[CANCER RESEARCH 59, 307–310, January 15, 1999]

Advances in Brief

Somatic Mutations in the Kinase Domain of the *Met*/Hepatocyte Growth Factor Receptor Gene in Childhood Hepatocellular Carcinomas[1]

Won Sang Park, Seung Myung Dong, Su Young Kim, Eun Young Na, Min Sun Shin, Jae Ho Pi, Bum Jun Kim, Jeong Hoon Bae, Young Ki Hong, Kyo Sun Lee, Sug Hyung Lee, Nam Jin Yoo, Ja June Jang, Svetlana Pack, Zhengping Zhuang, Laura Schmidt, Berton Zbar, and Jung Young Lee[2]

Department of Pathology and Cancer Research Institute, Catholic University Medical College, Seoul 137-701, Korea [W. S. P., S. M. D., S. Y. K., E. Y. N., M. S. S., J. H. P., B. J. K., J. H. B., Y. K. H., K. S. L., S. H. L., N. J. Y., J. Y. L.]; Department of Pathology, Seoul National University College of Medicine, Seoul 110-799, Korea [J. J. J.]; Laboratory of Pathology, National Cancer Institute, Bethesda, MD 20892 [S. P., Z. Z.]; Intramural Research Support Program, Science Applications International Corporation–Frederick [L. S.]; and Laboratory of Immunobiology, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, Maryland 21702 [B. Z.]

Abstract

The *MET* protooncogene encodes a transmembrane tyrosine kinase identified as the receptor of a polypeptide known as hepatocyte growth factor/scatter factor. We performed PCR-based single-strand conformational polymorphism and sequencing analysis of the tyrosine kinase domain of the *MET* gene (exon 15–19) in 75 primary liver cancers. Three missense mutations were detected exclusively in 10 childhood hepatocellular carcinomas (HCCs), while no mutations were detected in 16 adult HCCs, 21 cholangiocarcinomas, or 28 hepatoblastomas. The extremely short incubation period from hepatitis B virus infection to the genesis of childhood HCC as compared with the adult HCC suggests that there may be an additional mechanism that accelerates the carcinogenesis of childhood HCC. Our results indicate that mutations of the tyrosine kinase domain of the *MET* gene may be involved in the acceleration of the carcinogenesis in childhood HCC.

Introduction

The *MET* protooncogene encodes a transmembrane tyrosine kinase identified as the receptor of a polypeptide known as HGF/SF[3] (1). Binding with HGF/SF triggers tyrosine autophosphorylation of the intracellular domain in the MET receptor and induces a pleiotropic response in epithelial cells, including: (*a*) mitogenesis; (*b*) stimulation of cell motility; (*c*) dissociation of epithelial sheets; and (*d*) promotion of extracellular matrix invasion (2). In a normal liver, HGF/SF has been detected in bile duct epithelia, Ito cells, and endothelial cells of both the central lobular vein and the portal tract vessels, whereas MET receptor has been identified only in normal mature hepatocyte and in the "facultative stem cells" often referred to as oval cells (3). The latter are considered to be bipotential progenitor cells because they can be differentiated toward either bile duct epithelial cells or hepatocytes. Overexpression of MET protein has been documented in numerous human solid tumors including primary liver carcinomas such as HCC, hepatoblastoma, and intrahepatic cholangiocarcinoma (4–6). Activation of an oncogene is a frequent mechanism of tumorigenesis and may be accomplished by point mutation, rearrangement, or amplification. The human *MET* gene consists of 21 exons distributed over ~130 kb of genomic DNA located at chromosome 7q31. It encodes the extracellular ligand-binding domain containing exons 1–12, transmembrane domain containing exon 13, and intracellular kinase domain containing exons 15–21 (7, 8). Recently, trisomy of chromosome 7 and constitutively activating mutations of the *MET* gene have been identified in both hereditary and sporadic forms of papillary renal cell carcinoma (9, 10). All of these point mutations were missense mutations that localized to the tyrosine kinase domain of the MET receptor (9). All of these findings raise the possibility that mutations of the tyrosine kinase domain of the *MET* gene may also be associated with the development of primary liver carcinomas. It is an accepted fact that HBV plays an important role in the oncogenic process of HCC in children as well as adults (11–13). In adult HCC, it is well known that a minimum 20-year latency period is necessary for tumor development, whereas childhood HCC shows a minimum time period of 3 years (12); the reason for this short malignant transformation period after perinatal HBV infection in childhood HCC is still open to question (12, 13).

Here, we performed PCR-based SSCP and sequencing analysis of the tyrosine kinase domain of the *MET* gene (exon 15–19) in 75 malignant liver tumors; 10 childhood HCCs, 16 adult HCCs, 21 intrahepatic cholangiocarcinomas, and 28 hepatoblastomas. Our results indicate that mutations of the tyrosine kinase domain of the *MET* gene play an important role in the carcinogenesis of childhood HCC but not in adult HCC, hepatoblastoma, or intrahepatic cholangiocarcinoma of the liver.

Materials and Methods

Materials. This study was conducted on 75 primary liver carcinomas diagnosed at the Department of Pathology of the Catholic University Medical College or of Seoul National University Medical College, both in Seoul, Korea. We examined 26 HCCs (10 childhood and 16 adult-type), 28 hepatoblastomas, and 21 intrahepatic cholangiocarcinomas. Histological and DNA studies were performed on formalin-fixed, paraffin embedded tissue samples.

Microdissection. Tumor cells were selectively procured from H&E-stained slides using a 30 G1/2 hypodermic needle (Becton Dickinson, Franklin Lakes, NJ) affixed to a microdissection device (SPEM II; Simple, Precise and Economical Microdissection device, Sam Jung Co., Seoul, Korea), as described previously (14–16). We also obtained inflammatory cells or surrounding normal liver cells for corresponding normal DNA from the same slides in all cases.

DNA Extraction. DNA extraction was performed by a modified single-step DNA extraction method, as described previously (14–16).

SSCP and DNA Sequencing. Seven sets of primers covering the kinase domain of the *MET* gene (exon 15–19) were designed by using the OLIGO software program (version 5.0, National Bioscience Inc., Plymouth, MN) according to the cDNA and partial intron-exon boundary sequences of *MET* gene. The

Received 9/22/98; accepted 11/19/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by funding from the Good Health Research and Development Project of Ministry of Health and Welfare Republic of Korea (HMP-98-M-2-0018) and in part with Federal funds from the National Cancer Institute, NIH, under Contract No. NO1-CO-56000 (to L. S.).

[2] To whom requests for reprints should be addressed, at Department of Pathology, Catholic University Medical College, Seoul 137-701, Korea. Phone: 822-590-1190; Fax: 822-537-6586; Email: stingray@cmc.cuk.ac.kr.

[3] The abbreviations used are: HGF/SF, hepatocyte growth factor/scatter factor; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; SSCP, single-strand conformational polymorphism; HBs, hepatitis B surface antigen.

PCR primer pairs for the amplification of the kinase domain of the *MET* gene exon 15–19 were as follows: (*a*) 5′-CAGGCAGTGCAGCATGTAGTGA-3′ and 5′-TTCCTAATCTGCAAAGGCCAAAGA-3′ for exon 15; (*b*) 5′-GTTACGCAGTGCTAACCAA- GTT-3′ and 5′-TACTCCATGGTTAAATAAAATGCC-3′ for exon 16; (*c*) 5′-ATGAAGTTAATGTCTCCACCACTG-3′ and 5′-TTCACTTCGCAGGCAGATT-3′ for exon 17a; (*d*) 5′-CGAGGGAATCATCATGAAAGA-3′ and 5′-CAGTTAGTAAGCTTGGCAGTCAAC-3′ for exon 17b; (*e*) 5′-AAGTTCTGGGATTACAGGCTTGAG-3′ and 5′-TGGATTGTGGCACAGAGATTCT-3′ for exon 18; (*f*) 5′-TATTCTATTTCAGCCACGGGTAAT-3′ and 5′-CCA-AAGCCATCCACTTCACT-3′ for exon 19a; and (*g*) 5′-CCAGAGACATGTATGATAAAGAAT-3′ and 5′-AGGAGAAACTCAGAGATAACCA-3′ for exon 19b. Tumor and corresponding normal DNA from each slide were amplified in a thermal cycler (MJ Research Institute, Watertown, MA) with primers. Each PCR reaction was generally performed under standard conditions in a 10 μl reaction mixture containing 1 μl template DNA, 0.4 μM each primer, 125 μM each dNTP, 1.5 mM $MgCl_2$, 0.4 units *Taq* polymerase, 0.5 μCi [^{32}P]dCTP (Amersham, Buckinghamshire, United Kingdom), and 1 μl of 10× buffer. The reaction mixture was denatured for 5 min at 95°C and incubated for 35 cycles (denaturing at 95°C for 50 s, annealing at 50°C for 90 s and extending at 72°C for 90 s) with some variations in the annealing temperature. Final extension was continued for 10 min. The amplified DNA was mixed with an equal volume of formamide loading dye (95% formamide, 20 mM/liter EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol). The samples were denatured for 5 min at 95°C and loaded onto an MDE gel (AT Biochem, Malvern, PA) with 10% glycerol. After electrophoresis, the gels were transferred to 3 MM Whatmann paper, and autoradiography was performed using X-OMAT film. After the detection of abnormal bands by SSCP analysis, PCR was performed using DNA eluted from dried gels, and sequencing was done using Amplicycle Sequencing Kit (Perkin-Elmer, Branchburg, NJ). Normal and tumor DNA sequences were compared. All of the mutations were verified by repeated PCR and gel analyses using different SSCP gel conditions.

Results

Demographic Data. Among 16 cases of histologically proven adult HCCs, 11 patients were HBV-seropositive, 3 patients were Hepatitis C virus-seropositive, and the remaining 2 were anti-HBs-positive by the RIA method. The average age at diagnosis was 55.5 years (range, 42–72 years), and 13 patients had cirrhotic background. For the childhood HCC cases, HBs status and relevant clinical information are summarized in Table 1. HBV seropositivity was seen in 8 of 10 children with a 90% HBV-positivity rate in maternal serum; in the remaining 2 patients, anti-HBs antibodies were positive, which indicates past HBV infection. The age distribution at diagnosis was from 8 to 15 years (mean, 10.4 years). The histological patterns in all of the 10 cases were microtrabecular, and there was no fibrolamellar type. Only two patients had cirrhosis. There were 28 hepatoblastomas consisting of 20 epithelial, 7 mixed, and 1 undifferentiated type. The age distribution at diagnosis ranged from 1 month to 8 years with a mean of 22.4 months.

Mutation Analysis of the *MET* Gene. We detected three somatic mutations of the kinase domain in the *MET* gene in 10 childhood HCCs, as summarized in Table 1, and all of the three cases with aberrant bands and mutations are shown in Fig. 1. But no mutation was detected in 16 adult HCCs, 28 hepatoblastomas, or 21 intrahepatic cholangiocarcinomas. All of the mutations identified were missense mutations: a C to T transition at codon 1191 (Thr to Ile) in exon 17 in case 4, an A to G transition at codon 1262 (Lys to Arg) in exon 19 in case 6, and a G to A transition at codon 1268 (Met to Ile) in exon 19 in case 5.

Discussion

It is currently believed that gene amplification, rearrangement, or constitutive activating mutation of protooncogene encoding growth factor receptors may play an important role in the development of neoplasia by conferring a selective growth advantage. The *MET* gene is a receptor-type tyrosine kinase oncogene, originally identified as a transforming gene activated by rearrangement in cells treated with a chemical carcinogen (17). Increased levels of expression of HGF/SF and its specific receptor MET have been shown in several primary liver cancers such as HCC, cholangiocarcinoma, and hepatoblastoma (3–6). Recently, missense mutations— predicted to cause constitutive activation of the MET kinase in the absence of ligand—have also been identified in *MET* in patients with a hereditary or sporadic form of papillary renal cell carcinoma, which suggests that mutant MET might also be involved in other tumor development and progression (9, 18). It is not clear, however, whether activating mutations of *MET* are involved in the process of carcinogenesis of primary liver cancers in spite of its name, hepatocyte growth factor receptor. These facts led us to look for somatic mutations of the kinase domain of the *MET* gene in primary liver cancers. We designed seven sets of primers covering the kinase domain in *MET* and performed mutational analyses using PCR-based SSCP on our 75 primary liver cancer samples.

Mutations that activate receptor tyrosine kinases generally fall into two classes: (*a*) those that are localized to the extracellular or transmembrane domain and that promote receptor dimerization/oligomerization; and (*b*) those that are localized to the kinase domain and that alter catalytic activity or substrate specificity (8). We examined only those exons covering the kinase domain (exon15 to 19) because the sheer size of the *MET* coding region (consisting of 21 exons) makes the identification of the entire mutation labor-intensive and costly. We detected three missense mutations (T1191I, K1262R, and M1268I) in the kinase domain, and all of these mutations were exclusively in childhood HCC (3 of 10). There were no mutations in 21 cholangiocarcinomas, 28 hepatoblastomas, and 16 adult HCCs. Of these mutations, the K1262R and M1268I mutations fall within the COOH-terminal lobe of the kinase domain. This region of the molecule is believed to act as an intramolecular substrate that, in the absence of ligand, functions to inhibit enzymatic activity by blocking the active site (18, 19). It is possible that some of the mutations stimulate the kinase activity of MET by altering the structure of the intramolecular substrate such that it is constitutively disengaged from the active site.

Table 1 *Demographic data and MET mutations in 10 childhood HCCs*

							MET mutation			
Case no.	Sex	Age (yr)	Maternal HBV	HBV	Grade	Cirrhosis	Exon	Codon	Nucleotide	Amino acid
1	M	8	+	+	II	−				
2	M	12	+	+	II	−				
3	F	10	+	+	III	−				
4	M	12	+	+	II	−	17	1191	ACT → ATT	Thr → Ile
5	F	15	?	−	II–III	−	19	1268	ATG → ATA	Met → Ile
6	M	14	+	+	I–II	+	19	1262	AAG → AGG	Lys → Arg
7	M	10	+	+	II–III	−				
8	M	11	+	+	III–IV	+				
9	M	13	+	+	III–IV	−				
10	F	11	−	−	II–III	−				

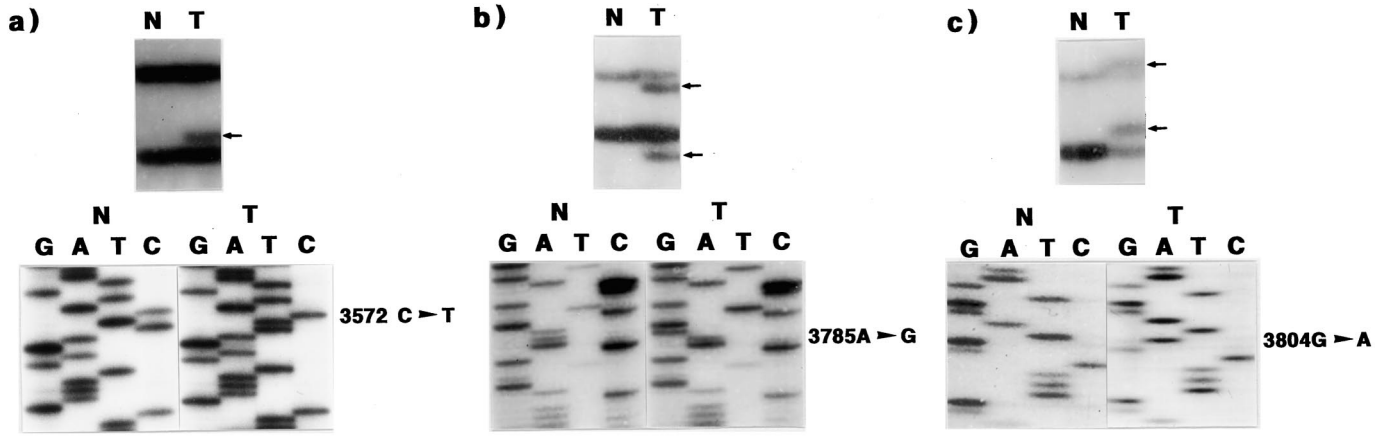

Fig. 1. The autoradiograms show SSCP and sequencing data in three cases of childhood HCC. All of the mutations were missense mutations: a C to T transition at codon T1191I on exon 17 in case 4 (*a*), an A to G transition at codon K1262R on exon 19 in case 6 (*b*), and a G to A transition at codon M1268I on exon 19 in case 5 (*c*).

In addition, the M1268 codon is highly conserved in a number of other tyrosine kinase receptors including c-Kit, Ret, PDGF-R, FGF-R, Ins-R, and EGF-R (18, 19). In fact, Ret receptor tyrosine kinase has been shown to be activated by amino acid change at methionine (M918), corresponding to the position of M1268 of MET, which leads to multiple endocrine neoplasia type 2B and sporadic medullary carcinoma of the thyroid gland (9). Additionally, this is the same location for a naturally occurring MET mutation found in papillary renal cell carcinoma, which was shown to be constitutively activating and transforming in NIH3T3 cells (18). Mutant T1191I is located in the subdomain span between NH_2- and COOH-terminal lobes of kinase domain (19), and the hydrophilic amino acid residue threonine is replaced by hydrophobic isoleucine.

The close relationship between HBV infection and HCC has been well documented by epidemiological case control and molecular biological studies (11–13). Because these infections are widespread in the Asian population, HCC occurs with a high incidence in Asia (11). In Korea, HCC is one of the most common malignant neoplasms, with an age-standardized incidence rate of 16.3% in male cancer patients and 5.6% in female patients, but remains an uncommon tumor in children (1.9% of all childhood malignant cases (20)). In the present study, adult HCC patients were found to have an 87.5% HBV and/or Hepatitis C virus. An 80% serum HbsAg positivity has been demonstrated in our childhood HCC patients, with a 90% positivity rate in maternal serum, which indicates that HBV transmission from the mother during perinatal period or early childhood is the most important mode of HBV infection in HCC patients in Korea. However, questions still remain to be answered concerning the relationship between HBV infection and the carcinogenesis of HCC. According to general opinion concerning the role of HBV in the oncogenesis of HCC, the incubation period is generally believed to be a minimum of 20 years (11, 12). This contrasts markedly to the average incubation period between the time of infection and the development of malignancy reported to be about 7 to 8 years in childhood HCC (12, 13). In our study, the mean age of childhood HCC is 10.4 years and the youngest child with HCC associated with HBV infection reported in the literature was 3 years old (12). In addition, a cirrhosis accompanying HCC in children is not common (2 of 10 in present study). Thus, this extremely short duration from HBV infection to the genesis of childhood HCC and the low incidence of cirrhosis in childhood HCC suggest that there may be an additional mechanism by which carcinogenesis in childhood HCC is accelerated when compared with adult HCC. We detected three mutations in the kinase domain of the *MET* gene that were exclusively observed in childhood HCC. These results suggest that mutant MET may be involved in the acceleration of the carcinogenesis process in childhood HCC.

Despite the small number of cases, the mutations in the kinase domain of the *MET* gene observed exclusively in childhood HCC suggest that mutant MET may play an important role in the carcinogenesis process of childhood HCC. However, additional studies in a large patient population and screening of the whole coding region of *MET* gene will be needed to verify these initial observations, and the identification of the biological function of MET will certainly broaden our understanding of the role of the *MET* oncogene in the pathogenesis of not only childhood HCC but also other tumors deserving consideration.

Acknowledgments

We thank Young Ho Han for help with English and Jong Won Kim and Se Young Kim for technical assistance.

References

1. Comoglio, P. M. Structure, biosynthesis and biochemical properties of the HGF receptor in normal and malignant cells. *In* I. D. Goldberg and E. M. Rosen (eds.), Hepatocyte Growth Factor-Scatter Factor (HGF-SF) and the C-*MET* receptor, pp. 131–165. Birkhäuser: Verlag Basel, 1993.
2. Weidner, K. M., Sachs, M., and Birchmeier, W. The *Met* receptor tyrosine kinase transduces motility, proliferation and morphogenic signals of scatter factor/hepatocyte growth factor in epithelial cells. J. Cell Biol., *121:* 145–154, 1993.
3. Grigioni, W. F., Fiorentino, M., D'Errico, A., Ponzetto, A., Crepaldi, T., Prat, M., and Comoglio, P. M. Overexpression of c-*met* protooncogene product and raised Ki67 index in hepatocellular carcinomas with respect to benign liver conditions. Hepatology, *21:* 1543–1546, 1995.
4. Suzuki, K., Hayashi, N., Yamada, Y., Yoshihara, H., Miyamoto, Y., Ito, Y., Ito, T., Katayama, K., Sasaki, Y., Ito, A., Kisida, Y., Kashiwagi, T., Fusamoto, H., and Kamada, T. Expression of the *c-met* protooncogene in human hepatocellular carcinoma. Hepatology, *20:* 1231–1236, 1994.
5. Von Schweinitz, D., Fuchs, J., Gluer, S., and Pietsch, T. The occurrence of liver growth factor in hepatoblastoma. Eur. J. Pediatr. Surg., *8:* 133–136, 1998.
6. Terada, T., Nakanuma, Y., and Sirica, A. E. Immunohistochemical demonstration of MET overexpression in human intrahepatic cholangiocarcinoma and hepatolithiasis. Hum. Pathol., *29:* 175–180, 1998.
7. Park, M., Dean, M., Kaul, K., Braun, M. J., Gonda, M. A., Van de Woude, G. F. Sequence of *MET* protooncogene cDNA has features characteristic of the tyrosine kinase family of growth-factor receptors. Proc. Natl. Acad. Sci. USA, *84:* 6379–6383, 1987.
8. Lin, J. C., Naujokas, M., Zhu, H., Nolet, S., Park, M. Intron-exon structure of the *MET* gene and cloning of an alternatively-spliced MET isoform reveals frequent exon-skipping of a single large internal exon. Oncogene, *16:* 833–842, 1998.
9. Schmidt, L, Duh, F. M., Chen, F., Kishida, T., Glenn, G., Choyke, P., Scherer, S. W., Zhuang, Z., Lubensky, I., Dean, M., Allikmets, R., Chidambaram, A., Bergerheim, U. R., Feltis, J. T., Casadevall, C., Zamarron, A., Bernues, M., Richard, S., Lips, C. J. M., Walter, M. M., Tsui, L. C., Geil, L., Orcutt, M. L., Stackhouse, T., Lipan, J., Slife, L., Brauch, H., Decker, J., Niehans, G., Hughson, M. D., Moch, H., Stokel, S., Lerman, M. I., Linehan, W. M., and Zbar, B. Germline and somatic mutations in the tyrosine kinase domain of the *MET* proto-oncogene in papillary renal carcinomas. Nat. Genet., *16:* 68–73, 1997.

10. Zhuang, Z., Park, W. S., Pack, S., Schmidt, L., Vortmeyer, A. O., Pak, E., Pham, T., Weil, R. J., Candidus, S., Lubensky, I. A., Linehan, W. M., Zbar, B., and Weirich, G. Trisomy 7-harbouring non-random duplication of the mutant *MET* allele in hereditary papillary renal carcinomas. Nat. Genet., *20:* 66–69, 1998.
11. Beasley, R. P. Hepatitis B virus as the etiologic agent in hepatocellular carcinoma: epidermiologic considerations. Hepatology, *2:* 21S–26S, 1982.
12. Chang, M. H., Chen D. S., Hsu, H. C., Hsu, H. Y., and Lee, C. Y. Maternal transmission of hepatitis B virus in childhood hepatocellular carcinoma. Cancer (Phila.), *64:* 2377–2380, 1989.
13. Cheah P. L., M, Looi, L. M., Lin, H. P., and Yap, S. F. Childhood primary hepatocellular carcinoma and hepatitis B virus infection. Cancer (Phila.), *65:* 174–176, 1990.
14. Lee, J. Y., Dong, S. M., Shin, M. S., Kim, S. Y., Lee, S. H., Kang, S. J., Lee, J. D., Kim, C. S., Kim, S. H., and Yoo, N. J. Genetic alterations of *p16*^{INK4a} and *p53* genes in sporadic dysplastic nevus. Biochem. Biophys. Res. Commun., *237:* 667–672, 1997.
15. Lee, J. Y., Dong, S. M., Kim, H. S., Kim, S. Y., Na, E. Y., Shin, M. S., Lee, S. H., Park, W. S., Kim, K. M., Lee, Y. S., Jang J. J., and Yoo, N. J. A distinct region of chromosome 19p13.3 associated with the sporadic form of adenoma malignum of the uterine cervix. Cancer Res., *58:* 1140–1143, 1998.
16. Lee, J. Y., Dong, S. M., Kim, S. Y., Yoo, N. J., Lee, S. H., and Park, W. S. A simple, precise and economical microdissection technique for analysis of genomic DNA from archival tissue sections. Virchows Arch., *433:* 305–309, 1998.
17. Cooper, C. S., Park, M., Blair, D. G., Tainsky, M. A., Huebner, K., Croce, C. M., and Van de Woude, G. F. Molecular cloning of a new transforming gene from a chemically transformed human cell line. Nature (Lond.), *311:* 29–33, 1984.
18. Jeffers, M., Schmidt, L., Nakaigawa, N., Webb, C. P., Weirich, G., Kishida, T., Zbar, B., and Vande Woude G. F. Activating mutations for the Met tyrosine kinase receptor in human cancer. Proc. Natl. Acad. Sci. USA, *94:* 11445–11450, 1997.
19. Hanks, S. K., and Hunter, T. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. FASEB J., *9:* 576–596, 1995.
20. Ministry of Health and Welfare Republic of Korea. Annual Report of Cancer Registry Program in the Republic of Korea, pp. 1–191. Seoul: Ministry of Health and Welfare Republic of Korea, 1997.

Somatic Mutations in the Kinase Domain of the *Met*/Hepatocyte Growth Factor Receptor Gene in Childhood Hepatocellular Carcinomas

Won Sang Park, Seung Myung Dong, Su Young Kim, et al.

Cancer Res 1999;59:307-310.