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c-Met Gene Amplification Is Associated with Advanced Stage Colorectal Cancer and Its Liver Metastases

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Abstract

The c-Met proto-oncogene encodes a receptor tyrosine kinase (TK) that promotes tumor invasive growth and metastasis. Recent studies have shown that presence of c-Met gene amplification is predictive for selective c-Met TK inhibitors in gastric cancer and lung cancer. In this study, we utilized a highly quantitative PCR/ligase detection reaction technique to quantify c-Met gene copy number in primary colorectal cancer (CRC) (N=247), liver metastases (N=147) and paired normal tissues. There were no differences in c-Met gene copy number between normal colonic mucosa and liver tissue. Mean c-Met gene copy number was significantly elevated in CRC compared to normal mucosa ($P < 0.001$) and in liver metastases compared to normal liver ($P < 0.001$) respectively. Furthermore, a significant increase in c-Met was seen in liver metastases compared to primary CRC ($P < 0.0001$). c-Met gene amplification was observed in 2% (3/177) of localized cancers, 9% (6/70) of cancers with distant metastases ($P < 0.02$) and in 18% (25/147) of liver metastases ($P < 0.01$). Among patients treated by liver resection, there was a trend toward worse 3-year survival associated with c-Met gene amplification ($P = 0.07$). Slight increases in c-Met copy number can be detected in localized CRCs, but gene amplification is largely restricted to Stage IV primary cancers and liver metastases. c-Met gene amplification is linked to metastatic progression and is a viable target for a significant subset of advanced CRC.

Keywords

colorectal cancer; liver metastasis; c-Met proto-oncogene; gene amplification; PCR-LDR

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1. INTRODUCTION

Colorectal cancer (CRC) is the fourth most common malignancy in the United States, with an estimated 153,760 new cases and 52,180 deaths in 2007 [1]. The main cause of CRC death is formation of hematogenous metastases. Thus, identification of patients at risk of developing distant metastases is fundamental for cancer management and prognosis. Prominent among cell metastasis-related molecules is the c-Met proto-oncogene product, which has been shown to stimulate cancer cell motility, invasion, and metastasis [2; 3].

The c-Met proto-oncogene belongs to the tyrosine kinase (TK) family of genes and codifies the hepatocyte growth factor receptor (HGFR). This proto-oncogene, initially discovered as a gene able to transform normal fibroblast cell lines [4], has subsequently identified in normal cells isolated from various mammalian tissues. The c-Met gene has been mapped to chromosome 7 at q-31 and encodes a 1408 amino acid transmembrane glycoprotein which specifically binds the hepatocyte growth factor/scatter factor (HGF/SF) [5]. Met-HGF/SF activation triggers a cascade of tyrosine phosphorylation and elicits mitogenic, motogenic, and morphogenic actions on a wide variety of target cells [6]. c-Met plays an important role both in physiological processes [2; 3; 6], such as embryological development, wound healing, tissue regeneration, morphogenic differentiation and pathological processes [2; 3; 6] including angiogenesis, scattering, cellular motility, growth, invasion, differentiation and migration [2; 3; 6].

In cancer, oncogenic activation of c-Met can occur by gene mutation, amplification and overexpression independent of binding to its ligand HGF [2]. Mutations in the tyrosine kinase and juxtamembrane domains can result in constitutive receptor activation. Mutations in c-Met have been identified in a few types of human cancers [3; 6], including papillary renal carcinomas, childhood hepatocellular carcinoma and head and neck squamous cell carcinomas. In CRC, we did not find c-Met mutations in 73 human CRC samples and seven CRC cell lines (unpublished results), which is consistent with findings from previous study [7]. c-Met overexpression at mRNA and protein levels have been reported in many varieties of human cancers [3; 6], including our previous reports in CRC [8; 9]. c-Met gene amplification has been reported in 10% to 20% of gastric cancers [10], in 4% of esophageal cancer [11] and 4% of lung cancers [12]. In CRC, Di Renzo, et al. reported that c-Met gene amplification was detected in 10% of primary CRC and in 89% (8/9) liver metastases by Southern blot hybridization [13]. However, this result could not be confirmed by others [14; 15].

c-Met gene amplification is receiving increased attention recently. The growing evidence has demonstrated that c-Met is an exciting novel drug target due to the success observed *in vitro*, *in vivo* and in preclinical studies [16]. Specifically, it has been shown that lung cancer and gastric cancer cell lines with c-Met gene amplification displayed significantly increased sensitivity to c-Met TK inhibitor [10; 17]. Treatment of c-Met amplified cancer cell lines with c-Met inhibitors leads to decreased cell growth, cell motility and eventual apoptosis [10; 17] suggesting that patients with amplified c-Met in their tumor may have clinical responses to c-Met inhibitors. Most recently, it is demonstrated that c-Met amplification contributes to non-small cell lung cancers (NSCLCs) acquired drug resistance to EGFR inhibitors, such as

gefitinib and erlotinib.[18] In addition, *in vitro* gefitinib sensitivity can be restored by blocking c-Met signaling [18].

The importance of the c-Met gene amplification in molecular target therapy as well as in acquired resistance to EGFR inhibitors prompted us to investigate the prevalence and clinical relevance of c-Met gene alteration in CRC. In this study, we analyzed large cohort of the frequency of c-Met amplification in different stages of primary CRC and liver metastases using a quantitative polymerase chain reaction / ligase detection reaction (PCR/LDR) technique developed in our laboratory.

2. Materials and Methods

2.1. Patient and Tissue Samples

After obtaining written consent, tissue samples were obtained immediately from patients undergoing surgery at Memorial Sloan-Kettering Cancer Center with the approval of the Institutional Review Board. The tissue samples were quick frozen in liquid nitrogen and stored at -80°C until processed. A total of 247 primary CRC and 147 liver metastases as well as corresponding normal tissues (mucosa or liver) were analyzed for c-Met amplification by PCR-LDR. In preparation for fluorescent in situ hybridization (FISH) analysis, frozen section tissue was embedded in O.C.T. (Miles, Elkhart, IN) and frozen in 2-methylbutane cooled with liquid nitrogen, as described below.

2.2 Cell lines and Culture Conditions

Seven commercially available human colorectal carcinoma cell lines COLO205, DLD1, HT29, LOVO, LS-180, SW480, and SW620 were purchased from American Type Culture Collection (ATCC, Manassas, VA). Gastric cancer cell line GTL-16 with well-documented c-Met gene amplification [19] as a positive control for c-Met amplification was obtained from Dr. Silvia Giordano.

LOVO was cultured in Ham's F12K medium. SW480 and SW620 were cultured in Leibovitz's L-15 medium, whereas HT-29 and LS180 were cultured in modified McCoy's 5A medium and Eagle in Earle's BSS with non-essential amino acids respectively. The other cell lines (COLO205, DLD1 and GTL-16) were cultured in RPMI 1640. All media were supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 IU/ml penicillin G. The cells were cultured at 37°C in a humidified incubator with 5% CO_2 . The cells were sub-cultured at sub-confluence by harvesting with a brief treatment with 0.25% trypsin–0.02% EDTA.

2.3. DNA Preparation from Fresh Tissues

Genomic DNA was extracted from frozen tumor tissues by standard proteinase K digestion and phenol/chloroform extraction as described previously [20]. In brief, the frozen tissue was macerated in liquid nitrogen and transfer to an Eppendorf tubes containing 500- μl digestion buffer (100mM NaCl, 10mM Tris HCL pH8.0, 25mM EDTA pH8.0, 0.5% SDS) and 500 μg proteinase K. The DNA was extracted with phenol/chloroform after incubation

overnight with shaking at 68°C and precipitated with ethanol. It was then resuspended in TE Buffer. The DNA concentration was determined by spectrophotometry.

2.4. Primer Selection

The software program Oligo (Molecular Biology Insights, Inc. MN) ^{was} used to design optimal PCR and LDR primers. The primers were selected from exon 15. PCR primers used for the analysis of c-Met amplification were forward primer: 5' CATCTCAGAACGGTTCATGCC - 3' and reverse primer: 5' - TGCACAATCAGGCTACTGGG - 3'.

For precise quantification of the native gene, 2 pairs of LDR primers are needed. One pair is specific for the wild-type fragment, and the other pair is specific for an internal standard (IS) that is identified to the wild-type (WT) fragment except for a 2 bp change. The 5' end of each upper primer is labeled with fluorescein. The LDR primers specific for the WT are as follows: upper: 5'-Fluor GTATCCTCTGACAGACATGTCCC-3' and lower, 5'-CCATCCTAACTAGTGGGGACTCTGA-3'. The LDR primers specific for IS are: upper, 5'-Fluor CCGGTATCCTCTGACAGACATGCCG -3' and lower 5'-GCATCCTAACTAGTGGGGACTCTGA- 3'. Thus the wild-type LDR product length is 48 bp and the internal standard LDR product length is 52 bp.

2.5. Polymerase Chain Reaction

PCR was performed using 100 ng of genomic DNA, in 50-μl reaction volumes, containing 1X PCR buffer (100 mM Tris-HCl, 15 mM MgCl₂, 500 mM KCl, pH 8.3), deoxynucleotide triphosphates (dNTP each at 0.2 mmol/L), 2 μM of each flanking primer primers and 1 unit of Taq polymerase. The reaction mixture was placed in a thermocycler (Hybaid, Ashford, UK). Programmable temperature cycling was performed with the following cycle profile: 94°C for 5 min and then 35 cycles each comprising denaturation for 1 min at 94°C, annealing for 1 min at 56°C, and extension for 1 min 30 sec at 72°C. After 35 cycles, the reaction tubes were kept for 10 min at 72°C and then at 4°C. The PCR products were fractionated on 2% agarose gel and visualized by ethidium bromide staining.

2.6. Cloning of c-Met Internal Standard PCR Products

A 2 bp substitution was introduced into the fragment amplified in the PCR described above using site-directed mutagenesis. The following PCR primer sequence was utilized for this purpose: 5'-atccatctcagaacgggtcatgccagaagtgcagtatcctcgacagacatgtccggcatcctaact-3'. The resultant mutant fragment contained a GG for CC substitution at positions 55 and 56 of the PCR product. This fragment was subsequently used as an internal standard.

The WT and the IS PCR fragment were agarose gel purified using QIAEX II agarose gel extraction kit (Qiagen, Valencia, CA) and then inserted into a using plasmid vector (TOPO cloning kit, Invitrogen, Carlsbad, CA) and transformed into *E. coli* TOP 10F' (Invitrogen, Carlsbad, CA) cells. After overnight incubation, the plasmid DNA was purified by Miniprep techniques, according to the manufacturer's instruction (Qiagen, Valencia, CA). Purified plasmid DNA concentrations were assessed by spectrophotometry at 260 nm and the DNA was then stored at -20°C. The plasmid containing the WT and the plasmid containing the IS

were used as standard for the quantitative PCR/LDR assay. The number of plasmid particles was calculated based on the OD₂₆₀ and the known molecular weight of the plasmid.

2.7. LDR for Wild-Type and Internal standard Fragment Discrimination

The LDR was carried out in a 25 µl reaction containing 1 X Taq DNA ligase buffer (20mM Tris-HCl, 25mM potassium acetate, 10mM magnesium acetate, 10mM dithiothreitol, 1mM NAD, 0.1% Triton X-100 pH 7.6@ 25°C), 20 unit of Taq DNA Ligase (New England BioLabs, Beverly, MA) and 2 µl of PCR product. The reactions were heated for 1.5 min at 94°C and then thermally cycled for 5 cycles (94 °C for 1 min and 65 °C for 4 min). Reactions were held at 4°C.

Three µl of the LDR products was mixed with 3 µl of loading buffer (50% formamide, 12.5 mM EDTA and 25mg/ml blue dextran) and then denatured at 95 °C for 2 min, chilled rapidly prior to loading on a 8 M urea-12.5% polyacrylamide gel and electrophoresed on an ABI 377 DNA Prism Sequencer (Applied Biosystems, Foster City, CA) at 1,400 volts for 2.5 hr. The presence of the ligated wild-type and internal standard LDR primers was evidenced by an electrophoretic mobility shift caused by successful ligation of a discriminating oligonucleotide to its fluorescently labeled paired primer. Genescan 350™ TAMRA size standard (Applied Biosystems, Foster City, CA) was used as a molecular weight marker. Fluorescent ligation products were quantified using the ABI Genescan analysis software (version 3.1, Applied Biosystems). Each sample was analyzed in duplicate and copy numbers determined from each corresponding standard curve.

2.8. Construction of the Standard Curve and c-Met Quantification

In each experiment a standard curve was constructed by serial dilution (1:5) of plasmid containing the WT fragment from 10⁷ to 10⁴ copies that was mixed with a fixed quantity of plasmid containing 10⁵ copies IS fragment. The PCR-LDR was performed as described. The WT-to-IS LDR product ratio is plotted on the Y axis against the known wild-type plasmid copy number on the X axis. An equation is generated by linear regression to describe this curve (SigmaPlot, version 5.0, Chicago, IL). These standard curves were used later to quantify the amount of c-Met gene copy number.

2.9. FISH Analysis

To validate PCR-LDR data, metaphases from seven colon cancer cell lines and touch preparations of 12 frozen colon cancers were subjected to FISH analysis using a c-Met probe in combination with a chromosome 7-specific centromere probe (CEP-7). The CEP7 probe was used as an internal control for chromosomes 7 aneusomy. The human bacterial artificial chromosome (BAC) clone RPC11-163C9 containing c-Met was obtained from the Invitrogen (Carlsbad, CA).

Metaphases from seven colon cancer cell lines were prepared for FISH analysis by standard methods.[21] Imprint touch preparations were done by lightly pressing a semi-thawed frozen tumor onto Vectabond-coated (Vector Laboratories, Burlingame, CA) microscope slides. After air drying, slides were fixed in cold Carnoy's solution (3:1 methanol/acetic acid) for 20 minutes. Samples were then fixed with 2% formalin with 50 mM MgCl₂ in PBS for 10

minutes, prehybridized in 2XSSC (30 minutes at 37 °C), dehydrated in cold graded ethanols and air dried [22].

FISH was performed according to standard procedures [21]. Briefly, The RP11-95120 and CEP7 probes were labeled by nick translation with biotin-(red) and digoxigenin- (green) dUTPs, respectively. About 50 ng of biotinylated probe and 25 ng of digoxigenin-labeled probe were mixed in 14 µl of hybridization mixture (containing 50% formamide, 2x SSC, 10% dextran sulphate, 0.1% SDA, 1X Denhardt's solution, 40mM phosphate pH7.0 and 1 µg human Cot I DNA), which was denatured at 65°C for 10 min. The slides containing metaphases from tumor cells were then denatured in 70% formamide at 65°C for 10 min and hybridized with pre-anneal probes at 37°C overnight. The biotin- and digoxigenin-labeled probes were detected consecutively using dual-color detection system. Counterstaining was performed with 4'6-diamidino-2-phenylindole (DAPI). Slides were mounted in Vectashield (Vector, Burlingame, CA). Signals were visualized with a Nikon Eclipse fluorescence microscope (Japan). Images were captured using the Metasystem software (Digital Scientific, Cambridge, United Kingdom).

2.10. Quantitation and Statistical Analysis

Each PCR contained 100 ng of the genomic DNA of interest and 10^5 copies of the internal standard. Therefore, the copy number of the IS in each reaction was the same as in the standard curve. The c-Met gene copy number was then derived by inserting the WT-to-IS LDR product ratio from each sample lane into the equation for the standard curve. The assay was performed in duplicate on all specimens. The gene copy number for each sample was the mean value of the duplicate assay.

c-Met gene amplification level was calculated for each specimen by dividing the absolute copy number for each tumor by the pooled mean copy number of the normal tissue. Tumors were considered to be amplified, if the gene copy number was 3 times the pooled mean of corresponding for normal tissue. Statistical analyses were performed using the SPSS 10.0 (SPSS Inc. Chicago, Illinois). A two-sided, P-value less than or equal to 0.05 was considered to indicate statistical significance. The Kaplan-Meier survival analysis was carried out using SPSS 10.0. Statistical significance of the difference between the survival curves for different groups of patients was assessed using χ^2 and log-rank tests.

RESULTS

3.1. Construction of the Standard Curve

The standard curves were used to quantify the amount of c-Met gene copy number. In each PCR-LDR assay, a new standard curve is constructed. A typical PCR-LDR gel imaging of serially diluted standard containing c-Met plasmid DNA (WT) gene copy number from 10^7 to 10^4 and fixed 10^5 gene copy number of plasmid DNA (IS) is shown in Figure. 1-A. The electropherogram of LDR was produced by the Genescan software (Figure. 1-B). The wild type to internal standard LDR product ratio was calculated by measuring the peak area of each standard WT (48 bp) and IS LDR product (52 bp). The WT to IS LDR product ratio is plotted on the Y axis and the known wild type plasmid copy number on the X axis (Figure.

1-C). The software (SigmaPlot, version 5.0) produced the standard curve by measuring the crossing point of each standard wild type plasmid copy number and plotting them against the logarithmic values of wild type to internal standard LDR product ratio. The standard curve using known plasmid c-Met gene copy number showed a high degree of linearity over the WT to IS LDR product ratio. The linear relationship was strong (The R^2 value for this curve is 0.99).

3.2. c-Met gene copy number in colon cancer cell lines detected by PCR-LDR and FISH

All seven colon cancer cell lines showed detectable copy number by PCR-LDR assay. PCR-LDR Genescan gel image from GTL-16 gastric cancer and seven colon cancer cell lines is shown in Figure. 2-B. A 48 bp LDR product corresponding to the c-Met gene was observed in all cell lines. The c-Met gene copy numbers were determined from each corresponding standard curve and displayed graphically (Figure. 2-A). The c-Met gene copy number per 100ng DNA in GTL-16 was 2,644,748, representing a 26.6 fold gene amplification compared to the mean c-Met copy number determined in sample of normal colonic mucosa. In colon cancer cell lines, c-Met gene copy number was varied from 199,465~160,385. The gene copy number was increased from 1.6 ~ 2.0 compared to normal colonic mucosa. To validate our PCR-LDR analysis data, we further investigated the c-Met gene copy number measured by two-color FISH. (Figure 2-C) Consistence with PCR-LDR, c-Met is highly amplified in gastric cancer cell line GTL-16. The seven colon cancer cell lines only showed euploidy or aneuploidy without c-Met amplification (Figure. 2) COLO205 and LS180 were diploid, SW620 was triploid, DLD1, HT29, SW480 were tetraploid, and LOVO was hexaploid.

3.3 c-Met Amplification in Primary Colorectal Cancer and Liver Metastases

Quantitative PCR-LDR was performed to evaluate c-Met gene copy number in 247 primary CRC, 202 corresponding colon mucosa and 147 CRC liver metastases as well as normal liver tissues. The top panel of Figure 3 shows an example of c-Met PCR-LDR in primary CRC (T) normal mucosa (N), liver metastases (LM), and normal liver (L). The normal colonic mucosa samples had an average gene copy number per 100ng DNA of $99,578 \pm 2,299$ (mean $\pm$ SE), whereas it was $127,962 \pm 5,584$ in CRC tissue samples. The mean c-Met gene copy number per 100ng DNA in normal liver tissues and paired liver metastases was $92,441 \pm 2,842$ and $196,888 \pm 16,320$, respectively. The distribution of PCR-LDR data is summarized in Figure. 4. There were no significant differences in the mean c-Met gene copy number between normal colonic mucosa and normal liver tissue ($99,578 \pm 2,299$ vs. $92,441 \pm 2,842$, $P > 0.05$, Figure.3). Furthermore, a significant increase c-Met gene number was seen in liver metastases compared to primary CRC (196888 ± 16320 vs. 127962 ± 5584 $P < 0.0001$).

Amplification of c-Met was defined as more than threefold c-Met gene copy number relative to the mean value of normal mucosa tissue samples. Based on this definition, 9 of the 247 (4%) examined showed c-Met amplification ranging from 3- to 9.5-fold. Seven CRC samples revealed three to five-fold amplification, and two tumors presented 5- to 10-fold amplification. c-Met gene amplification (≥ 3 fold compared to the mean of normal liver) was observed in 18% (25/147) liver metastases ranging from 3- to 14-fold (14 liver metastases

showed three to fivefold amplification, nine tumors presented 5- to 10-fold amplification, three liver metastases presented 10- to 14-amplification). Furthermore, when the mean value of c-Met copy number is compared for primary CRC and liver metastases, the difference is significant ($X^2=22.45$, $P<0.001$).

3.4. Validation of PCR/LDR data with FISH

The PCR-LDR results were further confirmed by comparing them with FISH in selected human tissues. None of 6 cases (3 CRCs and 3 liver metastases) without c-Met amplification detected by PCR-LDR show gene amplification by FISH (Figure. 3-A & C). However, in 6 specimens (3 CRCs and 3 liver metastases) with c-Met amplification determined by PCR-LDR, FISH analysis confirmed the quantitative PCR finding (Figure. 3-B & D) in all samples.

Relationships Between c-Met Amplification to Clinicopathological Parameters in Primary CRC

To further examine whether c-Met amplification is associated with clinicopathological characteristics, we investigated the clinical phenotype of patients whose tumor harbored c-Met amplification (Table 2). No significant associations were found between c-Met amplification and patient's gender ($P = 1.00$), age ($P = 0.230$), tumor location ($P = 0.286$), mucinous morphology ($P = 0.481$), tumor differentiation ($P = 0.752$) blood and/or lymphatic vessel invasion ($P = 0.203$), depth of tumor invasion (T status, $P=0.222$) or the status of lymph node metastases (N stage $P=1.000$) (Table 1). Although statistically not significant, c-Met gene amplification was only observed in T₃ CRC. Primary CRCs with distant metastases (M₁) were demonstrated to have a statistically significant difference of c-Met gene amplification compared to CRCs without distant metastases (M₀) ($P=0.017$). With regard to stage, c-Met amplification occurred mainly in stage IV (6/9). The correlation of c-Met amplification and CRC stage approached statistical significance ($p=0.061$).

3.5. Relationships between c-Met Amplification to Clinicopathological Parameters in Liver Metastases and Survival

There was no significant association of the c-Met amplification with any of the following clinicopathological parameters: gender, patient's age, the number of liver metastases, resection of liver metastases, status of extrahepatic spread, interval between CRC resection and liver metastases (Table 2). Because the prevalence of c-Met amplification was very low, we excluded primary CRC patients from the prognosis analysis.

To determine whether c-Met amplification affects the survival of patients with liver metastases, we prepared Kaplan-Meier survival curves and analyzed them statistically. Clinical follow-up data were available for all 147 liver metastases patients, of which 86 were treated with curative intent by hepatic resection. We compared the survival among all CRC liver metastases patients and 86 resectable liver metastases according to the c-Met amplification status. By log-rank tests, no difference was observed between c-Met amplification and 3 year overall survival in 147 liver metastases ($P = 0.59$). In the subgroup of 86 resectable liver metastases, sixteen (19%) of the samples showed c-Met gene amplification. According to Kaplan-Meier analysis, the 3-year survival in c-Met amplification group and no c-Met amplification group was 43.75% and 68.57%,

respectively. Log-rank tests showed that this difference only approaches significance ($P = 0.07$; Figure. 5).

4. DISCUSSION

Gene amplification is a critical mechanism by which tumors gain growth advantage and metastatic potential. Standard laboratory methods to study gene amplification such as, southern blot hybridization and FISH, however, are difficult for analysis of large series of samples. In addition, genomic arrays are only semi-quantitative and may miss narrow amplicons in a background of large chromosomal arm alterations. Therefore, the use of efficient and highly quantitative PCR techniques remains the most accurate method for detection of specific gene copy number aberrations. The PCR-LDR quantitative assay allows qualitative and quantitative analyses of alterations in DNA copy numbers [23]. The test is based on simultaneous PCR amplification of the target gene with a structurally altered internal standard. The ratio of the target gene to internal standard is constant throughout the PCR and is quantified after completion of PCR by LDR. By comparison to standard curves, the ratio can be translated into an absolute copy number of the target gene per nanogram of genomic DNA.

Our results have shown that PCR-LDR analysis gives highly reliable gene copy number data, with a several advantages compared with other methods. Quantitative PCR/LDR uses an internal standard that precisely mirrors the wild type fragment with the exception of a two-base pair substitution. Because the internal standard is present in every PCR, quantification is less susceptible to changes in PCR efficiency due to the inevitable small sample to sample variations found in clinical material. Therefore, this approach is particularly suitable for analysis of large number of clinical samples. Quantification of wild type and internal standard fragments by LDR relies on single-strand DNA recognition and avoids potential quantitative inaccuracies associated with heteroduplex formation. In addition, amplification by PCR primers followed by quantification by LDR primers provides exceedingly high specificity such that false signal from random priming or from homologous DNA sequences is eliminated. Detection of PCR-LDR products can be performed automatically without autoradiography. Linearity of fluorescence detection covers a much wider range than scanning of autoradiograms or ethidium bromide, resulting in an improved quality of data. PCR-LDR assay was previously used to detect Her 2/neu amplification in CRC and breast cancer in our laboratory [24; 25].

Our study is the largest cohort examination of c-Met gene copy number in human cancer and patient outcome to date. Nearly 400 tumor samples and matched normal tissues were evaluated by PCR-LDR analysis. We demonstrated that c-Met gene amplification is a relatively rare event (3.6%) in primary human CRC, with the majority of amplified case (6/9) occurring in primary cancer that presented with synchronous metastases within the liver (Stage IV). In addition, we demonstrated that c-Met amplification is statistically associated with distant metastases (M_1 stage). A closer evaluation of the phenotype associated with c-Met amplification in primary cancers shows that all 9 primary CRCs with c-Met amplification were deeply invasive into the intestinal wall (T_3 stage). Our observation is consistent with a prior report which showed high c-Met mRNA was correlated with depth of

tumor penetration [26]. It was reported that c-Met mRNA copies significantly correlated with CRC lymph-nodes metastases [26]. Our previously data demonstrated that c-Met protein expression by western blotting was correlated with advanced CRC stage and lymphovascular invasion [8]. However, in the present study, we did not find an association of c-Met amplification with lymph node metastases.

In order to understand the potential role of c-Met amplification in CRC liver metastases, we investigated the status of c-Met amplification in primary CRC as compared to liver metastases. There were no significant differences in the c-Met gene copy number between normal mucosa and liver. However, a significant increase c-Met gene copy number was seen in liver metastases compared to primary CRC ($P<0.0001$). Furthermore, c-Met gene amplification was observed almost five times more often (18%) in liver metastases than primary CRC. In two cases, paired tumor samples from primary colon cancer and synchronous liver metastasis were obtained. Interestingly, one showed c-Met amplification in liver metastases but not in primary colon cancer. These data strongly suggest that c-Met gene amplification is a late event in colorectal cancer progression that is associated with a high risk of hematogenous spread. Various *in vitro* and animal studies provide evidence that c-Met amplification or overexpression can facilitate colonization of the liver [27; 28]. A separate study of CRC xenografts demonstrated that elevated levels of c-Met transcripts were observed in all liver metastases as compared with the primary CRC growing in nude mice [29]. Herynk et al [30]. demonstrated that the highly metastatic human colorectal carcinoma cell line, KM20, with reduced c-Met expression by transfection with a c-Met-specific targeting ribozyme had significantly reduced growth rates and soft-agar colony-forming abilities *in vitro*. When the c-Met down-regulated cells were grown in the liver, both tumor incidence and growth rate were reduced. It has also been observed in adult T-cell leukemia (ATL) that ATL cells staining strongly positive for c-Met expression were seen in the perivascular area of liver [31]. Taken together, these data suggested that genetic alterations of c-Met provides a biologically significant survival and growth advantage within the liver, where there is high tissue expression of hepatocyte growth factor, the ligand for c-Met.

C-Met mutation, amplification and overexpression are demonstrated to have clinical significance in patients with different cancers [3; 6]. However, the correlation of c-Met amplification and clinical outcome in CRC liver metastases has not been previously reported. In current study, the number of primary CRC with c-Met amplification (3.6%) was too small for prognostic analyses. The prognostic significance of the c-Met amplification was retrospectively studied in two sets of liver metastases. In 147 CRC liver metastases patients, no significant difference was found between c-Met amplification group and no amplification group. In the subgroup of 86 resectable liver metastases, there was a trend toward a poor prognosis in patients with liver metastases harboring c-Met amplification. Thus, our data support the conclusion that the c-Met pathway plays a significant role in CRC liver metastasis.

The identification of key targets promoting metastasis is of great interest for the development of specific treatment strategies. Several groups have reported to develop novel therapeutic strategies for targeting c-Met *in vitro*, *in vivo* and in various stages of clinical

testing [32; 33; 34]. Recent evidence has demonstrated that c-Met amplification is critical for lung cancer and gastric cancer cell growth [10; 17]. Tumors that harbor amplified c-Met are potential targets for selective c-Met TK inhibitors [17]. The gastric cancer cell lines with c-Met gene amplification displayed high sensitivity to a specific c-Met TK inhibitor PHA-665752 [35]. In 5 of 5 gastric cancer cells with c-Met amplification, treatment with PHA-665752 resulted in a significant reduction in cell numbers, whereas treatment had no effect in any of the 12 cancer cells without c-Met amplification [10]. A similar results were reported in NSCLC. In c-Met amplified lung cancer cell lines EBC-1 and H1993, ShRNA-mediated Met knockdown induced significant growth inhibition, G₁-S arrest, and apoptosis, whereas it had little or no effect on the 7 lung cancer cell lines without c-Met amplification [17]. These data suggested that the patients with amplified c-Met in their tumor may have a significant response rates to c-Met inhibitors similar to those observed for Her2 amplification in breast cancer and EGFR in lung cancer [36] and metastatic CRC [37; 38]. Our current study demonstrated that c-Met amplification is present in about 18% of liver metastases patients, analysis of c-Met copy number in biopsy or resection samples may identify a subset of CRC liver metastases patients likely to respond to targeting amplified c-Met therapy.

EGFR interacts with signaling pathways affecting cellular growth, proliferation, and programmed cell death. EGFR inhibitors, erlotinib and gefitinib, are used to treat NSCLC with activating of EGFR [39]. Although most EGFR mutant NSCLCs initially respond well to EGFR inhibitors, resistance usually occurs in the majority of these patients within 6–12 months of the initiation of therapy [39]. In about 50% of these cases, resistance occurs because a single secondary mutation in EGFR exon 20, T790M, which recur after an initial response to EGFR inhibitors [39]. Most recently, Engelman and colleagues have identified that c-Met amplification is a new mechanism for some cases of acquired resistance to EGFR inhibitor therapy [18]. A genome-wide copy number analysis showed in the EGFR drug resistant cell line HCC827 GR, a marked focal amplification of the 7q31.1 to 7q33.3, which contains the c-Met was observed. Sequence analysis ruled out the presence of c-Met mutations in these cells. Although treating resistant cell lines with either gefitinib or a c-Met inhibitor did not stop tumor growth, treatment with combination of the two agents, the resistance was reversed. Therefore, it is suggested that combination therapies with c-Met inhibitors and EGFR inhibitors should be considered for patients whose tumors have become resistant to gefitinib or erlotinib.

Importantly, this resistance mechanism was also observed in NSCLC patients. Notably, 4 out of 18 (22%) NSCLCs that were resistant to gefitinib or erlotinib treatment showed c-Met amplification, and only one of these patients had a concomitant secondary mutation of EGFR. For eight patients, paired tumor specimens from before treatment and after the development of resistance to gefitinib were obtained. Of the eight paired tumor samples, two showed c-Met amplification in the resistant specimens but not in the before-treatment samples. The mechanism for development drug resistance after treatment with EGFR-directed therapies in CRC is not yet well understood. It will be interesting to explore whether c-Met amplification also occurs in CRC.

In conclusion, this study is the largest cohort examination of c-Met gene amplification in human cancer to date. The PCR-LDR assay provides important advantages for rapid, detection of DNA copy number. Using this method we demonstrated that c-Met amplification is rare in localized CRC but much more common in Stage IV primary cancers and liver metastases. Both association with advanced stage CRC and liver metastases suggest that c-Met gene amplification plays an important role in CRC progression and metastases. Higher incidence of c-Met amplification and poor prognosis in CRC liver metastases patients may have important clinical implications.

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Figure 1.

Standard curve of the c-Met gene copy number measurement by PCR/LDR. (A). GeneScan gel image of a typical standard curve. Internal standard (IS) plasmid concentration is fixed (10⁵), while serially diluted standard containing Wild type (WT) c-Met plasmid DNA gene copy number from 10⁷ to 10⁴. The first lane in each case is negative control (water without any added WT or IS c-Met DNA). c-Met IS plasmid DNA and gastric cancer cell line GTL-16 as a positive control were shown in the second and third lane. A red color 50-bp molecular ruler as a molecular marker is also shown. The next seven lanes depict the WT c-Met plasmid DNA gene copy number using 7 standards ranging from 10⁷ to 10⁴ standards. (B) Electropherograms of LDR reactions. The data were obtained by ABI 377 sequencer and analyzed with the GeneScan software. The peaks on the left and right are WT and IS c-Met

LDR products respectively. (C) A graphic representation of the same standard curve. WT to IS LDR product ratio is plotted on the Y axis vs. known wild type plasmid copy number on the X axis. The R^2 value (coefficient of correlation) for this curve is 0.99.

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Figure 2.

c-Met copy number status in cancer cell lines determined by PCR-LDR and FISH. Top: Bar graphs depict the c-Met gene copy number quantified by PCR-LDR assay. Amplification of c-Met was significantly higher in gastric cancer cell line GTL-16 than colon cancer cell lines. Middle: GeneScan gel image from cancer cell lines. A 48-bp band at the expected size for c-Met was observed in all cancer cell lines. A red color 50-bp molecular ruler as a molecular marker is also shown. C: Dual-color fluorescence in situ hybridization (FISH) on metaphase spread and interphase nuclei of cancer cell lines with a centromeric probe for

chromosome 7 (green color) in combination with a c-Met specific probe (red signals). DAPI (blue) was used as counterstain. C-Met was amplified in gastric cancer cell line GTL-16. All seven colon cancer cell lines show polyploidy.

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Figure 3.

Top: A representative PCR-LDR assay of 3 cases of colorectal cancer and 3 cases of liver metastases. A GeneScan gel images were generated by ABI 377 sequencer. A 48-bp band at the expected size for c-Met was observed in colorectal cancer (T) and normal mucosa (M) as well as in liver metastases (LM) and normal liver (NL). A red color 50-bp molecular ruler as a molecular marker is also shown. Bottom: Dual-color fluorescence in situ hybridization (FISH) on touch preparation sections with a centromeric probe for chromosome 7 (green color) in combination with a c-Met specific probe (red signals). DAPI (blue) was used as counterstain. Colon cancer (A) and liver metastases (C) without c-Met amplification detected by PCR-LDR do not show c-Met amplification by FISH. However, colon cancer (B) and liver metastases (D) with c-Met amplification detected by PCR-LDR, FISH analysis proved the gain c-met gene copy number.

Figure 4.

Scatter graph showing mean c-Met copy number measured by PCR-LDR. No difference was demonstrated between normal mucosa and liver. A significant increase of c-Met gene copy number is seen in cancer compared to corresponding normal tissue. In addition a significant increase of c-met gene copy number is seen in liver metastases compared to the primary colorectal cancer ($p<0.001$). Horizontal bars represent median values.

Figure 5. Kaplan-Meier analyses of overall survival in relation to c-Met gene amplification in CRC liver metastases. The 3 year overall survival time showed a slight trend toward a poor prognosis for c-Met amplification ($P=0.07$).

Characteristics of patients and correlation between c-Met gene amplification in primary colorectal cancer and clinicopathological parameters

Table 1

Parameter	Number of Investigable	(%)	Number of c-Met Amplification	P value
Total Patients	247	100	9	
<u>Gender</u>				
Female	118	47.8	4	1.000
Male	129	52.2	5	
<u>Age (Yr)</u>				
60	61	24.7	4	0.230
> 60	186	75.3	5	
<u>Tumor Location</u>				
Right	86	34.8	5	0.286
Left	22	8.9	0	
Sigmoid	57	23.1	3	
Rectum	82	33.2	1	
<u>Mucinous</u>				
No	166	67.2	5	0.481
Yes	81	32.8	4	
<u>Tumor Differentiation</u>				
Well	10	4.0	0	0.752
Moderate	199	80.6	8	
Poor	38	15.4	1	
<u>Blood and/or lymphatic Vessel Invasion</u>				
No	203	81.0	6	0.203
Yes	44	19.0	3	
<u>Depth of invasion</u>				
T ₁	10	4.0	0	0.222
T ₂	52	21.1	0	
T ₃	168	68.0	9	
T ₄	17	6.9	0	

Parameter	Number of Investigable	(%)	Number of c-Met Amplification	P value
<u>Lymph node metastases</u>				
N ₀	131	53.0	5	1.000
N ₁₋₃	116	47.0	4	
<u>Distant metastases</u>				
M ₀	177	71.7	3	0.017
M ₁₋₃	70	28.3	6	
<u>Clinical Stage</u>				
I	43	17.4	0	0.061
II	71	28.7	2	
III	63	25.5	1	
IV	70	28.4	6	

* T-test for comparison of two groups, F-test for comparison of more than 2 groups.

Characteristics of patients and correlation between c-Met gene amplification in colorectal cancer liver metastases and clinicopathological parameters

Table2

Parameter	Number of Investigable	(%)	Number of c-Met Amplification (%)	P value
Total Patients	147	100	26	17.8
<u>Gender</u>				
Female	58	39.5	9	15.5
Male	89	60.5	17	19.1
<u>Age (Yr)</u>				
60	71	48.3	10	14.8
> 60	76	51.7	16	21.1
<u>Liver Metastases</u>				
Single	62	42.2	13	21.0
Multiple	85	57.8	13	15.3
<u>Resectable</u>				
No	61	41.5	10	16.4
Yes	86	58.5	16	11.6
<u>Interval between CRC resection and liver metastases</u>				
No	63	42.8	13	20.6
Yes	84	57.2	13	15.5
<u>Extrahepatic spread</u>				
No	127	86.4	22	17.3
Yes	20	13.6	4	20.0

* T-test for comparison of two groups, F-test for comparison of more than 2 groups.