doi:10.4149/neo_2009_04_331

Low frequency mutation of the Ephrin receptor A3 gene in hepatocellular carcinoma

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Received October 10, 2008

EphA3 is a component of the Eph/ephrin tyrosine kinase system, which participates in vasculature development. This receptor/ligand system is associated with various signaling pathways related to cell growth and viability, cytoskeletal organization, cell migration, and anti-apoptosis. Accumulated evidence suggests that aberrant regulation of *EphA3* and its genetic alterations are implicated in the development and progression of various cancers. However, despite a high incidence of EphA3 over-expression, no such investigation has been performed in hepatocellular carcinoma. Thus, we investigated genetic alterations of the *EphA3* gene in 73 cases of hepatocellular carcinoma by single-strand conformational polymorphism and sequencing. One novel D219V missense mutation was found in the extracellular domain of *EphA3,* and two genetic alterations in the intracellular sterile-α-motif (SAM) domain of EphA3 appeared to be polymorphisms. Although the functional assessments of this mutant are incomplete, it is believed that this novel *EphA3* mutation may contribute to the development of hepatocellular carcinoma.

Key words: Eph receptor, EphA3, hepatocellular carcinoma, mutation

Hepatocellular carcinoma (HCC) is the fifth most common cancer, and its mortality is the third highest among cancers. In particular, it is one the main causes of cancer death, especially in Asia and Africa [1, 2]. Recent studies have found that genetic alterations of tumor suppressor genes or oncogenes, such as, *p53, β-catenin*, and *AXIN1*, are involved in hepatocarcinogenesis [3–5], but the frequencies of mutations in these genes appear to be low in HCC patients. Furthermore, it is unclear how these genetic changes reflect the clinical characteristics of individual HCC patients, which demonstrate that the molecular events underlying HCC remain substantially undetermined.

The Eph/ephrin system consists of Eph receptors and ephrin ligands and is one of the receptor tyrosine kinase systems involved in vascular organization. This system plays major roles in the induction of positional guidance cues on cells and in the control of the organization of arterio-venous asymmetry during vasculature development [6]. Eph receptors are transmembrane tyrosine kinases and have been categorized as A- and B- types based on their interactions with ephrin ligands [6, 7], which are also classified as A- or B- types based on their structural properties. The Eph/ephrin system is associated with various signaling pathways related to cell growth and viability, cytoskeletal organization, cell migration, and anti-apoptosis, whereas the major biological functions of this system are to mediate angiogenesis and cell-to-cell attachment or motility. Therefore, this tyrosine kinase system is thought to play primary roles in the developmental process, tumor cell proliferation, and migration [8].

Considerable evidence indicates that Eph receptors are mutated in various cancers, such as, breast cancer, lung cancer, colorectal cancer, and glioblastoma [9–13], and that these genetic alterations are cancer type specific. In addition, EphA3 expression is aberrantly regulated in various human cancers. In a previous study, the expression of EphA3 was found to be over-expressed by 1.6-fold on hepatocytes in HCC [14]. In addition, when we previously used a DNA microarray to assess the sequential expressions of genetic elements from the pre-cancer stage to overt HCC, EphA3 was found to be over-

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expressed in HCC tissues as compared with corresponding normal tissues [15], which suggested that EphA3 participates in hepatocarcinogenesis.

Thus, in the present study, we investigated somatic mutations of the *EphA3* gene in 73 hepatocellular carcinoma cases by using single strand conformation polymorphism (SSCP) and sequencing in order to determine whether genetic alterations of the *EphA3* gene are involved with the development and progression of HCC.

Materials and methods

Tissue Samples. Seventy-three frozen HCCs and their corresponding background liver tissue samples obtained from 73 patients at resection were evaluated. Approval was obtained from the institutional review board of Catholic University of Korea, College of Medicine, and informed consent was obtained beforehand in accord with the requirements of the Declaration of Helsinki. There was no evidence of familial cancer in any of the patients. Patients ages ranged from 26-89 years (average 51.6 years) and there were 61 men and 12 women. Background liver tissues demonstrated the presence of; cirrhosis in 50 (68.5%) cases, chronic hepatitis in 13 (17.8%) cases, and no-specific change in 10 (13.7%) cases. HBV was detected in 64 (86.5%) and HCV in 5 (6.8%). Histologically, 9, 32, and 32 samples were of Edmonson grades I, II, and III, respectively.

Table 1. Sequences of the primers used to amplify the coding region of the *EphA3* gene

Name of primer	Nucleotide sequence	Product size (bp)
E3-4F	5'-GCCCATTTACAGTGAAGAAT -3'	239
E3-4R	5'-GCTCAAAGAAAAATAGAGAAGG -3'	
E7-1F	5'-TCAGGTTTGAGTGGTTCAC -3'	187
E7-1R	5'-GGCTCGGATTTGGAATA -3'	
E10F	5'-TTGCGCCTTTCTTTCTTT -3'	182
E10R	5'-AAGTTGGCAGTAGGGTCATT -3'	
E11F	5'-ATTTTGTTGCTGTTCTGCTT -3'	248
E11R	5'-ACGGAAACACAGGTCTTATG -3'	
E12F	5'-CCTCAGGTAAATCCAACTA -3'	208
E12R	5'-GTCTCTAATGTTTTCAGCAA -3'	
E13-1F	5'-TGAACATTTGAAATGCTTCC -3'	187
E13-1R	5'-AGTCCGAAATCAGAAACCT -3'	
E13-2F	5'-CTGCTCGGAACATCTTGA -3'	201
E13-2R	5'-ACAACACGTTTTGGGTCA -3'	
E14F	5'-TGCATTGCTGATTTATGTAG -3'	240
E14R	5'-AGCTTGATCTTTTCATCTGA -3'	
E15-1F	5'-CTGAAAACTTCCTGGTTCCT -3'	166
E15-1R	5'-TCTGCTCAAACTTGGGTCT -3'	
E15-2F	5'-TTAAAGCTGTAGATGAGGGCTAT-3'	217
E15-2R	5'-TGCCAGATAACAAATTGAATG -3'	
E16F	5'-CTGATCTGTCTCCCTTTGGTGTT -3'	248
E16R	5'-AGCCAAGAAAGTTCAAAAGAATCCT -3'	
E17F	5'-CGAGGAAACCGATTCTTA -3'	215
E17R	5'-TCTGTCTGCAGGATGATG -3'	

DNA extraction. Frozen tissue samples were ground to fine powder in liquid nitrogen, and this powder was incubated overnight in 500 µl of lysis buffer (5 mM Tris-Cl pH8.0, 20 mM EDTA, 0.5% Triton X-100) containing 500 µg/ml of proteinase K (Takara Bio Inc., Shiga, Japan) at 50°C. Phenol: chloroform: isoamyl alcohol (25:24:1) solution (Sigma-Aldrich Corp., St. Louis, MO) was then added to each lysate, and after centrifugation (15,000 rpm, 4°C, 30 min), phenol: chloroform: isoamyl alcohol (25:24:1) solution was added to supernatants. DNA was precipitated with ethanol at -70°C and washed with 70% ethanol. Dried pellets were resuspended in 1X TE buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA).

Single strand conformation polymorphism and DNA sequencing. Genomic DNA samples from cancer cells and corresponding non-cancerous HCC tissues were amplified using twelve sets of primers covering exons which had previously been reported to harbor mutations and the kinase-domain coding regions (Table 1). Polymerase chain reactions (PCRs) were performed in 10 µl reaction mixtures containing 10 ng of template DNA, 0.1 mM of each deoxynucleotide triphosphate (Promega, Madison, WI), 1.5 mM of $MgCl_2$, 0.5 unit of Ampli Taq gold polymerase, and 1 µl of 10X buffer (Perkin-Elmer, Foster City, CA) and 1 µCi of [^{32}P]dCTP (Amersham, Bucks, UK). Reaction mixtures were initially denatured for 12 min at 95°C, then subjected to 40 amplification cycles (denaturing for 30 s at 95°C, annealing for 30 s at 49-54°C, and extension for 30 s at 72°C), and final extension was conducted for 5 min at 72°C. After amplification, PCR products were denatured for 5 min at 95°C in a 1:1 dilution of sample buffer containing 98% formamide/5mM/L NaOH and loaded onto SSCP gels (Mutation Detection Enhancement, FMC BioProduct, Rockland, ME, USA) with 10% glycerol. After electrophoresis, bands were transferred to 3 MM Whatman paper and dried. Autoradiography was performed using Kodak X-OMAT film (Eastman Kodak, Rochester, NY, USA). To detect mutations, DNAs showing mobility shifts were excised from dried SSCP gels and re-amplified over 40 cycles using the same primer sets. After electrophoresis in 2% agarose gels, PCR products were eluted from gels using Qiaquick gel extraction kits (Qiagen, Valencia, CA). Sequencing of PCR products was performed by COSMO (Seoul).

Results

The human *EphA3* gene consists of 17 exons, and the kinase domains of *EphA3* include exons 10 to 15. The majority of somatic mutations in *EphA3* previously found in human cancers are located in kinase domains. Thus, we examined genetic alterations of the *EphA3* gene in exons 3 and 7, which have been found to contain somatic mutations in different cancers by SSCP and direct sequencing methods. In the present study, we found three genetic alterations in these two exons of the *EphA3* gene (Table 2). Direct sequencing of an aberrantly migrating band in SSCP gel, resulted our identifying a missense mutation: a GAC to GTC transition (D219V) in *EphA3* (Fig.

Table 2. Genetic alterations of *EphA3* gene in hepatocellular carcinoma

Exon	No. of tissues	Nucleotide	Codon	Amino acid	Type
3	28T	A656T	GAC→GTC	D219V	Missense (Mutation)
16	2N,2T, 14N, 14T, 53N, 53T	T2770C	TGG →CGG	W924R	Non-synonymous (SNP)
		T2102C	GGT →GGC	G934G	Synonymous (SNP)

1A and B). Another two genetic alterations were found in exon 16, but both were appeared to be polymorphisms.

Discussion

EphA3 is a member of the ephrin receptor family, which composes the largest subgroup of the known receptor tyrosine kinases. Ephrin receptors and their ligands are essential for a variety of biological processes, and are implicated in tumor growth and survival [7]. *EphA3* maps to chromosome 3p11.2, a region frequently mutated in different cancers [16]. In previous reports, somatic mutations in *EphA3* have been described in lung and colon cancer [12, 13]. Here, we report for the first time an *EphA3* mutation in human hepatocellular carcinoma. This mutation (D219V) occurs in a cystein-rich linker region of the extracellular domain, which is evolutionally conserved and may be an important determinator of binding affinity between *EphA3* and its ligands.

In addition to the pivotal roles played by Eph receptors and their ligands in the regulations of embryonic patterning and vasculature development, it has also been suggested that they are involved in several signaling pathways related to cellular proliferation [7, 17]. For example, one member of the Eph receptors, EphA3, has been shown to induce the phosphorylation of c-Cbl proto-oncogene in Jurkat T cells, after stimulation by ephrinA1 ligand [18]. In addition, stimulation of EphA3 by ephrin-A5 causes loss of cell adhesion to fibronectin and the recruitment of adaptor protein CrkII to EphA3 [19, 20]. Furthermore, dysregulations of EphA3 expression in various cancers, such as, lung (1.1-fold), colon (4.4-fold), liver (1.6-fold), kidney (2.5-fold), melanoma, and sarcoma, have been reported [21]. Moreover, recently, EphA2 receptor antagonists have been found to have remarkable antiangiogenic and antitumor effects, which suggests that the EphA signaling pathway offers an attractive novel therapeutic target [22].

The *EphA3* gene is composed of 17 exons, and contains a single transmembrane domain with a glycosylated extracellular region comprised of a ligand-binding domain, a cystein-rich region, and two fibronectin type III domains [7, 8]. Somatic mutations in *EphA3* have been reported in the tyrosine kinase, SAM, and fibronetin type III domains and in the cystein-rich region. In the present study, we

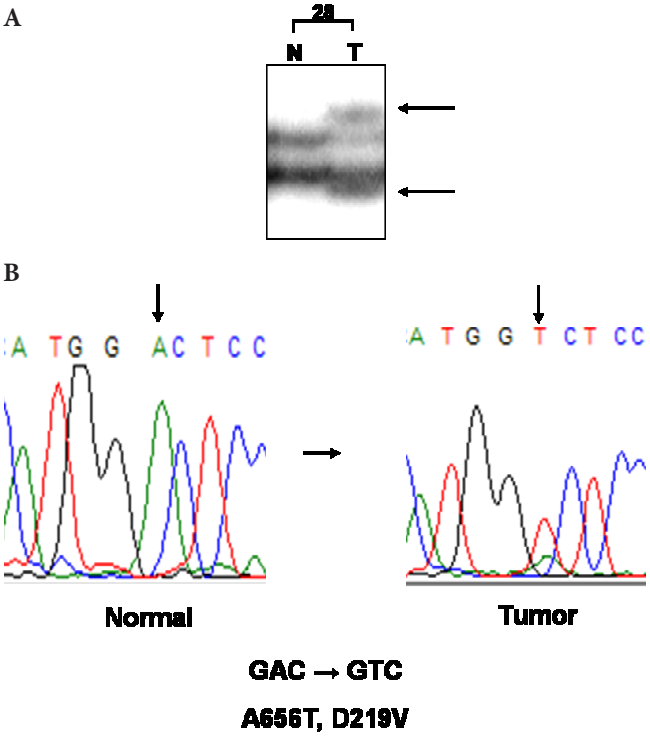

Figure 1. (A) Somatic mutation of the *EphA3* gene in human hepatocellular carcinoma. The nucleotide position of the mutation corresponds to the position of change in the coding sequence of the gene, where position 1 represents the A of the ATG. (Genbank Accession NM_005233.3). SSCP of DNAs obtained from cancer cells showed one aberrantly migrating band (arrowed) as compared with corresponding normal cells, indicating a genetic alteration in one allele. (B) Sequencing analysis identified the mutation to involve a GAC to GTC (D219V) change in exon 3 of the *EphA3* gene.

initially targeted exons of the kinase domain (exons 10–15) and some other exons that have been previously shown to harbor mutations (exon 3, 7, 16, 17). Although, we found no mutation in the kinase domain, we did find a novel mutation in *EphA3* in exon 3. Furthermore, this D219V mutation of *EphA3* gene has not been previously identified in human cancers. In a previous study, mutagenesis screening revealed that binding between an EphA3 mutant and ephrinA5 was impaired, which suggested the mutation existed in a non-functional domain [23]. Hence, it is possible that the D219V mutation affects the ligand-binding characteristics of EphA3. In addition, we identified two mutations in exon 16 (W924R, G934G), which belongs to the intracellular SAM domain of EphA3. However, unlike the D219V mutation these two single nucleotide polymorphisms of exon 16 have been reported (SNP ID: rs35124509; rs1054750).

Sumarizing, we found one missense mutation and two known SNPs in EphA3 in 73 hepatocellular carcinomas. Despite its low frequency of mutation, we suggest that mutation of *EphA3* contributes to the development and progression of hepatocellular carcinoma by altering receptor-ligand binding

affinities. Further functional analysis of the mutations identified in this study should broaden our understanding of the pathogenesis of hepatocellular carcinoma.

Acknowledgements. This work was supported by the Korean Ministry of the Environment via "The Eco-technopia 21 project" and by the Korean Science & Engineering Foundation (KOSEF) via the Cell Death Disease Research Center at The Catholic University of Korea.

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