Research Paper

Increased insulin mRNA binding protein-3 expression correlates with vascular enhancement of renal cell carcinoma by intravenous contrast-CT and is associated with bone metastasis

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ABSTRACT

Purpose: To: 1) assess the correlation between CT vascularity and a candidate molecular marker of RCC metastasis (insulin-like mRNA binding protein-3 (IMP3)); and 2) demonstrate the differential expression of IMP3 in high vs. low vascular tumors.

Experimental design: Retrospectively obtained contrast CT from 72 patients with primary RCC were used to establish threshold values for Low, Intermediate and High tumor vascularity. Paired histopathology specimens from 33 of these patients were used for immunohistochemistry (IHC) to correlate CT with IMP-3 expression. IMP-3 gene expression studies were performed on RCC and poorly vascular prostate cancer (PC) human bone metastases samples to confirm presence of IMP3 in metastatic samples from RCC. Gene expression studies were performed on RCC 786-O and PC3 cell lines to confirm the presence of high expression of IMP3 in the RCC cell line.

Results: IMP-3 expression positively correlated with CT vascular enhancement ($p < 0.01$). IMP3 expression by IHC was strongly positive in all RCC, but weak in PC bone metastases. Real time RT-PCR demonstrated a significant 4-fold increase in imp-3 expression in RCC 786-O vs. PC3 cells in vitro ($p < 0.001$).

Conclusion: Quantitation of pre-operative CT is a feasible method to phenotype primary RCC vascularity, which correlates with IMP-3 expression. In situ and cell line studies demonstrate an association between high IMP-3 expression and RCC bone metastasis. Studies aimed at defining the diagnostic potential of biomarkers for RCC bone metastasis, and functional significance of IMP-3 in RCC vascularity and tumor progression are warranted.

1. Introduction

There is an estimated 330,000 new cases of renal cell carcinoma (RCC) diagnosed each year worldwide, with over 100,000 deaths, and a rising incidence of 3% per year [1]. Although several advances in the treatment of metastatic RCC have occurred in the last decade, this disease remains one of the most deadly cancers with a 5-year survival rate of ~10% [2–4]. The primary treatment for localized RCC is surgical resection alone. Although systemic therapies in the form of receptor tyrosine kinase (RTK) inhibitors and targets of the mTOR protein have improved length of survival for patients found to have advanced metastatic disease, there is a

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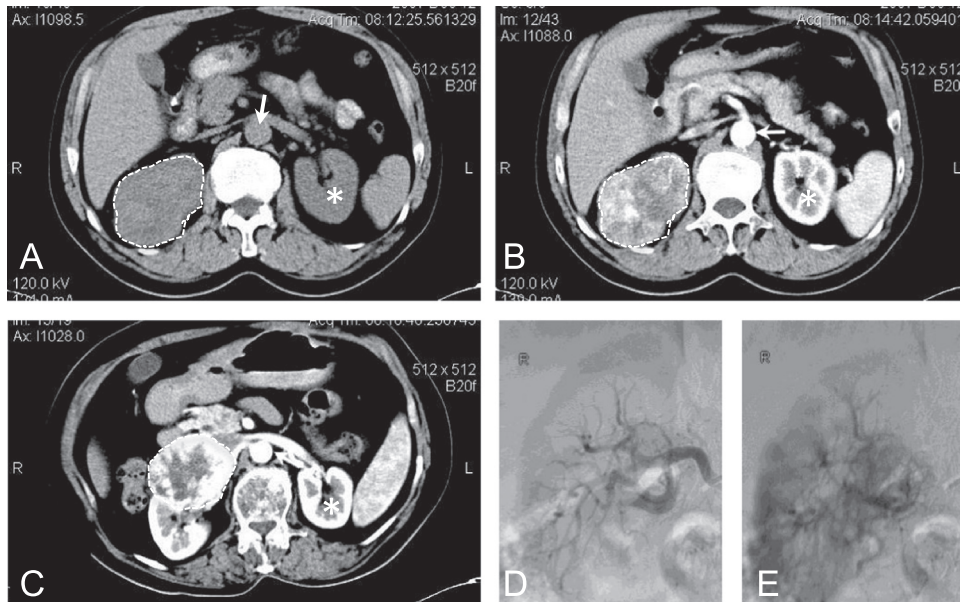[3] These authors contributed equally to this work.

Fig. 1. *Assessment of primary tumor vascularity via CT scan in patients with malignant renal cancer.* Contrast CT scan was performed on all patients in the study to assess the vascularity of their primary renal cancer (white dashed line). In the initial analysis, patients with low vascular tumor, as illustrated in images (A) and (B); versus patients with highly vascular tumors that went on to receive digital subtraction angiography (C–E). Shown are representative: (A) pre-contrast, and (B) early arterial phase images demonstrating the initial enhancement and uptake of contrast dye (Iohexol 300 mgl/ml) at 30 s of a patient with a low vascular tumor. (C) CT scan of late phase vascular uptake showing high contrast uptake in the highly vascular tumor, which warranted the subsequent DSA. The patient's DSA confirmed the presence of a major tumor feeding vessel as seen from image obtained immediately after injection (D; 1–2 s) and late phase (E; 10 s).

reluctance to start these treatments with significant side effects in patients with only localized disease [5,6], as many patients will not go on to develop metastatic disease if treated with surgery alone. Two important trends in RCC have been noted in the past decade. The first is that the incidence of localized disease has been increasing, despite a plateau in the number of abdominal CT scans performed that would normally detect such disease [7]. Secondly, the mortality rate for patients with localized disease has also increased. This is in the setting of no improvements in incidence or mortality rates for patients with advanced disease [7]. Despite our best efforts to detect and surgically treat early stage RCC, approximately 30% of patients will go on to develop advanced metastatic disease [1]. An area in which major improvements could be made is diagnostic radiology for those patients with localized disease and a high risk of developing metastases, as early-aggressive treatments could then be justified. To this end, we aim to identify novel-clinically relevant radiologic and molecular biomarkers that can differentiate the metastatic potential of RCC in patients with local disease. This may alter surveillance and possibly the indications for starting systemic treatment.

Our research in this area has been focused on understanding the highly vascular nature of RCC, which has been largely attributed to loss of function of the von Hippel-Lindau (VHL) gene and resultant vascular endothelial growth factor (VEGF) over-expression, as it is an early event during tumorgenesis and is the most common cause for inherited RCC [8–10]. However, therapies that specifically target VEGF and its receptor have failed to demonstrate significant efficacy in clinical trials [11,12]. Moreover, our observations in a murine xenograft model of bone metastasis demonstrated that the major difference between a highly vascular RCC cell line (786-O) and a prototypical avascular prostate cancer cell line (PC3) is the presence of large smooth muscle and pericyte lined blood vessels within the tumor [13], suggests that non-VEGF signaling pathways may be more important. To test this hypothesis, we performed a microarray analysis of 786-O vs. PC3 by using Affymetrix GeneChip Human Genome U133 Plus 2.0 Array (Chip

Lot# LE23BK05) and whole data set has been documented at NCBI GEO (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=qn-grecewnbothcv&acc=GSE61942) and a summary of the notable findings is presented in Supplementary Table 1.

Based on our search criteria for candidate molecular markers of RCC vascularity, we chose to focus on insulin-like growth factor II (IGF-II) mRNA binding protein 3 (IGF2BP3 or IMP3). IMP3 is an oncofetal mRNA-binding protein and has been recently described as an independent prognostic marker for renal cell carcinoma (RCC) distant metastasis, and is associated with shorter survival [14]. It also has been noted in other cancers to be associated with cell motility and trans-endothelial migration [15]. IMP3 is a member of the highly conserved family of proteins that have been found to be associated with mRNA transport, translation and turnover. Functionally, IMPs have been shown to modulate cell proliferation, adhesion, migration and invasion [16]. IMP3 expression is almost exclusively limited to embryonic development, as its expression in most adult tissues is undetectable. However, it has recently been found to have significant expression in malignant adult tissue, including RCC [17]. Although an association between the expression of IMP3 in RCC and prognosis has recently been discovered, the predictive studies of IMP3 in clinical practice have not been evaluated [17]. More specifically, the relationship between IMP3 expression and pre-operative imaging characteristics (i.e. computed tomography (CT), magnetic resonance image (MRI) or digital subtraction angiographic (DSA)) has yet to be investigated. To address this, we evaluated the relationship between IMP3 expression in primary RCC versus tumor vascularity quantified from the pre-operative intravenous contrast CT scan. We also evaluated the expression of IMP3 via IHC from samples of bone biopsies obtained from patients with metastatic RCC to bone and compared them to patients with metastatic prostate bone disease. The goals of this study were to determine whether vascularity as assesses by contrast CT is correlated to IMP3 expression and if IMP3 expression in tumor samples could be used to stratify metastatic disease risk assessment in RCC patients. These findings
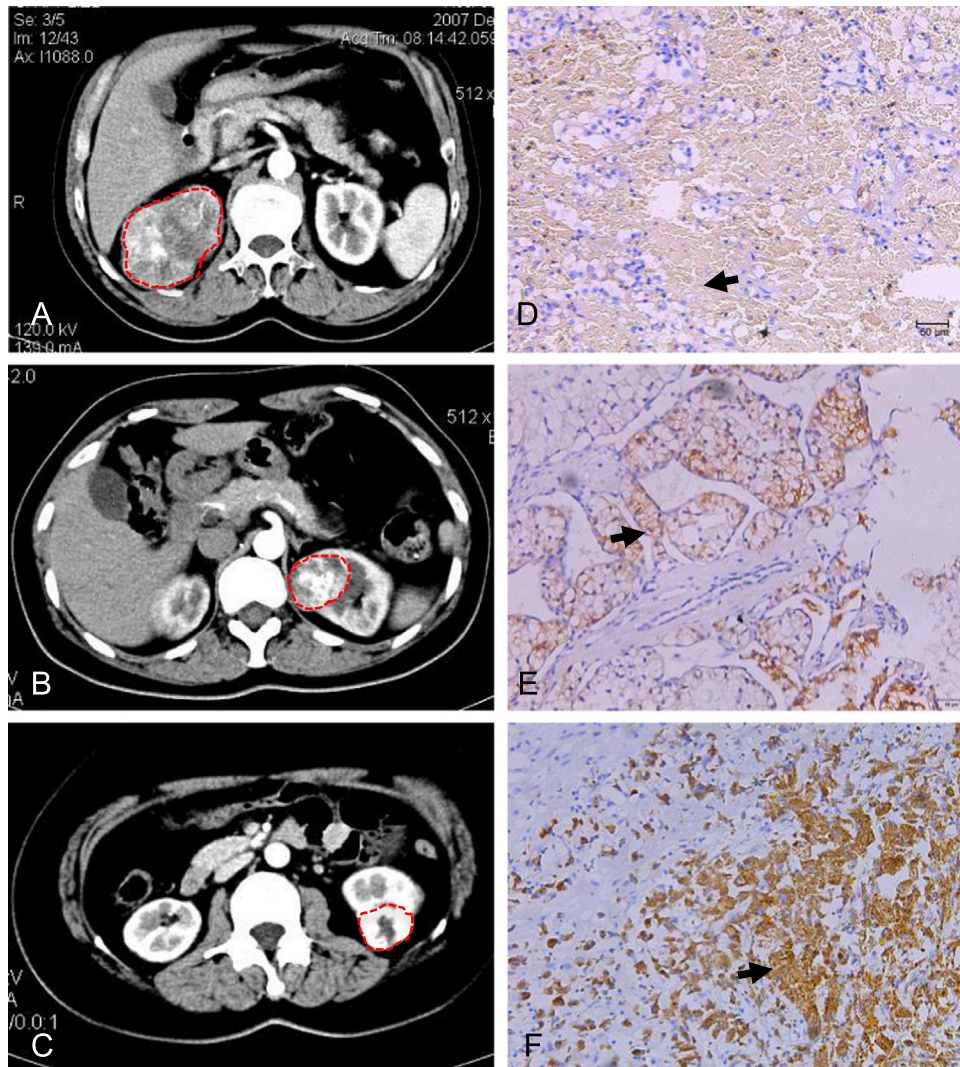
Fig. 2. Semiquantitative scoring systems to assess primary renal cancer vascularity via CT and IMP3 expression by immunohistochemistry (IHC). CT scans were obtained from the patients prior to their cancer surgery, and the resected tumor was harvested and processed for IHC with antibodies specific for IMP3. Representative early arterial phase images (A–C), with corresponding IMP3 IHC micrographs obtained at 200X (D–F) are presented to illustrate the semiquantitative scoring system used to correlate tumor vascularity with IMP3 expression as follows. Tumor vascularity, based on total Hounsfield Units (HU) of the segmented tumor (red line), was scored as: (A) Low (<20 HU); (B) Intermediate (20–40 HU); or (C) High (>40 HU). The intensity of IMP3 IHC staining (brown) was scored as: (D) Weak $+$; (E) Moderate $++$; or (F) Strong $+++$ as we have previously described (23). Note that the vascularity of the CT closely correlates with IHC staining for IMP3

could provide evidence to support early-aggressive systemic therapy, which is currently not the standard of care for most patients identified with RCC.

2. Materials and methods

2.1. Patient selection

The institutional research review boards at Zunyi Medical University and University of Rochester Medical Center approved this study. This was a retrospective study that (a) did not require participants to provide written or verbal informed consent to participate in this study as all (b) participants personal information were removed from data collection and (c) both IRBs from Zunyi Medical University and University of Rochester Medical Center approved the study design. A retrospective analysis was conducted of a consecutive series of 72 primary RCC patients who had histopathologic confirmation of RCC, and who were diagnosed and treated at the First Affiliated Hospital of Zunyi Medical

University (ZMU) and Zunyi Hospital between March 2003 and January 2010. All 72 patients had undergone pre-operative radiographic analysis with computed tomography (Siemens Somatom Sensation 16, Germany). Of the 72 initial patients with localized RCC who underwent CT analysis, 33 patients had paraffin blocks available for performing additional H&E staining and immunohistochemistry (IHC) for IMP3. Paraffin blocks were not available for the remaining 39 patients, and therefore IMP3 analysis was not performed on those tumors. Patients found to have markedly avid contrast uptake on CT, underwent digital subtraction angiography (DSA) and embolization. Seven of the 33 patients with IHC analysis underwent this treatment plan, and therefore had DSA imaging available for analysis in this study.

An additional eleven patients with metastatic RCC to bone and three patients with metastatic prostate cancer to bone from the University of Rochester Medical Center (URMC) underwent IHC analysis for IMP3 of their bone metastasis at the time of surgical stabilization for impending pathologic fracture. These patients were not included in the original 33 patients that underwent pre-operative abdominal CT, but were included for purposes of
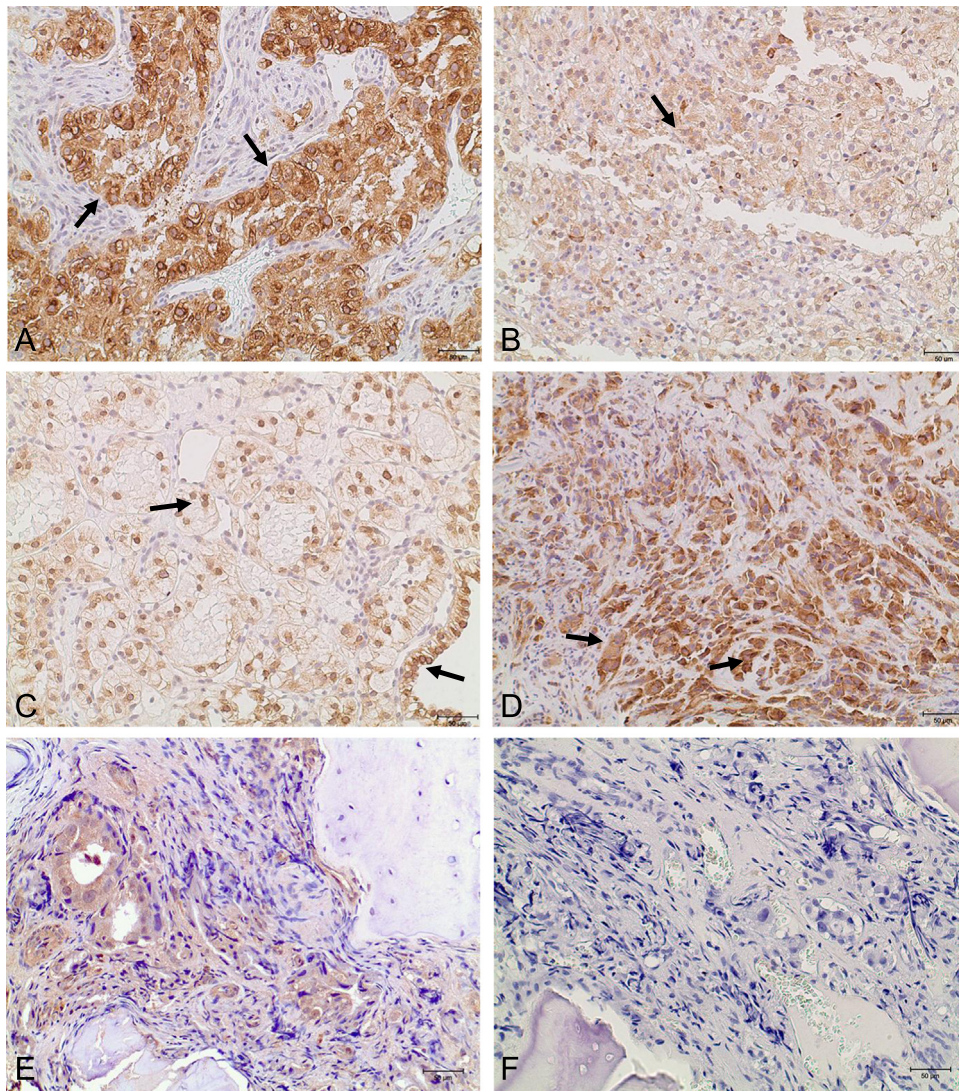
Fig. 3. *Differential IMP3 expression in renal cancer vs. prostate cancer bone metastasis.* IHC was performed on retrieval tissues obtained from patients with metastatic RCC in the iliac (A), sacrum (B), femur (C) and lumbar vertebra (D), and representative micrographs obtained at 200x are shown to illustrate the robust staining (brown) versus retrieval tissue from a patient with metastatic prostate cancer in spine immunostained for IMP3 (E) and treated with the secondary antibody only (negative control, F). Arrows indicated the strong IMP3 staining in the RCC bone metastases

assessing IMP3 expression in patients with bone metastatic disease from RCC.

2.2. CT protocol and CT data analysis

CT scanning was performed using a 16-MDCT (Siemens Somatom Sensation 16, Germany) within 2 weeks prior to surgical treatment. All images were acquired using a standardized renal mass protocol that did not change during the study. The CT (120.0 kV, 179.0 mA, W180/L40, DFOV 38.6 × 38.6 cm) protocol used for non-contrast images by an 8-mm scanning span per rotation and 5-mm intervals to cover the area of both sides of the kidneys from lower pole to diaphragm. After the acquisition of non-contrast images, arterial phase (40 s), nephrogenic phase (90 s), portal phase (120 s) and excretory phase (180 s) images were acquired, respectively, after the intravenous injection of contrast. Nonionic Iohexol (1.5 ml/kg, concentration of 300 mg/ml)-injection was employed using power injector (3.0 ml/s) via intermedian cubital vein for enhancement. DSA was performed following selective renal artery catheterization using 10–15 ml Iohexol (300 mg/ml) @ (5–7 ml/s). Images were routinely obtained

in an anterior–posterior projection for both early arterial phase and late arterial phase. (Fig. 1).

Regions of interest (ROI) for transverses CT images were manually drawn around the margins of the entire renal mass on multiple slices in the arterial/corticomedullary phase that excluded fat and gas areas [18,19] to measure Hounsfield attenuation values: 1) contrast enhancement within the tumor, 2) non-enhanced areas within the tumor, 3) the contralateral normal kidney; and 4) the abdominal aorta. The degree of tumor enhancement during the arterial/corticomedullary phase was calculated from an appropriately selected similar-sized region of interest (ROI): Tumor enhancement in Houndsfield Units (HU)= arterial/corticomedullary phase HU–baseline HU (pre-contrast portion of scan). Based on the enhancement data from Dr. Raman in Johns Hopkins University and others [18–21], the degree of tumor contrast enhancement was grouped as: Low < 20 HU; Intermediate 20–40 HU; and High > 40 HU. To assess tumor vascularity from the CT data, we performed volumetric rendering studies with Amira® software (Amira® V5.4.0, Visualization Sciences Group, Burlington MA). Once the kidney and tumor volumes were generated, the Amira® software was used to define the vascular and non-vascular components of the tumor by

Table 1
Clinical characteristics of the patient population who were included in the study.

	Dynamic CT enhancement scanning
Patient	72
Male	38(52.8%)
Female	34(47.2%)
Age (year)	53.1 ± 14.7
20–40	11(15.2%)
41–60	36(50.0%)
> 60	25(34.7%)
Clinical manifestations	
Hematuria	35(48.6%)
Pain	26(36.1%)
Abdomen mass	7(9.7%)
Renal palpable mass	5(6.9%)
No clinical symptoms at 1st visit	12(16.7%)
Surgery finding	
Right kidney	33(45.8%)
Left kidney	39(54.2%)
Single lesion	70(97.2%)
Upper pole	33(45.8%)
Pathological classification [30]	
Clear cell RCC	55(76.4%)
Multilocular clear cell renal cell carcinoma	5(6.9%)
Spindle cell carcinoma	5(6.9%)
Renal cell carcinoma, unclassified	4(5.6%)
Papillary transitional cell	3(4.2%)

contouring the 5 mm slices. Fig. 2 A–C shows representative examples of tumors determined to have low, intermediate and high enhancement as determined by this method.

2.3. Histology and immunohistochemistry

Hematoxylin and eosin (H&E) stained histology was performed on demineralized paraffin embedded tissue as previously described [22], and IHC was performed on parallel sections using a mouse anti-human IMP3 primary antibody (clone 69.1, Dako, Carpinteria, CA, USA), and a vectastain biotinylated universal secondary antibody (Dako, Carpinteria, CA, USA). Fig. 2 D–F illustrates representative tumors with Low, Intermediate and High IMP3 expression determined by this IHC method, with blinded semi-quantitative analysis as previously described [23]. All histological images were obtained using Axioskop 40 microscopy (Carl Zeiss AG, Goettingen, Germany) and Spot TR3^{TM} image system (V 4.5.9.9, Spot Image Solutions Inc., Michigan).

2.4. Cell culture, microarray and real time RT-PCR

786-O cells obtained from Dr. G. Wu [24] and PC3 cells from Dr. J. Lieberman were cultured in 37 °C, 5%CO$_2$ as described previous [13]. Total RNA was isolated using RNeasy mini purification kit (Qiagen 74104). Microarry was performed using Affymetrix GeneChip Human Genome U133 Plus 2.0 Array (Chip Lot #LE23BK05) (Supplementary Table 1). Real time RT-PCR was performed using Bio-Rad iScript TM cDNA synthesis Kit (Bio-Rad170-8842), and iQ^{TM} SYBR$^{®}$ Green supermix, and ΔCt was normalized to *gapdh*.

2.5. Statistical analysis

All statistical analysis was performed using Prism statistical package Version 4.0 (GraphPad, San Diego, CA) with *p* Values < 0.05 being considered statistically significant. A one-way analysis of variance (ANOVA) or Newman–Keuls multiple comparison test was utilized for volumetric enhancement data, PCR gene expression and microchip gene array expression analysis. A Fischer's exact test was used to determine statistical significance for the semiquatitative analysis of IHC for IMP3 and its correlation to tumor vascularity based on CT. Using the raw data, average and standard deviation HUs were calculated for the weak, intermediate and strong IMP3 expression levels. Students *T*-test was performed comparing means between the three groups as well for this data. Statistical significance is indicated in the artwork as well as captions of all the Figures.

3. Results

We performed immunohistochemistry for IMP3 on RCC and PC retrieval tissues from patients with bone metastases (Fig. 3). All of the 11 RCC bone metastases samples tested were highly positive (+ + +) for IMP3, while the non-neoplastic cells within the tumor parenchyma did not showed immunostaining for IMP3 (Fig. 3A–D). In comparison, the 3 PC bone metastases samples all had low (+) levels of IMP3 immunostaining (Fig. 3E).

To directly evaluate the IMP3 as a biomarker of RCC vascularity, we completed a retrospective analysis of 72 patients from ZMU who underwent contrast enhanced CT preoperatively. Table 1 provides a summary of patient characteristics for these 72 patients, including clinical manifestations and pathologic classification. As expected, the most common histologic type of RCC was clear cell (76.4%) and 97.2% were a single lesion. The 33 patients included in the IHC analysis had similar characteristics to the 72 patients as a whole. Fig. 1A and B and 1C–E shows images from a representative patient with low contrast uptake and high contrast uptake on CT respectively and subsequent DSA confirming the presence of large tumor feeding vessels in the high contrast uptake tumors, which were not seen in the low contrast uptake tumors. In order to assess the relationships between RCC primary tumor radiological characteristics and molecular markers, we first quantified the CT data to determine the volumes of the kidney, tumor and vascular vs. non-vascular regions of the tumor (Fig. 4). Interestingly, we found that the tumors with Intermediate enhancement (20–40 HU) were 2-fold larger than Low and High enhancing tumors, and that this increase in tumor size was significant in both vascular and non-vascular regions (Fig. 4B). Moreover, the volume of the Intermediate enhancing RCC accounted for ~70% of the kidney volume, while the Low and High enhancing tumors accounted for only ~40% and ~30% of kidney volume respectively (Fig. 4C). Further group analysis revealed that the High enhancing tumors were ~90% vascular, which was significantly greater than the ~70% vascularity observed in Intermediate tumors, which was significantly greater than the ~30% vascularity of the Low enhancement RCC (Fig. 4C). However, individual tumor volume did not correlate with tumor vascularity (data not shown). Finally, we directly compared the vascularity of the primary RCC tumors determined by CT to their IMP3 expression (Fig. 3D). The results demonstrated a significant correlation between HU and IMP3 immunostaining of the tumors.

4. Discussion

Metastatic RCC is a fatal disease with survival often measured in months, rather than years. Surgery alone is the standard of care for patients who present with isolated renal disease at the time of diagnosis. However, approximately 1/3 of these patients will go on to develop clinically detectable metastatic disease, which is then treated with systemic therapies that have been shown to prolong
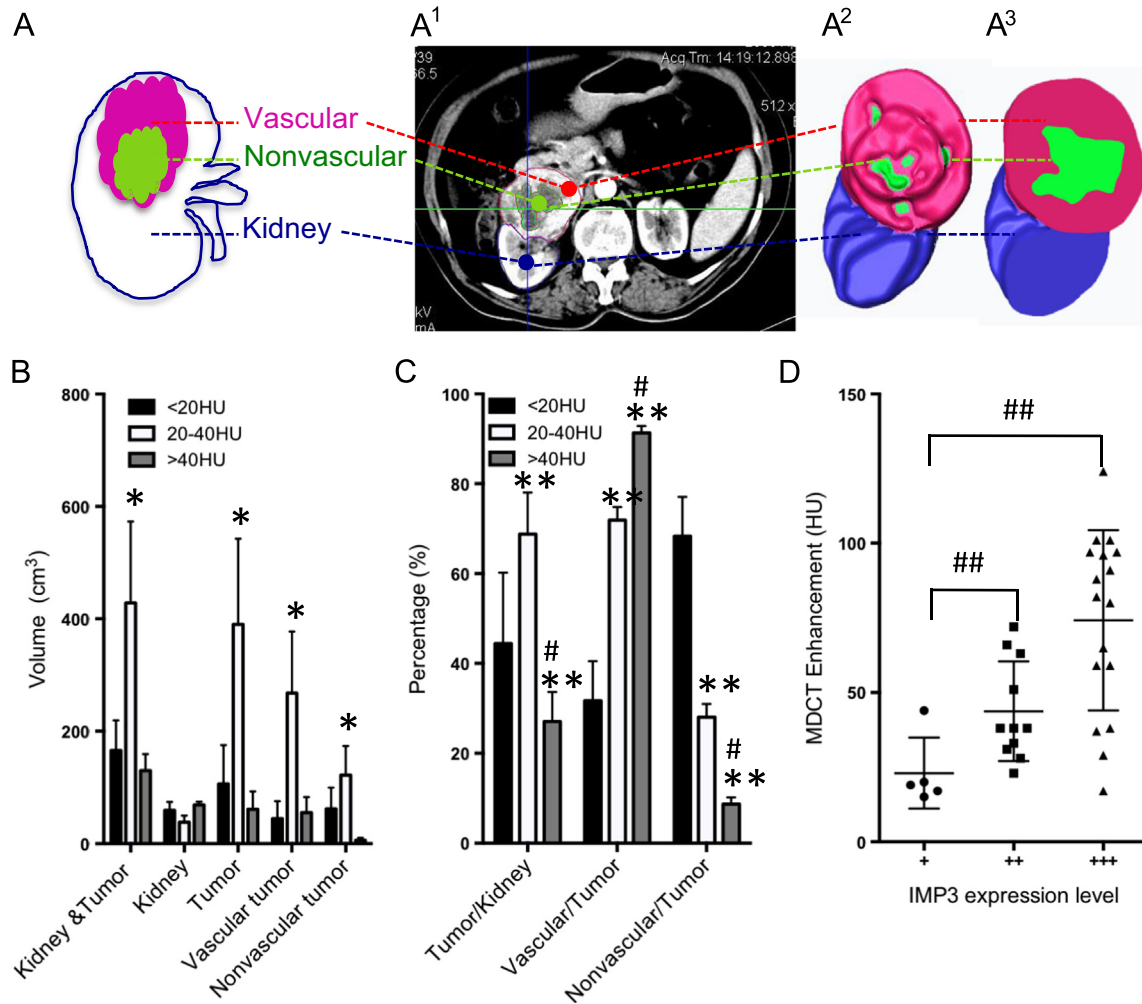
Fig. 4. *Use of volumetric contrast-enhance CT to correlate primary RCC vascularity with IMP3 expression in the tumor.* Amira 3-D visualization software was employed to quantify primary RCC tumor lesion volume within the kidney based on the anatomic structure of the contrast-enhanced CT attenuation obtained from the clinical radiology. (A) A cartoon illustration of the manual contouring of the tumor mass (pink) from the residue normal kidney (blue) tissue and non-enhanced tissue (green) within tumor is shown it illustrate the 2-D area measurements derived from the original CT section (A^1). These 2-D measurements were then reconstructed in 3-D to derive volumes (A^2), and were also used to visualize a sagittal section (A^3). Using these manipulations of the CT, the kidney, tumor and vascular/nonvascular tumor volumes were determined (B). Of note is that neither tumor size nor vascular tumor volume corresponded to gross lesion enhancement attenuation, as defined by Low (black bars), Intermediate (white bars) and High (gray bars) HU described in Fig. 2. However, as predicted, the vascular:tumor ratio significantly corresponded with the gross lesion enhancement attenuation (C). Direct semiquantitative assessment of tumor vascularity (CT enhancement) vs. IMP3 expression (IHC) as described in Fig. 2 was performed on 33 tumors in this study (D), which demonstrated a significant correlation (* $p < 0.05$ vs. Low; ** $p < 0.001$ vs. Low; # $p < 0.05$ vs. Intermediate; ## $p < 0.001$ vs. +)

survival by several months [25]. However, if identification of the subset of patients who are at high risk for developing metastatic disease could accurately be determined, systemic therapy could be initiated at the time of diagnosis with the goal of targeting the clinically undetectable micro-metastatic disease. Ultimately, as targeted systemic therapies continue to improve, this could enhance long-term overall survival in this population of patients.

To the end of identifying novel molecular targets to diagnose and treat RCC, our microarray studies identified IMP3 (Supplementary Table 1). IMP3 is a cytosolic protein that binds mRNA, and targets specific transcripts for processing within the cell. Although first identified as having high binding affinity for *igf2* mRNA, IMP3 also binds to many different mRNAs within the cell, and thereby influences gene expression, including genes involved in cellular migration and proliferation [16]. Interestingly, IMP3 is generally not expressed in normal adult tissues, but has been found to be present in several malignancies to varying degrees including RCC [16,26]. Thus, it has potential as a molecular diagnostic and targeted therapy if its role is determined to be essential for cancer progression. Consistent with this thinking, several prior studies

have shown that IMP3 expression is correlated with a poorer prognosis in patients with RCC [14,26]. Therefore, we aimed to assess the relationship between IMP expression, RCC metastatic potential and RCC vascularity as determined by contrast enhanced CT.

RCC is generally considered to be a vascular tumor both at the primary site and at the sites of metastasis, and its vascularity has been strongly implicated in the tumor's pathogenesis and invasive potential. Based on our initial investigations using microarray analysis and review of the current literature, we found that the IMP family of proteins may be implicated in RCC tumor virulence, and possibly vascular growth and development within the tumor. Further analysis by real-time RT-PCR (Supplementary Fig. 1) confirmed that IMP3 is highly expressed in RCC, and prompted further study of this protein and its role in the vascularity and metastatic potential of RCC.

Several studies have been performed by other groups that indicate the possibility of discriminating renal cell carcinoma subtypes using texture analysis or single phase corticomedullary contrast-enhanced CT [18–21]. These studies also found that CT

contrast enhancement was a valid method of representing tumor vascularity in either hetero- or homogeneously contrast-enhanced lesions. In order to directly assess the relationship between IMP3 expression in primary RCC and tumor vascularity, we first had to develop a quantitative outcome measure of tumor vascularity in vivo (Figs. 2 and 4A). Although several approaches to quantify tumor vascularity have been described, a consensus on the best method has not emerged [27]. As we were restricted to protocols that utilize clinical CT scans, we chose a method similar to that which we have described for other clinical conditions [28,29], but acknowledge that this approach still requires formal prospective studies for validation. Nevertheless, we find our contrast CT approach to be safe, feasible and readily translatable if future studies can validate its sensitivity and specificity.

One surprising result from our CT study was the lack of correlation between primary RCC tumor size and its vascularity. While the Low contrast enhancing tumors were 2-fold smaller than the Intermediate enhancing tumors as expected, the High contrast enhancing tumors were also significantly smaller than the Intermediate tumors (Fig. 4). Holding to the theory that RCC tumor vascularity is associated with virulence, our interpretation of this finding is that High contrast enhancing primary RCC tumors greater than 200 cm^3 are incompatible with human life, and thus none were observed in our study. However, consistent with the central hypothesis of this study, we found a strong association between primary RCC vascularity as measured by CT and IMP3 expression (Fig. 4D). Furthermore, IHC assessment of tissue from 11 patients with confirmed metastatic RCC to bone revealed that all of the metastatic tumors had strong IMP3 staining (Fig. 3). In contrast, we did not see strong IHC staining in poorly vascular metastatic bone-prostate cancer biopsies. Although our conclusions are limited by the small sample size, these findings suggest that IMP3 may play a critical role in tumor vascular development and potential to spread.

In summary, IMP3 is a protein that warrants further study to assess its role in the pathogenesis of metastatic RCC. Based on our findings, it may play a role in the vascular development of the tumor. We confirmed that contrast CT is a useful modality for detecting tumors that will have high IMP3 staining and can therefore be used as a relatively inexpensive and rapid method for assessing both vascularity and probability of high IMP3 expression. Moving forward, it will be useful to determine if CT and IMP3 can accurately predict risk of metastatic disease to other organs and to the bone in patients with primary RCC.

Acknowledgments

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jbo.2015.07.001.

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