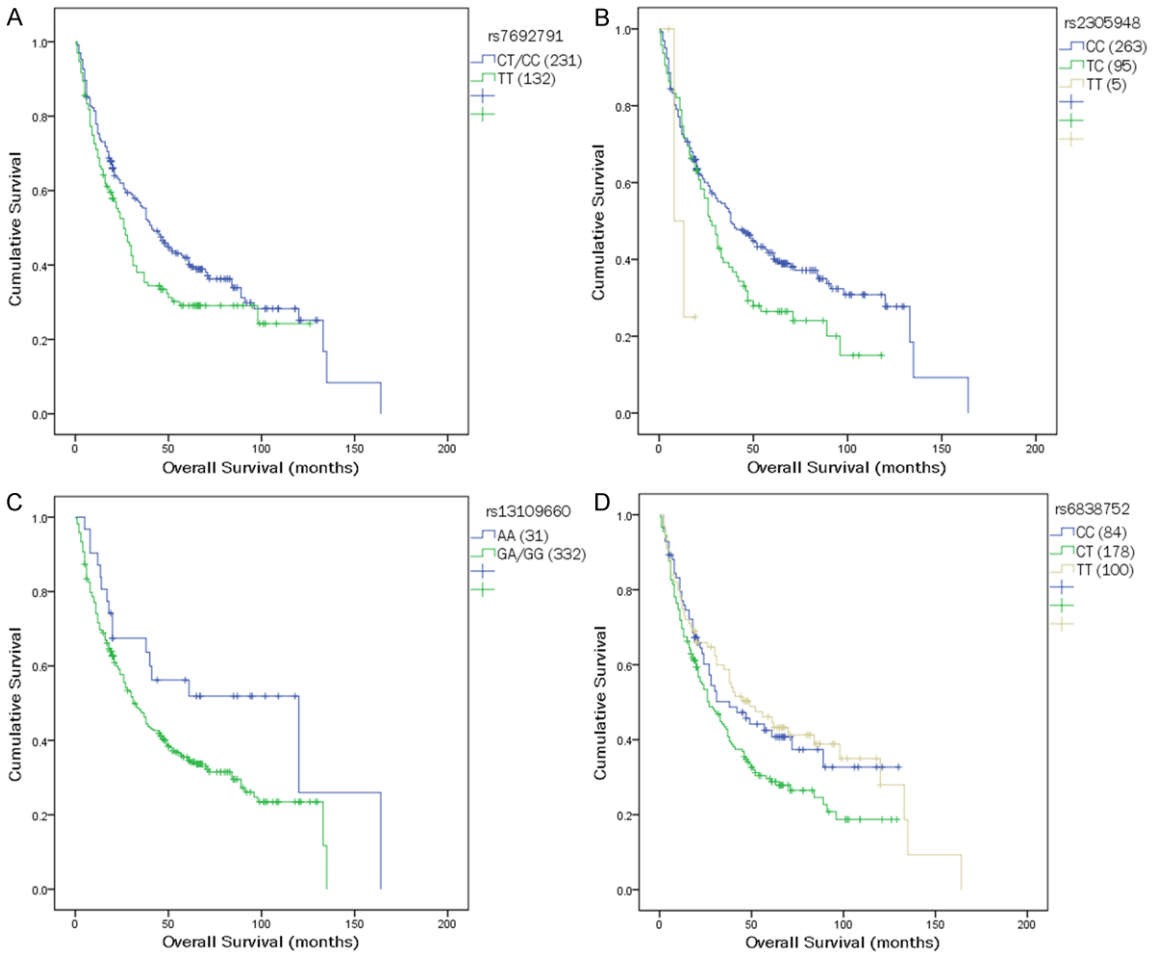

Figure 1. Kaplan-Meier survival curves of overall survival in HCC patients are shown for polymorphisms of (A) rs7692791, (B) rs2305948, (C) rs13109660, and (D) rs6838752.

estimated for the multivariate survival analyses by Cox proportional hazards regression models, adjusting for age, gender, family history, HBsAg and AFP.

Data analysis, with the exception of haplotype construction and haplotype frequency estimation, was performed with SPSS software version 19 (SPSS, Chicago, IL). All tests were two-sided and a $p<0.05$ was considered statistically significant.

Results

Patient characteristics and clinical outcomes

This study included 363 HCC patients with an overall median survival time (MST) of 34.0 months and median follow-up time of 53.0 months. At the time of analysis, 229 (63.1%) of the patients had died. The clinical pathologic characteristics and the association with OS and DFS are summarized in **Table 2**. By the Kaplan-Meier analysis, tumor capsule and TNM stage were significantly associated with OS and DFS (log-rank $p<0.001$). AFP was positive in 215 (60.2%) patients and shown to be related with both OS and DFS ($p=0.009$ and 0.007, respectively). In addition, large tumor size and differentiation were significantly associated with reduced OS and DFS, while venous invasion was a predictor for worse OS ($p=0.041$) and background cirrhosis for inferior DFS ($p=0.044$). The HBsAg of 304 (84.0%) cases was positive, but it didn't demonstrate a relationship with either OS or DFS in the present study.

As the clinicopathologic information from some patients was not available for several items, such as cirrhosis, venous invasion and TNM stage, and not all clinical factors above could

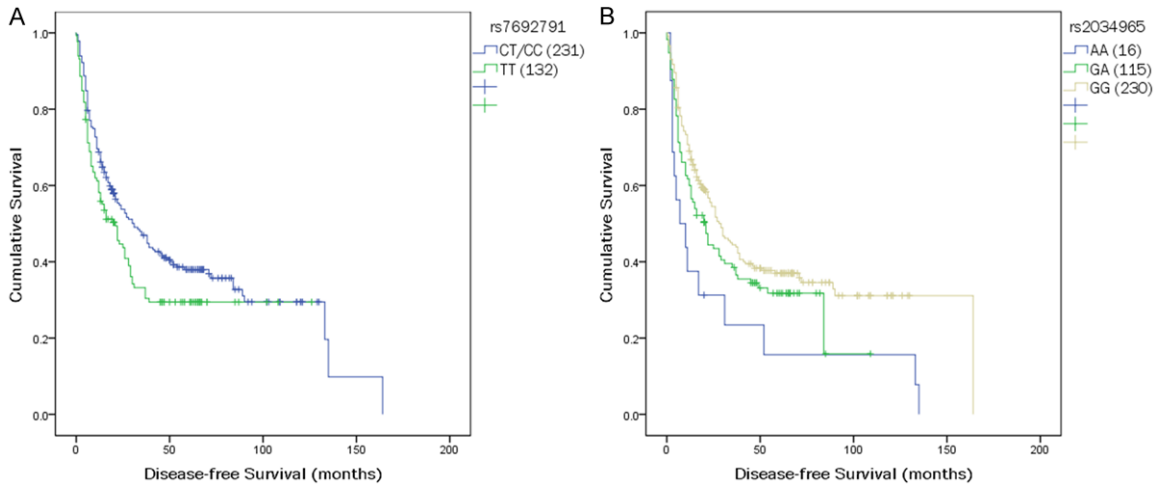

Figure 2. Kaplan-Meier survival curves of disease-free survival in HCC patients are shown for polymorphisms of (A) rs7692791 and (B) rs2034965.

be included in the subsequent multivariate analysis. Therefore we calculated HR and its corresponding *p*-value using Cox proportional hazard models, adjusted for age, gender, family history, HBsAg and AFP.

Genetic polymorphisms and HCC clinical outcomes

Table 3 shows the data for all the 36 SNPs among 4 genes (EGF, EGFR, VEGF and VEGFR2) analyzed for OS and DFS. In the univariate analysis, of all the 36 SNPs, 5 SNPs (rs7692791, rs2305948, rs13109660, rs6838752 and rs2034965), which are all resided in the VEGFR2 gene, were significantly associated with clinical outcomes. Overall, four SNPs (rs7692791, rs2305948, rs13109660, rs6838752) were associated with OS (**Table 3**; **Figure 1**); two SNPs (rs7692791 and rs2034965) were associated with DFS (**Table 3**; **Figure 2**). In particular, we observed VEGFR2 rs7692791 CC and CT genotype was significantly associated with improved OS (p=0.037; HR=0.751, 95% CI: 0.574-0.983) and DFS (p=0.043; HR=0.757, 95% CI: 0.579-0.991), compared with the CC/CT genotypes (**Table 4**), indicating that the rs7692791 variant T allele was significantly protective. On the contrary, we found a significant decreased OS for those carrying the TC genotype of rs2305948 (p=0.04; HR=1.349, 95% CI: 1.013-1.796) or rs6838752 (p=0.066; HR=1.371, 95% CI: 0.980-1.920, respectively). While rs13109660 AA genotype (p=0.033; HR=0.563, 95% CI: 0.333-0.954) was shown to result in a significant improvement in OS (log-rank p=0.029; **Figure 1**). However, none of the other 31 polymorphisms examined were significantly associated with OS or DFS (**Table 3**).

A multivariate analysis of genotype effects on survival was conducted using Cox proportional hazards models adjusted for available clinicopathologic variables. As shown in **Table 4**, only one SNP rs2034965 remained significant, with the AA genotype presenting an independent negative effect on DFS (p=0.009, HR=1.672, 95% CI: 1.136-2.460), compared to patients who had common homozygous genotype and heterozygous genotype. None of the genetic polymorphisms was identified as an independent prognostic factor for OS.

Furthermore, we examined the associations of the haplotypes with survival outcomes. When examining combinations of SNPs for the EGF, EGFR, VEGF, and VEGFR2, we attained 2 haplotypes of EGF, 2 haplotypes of EGFR, 2 haplotypes of VEGF, and 3 haplotypes of VEGFR2. The inferred haplotypes and their associations with OS and DFS are shown in **Table 5**. Consistent with the results of individual genotype analyses, none of the haplotypes carrying variant alleles from EGF, EGFR, and VEGF showed a significant association with OS or DFS. The most probable haplotype (CT) was from VEGFR2, which had an estimated frequency of 66.7 percent. However, even though each of the individual SNPs of rs7692791, rs2305948, rs13109660, rs6838752 and rs2034965

Table 4. Significant associations of SNPs in 4 EGFR pathway genes with prognosis of HCC patients

SNP	Outcome	Model	Log-rank p	Univariate analysis		Multivariate analysis	
				HR (95% CI)	p	HR (95% CI)*	p
rs7692791	OS	Dominant	0.035		0.037		0.174
		TT		1		1	
		CT/CC		0.751 (0.574-0.983)		0.815 (0.608-1.094)	
rs2305948	OS	General	0.038		0.027		0.185
		CC		1		1	
		TC		1.349 (1.013-1.796)	0.04	1.252 (0.913-1.716)	0.164
		TT		2.543 (0.804-8.040)	0.112	2.281 (0.695-7.484)	0.174
rs13109660	OS	Recessive	0.029		0.033		0.386
		GA/GG		1		1	
		AA		0.563 (0.333-0.954)		0.753 (0.398-1.428)	
rs6838752	OS	General	0.028		0.031		0.249
		CC		1		1	
		CT		1.371 (0.980-1.920)	0.066	1.325 (0.933-1.883)	0.116
		TT		0.939 (0.637-1.385)	0.752	1.112 (0.711-1.740)	0.643
rs7692791	DFS	Dominant	0.039		0.043		0.073
		TT		1		1	
		CT/CC		0.757 (0.579-0.991)		0.769 (0.578-1.025)	
rs2034965	DFS	General	0.017		0.021		0.024
		GG		1		1	
		GA		1.261 (0.953-1.669)	0.105	0.809 (0.627-1.044)	0.103
		AA		2.029 (1.169-3.522)	0.012	1.672 (1.136-2.460)	0.009

OS: overall survival; DFS: disease-free survival; HR: hazard ratio; CI: confidence interval. *Adjusted for age, gender, family history, HBsAg and AFP.

in VEGFR2 showed potential prognostic effect on HCC clinical outcomes, the VEGFR2 haplotypes were still significantly related with neither OS nor DFS.

Discussion

Although new treatment modalities changed the global approach to HCC, this disease still represents a therapeutic challenge. While some germline genetic factors have been suspected of playing an important role in prognosis, none have been firmly established [34, 35]. The EGFR system regulates important processes within the tumor microenvironment of autocrine and paracrine circuits, including tumor invasion and angiogenesis [6]. Previous studies reported that the intensity of EGF, EGFR, VEGF, VEGFR2 expression correlates with proliferative activity, stage, intrahepatic metastasis and carcinoma differentiation in HCC [20-22, 36, 37]. In addition, several observations suggested that polymorphisms of these genes might regulate angiogenesis and lymphangiogenesis and thus controlling tumor growth. However, most investigations into SNPs in EGFR pathway (EGF, EGFR, VEGF, and VEGFR2) genes have just focused on their effects on risk rather than prognosis of HCC [38, 39], or selected SNPs without a systematic method [27, 29, 40]. The aim of our study was to evaluate the role of EGFR, EGF, VEGF, VEGFR2 polymorphisms in determining the clinical outcomes of HCC patients. To the best of our knowledge, this is the first evidence showing the relationship between genetic variants of EGFR pathway genes and the prognosis of HCC patients. We found that in the VEGFR2 gene two non-synonymous SNPs, rs7692791 and rs2034965 were significantly associated with DFS and four SNPs (rs7692791, rs2305948, rs13109660, rs6838752) were associated with OS. Once prospectively validated, this finding could be used to predict which patients are at risk for poor clinical outcomes, and the analysis of VEGFR2 SNPs may help to identify HCC patients more likely to benefit from targeted inhibitor therapy [35].

Deregulation of EGF/EGFR signaling pathway is thought to be one of the most important factors in early hepatocarcinogenesis [36, 41-44].

Table 5. Associations between haplotypes in 4 EGFR pathway genes and prognosis of HCC patients

Haplotype	No. of alleles	Frequency (%)	No. of events	Log-rank p for OS	Log-rank p for DFS
EGF-1				0.864	0.648
Haplotype of rs11569017 A/G and rs3733625 A/G					
AG	463	64.5	289		
GG	155	21.6	105		
AA	100	13.9	62		
EGF-2				0.7	0.431
Haplotype of rs3756261 A/G and rs11568943 A/T					
AA	342	47.8	224		
GA	224	31.3	133		
AT	150	20.9	101		
EGFR-1				0.73	0.854
Haplotype of rs4947492 A/G and rs6965469 C/T					
AT	369	51	236		
GC	279	38.5	172		
GT	72	9.9	48		
AC	4	0.6	2		
EGFR-2				0.358	0.274
Haplotype of rs2293347 G/A and rs884225 G/A					
GG	350	48.9	226		
AA	212	29.6	127		
GA	153	21.4	100		
VEGF-1				0.528	0.583
Haplotype of rs699947 C/A, rs833061 C/T, rs2010963 G/C, rs1413711 C/T, rs833070 A/G, and rs3025000 C/T					
CTCGGT	303	42	200		
CTGGGC	211	29.2	128		
ACGAAC	168	23.3	100		
Others*	40	5.6	28		
VEGF-2				0.159	0.236
Haplotype of rs3025033 A/G, rs3025035 C/T, rs3025039 C/T, and rs10434 A/G					
ACCG	299	41.3	182		
ACCA	163	22.5	108		
GCTG	144	19.9	90		
ATCG	110	15.2	71		
Others*	8	1.1	7		
VEGFR2-1				0.488	0.198
Haplotype of rs1531289 G/A and rs2034965 A/G					
GA	341	47.4	216		
GG	237	32.9	144		
AA	142	19.7	96		
VEGFR2-2				0.378	0.254
Haplotype of rs6838752 C/T, rs17709898 A/G, and rs1870377 A/T					
CGA	334	46.3	209		
TAT	210	29.1	127		
TGT	160	22.2	111		
Others*	18	2.4	11		
VEGFR2-3				0.529	0.633
Haplotype of rs2071559 C/T and rs7667298 C/T					
CT	479	66.6	304		
TC	240	33.4	153		

*Others include rare haplotypes with frequencies less than 5%.

Membranous EGFR was observed in 40% HCC patients, and correlated with histological grade. Angiolymphatic invasion was more commonly seen in EGFR-positive cases [45]. A recent study showed that the EGFR signaling system stands at a crossroad between inflammatory signals and intracellular pathways associated with hepatocarcinogenesis. The EGFR ligand amphiregulin AR release and EGFR transactivation by TNF-alpha constitutes a novel link between inflammatory signals and pro-tumorigenic mechanisms in liver cells. Its sheddase ADAM17 increased in pre-neoplastic liver injury further supports its implication in hepatocarcinogenesis [46]. However, the association between the EGFR genotype and prognosis so far has not been described in HCC patients and results from EGF remain controversial [27, 29, 40]. In our study, none of the seven SNPs of EGF and six SNPs of EGFR investigated was associated with either DFS or OS in HCC patients. This result could be explained partly by the fact that though EGF is only part of a gene expression signature associated with poor OS in HCC patients [47, 48], EGF expression alone is not qualified enough to predict HCC prognosis, which is also affected by various clinicopathologic characteristics, such as cirrhosis, venous invasion and TNM stage. As most studies reported that the G allele of EGF rs4444903 was a risk factor for HCC, independently of ethnicity and etiology [39], the variants of EGF/EGFR signaling pathway are more likely to alter the susceptibility rather than prognosis of HCC.

HCC is a highly vascular tumor, which proliferates through angiogenic pathways mediated partly by VEGF and its multiple receptors including VEGFR2. Evidences from preclinical and clinical studies showed that there was a correlation between high VEGFR2 expression, both in tissues and serum, and the metastases or poor prognosis of HCC. VEGFR2 expression was significantly higher not only in the veins and sinusoids of poorly differentiated tumors, but also in the arteries of non-tumorous liver in HCC patients, suggesting that VEGFR2 expression is a feature of poor differentiation and tumor progression [49]. Another study reported that high VEGFR2 expression in HCC was related to large tumor diameter, poor differentiation, high serum alpha-fetoprotein, multifocal gross classification [50], and displayed a trend toward decreased OS [51]. On the other hand, the patients with a low serum level of VEGFR2 had better OS and DFS than those with a high serum level of VEGF [52]. Furthermore, the pretreatment serum level of VEGFR2 was an independent and significant prognostic factor of survival for HCC patients, and the serum VEGFR2 concentration decrease after transarterial chemoembolization (TACE) may predict favorable OS in patients with HCC [53]. However, limited information is available regarding the role of the VEGF system SNPs, especially its receptor VEGFR2, in HCC. The simultaneous presence of VEGFR2 and VEGF polymorphisms may confer an increased risk of HCC in patients with alcoholics presenting liver disease (ALD) [54]. Certain SNPs of VEGFR2 may affect treatment outcomes and toxicity in patients treated with sunitinib. One study reported that rs7692-791 of VEGFR2 was associated with poor OS among patients with gastric or biliary tract cancer who were treated with sunitinib [55]. VEGFR2 alleles C of rs2305948 and VEGF alleles C of rs699947, C of rs2010963 were significant predictors of DFS and OS at univariate analysis, but only rs2010963 resulted to be an independent factor influencing DFS and OS in multivariate analysis [56].

Given curative resection and postoperative treatment, including local radiofrequency ablation (RFA), TACE, radioembolization, and molecular targeted therapy, establishment of more precise prognostic determinants using molecular biology techniques is still warranted to make the best use of these options [57]. Particularly, evidences from studies on EGFR-tyrosine kinase inhibitors for several epithelial cancers are very encouraging. These inhibitors can block the expression of not only EGFR but also VEGF [58]. For example, Vandetanib, an inhibitor of VEGFR2 and EGFR, was showed to suppress tumor development and improve the prognosis of liver cancer [59]. In addition, concomitant inhibition of VEGFR2 and Raf will disrupt oncogenic signaling and efficiently reduce tumor growth and vascularization of HCC in Human HCC cell lines and endothelial cells [60]. Tyrosine kinase inhibitors of VEGFR2, such as sunitinib [61], *Sorafenib* [62], and foretinib [63], have shown promising preliminary efficacy in patients with HCC. Understanding how these genetic variants work on clinical outcomes of HCC patients may help

developing new drugs and achieving personalized therapeutic regimen.

In our study, only one SNP rs2034965 remained significant in the multivariate analysis, with the AA genotype presenting an independent negative effect on DFS. None of additional genetic polymorphisms reached significance and could be served as an independent prognostic factor for OS. Maybe we can find clues through our sample sources. We obtain blood samples from each HCC patient treated with surgery. However, the expression of VEGFR2 in tissues and serum may have different prognostic influence on HCC patients. Another explanation is that even though we selected and investigated these SNPs in a systematical way, due to limited techniques, labor and resources, we missed some key SNPs which play a predominant role in regulating the expression of the EGFR pathway genes. For this reason, we are not capable of concluding that the SNPs of these four genes are not associated with the prognosis of HCC at present. Instead, a more comprehensive analysis of polymorphisms in the EGFR pathway is imperative to illustrate the close correlation between EGFR pathway genes and HCC prognosis.

It is worth mentioning that there were a number of limitations in our study. Firstly, the cohort size was relatively small, and we didn't recruit enough cases for validation. The significant association found in the univariate analysis should be viewed as generating hypothesis or a clue for related researches afterwards. Therefore, larger well-designed longitudinal follow-up studies and functional evaluation are warranted to confirm these findings. Secondly, though several clinical and pathologic characteristics showed significant associations with OS and/or DFS, including tumor size, differentiation, tumor capsule, cirrhosis, venous invasion and TNM stage, it is regretful that we failed to collect adequate and accurate information of these factors in our study. In order to make the greatest use of the genotype polymorphisms information we got from the 363 HCC patients, we had to operate the multivariate analysis without adjusting all these potential prognostic factors. Future studies are essential to investigate the role of genetic polymorphisms in patients with more complete and comprehensive clinicalpathologic characteristics. Last but not the least, as mentioned above, all of our samples are blood from each HCC patients treated with surgery. This major drawback not only confined our results to the expression of EGFR pathway genes in serum rather than tissues, but also restricted criteria for patients who can be only treated with surgery. However, most patients with HCC are diagnosed at advanced stages when curative treatments, such as hepatic resection and liver transplantation, are not feasible [57]. Accordingly, analyses of tissue samples are urgent to figure out the unknown modulation of these genes in HCC survival.

In summary, our results demonstrated the potential use of VEGFR2 polymorphisms as prognostic markers for HCC patients. However, neither the SNPs in EGF, EGFR, and VEGF genes nor the haplotypes from the EGFR pathway genes were significantly associated with HCC prognosis.

Acknowledgements

We thank all the participants. This study was supported by the National Natural Science Foundation of China (31100895 and 81472618 to D.-K.J.), the National Key Sci-Tech Special Project of China (2008ZX10002-020 and 2013ZX10002010 to L.Y.), as well as supported from an intramural research grant for outstanding young scholar from Fudan University (to D.-K.J.), an intramural research grant for new young teachers from Fudan University (to D.-K.J.), an intramural research grant for promotion of the scientific research ability of young teachers from Fudan University (to D.-K.J.), an intramural research grant from Huashan Hospital, Fudan University (to J.X.), and an intramural research grant from Center for Genetic Epidemiology, Fudan University (to J.X.).

Disclosure of conflict of interest

There are no known conflicts of interest.

Address correspondence to: Dr. Deke Jiang, Dr. Jianfeng Xu or Dr. Long Yu, State Key Laboratory of Genetic Engineering, Center for Genetic Epidemiology, School of Life Sciences, Fudan University, 2005 Songhu Rd., Shanghai 200438, China. Tel: +86-21-51630617; E-mail: dekejiang@fudan.edu.cn (DKJ); jxu@wfubmc.edu (JFX); Tel: +86-21-5163-0580; E-mail: longyu@fudan.edu.cn (LY)

References

[1] Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 2010; 127: 2893-2917.

[2] Yang JD and Roberts LR. Hepatocellular carcinoma: A global view. Nat Rev Gastroenterol Hepatol 2010; 7: 448-458.

[3] Zhao B, Lu J, Yin J, Liu H, Guo X, Yang Y, Ge N, Zhu Y, Zhang H and Xing J. A functional polymorphism in PER3 gene is associated with prognosis in hepatocellular carcinoma. Liver Int 2012; 32: 1451-1459.

[4] Torzilli G, Belghiti J, Kokudo N, Takayama T, Capussotti L, Nuzzo G, Vauthey JN, Choti MA, De Santibanes E, Donadon M, Morenghi E and Makuuchi M. A snapshot of the effective indications and results of surgery for hepatocellular carcinoma in tertiary referral centers: is it adherent to the EASL/AASLD recommendations?: an observational study of the HCC East-West study group. Ann Surg 2013; 257: 929-937.

[5] Takayasu K, Arii S, Ikai I, Omata M, Okita K, Ichida T, Matsuyama Y, Nakanuma Y, Kojiro M, Makuuchi M and Yamaoka Y. Prospective cohort study of transarterial chemoembolization for unresectable hepatocellular carcinoma in 8510 patients. Gastroenterology 2006; 131: 461-469.

[6] De Luca A, Carotenuto A, Rachiglio A, Gallo M, Maiello MR, Aldinucci D, Pinto A and Normanno N. The role of the EGFR signaling in tumor microenvironment. J Cell Physiol 2008; 214: 559-567.

[7] Stoscheck CM and King LE Jr. Role of epidermal growth factor in carcinogenesis. Cancer Res 1986; 46: 1030-1037.

[8] Hansen TF, Sorensen FB, Spindler KL, Olsen DA, Andersen RF, Lindebjerg J, Brandslund I and Jakobsen A. Microvessel density and the association with single nucleotide polymorphisms of the vascular endothelial growth factor receptor 2 in patients with colorectal cancer. Virchows Arch 2010; 456: 251-260.

[9] Kang D, Gridley G, Huang WY, Engel LS, Winn DM, Brown LM, Bravo-Otero E, Wu T, Diehl SR and Hayes RB. Microsatellite polymorphisms in the epidermal growth factor receptor (EGFR) gene and the transforming growth factor-alpha (TGFA) gene and risk of oral cancer in Puerto Rico. Pharmacogenet Genomics 2005; 15: 343-347.

[10] Brandt B, Hermann S, Straif K, Tidow N, Buerger H and Chang-Claude J. Modification of breast cancer risk in young women by a polymorphic sequence in the egfr gene. Cancer Res 2004; 64: 7-12.

[11] Choi JE, Park SH, Kim KM, Lee WK, Kam S, Cha SI, Kim CH, Kang YM, Kim YC, Han SB, Jung TH and Park JY. Polymorphisms in the epidermal growth factor receptor gene and the risk of primary lung cancer: a case-control study. BMC Cancer 2007; 7: 199.

[12] Zhou Q, Cheung YB, Jada SR, Lim WT, Kuo WL, Gray JW, Lee AS and Chowbay B. EGFR Intron 1 polymorphism in Asian Populations and its correlation with EGFR gene expression and amplification in breast tumor tissues. Cancer Biol Ther 2006; 5: 1445-1449.

[13] Zhang W, Stoehlmacher J, Park DJ, Yang D, Borchard E, Gil J, Tsao-Wei DD, Yun J, Gordon M, Press OA, Rhodes K, Groshen S and Lenz HJ. Gene polymorphisms of epidermal growth factor receptor and its downstream effector, interleukin-8, predict oxaliplatin efficacy in patients with advanced colorectal cancer. Clin Colorectal Cancer 2005; 5: 124-131.

[14] Forsti A, Jin Q, Altieri A, Johansson R, Wagner K, Enquist K, Grzybowska E, Pamula J, Pekala W, Hallmans G, Lenner P and Hemminki K. Polymorphisms in the KDR and POSTN genes: association with breast cancer susceptibility and prognosis. Breast Cancer Res Treat 2007; 101: 83-93.

[15] Ferrara N, Gerber HP and LeCouter J. The biology of VEGF and its receptors. Nat Med 2003; 9: 669-676.

[16] Delongchamps NB, Peyromaure M and Dinh-Xuan AT. Role of vascular endothelial growth factor in prostate cancer. Urology 2006; 68: 244-248.

[17] Gasparini G. Prognostic value of vascular endothelial growth factor in breast cancer. Oncologist 2000; 5 Suppl 1: 37-44.

[18] Giatromanolaki A, Sivridis E and Koukourakis MI. Angiogenesis in colorectal cancer: prognostic and therapeutic implications. Am J Clin Oncol 2006; 29: 408-417.

[19] Rudlowski C, Pickart AK, Fuhljahn C, Friepoertner T, Schlehe B, Biesterfeld S and Schroeder W. Prognostic significance of vascular endothelial growth factor expression in ovarian cancer patients: a long-term follow-up. Int J Gynecol Cancer 2006; 16 Suppl 1: 183-189.

[20] Iavarone M, Lampertico P, Iannuzzi F, Manenti E, Donato MF, Arosio E, Bertolini F, Primignani M, Sangiovanni A and Colombo M. Increased expression of vascular endothelial growth factor in small hepatocellular carcinoma. J Viral Hepat 2007; 14: 133-139.

[21] Park YN, Kim YB, Yang KM and Park C. Increased expression of vascular endothelial growth factor and angiogenesis in the early stage of multistep hepatocarcinogenesis. Arch Pathol Lab Med 2000; 124: 1061-1065.

[22] Suzuki K, Hayashi N, Miyamoto Y, Yamamoto M, Ohkawa K, Ito Y, Sasaki Y, Yamaguchi Y, Nakase H, Noda K, Enomoto N, Arai K, Yamada Y, Yoshihara H, Tujimura T, Kawano K, Yoshikawa K and Kamada T. Expression of vascular permeability factor/vascular endothelial growth factor in human hepatocellular carcinoma. Cancer Res 1996; 56: 3004-3009.

[23] Jinno K, Tanimizu M, Hyodo I, Nishikawa Y, Hosokawa Y, Doi T, Endo H, Yamashita T and Okada Y. Circulating vascular endothelial growth factor (VEGF) is a possible tumor marker for metastasis in human hepatocellular carcinoma. J Gastroenterol 1998; 33: 376-382.

[24] Poon RT, Lau C, Pang R, Ng KK, Yuen J and Fan ST. High serum vascular endothelial growth factor levels predict poor prognosis after radiofrequency ablation of hepatocellular carcinoma: importance of tumor biomarker in ablative therapies. Ann Surg Oncol 2007; 14: 1835-1845.

[25] Schneider BP, Radovich M and Miller KD. The role of vascular endothelial growth factor genetic variability in cancer. Clin Cancer Res 2009; 15: 5297-5302.

[26] Giacalone A, Montalto G, Giannitrapani L, Balasus D, Terranova A, Cervello M, Soresi M and Marasa L. Association between single nucleotide polymorphisms in the cyclooxygenase-2, tumor necrosis factor-alpha, and vascular endothelial growth factor-A genes, and susceptibility to hepatocellular carcinoma. OMICS 2011; 15: 193-196.

[27] Suenaga M, Yamada S, Fujii T, Fuchs BC, Okumura N, Kanda M, Kobayashi D, Tanaka C, Nakayama G, Sugimoto H, Koike M, Nomoto S, Fujiwara M, Takeda S, Hayashi K, Tanabe KK, Goto H and Kodera Y. A functional polymorphism in the epidermal growth factor gene predicts hepatocellular carcinoma risk in Japanese hepatitis C patients. Onco Targets Ther 2013; 6: 1805-1812.

[28] Kong SY, Park JW, Lee JA, Park JE, Park KW, Hong EK and Kim CM. Association between vascular endothelial growth factor gene polymorphisms and survival in hepatocellular carcinoma patients. Hepatology 2007; 46: 446-455.

[29] Wu LM, Xie HY, Zhou L, Yang Z, Zhang F and Zheng SS. A single nucleotide polymorphism in the vascular endothelial growth factor gene is associated with recurrence of hepatocellular carcinoma after transplantation. Arch Med Res 2009; 40: 565-570.

[30] The International HapMap Project. Nature 2003; 426: 789-796.

[31] Sherry ST, Ward M and Sirotkin K. dbSNP-database for single nucleotide polymorphisms and other classes of minor genetic variation. Genome Res 1999; 9: 677-679.

[32] A haplotype map of the human genome. Nature 2005; 437: 1299-1320.

[33] Stephens M and Donnelly P. A comparison of bayesian methods for haplotype reconstruction from population genotype data. Am J Hum Genet 2003; 73: 1162-1169.

[34] Jin F, Xiong WJ, Jing JC, Feng Z, Qu LS and Shen XZ. Evaluation of the association studies of single nucleotide polymorphisms and hepatocellular carcinoma: a systematic review. J Cancer Res Clin Oncol 2011; 137: 1095-1104.

[35] Nishida N and Kudo M. Recent advancements in comprehensive genetic analyses for human hepatocellular carcinoma. Oncology 2013; 84 Suppl 1: 93-97.

[36] Ito Y, Takeda T, Sasaki Y, Sakon M, Yamada T, Ishiguro S, Imaoka S, Tsujimoto M, Higashiyama S, Monden M and Matsuura N. Expression and clinical significance of the erbB family in intrahepatic cholangiocellular carcinoma. Pathol Res Pract 2001; 197: 95-100.

[37] Zhong JH, You XM, Gong WF, Ma L, Zhang Y, Mo QG, Wu LC, Xiao J and Li LQ. Epidermal growth factor gene polymorphism and risk of hepatocellular carcinoma: a meta-analysis. PLoS One 2012; 7: e32159.

[38] Abu DBK, Yang M, Fuchs BC, Karl DL, Yamada S, Sninsky JJ, O'Brien TR, Dienstag JL, Tanabe KK and Chung RT. A functional polymorphism in the epidermal growth factor gene is associated with risk for hepatocellular carcinoma. Gastroenterology 2011; 141: 141-149.

[39] Yang Z, Wu Q, Shi Y, Nie Y, Wu K and Fan D. Epidermal growth factor 61A>G polymorphism is associated with risk of hepatocellular carcinoma: a meta-analysis. Genet Test Mol Biomarkers 2012; 16: 1086-1091.

[40] Yoshiya S, Fujimoto Y, Bekki Y, Konishi H, Yamashita Y, Ikegami T, Yoshizumi T, Shirabe K, Oda Y and Maehara Y. Impact of epidermal growth factor single-nucleotide polymorphism on recurrence of hepatocellular carcinoma after hepatectomy in patients with chronic hepatitis C virus infection. Cancer Sci 2014; 105: 646-650.

[41] Komuves LG, Feren A, Jones AL and Fodor E. Expression of epidermal growth factor and its receptor in cirrhotic liver disease. J Histochem Cytochem 2000; 48: 821-830.

[42] Morimitsu Y, Hsia CC, Kojiro M and Tabor E. Nodules of less-differentiated tumor within or adjacent to hepatocellular carcinoma: relative expression of transforming growth factor-alpha and its receptor in the different areas of tumor. Hum Pathol 1995; 26: 1126-1132.

[43] Lin SY, Makino K, Xia W, Matin A, Wen Y, Kwong KY, Bourguignon L and Hung MC. Nuclear lo-

calization of EGF receptor and its potential new role as a transcription factor. Nat Cell Biol 2001; 3: 802-808.

[44] Berasain C and Avila MA. The EGFR signalling system in the liver: from hepatoprotection to hepatocarcinogenesis. J Gastroenterol 2014; 49: 9-23.

[45] Bassullu N, Turkmen I, Dayangac M, Yagiz Korkmaz P, Yasar R, Akyildiz M, Yaprak O, Tokat Y, Yuzer Y and Bulbul Dogusoy G. The Predictive and Prognostic Significance of c-erb-B2, EGFR, PTEN, mTOR, PI3K, p27, and ERCC1 Expression in Hepatocellular Carcinoma. Hepat Mon 2012; 12: e7492.

[46] Berasain C, Nicou A, Garcia-Irigoyen O, Latasa MU, Urtasun R, Elizalde M, Salis F, Perugorria MJ, Prieto J, Recio JA, Corrales FJ and Avila MA. Epidermal growth factor receptor signaling in hepatocellular carcinoma: inflammatory activation and a new intracellular regulatory mechanism. Dig Dis 2012; 30: 524-531.

[47] Hoshida Y, Villanueva A, Kobayashi M, Peix J, Chiang DY, Camargo A, Gupta S, Moore J, Wrobel MJ, Lerner J, Reich M, Chan JA, Glickman JN, Ikeda K, Hashimoto M, Watanabe G, Daidone MG, Roayaie S, Schwartz M, Thung S, Salvesen HB, Gabriel S, Mazzaferro V, Bruix J, Friedman SL, Kumada H, Llovet JM and Golub TR. Gene expression in fixed tissues and outcome in hepatocellular carcinoma. N Engl J Med 2008; 359: 1995-2004.

[48] Hoshida Y, Villanueva A, Sangiovanni A, Sole M, Hur C, Andersson KL, Chung RT, Gould J, Kojima K, Gupta S, Taylor B, Crenshaw A, Gabriel S, Minguez B, Iavarone M, Friedman SL, Colombo M, Llovet JM and Golub TR. Prognostic gene expression signature for patients with hepatitis C-related early-stage cirrhosis. Gastroenterology 2013; 144: 1024-1030.

[49] Aucejo F, Kim R, Zein N, Quintini C, Uso TD, Lopez R, Eghtesad B, Fung J, Miller C and Yerian L. Vascular endothelial growth factor receptor 2 expression in non-tumorous cirrhotic liver is higher when hepatoma is beyond Milan criteria. Liver Transpl 2009; 15: 169-176.

[50] Huang J, Zhang X, Tang Q, Zhang F, Li Y, Feng Z and Zhu J. Prognostic significance and potential therapeutic target of VEGFR2 in hepatocellular carcinoma. J Clin Pathol 2011; 64: 343-348.

[51] Patel SH, Kneuertz PJ, Delgado M, Kooby DA, Staley CA 3rd, El-Rayes BF, Kauh JS, Sarmiento JM, Hanish S, Cohen C, Farris AB 3rd and Maithel SK. Clinically relevant biomarkers to select patients for targeted inhibitor therapy after resection of hepatocellular carcinoma. Ann Surg Oncol 2011; 18: 3384-3390.

[52] Zheng YB, Meng QW, Zhao W, Liu B, Huang JW, He X, Li Y, Hu BS and Lu LG. Prognostic value of serum vascular endothelial growth factor receptor 2 response in patients with hepatocellular carcinoma undergoing transarterial chemoembolization. Med Oncol 2014; 31: 843.

[53] Meng QW, Li Y, Hu BS, Shao PJ, Zhan MX, Yu XY and Lu LG. [Prognostic significance of serum level of vascular endothelial growth factor receptor-2 in hepatocellular carcinoma patients after transcatheter arterial chemoembolization]. Zhonghua Yi Xue Za Zhi 2013; 93: 341-344.

[54] Machado MV, Janeiro A, Miltenberger-Miltenyi G and Cortez-Pinto H. Genetic polymorphisms of proangiogenic factors seem to favor hepatocellular carcinoma development in alcoholic cirrhosis. Eur J Gastroenterol Hepatol 2014; 26: 438-443.

[55] Maeng CH, Yi JH, Lee J, Hong JY, Choi MK, Jung HA, Park JO, Park SH, Park YS, Kang WK and Lim HY. Effects of single nucleotide polymorphisms on treatment outcomes and toxicity in patients treated with sunitinib. Anticancer Res 2013; 33: 4619-4626.

[56] Scartozzi M, Faloppi L, Svegliati Baroni G, Loretelli C, Piscaglia F, Iavarone M, Toniutto P, Fava G, De Minicis S, Mandolesi A, Bianconi M, Giampieri R, Granito A, Facchetti F, Bitetto D, Marinelli S, Venerandi L, Vavassori S, Gemini S, D'Errico A, Colombo M, Bolondi L, Bearzi I, Benedetti A and Cascinu S. VEGF and VEGFR genotyping in the prediction of clinical outcome for HCC patients receiving sorafenib: the ALICE-1 study. Int J Cancer 2014; 135: 1247-1256.

[57] Forner A, Llovet JM and Bruix J. Hepatocellular carcinoma. Lancet 2012; 379: 1245-1255.

[58] Pore N, Jiang Z, Gupta A, Cerniglia G, Kao GD and Maity A. EGFR tyrosine kinase inhibitors decrease VEGF expression by both hypoxia-inducible factor (HIF)-1-independent and HIF-1-dependent mechanisms. Cancer Res 2006; 66: 3197-3204.

[59] Inoue K, Torimura T, Nakamura T, Iwamoto H, Masuda H, Abe M, Hashimoto O, Koga H, Ueno T, Yano H and Sata M. Vandetanib, an inhibitor of VEGF receptor-2 and EGF receptor, suppresses tumor development and improves prognosis of liver cancer in mice. Clin Cancer Res 2012; 18: 3924-3933.

[60] Lang SA, Brecht I, Moser C, Obed A, Batt D, Schlitt HJ, Geissler EK and Stoeltzing O. Dual inhibition of Raf and VEGFR2 reduces growth and vascularization of hepatocellular carcinoma in an experimental model. Langenbecks Arch Surg 2008; 393: 333-341.

[61] Zhu AX, Duda DG, Sahani DV and Jain RK. Development of sunitinib in hepatocellular carcinoma: rationale, early clinical experience, and correlative studies. Cancer J 2009; 15: 263-268.

[62] Yu W, Gu K, Yu Z, Yuan D, He M, Ma N, Lai S, Zhao J, Ren Z, Zhang X, Shao C and Jiang GL. Sorafenib potentiates irradiation effect in hepatocellular carcinoma in vitro and in vivo. Cancer Lett 2013; 329: 109-117.

[63] Huynh H, Ong R and Soo KC. Foretinib demonstrates anti-tumor activity and improves overall survival in preclinical models of hepatocellular carcinoma. Angiogenesis 2012; 15: 59-70.