Oncogene (1999) 18, 2343–2350

http://www.stockton-press.co.uk/onc

Novel mutations of the MET proto-oncogene in papillary renal carcinomas

Laura Schmidt*[,1], Kerstin Junker[13], Noboru Nakaigawa[2], Tracy Kinjerski[1], Gregor Weirich[2], Maria Miller[3], Irina Lubensky[6], Hartmut PH Neumann[7], Hiltrud Brauch[8], Johann Decker[9], Cathy Vocke[5], James A Brown[14], Robert Jenkins[10], Stephane Richard[12], Ulf Bergerheim[11], Bernard Gerrard[1], Michael Dean[4], W Marston Linehan[5] and Berton Zbar[2]

[1]Intramural Research Support Program, SAIC Frederick, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Maryland 21702, USA; [2]Laboratory of Immunobiology, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Maryland 21702, USA; [3]Macromolecular Structure Lab, ABL-Basic Research Program, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Maryland 21702, USA; [4]Laboratory of Genomic Diversity, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Maryland 21702, USA; [5]Urologic Oncology Branch, National Institutes of Health, Bethesda, Maryland 20892, USA; [6]Laboratory of Pathology, National Institutes of Health, Bethesda, Maryland 20892, USA; [7]Albert-Ludwigs-University, 79106 Freiburg, Germany; [8]University Women's Clinic, Hamburg-Eppendorf (UKE), 20246 Hamburg, Germany; [9]Johannes-Gutenberg University of Mainz, D-55131 Mainz, Germany; [10]Division of Laboratory Genetics, Mayo Clinic, Rochester, Minnesota 55905, USA; [11]Karolinska Institute, Stockholm, Sweden; [12]Laboratory of Neuro-Oncology, EPHE, and Department of Urology, Bicetre Hospital, Paris, France; [13]Department of Urology, Freidrich-Schiller University, 07743 Jena, Germany; [14]Urology Department, David Grant Medical Center, Travis AFB, California 94535, USA

Hereditary papillary renal carcinoma (HPRC) is characterized by multiple, bilateral papillary renal carcinomas. Previously, we demonstrated missense mutations in the tyrosine kinase domain of the MET proto-oncogene in HPRC and a subset of sporadic papillary renal carcinomas. In this study, we screened a large panel of sporadic papillary renal carcinomas and various solid tumors for mutations in the MET proto-oncogene. Summarizing these and previous results, mutations of the MET proto-oncogene were detected in 17/129 sporadic papillary renal carcinomas but not in other solid tumors. We detected five novel missense mutations; three of five mutations were located in the ATP-binding region of the tyrosine kinase domain of MET. One novel mutation in MET, V1110I, was located at a codon homologous to an activating mutation in the c-*erb*B proto-oncogene. These mutations caused constitutive phosphorylation of MET when transfected into NIH3T3 cells. Molecular modeling studies suggest that these activating mutations interfere with the intrasteric mechanism of tyrosine kinase autoinhibition and facilitate transition to the active form of the MET kinase. The low frequency of MET mutations in noninherited papillary renal carcinomas (PRC) suggests that noninherited PRC may develop by a different mechanism than hereditary papillary renal carcinoma.

Keywords: papillary renal carcinoma; MET proto-oncogene mutations; receptor tyrosine kinase

Introduction

The MET proto-oncogene encodes a receptor tyrosine kinase that is widely expressed in epithelial cells; the ligand for the MET receptor is hepatocyte growth factor/scatter factor (HGF/SF). Increased expression of the MET proto-oncogene has been found in a number of human cancers implicating MET in their pathogenesis (Jeffers *et al*., 1996; Di Renzo *et al*., 1995). These studies have not determined whether the MET proto-oncogene was involved in the origin or progression of these neoplasms. The recent demonstration of germline and somatic mutations in the MET proto-oncogene in papillary renal carcinomas directly implicated the MET gene in the pathogenesis of papillary renal cancer. Schmidt *et al*. (1997) found missense mutations in the tyrosine kinase domain of the MET proto-oncogene in families with papillary renal carcinomas and in a subset of sporadic papillary renal carcinomas. Studies by Jeffers *et al*. (1997) demonstrated that the observed mutations in MET produced constitutive phosphorylation of the MET protein, and malignant transformation of NIH3T3 cells.

To determine the frequency of MET proto-oncogene mutations in sporadic papillary renal carcinomas, to determine whether mutations in the MET proto-oncogene play a role in the pathogenesis of other solid tumors, and to identify additional mutations, we performed mutation analysis on a large panel of sporadic papillary renal carcinomas and various solid tumors. The results indicate that mutations in the MET proto-oncogene are uncommon in sporadic papillary renal carcinomas and not detected in the panel of solid tumors. Five novel missense mutations in MET were detected. Molecular modeling studies were performed to predict the effect these mutations would have on the regulation of the kinase activity of the MET protein.

Results

Frequency of detection of MET mutations in sporadic papillary renal carcinomas and metanephric adenomas

We tested 129 sporadic papillary renal carcinomas, and two metanephric adenomas for mutations in the MET gene. The sporadic papillary renal carcinoma panel included 79 matched normal and tumor DNAs, seven

*Correspondence: LS Schmidt, Laboratory of Immunobiology, NCI-FCRDC, Bldg 560 Rm 12-29, Frederick, Maryland 21702, USA
Received 12 August 1998; revised 28 October 1998; accepted 29 October 1998

papillary renal carcinoma cell lines, and 43 paraffin-embedded or frozen tumor samples from patients with multiple or single papillary renal carcinomas and no family history of renal neoplasia. Summarizing these and previously reported data (Schmidt *et al*., 1997, 1998), MET mutations were detected in 17/129 (13%) sporadic papillary renal carcinoma samples (Table 1). Eight of the 17 apparent sporadic MET mutations were germline, even though there was no known family history of renal cancer in these patients.

Novel mutations in the MET proto-oncogene

In this study we identified five novel missense mutations in the MET proto-oncogene in the sporadic papillary renal carcinoma panel. Mutations and clinical details describing these patients are summarized in Table 2.

MET V1110I We identified three apparently unrelated individuals with papillary renal carcinoma (patients 5946, 5274 and 6088) and the identical germline MET proto-oncogene mutation changing a valine to an isoleucine at codon 1110. The patients with the V1110I mutation were from the United States, Sweden and Germany. The V1110I change was not detected in 300 normal chromosomes making it unlikely that this change was a rare polymorphism. It was not possible to test whether the mutation segregated with the disease because there was only one affected individual in each family. The V1110I mutation was located in the highly conserved, glycine-rich, ATP-binding region of the tyrosine kinase domain. Previously, an activating mutation was detected at an homologous codon in the chicken proto-oncogene, c-*erb*B (V157I) (Figure 1) (Shu *et al*., 1990, 1991, 1994). The amino acid changes, valine→isoleucine, were identical in the MET and c-*erb*B mutations.

MET H1112L and H1112Y Previously, a mutation in MET codon H1112 (H1112R) was found to predispose to HPRC in two large North American families (Schmidt *et al*., 1998). In this study, we identified two additional missense mutations in codon 1112, H1112L and H1112Y. MET mutation H1112Y was detected in the germline of patient 6285 and as a somatic change in the papillary renal carcinoma of patient 5458. MET mutation H1112L was detected as a somatic change in the papillary renal carcinoma of patient 6134. The H1112L and H1112Y mutations were not detected in 300 normal chromosomes making it unlikely that these changes were rare polymorphisms. The H1112 mutations were immediately adjacent to the ATP-binding domain (GXGXXGXV) and the V110I mutation (Figure 1).

MET H1124D A new mutation in codon 1124, H1124D, was identified in a sporadic papillary renal tumor from archival tissue. (Patient 6052). No accompanying normal was available for comparison. This change was not observed in 300 normal chromosomes. The location of this mutation is in proximity to the conserved, ATP binding region and adjacent to two highly conserved amino acids, alanine 1126 and lysine 1128 (Figure 1).

MET Y1248D Missense mutations were detected previously in MET codon 1248. (Y1248C and Y1248H) (Schmidt *et al*., 1997). In this study, we detected another germline mutation in codon 1248, Y1248D, in patient 6059, which was not found in 300 normal chromosomes.

Table 1 Frequency of MET mutations in sporadic papillary renal carcinomas and other solid tumors

Tumor	*Frequency MET mutation*
Sporadic papillary renal carcinoma	17/129 (13%)
Metanephric adenoma	0/2
Pancreatic carcinoma	0/11
Colon carcinoma	0/20
Malignant melanoma	0/17
Papillary thyroid carcinoma	0/17
Wilms' tumor	0/52
Gliomas	0/42
Bladder	0/11
Prostate	0/29

Table 2 Clinical information for patients wtih novel MET mutations

Patient	*Mutation*	*Germline or somatic*	*Family history*	*Age of onset*	*Unilateral or bilateral*
5946	V1110I	G	Yes	43	B
5274	V1110I	G	No	39	B
6088	V1110I	G	No	55	U
6134	H1112L	S	No	50	U
6285	H1112Y	G	No	47	B
5458	H1112Y	S	No	56	U
6052	H1124D	S*	N/A	N/A	N/A
6059	V1248D	G	No	52	B

*No normal tissue was available for analysis

A MET mutation in a renal papillary neoplasm

Metanephric adenoma is a recently recognized, benign, papillary renal tumor characterized by trisomy of chromosomes 7 and 17 (Brown *et al*., 1997; Grignon and Eble, 1998). No MET mutations were detected in two metanephric adenomas analysed in this study. A third sample, 6046, a renal papillary neoplasm, was examined histologically by three pathologists who disagreed on the diagnosis of metanephric adenoma. MET mutation Y1248C was detected in sample 6046. Normal DNA was not available for comparison.

A French patient, 6067, with papillary renal carcinoma and no known family history of kidney cancer, was found to have a germline MET mutation, V1238I, previously identified in a Spanish HPRC family (Schmidt *et al*., 1997).

A MET polymorphism, T1010I

We detected a C to T sequence change at nt 3223 (T1010I) in exon 14 in the papillary renal carcinoma cell line ACHN (Borden *et al*., 1982), and in the germline of a patient with a family history of papillary renal carcinoma. This change was located in the juxtamembrane region of the intracellular portion of MET. The T1010I change did not segregate with the

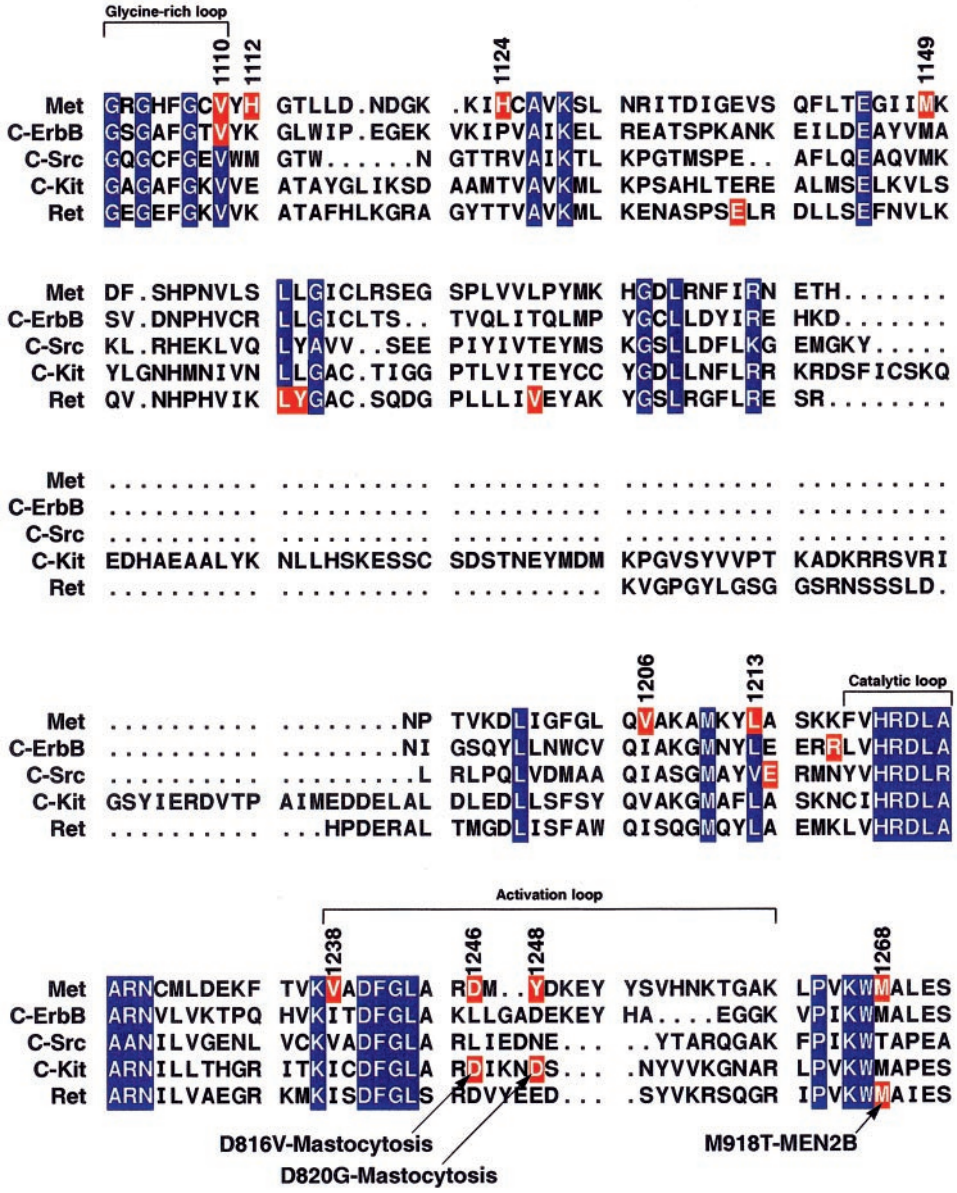

Figure 1 Partial amino acid sequence of the tyrosine kinase domain of MET illustrating location of germline and somatic mutations in papillary renal carcinomas. The amino acid sequences of the homologous regions of c-*erb*B, c-*src*, c-Kit and RET are shown for comparison. The residues in MET indicated by red were mutated in the germline or in sporadic papillary renal tumors. The codons in blue were conserved. The locations of the RET mutations, E768D, L790F, Y791F, V804L and M918T, are shown; the locations of the mutations in c-Kit that produce mastocytosis with an associated hematologic disorder, D816V and D820G, are indicated; the location of the V157I mutation in c-*erb*B that produces malignancy in chickens is shown (Berndt *et al*., 1998; Fattoroso *et al*., 1998; Levy *et al*., 1986; Pignon, 1997; Shu *et al*., 1990). Also shown is the E378G mutation which converts $p60^{src}$ into a transforming virus (Hanafusa *et al*., 1986; Levy *et al*., 1986)

disease (inherited from the unaffected parent) and may represent a rare polymorphism. The amino acid change T1010I was not found in 150 normal chromosomes.

Evaluation of various human tumors for mutations in the MET proto-oncogene

Previously, we observed that some patients with a germline MET mutation (H1112R) had other nonrenal malignancies (pancreatic carcinoma, malignant melanoma, carcinoma of the common bile duct) (Zbar *et al*., 1994; Zbar and Lerman, 1998, in press; Schmidt *et al*., 1997, 1998). Other investigators had found that breast, prostate and ovarian carcinomas were characterized by a loss of heterozygosity that mapped to chromosome 7q31, the region containing the MET proto-oncogene (Lin *et al*., 1996; Zenklusen *et al*., 1994, 1995; Huang *et al*., 1998). These studies raised the possibility that mutations in the MET proto-oncogene might play a role in the pathogenesis of certain solid tumors.

We screened 199 sporadic human tumors for mutations in the tyrosine kinase domain of the MET proto-oncogene (Table 1) by SSCP or DHPLC. Gliomas, prostate and Wilms' tumors were selected for study because they have been found to have trisomy of chromosome 7, a genetic change consistently found in papillary renal carcinomas. Malignant melanoma, pancreatic cancer and colon cancer were tested because they have been found as secondary

malignancies in some HPRC family members. In addition, we tested tumors from bladder and thyroid, neoplasms which overexpress MET. No mutations in the MET proto-oncogene were detected in these samples.

Functional studies of novel mutations in the MET proto-oncogene

Previously, we demonstrated that missense mutations in the tyrosine kinase domain of the MET proto-oncogene, when introduced into mouse MET cDNA, caused constitutive phosphorylation of the MET protein and malignant transformation of NIH3T3 cells (Jeffers *et al*., 1997). In this study we evaluated the ability of the MET mutations described in this report to cause constitutive phosphorylation of MET and malignant transformation of NIH3T3 cells.

Western analysis of the mutant MET transfected NIH3T3 cell lines is shown in Figure 2. Constitutive phosphorylation of MET protein was stimulated by the V1110I, H1112L and H1124D mutations at levels comparable to the weakly activating MET mutant, M1149T, described by Jeffers *et al*. (1997) and included for comparison (Figure 2a). The Y1248D and H1112Y mutations resulted in intermediate levels of constitutive phosphorylation, comparable to Y1248H described by Jeffers *et al*. (1997) (Figure 2a). No phosphorylation of MET was seen in the cell lines transfected with wild type MET or empty vector. Western analysis of the mutant MET transfected NIH3T3 cell lines revealed MET protein expression at levels comparable to wild type for all five novel mutations (Figure 2b).

These new MET mutations, when introduced into mouse MET cDNA and transfected into NIH3T3 cells, stimulated focus formation. Mutants Y1248D, V1110I, H1112Y, H1112L and H1124D produced an average of 2, 12, 30, 36 and 36 foci per μg DNA, respectively, from three separate experiments (data not shown). No foci were produced in transfections with the wild type MET construct or empty vector. It should be noted that when MET T1010I, thought to be a rare polymorphism, was introduced into NIH3T3 cells, neither focus formation nor constitutive MET phosphorylation was observed (data not shown).

Predicted effect of MET mutation on the activation of the MET protein based on molecular modeling studies

To study the mechanism by which missense mutations in the MET proto-oncogene produce constitutive activation, we constructed a three dimensional model (3-D) of the catalytic core of the MET tyrosine kinase domain (amino acid residues 1096 – 1355) based on the crystal structure of the unphosphorylated form of the insulin receptor (IRK) (Hubbard *et al*., 1994). Sequence identity of 40% in this region and the presence of three critical tyrosine residues in the activation loop of these two receptors suggests that the IRK structure should be a good approximation of MET. The model can be superimposed on the structure of IRK with the root mean square difference (rmsd) of 0.66 Å between 249 equivalent C-α pairs. The tyrosine kinase domain is divided into amino terminal and carboxy terminal lobes separated by a large cleft (Figure 3a). In the catalytically inactive, unphosphory-

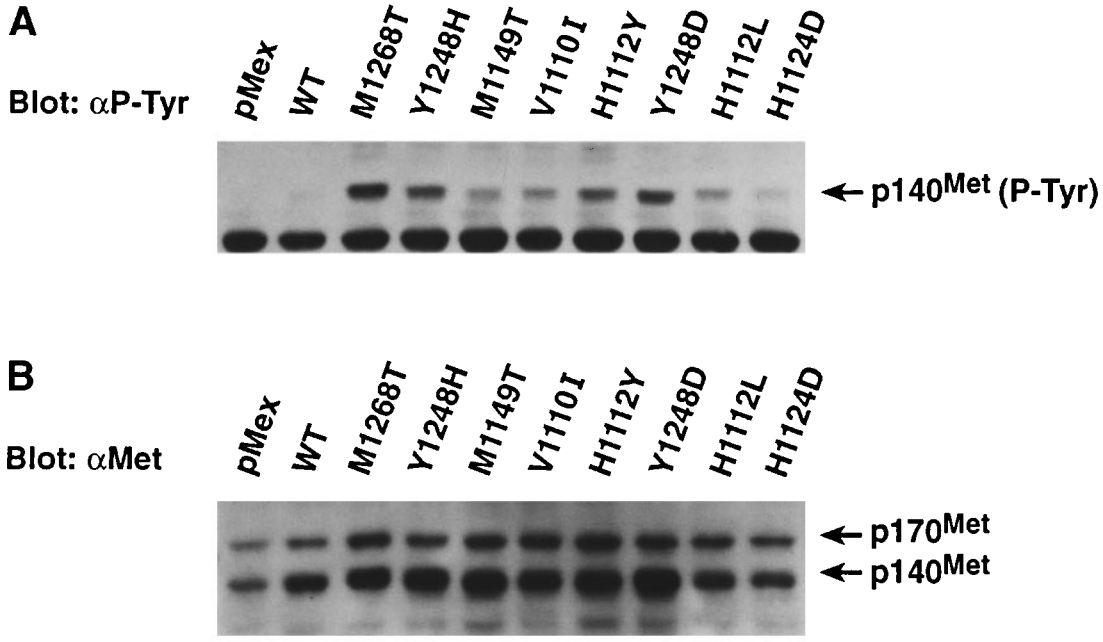

Figure 2 MET protein expression and autophosphorylation levels of wild type and mutant MET in NIH3T3 cells. Control samples are NIH3T3 cells stably transfected with empty vector, pMex; wild type cells are transfected with vector expressing wild type mouse MET; all other samples are from cells stably transfected with the indicated mouse MET mutants. Examples of strongly activating, moderately activating and weakly activating MET mutants (M1268T, Y1248H, M1149T, respectively) described by Jeffers *et al*. (1997), were included for comparison. (**a**) Cell lysates were prepared, resolved on 8% PAGE and electrotransferred to membrane. After blocking with 5% albumin, Western analysis was performed with anti-phosphotyrosine antibody. (**b**) The membrane was stripped and reprobed with anti-MET antibody. The ECL detection system was used for both experiments

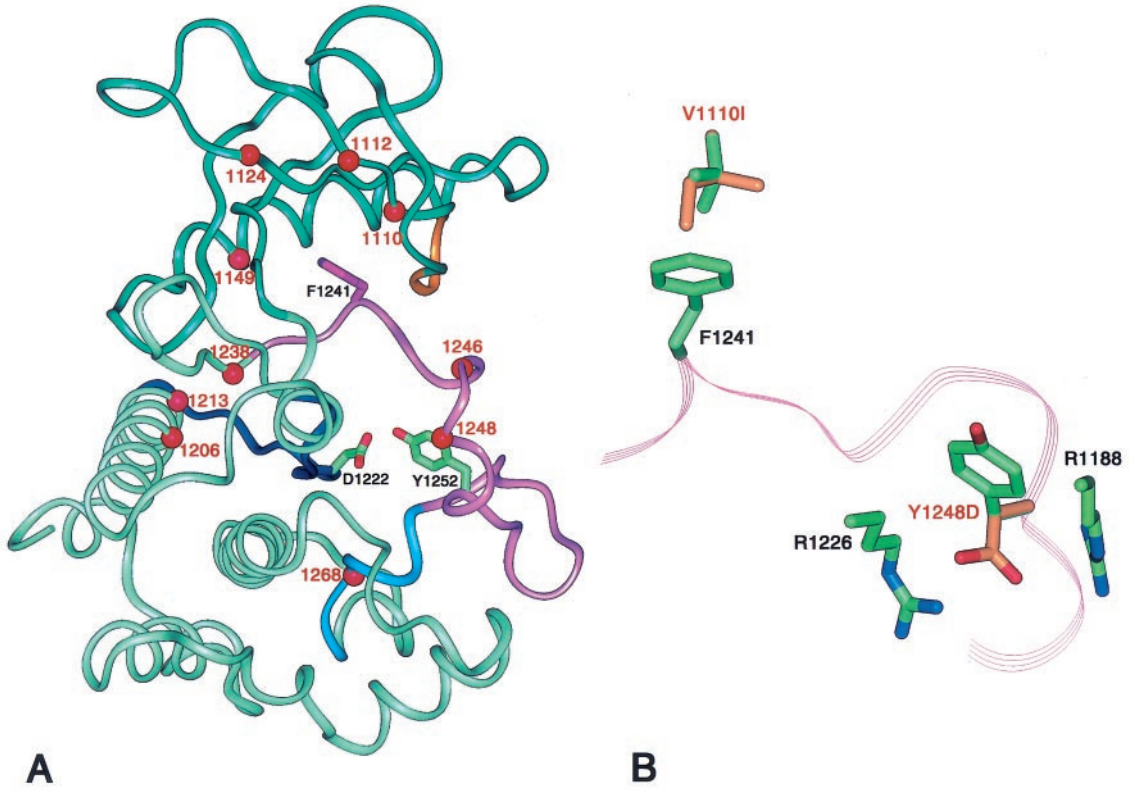

Figure 3 3D model of the kinase domain of MET in the autoinhibited form. (**a**) Ribbon representation of the protein backbone. N-terminal and C-terminal lobes are shown in dark and light green, respectively; the glycine rich, ATP binding loop in orange, the catalytic loop in dark blue, the activation loop in magenta, the P+1 loop in blue. The position of MET activating mutations are marked as red dots. Numbers in red are sites of MET mutations. Numbers in black are critical amino acid residues. (**b**) Selected side chains of wild-type (green) and mutated (orange) MET residues. The replacement of valine 1110 with isoleucine results in a steric clash with phenylalanine 1241. Substitution of tyrosine 1248 with aspartic acid eliminates the hydrophobic interactions of the phenolic ring of Y1248 with the aliphatic side chains of arginines 1226 and 1188, which stabilize the inhibitory conformation of the activation loop. Both mutations will facilitate the transition to the active form of the enzyme

lated form, access to the ATP and substrate binding sites is blocked by the activation loop. Y1252 is hydrogen bonded to the catalytic aspartic acid, D1222, while the side chain of Y1253 resides in a substrate binding pocket formed mainly by residues from the P+1 loop. F1241 from the kinase conserved triplet DFG occupies the adenine binding pocket, and together with G1242 create a steric hindrance which forces the two lobes apart. This conformation of the activation loop is further stabilized by hydrophobic interactions of the phenolic ring of Y1248 with the alkyl portions of arginines 1226 and 1188 (Figure 3b) and hydrogen bonds through its hydroxyl group to A1182 and N1185 (data not shown).

The proposed mechanism of autoregulation of kinase activity assumes that the activation loop can adopt an alternative conformation which allows for the transition to the active form of the enzyme (Hubbard *et al*., 1994). Trans-phosphorylation of tyrosines in the activation loop strongly shifts the equilibrium toward the active conformation.

Mutations may activate the MET kinase by destabilizing the inhibitory conformation of the activation loop. For example, as shown in Figure 3b, the replacement of valine 1110 with isoleucine results in a steric clash with phenylalanine 1241 forcing it out of the adenine binding pocket, and releasing the steric hindrance which prevents rearrangement of the two lobes. Substitution of the phenolic ring of the buried Y1248 by polar side chains of aspartic acid, histidine or cysteine would also strongly favor the activated form (Figure 3b). As revealed by the crystal structure of the activated IRK, the phosphotyrosine at this codon is fully exposed to the cytosol (Hubbard, 1997).

Discussion

We screened a panel of sporadic papillary renal carcinomas and solid tumors to determine the frequency of mutation of the MET proto-oncogene in sporadic papillary renal carcinoma and in various nonrenal tumors, and to identify novel mutations in the MET proto-oncogene. Summarizing these and our previous results, we detected a total of 17 mutations in 129 (13%) sporadic papillary renal carcinomas and 0 mutations in two metanephric adenomas and 199 solid tumors (Tables 1 and 3).

In our previous report, mutation analysis was concentrated in exons 16–19 of the tyrosine kinase domain (Schmidt *et al*., 1997). However it was possible that we failed to detect MET mutations located outside the tyrosine kinase domain. Therefore, the cysteine-rich extracellular domain (exons 5 and 7), the transmembrane region (exon 13) and intracellular portion of the protein (exons 14–20), were sequenced in their entirety

Table 3 Summary of MET proto-oncogene mutations in papillary renal carcinoma

DNA#	*Exon*	*Mutation*	*Codon*
Somatic			
5458	16	C3528T	H1112Y
6052	16	C3564G	H1124D
6134	16	A3529T	H1112L
5468	18	C3831G	L1213V
4768	19	G3930C	D1246H
4769	19	T3936C	Y1248H
6046	19	A3937G	Y1248C
5422, 5434	19	T3997C	M1268T
Germline			
5946, 5274, 6088	16	G3522A	V1110I
6285	16	C3528T	H1112Y
5161, 4599, 5976	16	A3529G	H1112R
4374, 4762	17	T3640C	M1149T
5269	18	G3810T	V1206L
5243, 6067	19	G3906A	V1238I
5928	19	G3930A	D1246N
6059	19	T3936G	Y1248D
5456	19	A3937G	Y1248C
6082	19	T3997C	M1268T
MET polymorphism			
ACHN, 5242	14	C3223T	T1010I

*Samples analysed in this study, in which mutations were identified, are underlined. Mutations in the remaining samples were previously reported in Schmidt *et al*., 1997, 1998

in 79 sporadic papillary renal carcinomas and exons 14–19 were sequenced in 43 paraffin block DNAs and seven papillary renal cell lines. The results of this study suggest that MET mutations occur in only a small proportion of sporadic papillary renal carcinomas. Independent studies suggest that sporadic papillary renal carcinoma is genetically heterogeneous, and may also be caused by mutations in the TFE3 and PPRC genes (Sidhar *et al*., 1996; Weterman *et al*., 1996).

Trisomy 7 is a characteristic of papillary renal carcinomas, other neoplasms and some normal tissues (Kovacs, 1993; Ermis *et al*., 1995). Recently, studies of tumors in patients with hereditary papillary renal carcinoma showed that the chromosome 7 carrying the inherited mutant MET allele was duplicated in these tumors (Fischer *et al*., 1998; Zhuang *et al*., 1998). This observation raised the possibility that the MET gene might be mutated and duplicated in tumors characterized by trisomy 7. Our data which show MET mutations in 13% of sporadic papillary renal carcinomas and data of Kovacs (1993), showing trisomy 7 in 95% of papillary renal carcinomas indicate that most papillary renal carcinomas are characterized by trisomy 7 without MET mutations. Since polysomy of chromosome 7 has been associated with a proliferative advantage in normal cells (Ermis *et al*., 1995), the increased dosage of MET and its ligand (both located on chromosome 7) may be responsible for a growth advantage of papillary renal carcinoma cells with trisomy 7, but without MET mutation. The low frequency of MET mutations in sporadic PRC is underscored by the fact that eight of 17 mutations identified in our studies were germline (with no known family history of disease) and may represent examples of *de novo* mutational events.

We did not detect mutations in the MET gene in 199 nonrenal solid tumors. MET mutations may be present in other tumor types not included in our panel, or may be present in some of these tumors at a low percentage, undetectable in our relatively small sample set. It also may be possible that mutations exist in regions of the MET gene not screened in this study.

Metanephric adenoma is a recently recognized benign tumor of the kidney. Brown *et al*. (1997) identified trisomy of chromosomes 7 and 17 in these tumors. We tested two metanephric adenomas and found no mutations in the MET proto-oncogene.

In this study we report five novel mutations identified in a large panel of sporadic papillary renal carcinomas, which were shown to be transforming and cause constitutive phosphorylation of MET. These new mutations may best be analysed in the context of previously identified mutations in the MET proto-oncogene. The 15 different mutations detected to date were missense and were located in exons 16–19 of the MET proto-oncogene in the tyrosine kinase domain. These 15 different mutations involved ten codons: V1110, H1112, H1124, M1149, V1206, L1213, V1238, D1246, Y1248 and M1268. Codons V1110, H1112, V1238, D1246, Y1248 and M1268 were mutated in more than one sample. Codons H1112 and Y1248 were altered by three different missense mutations. These codons appear to be particularly critical in regulating tyrosine kinase activity because each of these codons was mutated to three different amino acids and still produced malignant disease. Mutations were clustered in the activation loop and the glycine rich-ATP binding region.

We previously reported the presence of striking homologies between MET proto-oncogene mutations and mutations in other receptor tyrosine kinases (Schmidt *et al*., 1997; Zbar *et al*., 1998). MET mutation M1268T is homologous to RET mutation M918T (the RET M918T mutation is responsible for most cases of multiple endocrine neoplasia type 2B); MET proto-oncogene mutations D1246H and D1246N are homologous to c-KIT mutation D816V (the c-Kit mutation D816V is responsible for systemic mastocytosis).

Here, we report another striking homology between an activating mutation in MET, V1110I, and a corresponding activating mutation in c-*erb*B, V157I (Shu *et al*., 1990). Both mutations change a valine to an isoleucine in the highly conserved glycine rich ATP-binding pocket. Detailed studies of the c-*erb*B mutation, V157I, have been performed (Shu *et al*., 1990, 1991, 1994). These observations demonstrate that there are several conserved codons in the tyrosine kinase domain which, when mutated, lead to uncontrolled cellular proliferation. The identification of such codons may be important in analysing the role of other tyrosine kinases in malignancy (Santoro *et al*., 1998).

Regulation of kinase activity by autophosphorylation of tyrosines in the activation loop is a common regulatory mechanism used by several receptor tyrosine kinases (RTK). Autophosphorylation occurs in trans and requires ligand-induced receptor dimerization (Ullrich and Schlessinger, 1990). Ligand-independent activating mutations within the catalytic core of RTKs appear to interfere with the intrasteric mechanism of autoinhibition. This can occur either by a direct alteration of contacts between the residues of the activation loop and those from the main body of the catalytic domain, or by increasing flexibility at critical

points of the structure to facilitate subdomain movement. Molecular modeling studies described here suggest this type of activating mechanism for MET mutations in papillary renal carcinoma. Further biochemical studies will provide the experimental evidence to support this model of mutation-induced MET RTK activation.

Materials and methods

Tumor tissues

The panel of papillary renal carcinomas included 79 tumor DNAs with matching normal DNA, 21 paraffin-embedded tumor samples from patients with multiple or single papillary renal tumors, 22 paraffin-embedded or frozen tumor samples from patients with bilateral papillary renal carcinoma without a family history of renal neoplasia, and seven papillary renal carcinoma cell lines. The two metanephric adenomas and 199 nonrenal solid tumor DNAs were obtained from collaborators listed in acknowledgements.

Mutation analysis of the MET gene

We sequenced the cyteine-rich extracellular region (exons 5 and 7) and the intracellular portion (exons 13–20) of the MET proto-oncogene in 79 sporadic papillary renal carcinomas. (Exons 16–19 were sequenced in 60 of 79 samples for a previous study.) DNA from an additional 43 papillary renal carcinomas and seven PRC cell lines were sequenced in exons 14–19. DNAs from two metanephric adenomas and 199 solid nonrenal tumors were screened for mutations in exons 14–19 in the MET proto-oncogene by PCR-based single strand conformation polymorphism (PCR–SSCP) (Schmidt *et al*., 1993, 1997), or by denaturing high performance liquid chromatography (DHPLC).

Nucleotides were numbered according to the scheme in GenBank Accession number J02958 with nucleotide −194 in the cDNA (Park *et al*., 1987) given the number 1. Amino acids were numbered as in the reference by Park *et al*. (1987). The primers used for amplification of MET exons for PCR were described previously (Duh *et al*., 1997). PCR products from samples that showed an aberrant SSCP or DHPLC shift were sequenced on both strands from two independent PCR reactions as described previously (Schmidt *et al*., 1995).

DHPLC was performed as described (Underhill *et al*., 1996). Products were run on a Varian/Rainin machine using a DNASep column (Transgenomics, San Jose, CA, USA) and the DNA was detected by UV fluorescence at 254 nm. Samples were run in 0.1 M TEAA pH 7 buffer containing acetonitrile. Samples were injected at 17.5% acetonitrile, raised to 30.5% at 1 min, to 34% over the next 4 min and to 50% for the last 2 min. Melting temperatures for heteroduplexes formed between mutant and wild type strands of the PCR products were calculated using a program developed by Peter Oefner available at http:.//lotka.standford. edu.

NIH3T3 transformation assay NIH3T3 cells (CRL 1658) were obtained from the American Type Culture Collection, (Rockville, MD, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% calf serum (Life Technologies Gaithersburg, MD, USA). The pMB1 expression vector containing the wild-type mouse MET cDNA (pMB11) was used for mutation construction (Jeffers *et al*., 1997). To construct the MET mutants, the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) was used with pMB11 as the template. Mutations were verified by sequencing both strands of DNA in the region of interest.

Transfections were performed as previously described with a plasmid containing the mutant or wild-type MET construct, a plasmid conferring resistance to G418 (pSV2neo) and Lipofectamine (Life Technologies, Gaithersburg, MD, USA). Focus formation assays were performed as described (Jeffers *et al*., 1997).

MET phosphorylation and expression

The NIH3T3 cell lines transfected with mouse MET mutant constructs were grown in DMEM/10% calf serum (Life Technologies, Gaithersburg, MD, USA) supplemented with Geneticin (G418; Life Technologies). Pools of 100 or more cells were used for expression and phosphorylation studies. Western analysis was performed as described (Jeffers *et al*., 1997). SDS–PAGE (8%) was performed under reducing conditions and protein was electrotransferred to Immobilon-P (Millipore, Bedford, MA, USA). Western blotting was performed using the anti-MET antibody SP260 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or the anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology, Lake Placid, NY, USA) and the ECL detection system (Amersham, Arlington Heights, III, USA).

Molecular modeling

To study the mechanism of ligand-independent MET activation by mutations found in patients with sporadic papillary renal carcinoma, homology modeling using crystal structure coordinates of the insulin receptor (1IRK) (40% sequence identity with MET) from the Brookhaven Protein Data Bank was performed with the program package LOOK version 2.0 (Molecular Applications Group, Palo Alto, CA, USA). INSIGHT II version 95.0 (Biosym/MSI, San Diego, CA, USA) was used for the analysis of the model, side chain manipulations and preparation of illustrations.

Acknowledgements

We thank Drs Scott Kern, Bert Vogelstein, Marcel Mannens, Marius Skudlinski, Gloria Niehans, Holger Moch, Webster Cavanee, Sandra Bigner, Burt Feuerstein, McClellan Walther, Kimberly Newsom Johnson/Vanderbilt Tissue Procurement Lab, Sharon Howard/Fox Chase Cancer Center, Paolo Radice, Michael Hughson, Masahiro Yao, Suzanne Topalian, Virginia LiVolsi, and Bruce Weintraub for providing tumor samples or DNA for mutation analysis. In addition we thank Dr Gerd Riedasch, Department of Urology, Rupert-Karls-University, Heidelberg, Germany for clinical evaluation of PRC patients and Dr JA Brown, Mayo Clinic (Rochester, MN, USA) for karyotypic and histologic analyses of metanephric adenomas. This project has been funded in whole or in part with Federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. NO1-CO-56000 and under contract with ABL (MM). The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government. The publisher or recipient acknowledges right of the US Government to retain a nonexclusive, royalty-free license in and to any copyright covering the article.

References

Berndt I, Reuter M, Saller B, Frank-Raue K, Groth P, Grubendorf F, Raue F, Ritter MM and Hoppner W. (1998). *J. Clin. Endo. Metab.,* **83,** 770–774.

Borden EC, Hogan TF and Voelkel JG. (1982). *Cancer Res.,* **42,** 4948–4953.

Brown JA, Anderl KL, Borell TJ, Qian J, Bostwick DJ and Jenkins RB. (1997). *J. Urology,* **158,** 370–374.

Di Renzo MF, Olivero M, Serini G, Orlandi F, Pilotti S, Belfiore A, Costantino A, Vigneri R, Angeli A, Pierotti MA and Comoglio PM. (1995). *J. Endocrinol. Invest.,* **18,** 134–139.

Duh F-M, Scherer SW, Tsui L-C, Lerman M, Zbar B and Schmidt L. (1997). *Oncogene,* **15,** 1583–1586.

Ermis A, Henn W, Remberger K, Hopf C, Hopf T and Zang KD. (1995). *Human Genet.,* **96,** 651–654.

Fattoruso O, Quadro L, Libroia A, Verga U, Lupoli G, Cascone E and Colantuoni V. (1998). *Human Mutation Supplement,* **1,** S167–S171.

Fischer J, Palmedo G and Kovacs G. (1998). *Proc. Amer. Assoc. Can. Res.,* **39,** 130.

Grignon DJ and Eble JN. (1998). *Semin. Diagnos. Pathol.,* **15,** 41–53.

Hanafusa H. (1986). In: *Oncogenes and Growth Control.* Kahn P and Graf T. (ed.). Springer-Verlag: Berlin, Heidelberg. pp. 100–105.

Huang H, Qian C, Jenkins RB and Smith DI. (1998). *Genes, Chromosomes and Cancer,* **21,** 152–159.

Hubbard SR, Wei L, Ellis L and Hendrikson WA. (1994). *Nature,* **372,** 746–754.

Hubbard SR. (1997). *EMBO J.,* **16,** 5572–5581.

Jeffers M, Schmidt L, Nakaigawa N, Webb CP, Weirich G, Kishida T, Zbar B and Vande Woude GF. (1997). *Proc. Natl. Acad. Sci. USA,* **94,** 11445–11450.

Jeffers M, Rong S and Vande Woude GF. (1996). *J. Mol. Med.,* **74,** 505–513.

Kovacs G. (1993). *Adv. Cancer Res.,* **62,** 89–124.

Levy JB, Iba H and Hanafusa H. (1986). *Proc. Natl. Acad. Sci. USA,* **83,** 4228–4232.

Lin JC, Scherer SW, Tougar L, Traverso G, Tsui L-C, Andrulis IL, Jothy S and Park M. (1996). *Oncogene,* **13,** 2001–2008.

Park M, Dean M, Kaul K, Braun MJ, Gonda MA and Vande Woude GF. (1987). *Proc. Nat. Acad. Sci. USA,* **84,** 6379–6383.

Pignon J-M. (1997). *Hematol. Cell Ther.,* **39,** 114–116.

Santoro MM, Penengo L, Minetto M, Orecchia S, Cili M and Gaudino G. (1998). *Oncogene,* **17,** 741–749.

Schmidt L, Li F, Brown RS, Berg S, Chen F, Wei M-W, Tory K, Lerman MI and Zbar B. (1995). *Cancer J. Sci. Amer.,* **1,** 191–196.

Schmidt L, Duh F-M, Chen F, Kishida T, Glenn G, Choyke P, Scherer S, Zhuang Z, Lubensky I, Dean M, Allikmets R, Chidambaram A, Bergerheim UR, Feltis JT, Casadevall C, Zamarron A, Bernues M, Richard S, Lips CJM, Walther MM, Tsui L-C, Geil L, Orcutt ML, Stackhouse T, Lipan J, Slife L, Brauch H, Decker J, Niehans G, Hughson MD, Moch H, Storkel S, Lerman MI, Linehan WM and Zbar B. (1997). *Nature Genet.,* **16,** 68–73.

Schmidt L, Li H, Wei M-H, Lerman MI, Zbar B and Tory K. (1993). *Human Mol. Genet.,* **2,** 817–818.

Schmidt L, Junker K, Weirich G, Glenn G, Choyke P, Lubensky I, Zhuang Z, Jeffers M, Vande Woude G, Neumann H, Walther M, Linehan WM and Zbar B. (1998). *Cancer Res.,* **58,** 1719–1722.

Shu H-K, Pelley RJ and Kung H-J. (1990). *Proc. Natl. Acad. Sci. USA,* **87,** 9103–9107.

Shu H-K, Pelley RJ and Kung HJ. (1991). *Virology,* **65,** 6173–6180.

Shu H-K, Chang C-M, Lakshmeswari R, Ling L, Castellano CH, Walterm E, Pelley RJ and Kung H-J. (1994). *Mol. Cell Biol.,* **14,** 6868–6878.

Sidhar SK, Clark J, Gill S, Hamoudi R, Crew AJ, Gwillian R, Ross M, Linehan WM, Birdsall S, Shipley J and Cooper CS. (1996). *Hum. Mol. Genet.,* **5,** 1333–1338.

Weterman MA, Wilbrink M and van Kessel AG. (1996). *Proc. Natl. Acad. Sci. USA,* **93,** 15294–15298.

Ullrich A and Schlessinger J. (1990). *Cell,* **61,** 203–212.

Underhill PA, Jin L, Zemano R, Oefner PJ and Cavalli-Sforza LL. (1996). *Proc. Natl. Acad. Sci. USA,* **93,** 196–200.

Zbar B, Tory K, Merino M, Schmidt L, Glenn G, Choyke P, Walther MM and Linehan WM. (1994). *J. Urol.,* **15,** 561–566.

Zbar B and Lerman M. (1998). *Adv. Cancer Res.,* **75,** 163–201.

Zenklusen JC, Thompson JC, Troncoso P, Kagan J and Conti CJ. (1994). *Cancer Res.,* **54,** 6370–6373.

Zenklusen JC, Weitzel JN, Ball HG and Conti JC. (1995). *Oncogene,* **11,** 359–363.

Zhuang Z, Park WS, Pack S, Schmidt L, Vortmeyer AO, Pak E, Pham T, Candidas S, Weil RJ, Lubensky I, Linehan WM, Zbar B and Weirich G. (1998). *Nature Gen.,* **20,** 66–69.