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Validation and Genomic Interrogation of the *MET* Variant rs11762213 as a Predictor of Adverse Outcomes in Clear Cell Renal Cell Carcinoma

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Abstract

Background—The exonic single nucleotide variant rs11762213 located in the *MET* oncogene has recently been identified as a prognostic marker in clear cell renal cell carcinoma (ccRCC). We validated this finding using the Cancer Genome Atlas cohort and explored the biologic implications.

Methods—Genotype status for rs11762213 was available for 272 patients. Paired tumor-normal data, genomic data and clinical information were acquired from ccRCC TCGA datasets. Cancer specific survival (CSS) was analyzed using the competing risk method and Cox proportional hazard regression was used for analysis of time to recurrence (TTR). Multivariate competing risk models were fitted to adjust for the validated Mayo Clinic Stage, Size, Grade and Necrosis (SSIGN) score.

Results—The variant allele of rs11762213 was detected in 10.3% of the cohort. After adjusting for SSIGN score, the risk allele remained a significant predictor for adverse CSS ($p < 0.0001$; Odds Ratio [OR] 3.88, 95% confidence interval [CI] 1.99–7.56) and for TTR ($p = 0.003$; OR 2.97; 95% CI 1.43–6.2). Mapping of rs11762213 to regulatory regions within the genome suggests that it may impact a DNA enhancer region. RNA and protein sequencing data for *MET* did not reveal differences in steady-state expression when stratified by risk allele.

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Conclusions—The exonic *MET* variant rs11762213 is an independent predictor of adverse CSS and TTR in ccRCC and should be integrated into clinical practice for prognostic stratification. Genomic analysis suggests that the SNP may affect an enhancer region located in the coding region of *MET*. Further biological mechanistic interrogation is currently underway.

Keywords

MET oncogene; Biomarker; Renal cell carcinoma; TCGA; Variant

INTRODUCTION

There are diverse molecular mechanisms directing the pathogenesis of renal cell carcinoma (RCC). These occur on both the germline and tumor level and influence numerous cellular pathways including those directing angiogenesis, glucose metabolism, and chromatin configuration [1]. In the last few years, the oncogenome of RCC has been the focus of much research effort, leading to the discovery of recurrent alterations in clear cell RCC (ccRCC) [2, 3]. For example, somatic mutations leading to loss of function of tumor suppressor genes *BAP1*, *SETD2*, and *PBRM1* (in addition to the already known *VHL* gene) have been shown to drive renal tumors toward more aggressive behavior; specifically, they are associated with advanced tumor grade and stage as well as increased risk of recurrence after definitive therapy [4, 5].

Prediction models are important tools to use when counseling patients on their disease and tailoring case-specific treatment recommendations, yet clinical outcomes of RCC patients are highly variable and difficult to predict, even with the most comprehensive prognostic models [6, 7]. While somatic alterations in an RCC tumor may prove informative, germline variants are particularly attractive markers. Unlike somatic alterations, germline variants are static, simple to ascertain from peripheral blood, and not subject to intratumoral heterogeneity [8].

Single nuclear polymorphisms (SNPs) are single base pair variants found in germline DNA throughout the genome. These inherited sequence modifications are present in exons, introns, and intergenic segments and may modulate variable downstream effects [9]. Multiple germline polymorphisms have been identified in prostate and breast cancer patients that are associated with tumor recurrence and survival [10, 11]. Until recently, no SNPs have been identified that are associated with RCC clinical outcomes.

Schutz et al recently identified a novel SNP in the coding region of the *MET* oncogene (rs11762213) that is associated with an increased risk of recurrence and worse cancer specific survival following nephrectomy [12]. Median recurrence free survival for carriers of the rare allele of this SNP was 19 months compared to 50 months which was observed in non-carriers. Occurring at a minor allele frequency of approximately 10% in a European-ancestry population, this detectable SNP could potentially be integrated into prognostic models aiding in patient counseling and may even influence future development of novel agents for targeted therapy, especially considering that *MET* inhibitors are in clinical trials. The aim of our current study is both to validate the prognostic significance of the rs11762213 polymorphism in a high risk RCC cohort as well as to explore the potential

biological mechanisms of the variant using the rich genomic data of the Cancer Genome Atlas (TCGA) project and other publically available data sets.

MATERIAL AND METHODS

Paired tumor-normal materials, genomic data, and clinical information were acquired by our ccRCC TCGA Consortium. This multi-institutional effort included clinical and pathologic information on 446 retrospectively identified patients who underwent either radical or partial nephrectomy for sporadic ccRCC from 1998-2010. Since rs11762213 is an exonic variant, we extracted genotype data on it from the available variant call file (VCF) provided by TCGA for each tumor/normal pair (n=272). These data were first generated as part of the comprehensive characterization of the clear cell renal cell carcinoma genome in TCGA are available via NIH's database of Genotypes and Phenotypes (dbGaP) through accession number phs000178.v8.p7 [13]. We used the observed allelic fraction of variant alleles in the germline sample as reported in the VCF to determine genotype. An allelic fraction between 30% and 70% was taken to be heterozygous, and an allelic fraction greater than 80% was taken to be homozygous for the variant allele. Allelic fractions in the indeterminate ranges (20%-30% or 70%-80%) were taken to be no calls. To estimate the quality of this approach, we compared the VCF-derived genotype for all variants present on the Affymetrix SNP 6.0 array with the genotype as called from the Affymetrix SNP on the matched germline sample from the same individual (see below). Overall, 2.3% discordance was observed. After removing 17 individuals for which the discordance rate was >10%, the overall discordance rate was lowered to 1% in the remaining 262 individuals. Using the above algorithm, we called the genotype for rs11762213 in 272 individuals (including 25 for which Affymetrix SNP 6 data was not available for comparison, but excluding 15 patients that were used in the previously published *MET* analysis).

To conduct the GWAS for SNPs associated with survival time, we used SNP genotypes calls made from a large set of Affymetrix SNP 6.0 CEL files from TCGA and other projects. Specifically, all available CEL files from germline (either blood or adjacent normal) TCGA samples as of September 2011 were downloaded. Genotypes in these files were called jointly with other datasets from dbGaP and elsewhere using the BirdSeed algorithm as implemented in the Affymetrix Power Tools software. Only those samples from the TCGA KIRC study were extracted, and principal components analysis was conducted using smartpca [14] to identify the top 5 principal components of genetic ancestry. We restricted analysis to individuals with AJCC Stage 3 disease prior to surgery. We asked if each SNP with a minor allele frequency greater than 10% was associated with overall survival time, adjusting for the top 5 principal components. This analysis was done using plink to call a custom R package that conducts survival analysis [15].

Mutation, mRNA expression, methylation and protein data were downloaded through the TCGA web portal (<http://tcga-data.nci.nih.gov/tcga/findArchives.htm>). Clinical information was obtained from the file entitled "KIRC+Clinical+Data+Jul-31-2012" encompassing the most recent follow up information ("Max Followup").

Statistical Analysis

Associations between binary variables, and between binary and continuous variables, were assessed using Fisher's Exact and Wilcoxon Exact two tailed tests, respectively. Kaplan-Meier method was used to estimate the survival probabilities. Cancer specific survival (CSS) was analyzed using the competing risk method, utilizing the “cmprsk” R package. Cox proportional hazard regression was used for analysis of time to recurrence. Multivariate competing risk models were also fitted in order to adjust for clinical covariates using the validated SSIGN prognostic model which include pathologic stage, nuclear grade, node and distant metastasis status, tumor size and presence of necrosis. Concordance index (c-index) was calculated to assess the predictive accuracy of models with and without SNP. While we tested the difference in c-indices between the two models; the incremental value of the SNP is better assessed using adjusted p-value from the multivariate Cox model [16].

Deceased patients were considered dead from renal cancer-related causes if the field “Composite Tumor Status” = “WITH TUMOR” at the time of death, or they had metastatic disease at presentation (M1), or, if “Composite Tumor Status” was unavailable, they had lymph node disease (N1) or died within 2 years of surgery. Time to recurrence for patients who are N0/NX and M0 is defined as time between surgery and recurrence or day of last follow up. Patients with multiple kidney tumors at surgery were excluded from this analysis. Patients without recurrence date but whose “Composite Tumor Status” is “WITH TUMOR” were considered as recurrences at the last day of follow up or day of death.

Methods of SNP analysis

To identify SNPs in linkage disequilibrium (LD) with rs11762213, we used data from the 1000 Genomes Project. Specifically, we used the vcftools software to identify all SNPs in LD with rs11762213 ($r^2 > 0.8$) using the phased haplotype data restricted to those individuals primarily of European (EUR) ancestry.

Methods of TCGA analysis

Expression data was downloaded through the TCGA data portal: (<http://tcga-data.nci.nih.gov/tcga/findArchives.htm>). The Mann Whitney U test was used to evaluate *MET* normalized mRNA and protein expression by genotype.

RESULTS

Validation of rs11762213 as a predictor of adverse outcomes in the TCGA data set

Demographic and clinical information of the cohort can be seen in Table 1. At least one copy of the risk allele was present in 10.3% of the cohort (dominant model). Patients with the risk genotype (at least one copy of the risk allele) were more likely to have higher Fuhrman nuclear grade tumors ($p=0.03$) and trended toward higher AJCC stage ($p=0.07$). The 5 year cumulative incidence of death from disease was 56.4% for patients with risk genotype, and 22.5% for remaining patients. The SSIGN score adjusted cancer specific survival was significantly worse in the risk genotype cohort (HR 3.88 95% CI (1.99-7.56); $p<0.0001$) as was overall survival (HR 2.66 95% CI (1.4-4.9); $p=0.002$). The median length of follow up for survivors was 37.4 months for patients with the risk genotype vs 19.8

months for the remaining ($p=0.33$). Time to recurrence data was available for 217 patients. The 5 year recurrence free survival among patients with and without risk genotype was 48% and 76% respectively. Again, patients with the risk genotype were more likely to develop tumor recurrence adjusting for SSIGN score (HR 2.97 95% CI(1.43-6.2); $p=0.003$) (Table 2, Figure 1). To determine the impact of the risk allele on the predictive accuracy of the survival and recurrence model we calculated the impact of the genotype on the c-Index. For death from disease, the c-index using SSIGN score alone is 0.845, and increases to 0.866 when adding the genotype status. Similar improvement was seen in the c-index for time to recurrence with the c-index for TTR and SSIGN alone, of 0.719 and increasing to 0.738 when adding genotype.

Genome-wide interrogation of other survival-associated variants

After validating the clinical significance of rs11762213 in aiding prognostic models of RCC we then explored the genomic impact using the available data from the TCGA data set. Because Schutz et al identified the SNP through targeted panel of 70 genes involved in RCC pathogenesis, we first explored whether any additional SNP genome wide was associated with cancer specific death using the entire TCGA cohort in individuals with AJCC Stage 3 disease. Of these 331 individuals (AJCC Stage 3), 48 had disease-specific death. We asked if any SNP with a minor allele frequency greater than 10% was associated with survival in this cohort, after adjusting for the top 5 principal components of genetic variation. After adjusting for multiple testing, no SNP was significant. The lowest p-value observed, $1.6e-06$, was to be expected given the 559,215 tests conducted (Figure 2).

Next, we determined whether the *MET* SNP was in linkage disequilibrium (LD) with any other variants in the region. Only three additional variants were found to be in LD at a threshold of $r^2>0.8$ and all were in intronic regions of *MET* (Supplemental Figure 1).

rs11762213 maps to an enhancer regions of *MET*

Given that rs11762213 is in the coding region of a well-known proto-oncogene, yet does not alter the amino acid sequence, we hypothesized that the variant impacted gene regulation. Using publically available regulatory domain data from the ENCODE project [17] we mapped rs11762213 and the three other SNPs in LD with it onto DNase I Hypersensitivity Clusters in 125 cell types (Figure 3). Intriguingly, rs11762213 alone mapped to a highly conserved DNase hypersensitivity site suggesting its active role in gene expression regulation. To further characterize the functional significance of rs11762213 we used our previously generated genome-wide chromatin annotation maps [18, 19] using cultured human proximal tubular epithelial cells (HKC8) and overlaid them with previously generated gene regulatory annotation maps from a panel of ChIP-seq data using the hidden Markov model-based ChromHMM chromatin segmentation program (Figure 4) [20]. Notably, rs11762213 maps to an H3K4me1 histone modification mark, which serves as an enhancer marker and is therefore consistent with the hypothesis that the SNP has a regulatory function.

Next, we assessed the impact of rs11762213 on *MET* steady-state mRNA and protein tumor expression using available RNA seq data and reverse phase protein array (RPPA) data from

the TCGA. We found no difference in tumor *MET* expression by SNP status ($p=0.47$ Mann Whitney) including all detected *MET* isoforms ($n=18$) (Supplemental figure 2A). Since rs11762213 is located in the coding region of exon 2 we further explored exon level expression differences by genotype and again did not find any difference by genotype for exon 2 *MET* expression ($p=0.29$) of any other exon (Supplemental figure 3). Additionally, we did not see differences in tumor *MET* protein ($p=0.88$) or *MET* phospho-protein expression (Y1235) (Supplemental figure 2B and 2C). Finally we investigated the adjacent normal kidney mRNA expression by genotype which was available for a subset of the TCGA cohort ($n=61$) (Supplemental figure 2D). rs11762213 was associated with higher normal tissue *MET* expression ($p=0.019$), however, in an independent normal kidney Affymetrix mRNA array data set this finding did not validate ($n=95$) (data not shown).

DISCUSSION

c-Met is a proto-oncogene whose protein product is a critical transmembrane receptor tyrosine kinase for the hepatocyte growth factor. *MET* is involved in the pathogenesis of RCC, usually cooperating with VEGFR to promote tumor growth and is thought to be induced by hypoxia-inducible factor 1 α [21, 22]. Several investigators have shown that increased *MET* activity correlates with aggressive disease behavior in RCC including ccRCC [23, 24]. More importantly higher *MET* activity may predict response to *MET* inhibition [25]. Further, upregulation of *MET* is thought to mediate VEGFR inhibitor resistance [26].

Our data validates the recently published finding that the *MET* variant rs11762213 is predictive of worse clinical outcomes. We extend the clinical utility of this variant by showing that it retains its independent prognostic effect on survival even on multivariate analysis for cancer specific (HR 3.88 95% CI (1.99-7.56); $p<0.0001$) and overall survival (HR 2.66 95% CI (1.4-4.9); $p=0.002$). We found the same to apply to time to recurrence (HR 2.97 95% CI (1.43-6.2); $p=0.003$). These HR's were similar to those seen by Schutz et al. (3.52 (1.32-9.38) for overall survival and 2.45 (1.01-5.95) for recurrence free survival).

The additional knowledge of genotype status could serve several clinical purposes. For patients who have undergone surgical resection, it can influence surveillance patterns and enrollment in adjuvant trials which are currently dictated by tumor stage alone. The fact that the biomarker is a germline variant makes this particularly attractive given the practical difficulties of sampling multiple tumors regions to address tumor heterogeneity when investigating acquired somatic mutations. In the preoperative setting, its utility includes the ability perform “liquid biopsies”, which might be particularly helpful in patients being considered for active surveillance or other forms of non-definitive management.

Our use of TCGA data allowed us to explore and address many of the questions brought up by Schutz et al's initial discovery and external validation of the variant including the potential effects on *MET*. Using genome-wide linkage disequilibrium analysis from individuals of European ancestry in The 1000 Genomes Project we were able to determine whether this variant was merely a surrogate for another causal allele. Only three other variants were found to have an $r^2>0.8$, all were in intronic regions of *MET*, and none

mapped to regulatory domains. Utilizing publicly available and experimentally derived data, we were able to show that rs11762213 alone is found in a regulatory element of *MET* likely in an enhancer domain, which has implications on its interaction both with *MET* itself and potentially other cis-acting target genes. Our analysis of mRNA and protein expression utilizing RNAseq and RPPA data did not show an effect on tumor steady-state expression, and while we did find an effect on normal kidney mRNA expression we were unable to validate that in an external cohort although the direction of effect was the same (data not shown). We and others have recently shown that risk alleles that physically interact with oncogenes such as *MYC* may not have effect on steady state gene expression [27]. Steady state levels of RNA or protein at a single time point may not adequately capture *MET*'s impact on tumor aggressiveness. Because the allele does not seem to have an impact on disease risk (data not shown) but rather on tumor biology, specifically metastasis, the effects on *MET* expression may occur at some time point after malignant transformation. Moreover, expression differences may only be present in a subpopulation of cells (for example, stem cells). Further, the impact may be more pronounced on normal renal tissue, which has implications on tumor stromal interactions, a well-known phenomenon in renal cell carcinoma. Given the SNP's effect on a potentially targetable oncogene, assessment of the genotype response to targeted *MET* inhibition is critical given the recent evidence of improved response rates in papillary renal cell carcinoma patients with germline *MET* mutations [28] and evidence of activity in heavily pretreated patients with ccRCC as well [29]. Given the potential role for *MET* activation and VEGF inhibitor resistance, it would be valuable to determine whether rs11762213 predicts poor response to standard first line therapy for ccRCC. Indeed, the NCI has recently finished accruing a phase 2 trial to compare cabozantinib to sunitinib in untreated locally advanced or metastatic RCC (NCT01835158).

Intriguingly, we found that rs11762113 maps to an H3K4me1 histone modification mark. RCC was recently shown to have a preferential enrichment for aberrant methylation in kidney specific enhancer regions associated with H3K4Me1 marks which also predicted for poor prognosis (PMID 24916699).

Limitations of our study include the fact that the median length of followup differed between our SNP groups (37.4 vs 19.8 months) although this was not statistically significant ($p=0.33$).

CONCLUSIONS

In sum we have externally validated and proven the clinical utility of the *MET* variant rs11762213 on disease behavior in ccRCC and established its ability to improve prognostic models. We demonstrate that this specific variant is the likely causal SNP and provide computational evidence that it affects a regulatory domain within a well-known and well characterized oncogene.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Cancer specific survival (CSS) and Time to Recurrence (TTR) curves stratified by genotype status.

Figure 2. Manhattan plot indicating SNPs correlated with cancer specific death in the entire TCGA cohort of AJCC Stage 3 disease. Red line indicates necessary p value needed to achieve statistical significance given the multiple tests performed ($n=559,215$; $p<8.9\times 10^{-8}$).

Figure 3.

Genomic location of rs11762213 and the 3 additional SNPs in linkage disequilibrium ($r^2 > 0.8$). Only rs11762213 maps to a coding regions within MET as well as a highly conserved region across species (bottom rows). Publically available data from the ENCODE shows that rs11762213 maps to a DNase I hypersensitivity cluster (a short region of open chromatin available for binding of proteins such as transcription factors) across 125 cell types.

Figure 4. rs11762213 maps to an H3K4me1 histone modification mark as determined by previously generated gene regulatory annotation maps from a panel of ChIP-seq data using the hidden Markov model-based ChromHMM chromatin segmentation program.

Table 1

Clinical Characteristics of the TCGA Cohort

		Risk Allele Present (N=28)		Risk Allele Absent (N=244)	
Gender	Male (%)	17	61%	155	64%
	Female (%)	11	39%	89	36%
Median age years (IQR)		66.5	(56,73)	60	(51,69)
Race	White	26	93%	224	92%
	Black	2	7%	10	4%
	Asian	0	-	6	2%
	NA	0	-	4	2%
Tumor Grade	1	0	-	6	2%
	2	7	25%	108	44%
	3	15	54%	92	38%
	4	6	21%	37	15%
	NA	0	-	1	0%
Median tumor maximal diameter cm (IQR)		6	(5.1,10.3)	5.5	(3.6,8)
T Stage	T1	11	39%	130	53%
	T2	3	11%	29	12%
	T3	12	43%	84	34%
	T4	2	7%	1	0%
N Stage	N0	15	54%	106	43%
	N1	1	4%	10	4%
	NX	12	43%	128	52%
M Stage	M0	24	86%	208	85%
	M1	4	14%	36	15%
AJCC Stage	Stage I	10	36%	126	51%
	Stage II	2	7%	24	11%
	Stage III	10	36%	58	24%
	Stage V	6	21%	36	14%
Median FU for surviving patients in months		37.4		19.8	

Table 2Association between outcomes and variants of the *MET* polymorphism rs11762213

CSS		5 year cumulative incidence rate (%; (95% CI)	SSIGN Adjusted Cancer specific Survival	
	N patients (N events)		HR (95%CI)	P value
Overall	272 (56)	27.7 (22.8, 32.8)	..	..
GG	244 (44)	22.5 (16, 30)	..	..
AA/AG	28 (12)	56.4 (31, 75.6)	3.88 (1.99-7.56)	p<0.0001
TTR		5 year recurrence free survival rate (%; (95% CI)	SSIGN Adjusted Time to Recurrence	
	N patients (N events)		HR (95%CI)	P value
Overall	217 (43)	74 (68,80)	..	..
GG	194 (32)	76 (68, 84.8)	..	..
AA/AG	23 (11)	48 (28.3, 81.5)	2.97 (1.43-6.2)	p=0.003