Detection of MET oncogene amplification in hepatocellular carcinomas by comparative genomic hybridization on microarrays

Research Article

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Summary

The oncogene MET localized on human chromosome 7q21-31 encodes a transmembrane protein with tyrosine kinase activity and is believed to be implicated in progression of colorectal cancer. The aims of the study were to determine whether overexpression and amplification of the MET oncogene confers a selective growth advantage to hepatocellular carcinomas. Comparative genomic hybridization on microarrays was used in the analysis of DNA from 32 liver tumors (6 hepatocellular carcinoma; 16 colorectal liver metastases; 3 cholangiocarcinomas; 2 adenomas; 2 fibrolamellar; 3 unclassified) to screen for sequence copy number changes. The results revealed a MET gene amplification in hepatocellular carcinoma, cholangiocarcinoma, and colorectal liver metastases tumors. Moreover, one of the patients with hepatocellular carcinoma showed MET amplifications in both tumor and non-tumor samples, with the tumor having approximately 12.8 copies of the MET target locus per cell. These findings suggest that amplifications in the MET gene may play an important role in hepatocarcinogenesis.

I. Introduction

The oncogene MET, localized on human chromosome 7q21-31 by in situ hybridization (Dean et al, 1985), encodes a transmembrane protein with tyrosine kinase activity (Dean et al, 1985; Park et al 1996). It was shown that this protein is the receptor of hepatocyte growth factor (HGF)/ Scatter factor (Giordano et al, 1989; Bottaro et al, 1991), and the signals of HGF are transduced through the receptor tyrosine kinase encoded by the MET proto-oncogene. The MET gene can be activated by the formation of a chimeric gene through fusing the translocated promoter region (TPR) on chromosome 1 to the N-terminally truncated MET kinase domain (Park et al, 1996). Gene amplification and mutation may be another path to MET proto-oncogene activation, since MET gene amplifications have been reported in human gastric carcinomas (Soman et al, 1990; Ponzetto et al, 1991) and gliomas (Fischer et al 1995). Furthermore, MET gene amplification and the resulting over-expression are believed to be involved in progression of colorectal cancer (Di Renzo et al, 1995).

Human hepatocellular carcinoma (HCC) is one of the most common and devastating cancers with a poor prognosis. It has been widely considered that hepatitis B virus (HBV) and environmental agents such as aflatoxin B1 are major risk factors. However, the molecular mechanism of hepatocarcinogenesis is poorly understood. Loss of heterozygosity (LOH) has been reported for several genomic loci, such as the region surrounding RB1 on 13q (Nishida et al, 1992; Zhang et al, 1994), or sequences on 11p (Rogler et al, 1985), and 6q (De Souza

et al, 1995). Mutation of the p53 gene was detected in approximately 36% of advanced HCC (Murakami et al, 1991) and was also implicated in tumor progression (Teramoto et al, 1994). Overexpression was reported for several oncogenes such as N-ras, c-myc and fos (Arbuthnot et al, 1991). However, oncogene amplification appears to be rarely the underlying mechanism of cancer development in these cases. Amplifications associated with HCC have been found on 11q13 (Nishida et al, 1994), involving both INT2 and cyclin D1. Nishida and colleagues (1994) showed that the cyclin D1 gene was amplified 3 to 16 fold in about 11% of HCC samples analyzed, with a concomitant 6 to 10 fold overexpression. Based on this finding they suggested that amplification and overexpression of the cyclin D1 gene might be responsible for rapid growth of a subset of HCC.

The rapid emergence of microarray technology has allowed new approaches to tumor analysis. The most common application of this technology has focused on the use of cDNA arrays for the large-scale analysis of gene expression to monitor tumor progression (Sgroi et al, 1999) or for cancer typing (Anbazhagan et al, 1999). Oligonucleotide arrays have enabled rapid re-sequencing for genotyping or point mutation analysis, such as p53 mutation detection (Hacia, 1999). Applying Comparative Genomic Hybridization (CGH) to microarrays of large genomic clones has also been successful, allowing the detection of gross chromosomal abnormalities that result in copy number changes for a given sequence, such as gene amplifications or deletions (e.g. LOH), (Solinas-Toldo et al, 1997; Pinkel et al, 1998; Muller, 2001). Such sub-chromosomal aneuploidies are known to be fundamental causes of cancer and many other human diseases, often leading to the over- or under-expression of genes.

II. Materials and methods

We have developed a CGH-based microarray system (Genosensor System) and a microarray to specifically detect abnormalities of 52 genomic loci that have been associated with formation of various human solid tumors (Müller et al, 2002). The arrays consist of 3 repeats each of 52 P1, PAC or BAC clone DNAs that are arrayed on a chromium-coated glass surface. For hybridization to this array, genomic DNA samples were extracted from human liver tumors or from histopathologically non-tumor liver sections from the same patient. After purification (Gentra Kits, Gentra Systems, Inc., Minneapolis, MN), the genomic DNA samples were then labeled by nick translation (Nicktranslation Kit, Vysis, Inc., Downers Grove, IL) in the presence of Spectrum-Green dUTP (green fluorophore). Genomic DNA from a normal human male donor was chemically labeled with a red fluorophore (Vysis, Inc., Downers Grove, IL), and served as a reference. The test probe (green) and reference probe (red) were then mixed with unlabeled human cot-1 DNA and co-hybridized to the microarrays. After removal of un-hybridized probes, the array was imaged by a multi-color CCD based image analysis system, and fluorescence intensities were determined for each target spot. Under the assumption that the hybridization kinetics for a given sequence are equal for the test and reference DNA, the signal intensity is proportional to the copy number of that sequence in the hybridization mixture. The test/reference intensity ratio for each target genomic locus (average of 3 spots)

was normalized by dividing with the average ratio of all "normal" targets, resulting in an estimate for the copy number change of that specific sequence compared to the rest of the genome.

III. Results

As shown in **Figure 1**, the DNA extracted from the tumor tissue of HCC patient #21 was found to have an average normalized ratio of 4.2 ± 1 by Genosensor analysis for the MET target locus (average of 5 experiments), and 6.4 ± 0.8 by Southern analysis (3 experiments; see below). Since the reference sample used here was from a normal human male and has 2 copies of the MET sequence, this ratio suggests that there are on average between 8.4 to 12.8 copies per cell (4.2 or 6.4 x 2) of the MET gene in the HCC tumor sample. This amplification is considered a significant finding, as it is the first time to be reported in this type of cancer.

Since microarray or Southern analysis yields an estimate for the copy number of a sequence averaged over all cells from which the DNA was extracted, the MET amplification level was confirmed by fluorescent in situ hybridization (FISH). The tumor tissue from the same HCC patient was formalin-fixed, paraffin-embedded, and sectioned. FISH was performed with SpectrumGreen labeled DNA from a BAC clone containing the MET gene. SpectrumOrange labeled CEP 7 DNA (containing chromosome 7-specific centromere DNA sequences; Vysis) was co-hybridized as a control. The signal for both, the MET gene and chromosome 7 were counted under a fluorescent microscope after counterstaining with DAPI. As expected, the majority (60%) of the cells contained 2 copies of chromosome 7 per nucleus, while approximately 40 % of cells have an average of 25 copies of MET (**Figure 2**). Since the remaining 60% of cells have only 2 copies of the MET gene, the DNA extracted from this tumor section should have 11 copies of the MET gene, which is in good agreement with the microarray and Southern data.

For further confirmation and comparisons additional Southern blot analyses were carried out with EcoR1-digested DNA from 32 tumor samples including 6 HCC, 16 colorectal liver metastasis (CLM), 3 cholangiocarcinomas, 2 adenomas, 2 fibrolamellar (HCC variant), and 3 unclassified liver tumors. Normal human genomic DNA (control) and DNA from the non-tumor liver tissue of HCC patient #21 were also included in the Southern blot analyses. A 360bp DNA fragment (1) was amplified by polymerase chain reaction (PCR) in the presence of the following pair of primers, primer H1: 5'-TCTTGATTACCTGCATTTGC-3' and primer H2: 5'-TGGGGCAAGAAGGCCTCTCT-3' from a BAC clone containing the entire MET gene. The 360bp MET probe was labeled by PCR in the presence of ^{32}P-labelled dCTP and hybridized to the Southern blot. A probe generated from a genomic clone on 11q13 was re-hybridized to the same Southern blot for normalization, after the MET probe was stripped from the blot.
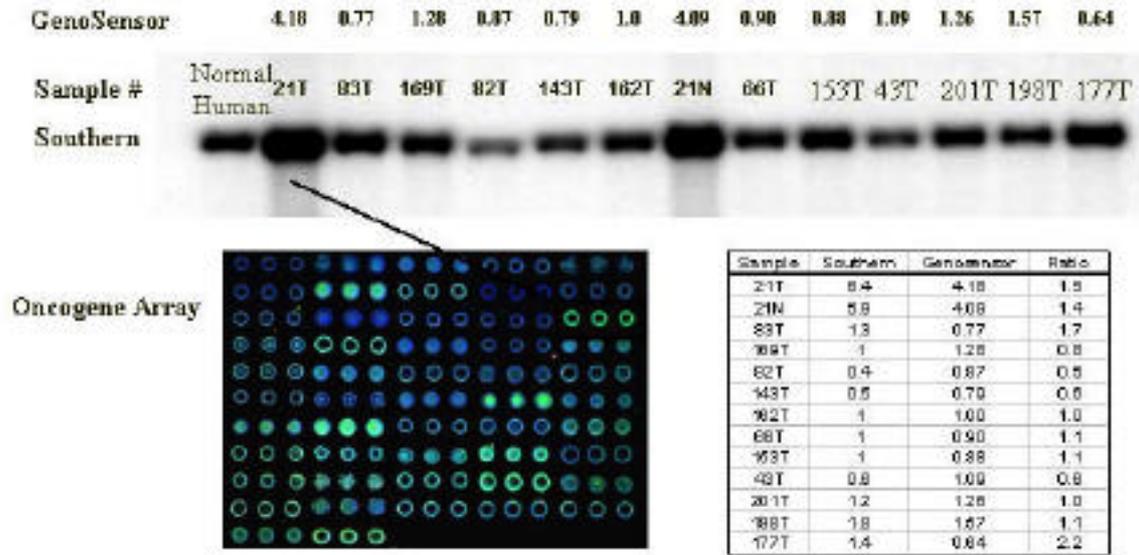
GenoSensor	4.18	0.77	1.28	0.87	0.79	1.0	4.09	0.90	0.88	1.09	1.26	1.57	0.64

Sample # — Normal Human, 21T, 83T, 169T, 82T, 143T, 162T, 21N, 66T, 153T, 43T, 201T, 198T, 177T

Sample	Southern	Genosensor	Ratio
21T	6.4	4.18	1.5
21N	5.9	4.09	1.4
83T	1.3	0.77	1.7
169T	1	1.28	0.8
82T	0.4	0.87	0.5
143T	0.5	0.79	0.6
162T	1	1.00	1.0
66T	1	0.90	1.1
153T	1	0.88	1.1
43T	0.8	1.00	0.8
201T	1.2	1.26	1.0
198T	1.8	1.57	1.1
177T	1.4	0.64	2.2

Figure 1: Genosensor and Southern analysis of HCC samples. Genomic DNA (8 μg for DNA from tumor tissue and 8 μg for DNA from normal tissue) was digested with *Eco* R1, run on agarose gels and blotted. Southern hybridization was performed with a P32 labeled 360 bp MET probe as described in the text. A composite image (red, green and blue) of a Gneosensor oncogene array after hybridization with a mixture of sample 21T DNA (green) and normal refernce DNA (red) is shown after counterstaining with DAPI.

The level of MET gene amplification was determined using a PhosphoImager (Molecular Dynamics). Some of the results are shown in **Figure 1**. Among the 6 HCC samples analysed, 2 MET gene amplifications were observed (6.4 and 2.5 fold after normalization). MET gene amplifications were also observed in the cholangiocarcinoma and CLM samples. Two of the three cholangiocarcinoma patients had MET gene amplifications in their tumour specimens at a level of 6.5 fold and 1.6 fold, respectively. Of the 16 patients with CLM, three had MET gene amplifications of 2.3, 2.1 and 1.8 fold, respectively. Of specific interest is the finding that both, the tumor as well as non-tumor tissues from the same HCC patient (No. 21) showed a similar level of MET amplification (6.4 fold and 6.1 fold, respectively), suggesting that MET amplification may precede malignant histopathological changes. This patient developed HCC in the background of a cirrhotic liver complicating hepatitis C infection. Liver cirrhosis provides a pre-malignant field change for HCC development.

IV. Discussion

Hepatocyte growth factor (HGF) plays an important role in the growth, progression and angiogenesis of various tumors and is known to specifically promote hepatocyte proliferation and liver regeneration. In addition, it may also be involved in tumor invasion and progression (Tamatani et al, 1999). Overexpression and amplification of the HGF receptor (MET gene) have been implicated in progression of colorectal cancer (Di Renzo et al, 1995), by a mechanism where the elevated level of the MET gene product confers a selective growth advantage to tumor cells (Di Renzo et al, 1991). In the context of this information, our finding of amplifications of the MET oncogene in hepatocellular carcinomas strongly suggests a role here as well.

This finding in combination with multiple other reports of cancer associated gene amplifications underscores the need for a rapid, quantitative detection method for such genetic changes. The microarray based method described here is consistent (within a factor of 2) with other established methods (FISH, Southern blotting), and therefore suitable for the screening of gene amplifications. Since this method is non-radioactive, simpler, faster, and more economical than either FISH or Southern, especially when the mutated genetic locus is not known, it lends itself to applications in clinical diagnostics.
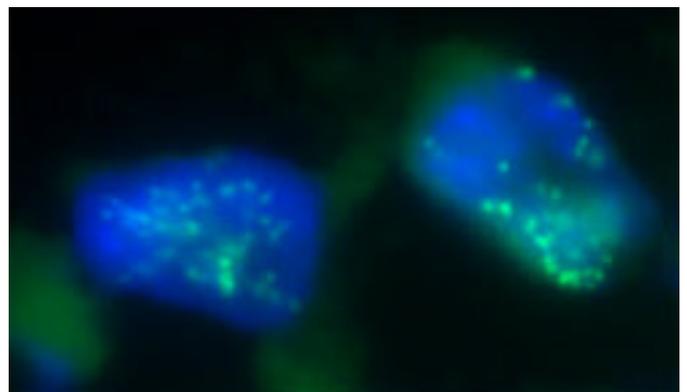

Figure 2: FISH on interphase nuclei of patient #21. FISH was performed on formalin-fixed, de-parafinized tumor tissue sections. A BAC clone containing the MET gene was labeled with SpectrumGreen by nick translation and used as a probe. A SpectrumOrange labeled chromosome 7-specific centromere probe (CEP7; Vysis Inc.) was co-hybridized as reference.

Acknowledgments

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