The Gulf Journal of Oncology

Indexed By PubMed and Medline Database

Issue 42, May 2023
ISSN No. 2078-2101

8th Gulf Week for Cancer Awareness, 1-7 February 2023

The Official Journal of the Gulf Federation For Cancer Control

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Original Article

Variants of Human Mucin Genes in Clear Cell Renal Cell Carcinoma and their Potential Prognostic and Predictive Values

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Abstract

Background: There is no reliable prognostic and predictive biomarkers for clear cell renal cell carcinoma (cc–RCC).

Methods: DNA from 47 cc–RCC tissue samples were sequenced using next generation sequencing and a customized gene panel testing for tumor–driver genes including 19 Mucin genes.

Results: Distinctive variants in 12 Mucin genes were present in all samples. These genes are: MUC2, MUC3A, MUC4, MUC5AC, MUC5B, MUC6, MUC7, MUC12, MUC16, MUC17, MUC19, and MUC22. The numbers of distinctive and non–distinctive variants were counted for each sample. The median number of variants was 455. High variant number (HVN) (>455) was associated with shorter overall survival compared to low variant number (≤455) [Median 50 months vs. not reached; P=0.041]. In the 11 patients who received anti–angiogenic tyrosine kinase inhibitors (TKIs), HVN was associated with a trend of shorter progression free survival.

Conclusion: Alterations in Mucin family genes are common in ccRCC. HVN is associated with worse prognosis and may predict decreased benefit from anti–angiogenic TKIs.

Key words: Mucin; Variants; Renal cell carcinoma; Biomarker; Tyrosine kinase inhibitors

Introduction

Renal cell carcinoma (RCC) accounts for the majority of malignancies affecting the kidneys and represents approximately 2%–4% of all malignancies[1].

The typical histology is adenocarcinoma arising from the renal tubules and the majority of cases are of the clear–cell carcinoma subtype (ccRCC)[2].

The Cancer Genome Atlas identified the commonly altered genes in ccRCC such as VHL, PBRM1, BAP1, SETD2 and components of the PI3K/Akt pathway[3].

Clinical prognostic criteria such as the University of California Los Angeles Integrated Staging System (UISS) International, Metastatic Renal Cell Carcinoma Database Consortium (IMDC) and Memorial Sloan Kettering Cancer Center (MSKCC) are established risk models used in clinical trials and in day to day clinical practice[4].

However, the search for more accurate molecular prognostic and predictive biomarkers remains elusive. Next generation Sequencing (NGS) has revolutionized genomic characterization in research and routine practice settings.

In the initial screening phase, NGS of 83 genes was performed on 47 samples of ccRCC to provide an overview of the diversity and to identify genes of potential interest for subsequent prognostic and predictive analysis. We found frequent distinctive potential significant variants in 13 Mucin genes in all the samples. Therefore, a second analytical phase was pursued which is the subject of this report. The main aim of the study is to investigate the prognostic value

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of Mucin gene variants. A secondary aim is to explore the therapeutic predictive value of these gene variants.

Materials and Methods:

Samples collection and processing

Retrospective search was conducted for specimens with the diagnosis of RCC archived in the department of pathology and laboratory medicine, King Faisal Specialist Hospital and Research center (KFSH&RC) between the years 2005–2009 and 2013–2015. Cases of ccRCC diagnosed from nephrectomy samples were identified to be included in this project. Hematoxylin and Eosin (H&E) slides were examined and screened by one pathologist. Tissue blocks with high tumor volume (>80% viable tumor) were selected for further processing while those with extensive necrosis (>50%) and/or those with features of poor fixation were deemed not suitable for the study. Seven tissue rolls of 10 um thickness were obtained from each Formalin–Fixed Paraffin–Embedded (FFPE) tissue block and placed in sterile DNA/RNA free 1.5 ml centrifuge tubes.

Institutional Review Board at KFSH&RC approval was obtained and the written consent was waived due to the retrospective archival nature of this study.

DNA extraction, quantification and sequencing

DNA extraction was performed using GeneRead DNA FFPE kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA was quantified by using NanoDrop™ 2000c Spectrophotometers and Qubit® fluorometer 0.3 instrument with Qubit® dsDNA HS assay kit (Invitrogen/Life Technologies, Carlsbad, CA, USA).

Ion Proton™ System was used to sequence the amplified 30 ng DNA samples according to the manufacturer's instructions for a customized cancer panel of 83 genes that included 19 Mucin genes (Ampliseq™, Life Technologies). The specimens were batched (12 per pool) to obtain aminimum coverage of 10x per run.

Analysis of NGS output (Bioinformatics)

Primary analysis:

Individual samples were filtered by integrity parameters: on target reads (>80%), mean depth (>300) and coverage uniformity (>60%). Primary analysis was done using Torrent Suite Software. First line of variants annotation was achieved by the Saudi Human Genome Program (SHGP) pipeline[5].

Somatic variants:

Due to the lack of paired tissue analysis, we filtered the somatic alterations following Jones, Siân, et al protocol[6]. Subsequently, a somatic score was developed based on the following criteria: (a) availability in general population (1000G & gnomad), (b) tumor specific (COSMIC) databases, type of mutation (splicing, stop gain or frame–shift) & gene–cancer association (tumor suppressor), and (c) gain of function possibility for oncogenes (intOgen). The higher the score the more likelihood the change was somatic.

We didn't follow the American College of Medical Genetics and Genomics (ACMG) guidelines for variants classification because of our interest in the somatic alterations. Hence, likely significant variants were classified based on consensus among multiple prediction scores (SIFT, PolyPhen, MutationTaster, MetaSVM, MCAP, CADD), mutation effect (non–synonymous, frame–shift, nonsense and stop–loss) and mutation location (splicing, exonic).

Results:

A total of 115 archival samples were identified of which 47 fulfilled the inclusion criteria and underwent successful NGS sequencing (Fig 1). Patients' characteristics are depicted in table 1. We identified distinctive potential significant variants in 13 genes (12 Mucins and CR1) in all screened samples. The variants counts (distinctive and non–distinctive) were particularly higher in twelve genes, all of which are Mucins: MUC2, MUC3A, MUC4, MUC5AC, MUC5B, MUC6, MUC7, MUC12, MUC16, MUC17, MUC19, and MUC22.

The numbers of all potential significant variants (distinctive and non–distinctive) in these 12 genes were counted for each sample. Median number of variants was 455 (range: 351–542) and was selected as a cut off to define cases with high variant number (HVN [> 455 variants]) and low variant number (LVN [≤455 variants]). For the whole cohort, HVN was associated with shorter overall survival (OS) compared to LVN (Median 50 months vs. not reached; Log Rank P=0.041) (Fig 2). In the 11 patients who received first line anti–angiogenic tyrosine kinase inhibitors (TKIs), HVN was associated with a trend of shorter progression free survival (Median 5 vs. 10 months; Log Rank P=not significant) (Fig 3).

Discussion:

Mucins are high molecular weight glycoproteins produced by epithelial cells with oligosaccharides attached

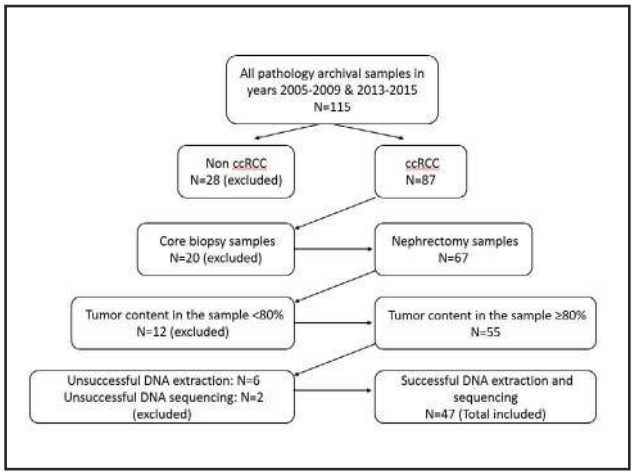

Figure 1. Study flow chart illustrating the excluded and included samples.

	Number (%)
Gender Male Female	 35 (74.5) 12 (25.5)
Median age (range)	56 (14–80) years
Stage at initial presentation Non–metastatic (stage I–III) Metastatic (stage IV)	 37 (78.7) 10 (21.3)
Stage during the course of follow up Non–metastatic (stage I–III) Metastatic (stage IV)	 28 (59.6) 19 (40.4)
First line Tyrosine Kinase Inhibitor treatment Yes No	 11 (23.4) 36 (76.6)
Survival at time of data analysis Alive Dead Lost to follow up	 22 (46.8) 14 (29.8) 11 (23.4)

Table 1. Patients' characteristics

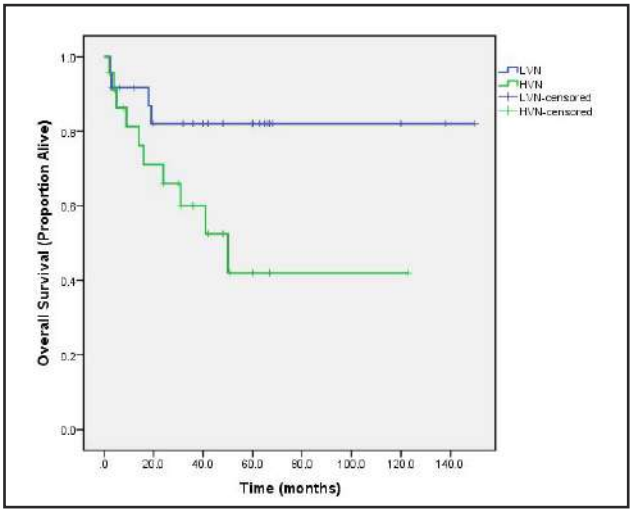

Figure 2. Overall survival for patients with low variants number (LVN: ≤455) and high variants number (HVN: >455)

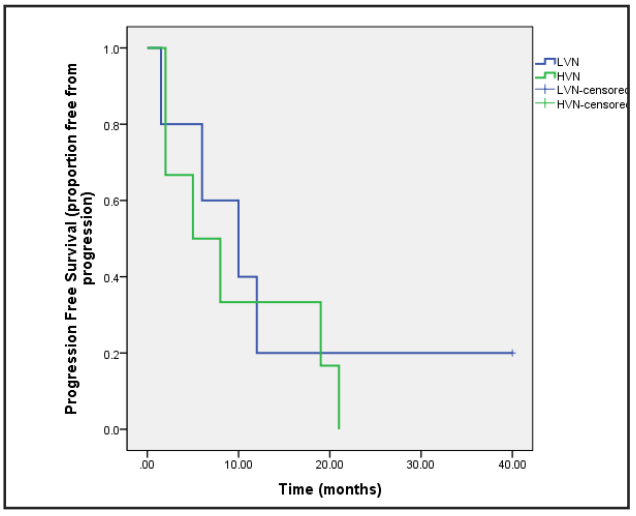

Figure 3. Progression free survival on first line anti–angiogenic tyrosine kinase inhibitors for patients (n=11) with low variants number (LVN: ≤455) and high variants number (HVN: >455)

to serine or threonine residues of the core protein backbone by O–glycosidic linkages. Mucins are classified into 11 membrane–bound mucins (MUC1, MUC3A &3B, MUC4, MUC12, MUC13, MUC15, MUC16, MUC17, MUC20 and MUC21) and seven secreted mucins (MUC2, MUC5AC, MUC5B, MUC6, MUC7, MUC8 and MUC19). Physiologically, mucins play roles in physical barrier, maintenance of environment and regulatory interactions with intracellular (such as growth receptors) and extracellular structures [7,8].

Cancer cells express aberrant mucins probably due to deregulation of expression of mucin core proteins and their regulatory enzymes during the process of cellular oncogenesis. These aberrant mucins are hypothesized to promote cancer progression, invasion and metastases. The widely used serum diagnostic assays for CA19–9 and CA125 recognize epitopes that are found on mucins and are clinically used to study tumor burden in patients with pancreatic and ovarian cancers respectively[7].

Majority of the published literature focused on studying mucin expression by immunohistochemistry (IHC) or In situ Hybridization (ISH) and mostly, each studied one or few mucin family members. Our work is the first to utilize NGS to report on a long list of mucin genes alterations in ccRCC. In addition, it is the first time such alterations are described in a collective manner and tested for possible prognostic value.

We found the existence of distinctive, likely significant variants in Mucins among all the studied samples highly suggesting their association with ccRCC tumorigenesis. Our results are in line with those of previously reported studies using ISH, IHC and reverse transcriptase polymerase chain reaction (RT–PCR). Leroy et al reported over–expression of

MUC3, MUC11, MUC12 in 29 RCC samples when compared with 15 normal kidney tissue[9].

Indeed, a number of MUC genes have been associated with the clinical outcome when studied individually. The number of the genetic variants is likely to reflect the genetic state during oncogenesis and cancer progression. Thus it may indicate tumor aggressiveness and the likelihood of worse clinical outcome. We found a significant relation between mucin genes HVN and poor survival (Fig 2). A number of reports in the literature demonstrated the prognostic value of various MUC genes when studied individually mostly using IHC techniques. Higher MUC13 expression showed positive association with higher tumor grade and shorter OS and relapse free survival (RFS) [10,11]. MUC7 expression was found to be an independent predictive factor for OS and RFS[12]. In 602 ccRCC samples, higher MUC5AC was associated with poor pathological features and worse clinical outcome[13].

Cancer is a multi–step process during which cells acquire a series of genetic and epigenetic alterations that eventually lead to uncontrolled cell growth and division, de–differentiation, invasion and loss of apoptosis[14]. Tumor mutational burden (TMB) defined as the total number of mutations found in the DNA of cancer cells has been proposed as a potential prognostic biomarker. Recently, the Cancer Genome Atlas database was used to study cancer samples of 6035 patients representing 20 solid tumor types. The investigators classified 8 solid tumor types (including ccRCC) as TMB–worse in which patients with high TMB had a poorer prognosis compared with those with low TMB[15].

To our knowledge, this study is the first to report the collective number of variants of many mucin genes in ccRCC and correlates it with the outcome. In addition, such approach may prove helpful as a biomarker to predict the clinical outcome of patients receiving first line TKIs (Fig 3). It is worth mentioning in this context that an in–vitro study found that MUC13 promoted the growth of 769–P and 786–O RCC cells and that silencing with TKIs (sunitinib and sorafenib) substantially impaired cell proliferation and migration, and greatly enhanced cell death[11].

Limitations of our study include (a) relatively small sample size and even a smaller number of patients who received first line TKIs, (b) our approach of identifying somatic variants relies on current knowledge in public databases which is dynamic and changeable, thus affecting the variant count. Additionally, dichotomizing the number of variants at the median value was a good indicator of the outcome as shown in our study. However, the number of variants is likely to change (increase) overtime as more variants will be discovered, making the median a moving target that is difficult to apply in clinical practice[16].

Conclusion:

This study shows that many mucin genes are mutated in ccRCC and the collective number of these genes variants is inversely related to the clinical outcome. Further larger studies are needed to confirm this finding and explore the association with response to TKIs and other therapies.

Acknowledgments:

No acknowledgments are relevant to this study.

Funding:

The study is conducted in the laboratories of the research centre at the KFSH&RC (Jeddah Branch). The bioinformatics analysis was performed through the Saudi Human Genome Project pipeline.

Conflict of Interests Statement:

All authors declare no conflicts of interest.

Ethical Considerations:

Our study was approved by the Institutional Review Board (IRB) at King Faisal Specialist Hospital & Research Centre – Jeddah (approval no. 2016–57). The written consent was waived by the IRB due to the retrospective archival nature of this study.

Authors Contribution:

Conception and design of the study: JZ and TMS. Acquisition of clinical data: JZ and SI. Analysis and interpretation of clinical data: JZ. Analysis and interpretation of molecular data: TMS and JZ. Identifying and processing archival pathology samples: AM. DNA extraction, next generation sequencing and administrative logistical support: MAB. Major role in drafting and revising the manuscript: JZ. Supportive role in drafting the manuscript: TMS, AM, MAB. Final approval of the version to be published: JZ, TMS, AM, MAB and SI.

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