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Race Disparities in Peptide Profiles of North American and Kenyan Wilms Tumor Specimens

Jaime M Libes, MD¹, Erin H Seeley, PhD², Ming Li, PhD³, Jason R Axt, MD⁴, Janene Pierce, MS⁴, Hernan Correa, MD⁵, Mark Newton, MD^{6,7}, Erik Hansen, MD^{4,7}, Audra Judd², Hayes McDonald, PhD¹¹, Richard M Caprioli, PhD², Arlene Naranjo, PhD¹², Vicki Huff, PhD¹², James A O'Neill Jr, MD, FACS^{4,7}, Harold N Lovvorn III, MD, FACS⁴, and on behalf of the Kenyan Wilms Tumor Consortium

¹Division of Pediatric Hematology and Oncology, Vanderbilt University School of Medicine

²Mass Spectrometry Research Center Tissue Core, Department of Biochemistry, Vanderbilt University School of Medicine

³Department of Biostatistics, Vanderbilt University School of Medicine

⁴Department of Pediatric Surgery, Vanderbilt University School of Medicine

⁵Department of Pathology, Vanderbilt University School of Medicine

⁶Division of Pediatric Anesthesiology, Vanderbilt University School of Medicine

⁷Kijabe Mission Hospital (Kenya)

¹¹Mass Spectrometry Research Center Proteomics, Department of Biochemistry, Vanderbilt University School of Medicine

¹²Children's Oncology Group Statistics and Data Center, Department of Biostatistics, University of Florida

Abstract

Background—Wilms tumor (WT) is the most common childhood kidney cancer worldwide and arises in children of black African ancestry with greater frequency and severity than other race groups. A biologic basis for this pediatric cancer disparity has not been previously determined. We hypothesized that unique molecular fingerprints might underlie the variable incidence and distinct disease characteristics of WT observed between race groups.

Study Design—To evaluate molecular disparities between WTs of different race groups, the Children's Oncology Group provided 80 favorable histology specimens divided evenly between black and white patients and matched for disease characteristics. As a surrogate of sub-Saharan African patients, we also analyzed 18 Kenyan WT specimens. Tissues were probed for peptide

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Correspondence address: Harold N. Lovvorn, III, MD, The Monroe Carell, Jr. Children's Hospital at Vanderbilt, Vanderbilt University School of Medicine, Department of Pediatric Surgery, 2200 Children's Way, Doctor's Office Tower, Suite 7102, Nashville, TN 37232-9780, harold.lovvorn@vanderbilt.edu, Phone: 615-936-1050, Facsimile: 615-936-1046. The members of the Kenyan Wilms Tumor Consortium are listed in the Appendix.

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profiles using MALDI-TOF imaging mass spectrometry. To control for histologic variability within and between specimens, cellular regions were analyzed separately as triphasic (containing blastema, epithelia, and stroma), blastema-only, and stroma-only. Data were queried using ClinProTools and statistically analyzed.

Results—Peptide profiles, detected in triphasic WT regions, recognized race with good accuracy, which increased for blastema- or stroma-only regions. Peptide profiles from North American WTs differed between black and white race groups but were far more similar in composition than Kenyan specimens. Individual peptides were identified that also associated with WT patient and disease characteristics (eg, treatment failure and stage). Statistically significant peptide fragments were used to sequence proteins, revealing specific cellular signaling pathways and candidate drug targets.

Conclusions—WT specimens arising among different race groups show unique molecular fingerprints that could explain disparate incidences and biological behavior and that could reveal novel therapeutic targets.

INTRODUCTION

Wilms tumor (WT) is the most common childhood kidney cancer worldwide, and its incidence varies according to race.(1–5) When comparing North American race groups, black children of sub-Saharan African ancestry develop WT at the highest rate (11 cases per million children under 15 years of age); white children show an intermediate rate (8.5 cases per million under 15 years); and children of Asian descent show the lowest rate (3 cases per million under 15 years).(4, 6, 7) Moreover, in the seminal analysis of the Greater Delaware Valley Pediatric Tumor Registry (i.e., the region surrounding Philadelphia, PA), black children not only developed WT at a nearly two-fold greater rate than whites, but also showed a higher frequency of congenital anomalies known to cluster with WT, which since have been linked to specific genetic mutations (e.g., loss of heterozygosity at *WT1* and loss of imprinting at *H19-IGF2* or *WT2*).(8, 9) This cancer predisposition appears to follow black children through generations regardless of geographical immigration from sub-Saharan Africa.(10) Importantly, WT continues to burden black children born and living in developing or low income African countries where resources are often limited to treat this disease effectively, abandonment of care is common, and therapy does not follow standardized protocols.(11–15) For example, WT is the third most commonly diagnosed childhood cancer in Kenya, behind only lymphoma and leukemia, and where, alarmingly, two-year survival currently approaches 50% at best.(11) In contrast, owing to treatment optimization strategies over the past four decades, WT survival in North America and other developed nations now exceeds 90% at five years.(16) Taken together, these observations suggest that black children may indeed harbor a heritable molecular predisposition to develop WT. Despite consistent epidemiologic documentation of this childhood cancer disparity both in North America and globally, little has been published regarding a potential biologic basis to explain the different incidence rates according to race and ancestry.

Although race disparities in survival from WT previously existed in the U.S., as black children typically fared worse, current outcomes comparing blacks to whites now have approximated one another.(4, 5, 17, 18) Nevertheless, the disparity in incidence remains, suggesting a biological predisposition. Societal and socioeconomic barriers likely explain in part the once disparate outcomes between black and white WT patients, yet genetic and molecular events more probably influence the recognized, increased risk among black children to develop this potentially lethal childhood cancer.

The Children's Oncology Group (COG) 2013 Blueprint for Research of Renal Tumors calls for investigators to identify new targets and strategies in WT therapy to close the persistent

survival gap, to reduce late toxic side effects for survivors, and to optimize therapy for children having treatment-resistant favorable histology (FH) disease.(19) One current focus in translational cancer biology is to personalize therapy for individual patients having unique genetic mutations and potential pharmaco-genetic variations in responsiveness to established standard therapies. Clues to personalize therapy for any cancer, and specifically WT, may well originate with race group status, if core biological differences are found to exist.

The purpose of this study was to explore at the molecular level the well-documented disparity in WT incidence that separates along race group status but for which a biologic explanation remains unknown. In a previous pilot study seeking to explain potential biological differences in WT between race groups, we observed unique peptide profiles that may discriminate specimens according to race, but the power of that study was too low to draw substantial conclusions or to identify specific mechanistic pathways.(20) These current studies were designed to build on those preliminary findings and to test the hypotheses that WT specimens express a molecular fingerprint specific to race and that these profiles might reveal candidate targets as an initial step to personalize therapy.

METHODS

North American Wilms tumor (NAWT) specimens

To compare peptide composition between black and white WT patients residing in North America, the COG provided 80 favorable histology (FH) specimens (i.e., no anaplasia) divided evenly between either race group (i.e., n=40), and relatively evenly between treatment success (n=42) and failure (n=38; Table 1). The COG matched these FH WT specimens for age, gender, stage, and loss of heterozygosity (LOH) at 1p and 16q, which, when found in combination, portend a significantly worse outcome, increase risk stratification, and call for intensified therapy. Because disease-relapse numerically plagues far more children having FH WT, nearly half of whom will die from refractory treatment resistance, we chose to evaluate only FH WT.(21)

Kenyan Wilms tumor (KWT) specimens

To evaluate peptide profiles in WT specimens originating from a genetically more isolated and uniform sub-Saharan black population, we acquired tissue samples from 101 WT patients who had been treated from 2008 to the present at four collaborating institutions in Kenya (Kenyatta National Hospital of the University of Nairobi, Moi University Teaching and Referral Hospital, Kijabe Mission Hospital, and Tenwek Mission Hospital).(11) To ensure proper fixation and suitability for imaging mass spectrometry, specimens were re-embedded in paraffin as indicated, sectioned, and reviewed for histologic quality assurance (QA) after hematoxylin and eosin (H&E) staining. KWT specimens were chosen for tissue viability, and those showing diffuse necrosis, apoptosis or treatment effect were excluded. Eighteen of the KWT specimens (15 favorable and 3 unfavorable histology) met these QA criteria and were analyzed using imaging mass spectrometry.

Imaging Mass Spectrometry (IMS)

To identify unique biological features of FH WT that separate according to race group, we compared the proteome between black and white races of NAWT and KWT patients. Using MALDI-TOF-IMS as previously described,(20) we analyzed a single tissue section from each specimen block of the 40 black NAWT, the 40 white NAWT, and the 18 KWT specimens. Three 6 µm serial sections were cut from each specimen block, and the middle section was H&E stained. Photomicrographs of these 98 H&E slides were marked with multiple 300µm diameter circles on histologic regions of interest (Mirax Desk, 3D Histech, Budapest, Hungary).(20) To control for peptide profiles varying according to the different

WT cellular compartments, histologic regions were labeled separately as “triphasic”, “blastema”, and “stroma”. Next, corresponding tissue sections for IMS analysis were deparaffinized and subjected to heatinduced antigen retrieval, and the annotated H&E stained images were digitally registered to the unstained sections. Coordinates of the histological annotations were transferred to an acoustic robotic microspotter (Portrait 630, Labcyte, Inc., Sunnyvale, CA) for deposition of trypsin (0.076 µg/µl in 100 mM ammonium bicarbonate, pH 7.6 containing 9% acetonitrile) and α-cyano-4-hydroxycinnamic acid matrix (10mg/ml in 50:49.9:0.1 acetonitrile:water:trifluoroacetic acid) onto corresponding unstained serial sections. A total of 40 trypsin droplets and 72 matrix droplets (~160 pL each) were deposited at each location. Spectra were collected using a Bruker AutoFlex Speed mass spectrometer equipped with a SmartBeam™ laser (Bruker Daltonics, Billerica, MA). Each spectrum was the sum of 1600 laser shots collected throughout the entire area of each matrix spot. The data were preprocessed (baseline subtraction, noise level estimation, and alignment to common peaks). Peak picking was performed manually to assure proper peak boundaries and selection of only the *monoisotopic* peak. Unsupervised clustering and principal component analysis were performed to classify peptide spectra in NAWT and KWT. To control for differences in peptide spectra between histologic compartments, comparisons were made between specific cell types only (e.g., blastema versus blastema). All experiments were completed in duplicate.

Protein sequencing

To identify and sequence proteins from the statistically significant peptide profiles (having a $p < 0.05$ for each comparison), additional tryptic digestions were carried out on blastema and stroma regions from two different NAWT specimens. Polyacrylamide hydrogels (~1.5 mm diameter), swelled with a buffered trypsin solution, were placed onto the tissue sections containing cellular regions of interest and incubated in a humidity chamber for 5 hours at 50°C. The hydrogels were removed from the tissue, and the digested peptides were extracted using acetonitrile. Extracts were dried in a speedvac and reconstituted in 0.1% formic acid and analyzed by a 70-minute data dependent LC-MS/MS analysis. Briefly, peptides were autosampled (NanoAcquity – Waters, Milford, MA) on a 200 mm by 0.1 mm (Jupiter 3 micron, 300A) self-packed analytical column coupled directly to an LTQ Ion Trap (ThermoFisher, Waltham, MA) using a nanoelectrospray source and resolved using an aqueous to organic gradient. A series of a full scan mass spectrum, followed by 5 data-dependent tandem mass spectra (MS/MS), was collected throughout the run, and dynamic exclusion was enabled to minimize acquisition of redundant spectra. MS/MS spectra were searched via SEQUEST against a human database (UniprotKB – reference proteome set) that also contained reversed version for each of the entries (<http://www.ncbi.nlm.nih.gov/pubmed/7741214>). Identifications were filtered and collated at the protein level using Scaffold (Proteome Software).

Protein validation

To validate the statistically significant peptides and other proteins of interest sequenced from the MALDI-TOF-IMS analyses, real-time PCR was performed on an independent set of snap frozen WT and adjacent kidney specimens that had been archived previously in our laboratory tissue repository. Total RNA was isolated from snap frozen tissue of 20 consecutive treatment-naïve WTs and 12 adjacent kidney specimens using RNeasy (Qiagen, Valencia, CA). 3µg of RNA were reverse transcribed using Superscript II (Life Technologies) and oligo(dT) primers (Applied Biosystems). Quantitative real-time PCR was performed using iQ SYBR Green Master Mix (Bio-Rad, Hercules, CA) on a Bio-Rad iCycler. The cellular biology of ipsilateral kidney tissue arising adjacent to a WT may not be truly normal, due to cancer field effects and/or to physical compression and relative ischemia from the often-massive

size of these lesions. Real-time PCR results for each gene of interest were normalized to the mRNA content of the housekeeping gene, *GAPDH*, for all conditions. Primer sequences for the specific proteins of interest validated are shown in Table 2.

Statistics

The MALDI-TOF-IMS data were first visualized and preprocessed (baseline subtraction, noise level estimation, and alignment to common peaks) using ClinProTools software (Buker Daltonics, Billerica, MA). Peak picking was performed manually to assure proper peak boundaries and selection of *only* the monoisotopic peak, and integrated peak areas were used for further analysis. Unsupervised clustering and principal component analysis were performed to classify peptide spectra in NAWT and KWT. To control for differences in peptide spectra between histologic compartments, comparisons were made between specific cell compartments only (e.g., blastema versus blastema). The primary research question was to identify any potential peptide profiles that might differentiate race status between North American black, white, and Kenyan children having WT. Initial race group analyses were carried out in ClinProTools to generate classification algorithms that could differentiate the race groups. A support vector machine algorithm was built for each of the cellular compartments. In lieu of an independent validation set, a leave-20%-out cross validation was used over 10 iterations to estimate the classification accuracy of the model. These classification models consisted of 25 peaks for each of the stroma and the blastema, and 19 peaks for the triphasic histology. A univariate approach was adopted to select the top biomarker candidates, or the “winner” (i.e., the most statistically significant), for each patient and disease characteristic evaluated. To handle peptide abundance values, expressed as MS peak intensity with repeated measurements for each patient, a linear mixed effect model was fitted to estimate the racial effect, as well as to model the correlated data structure appropriately. P-values were obtained for each comparison based on the likelihood ratio test. Rate ratio was also generated from the ratio of expected values for two race groups. Given the exploratory nature of the current statistical analysis, we did not apply a false discovery, rate-controlling procedure to test the multiple hypotheses. Additionally, although the mean intensity of peptide expression was determined to be statistically different between comparison groups, further study is warranted to validate these findings.

RESULTS

Race disparities in peptide profiles of Wilms tumor

MALDI-TOF-IMS was able to detect unique peptide profiles, extracted from triphasic histologic regions (i.e., containing blastema, epithelia, and stroma), which could classify WT specimens as arising from black or white North American and from black Kenyan children with good recognition capability (Table 3). Recognition improved when comparisons were restricted to and controlled for specific WT cellular compartments (e.g., blastema or stroma). Specifically, to determine peptides that could distinguish race groups, a support vector machine algorithm was developed for each histologic region profiled. When examining triphasic regions, an algorithm consisting of 19 peptides was able to classify the races in a leave-20%-out cross validation achieving an overall accuracy of 70.9%. When blastema alone was examined, an accuracy of 75.5% was achieved using a classifier of 25 peptides. Within stroma, an accuracy of 81.3% was achieved also using a classifier of 25 peptides.

Applying an unsupervised principal component analysis, three clusters of peptides were able to differentiate the three race groups according to triphasic, blastema, and stroma histologies (Figure 1 A, C, E). Specific and paired peptide peaks were able further to separate WT specimens according to the three race groups analyzed (Figure 1 B, D, F). Because

recognition capability of peptides to differentiate race groups improved when analyses were restricted to specific WT cellular compartments, either blastema or stroma, we focused our remaining supervised analyses on these two histologies. When comparing the total number of statistically significant peptide profiles that differed between the three race groups, interestingly, WT specimens of the two North American race groups (i.e., black and white) were most similar in molecular composition for both the blastema and stroma compartments (Table 4). Unexpectedly, WT specimens arising from North American black and Kenyan children showed the most disparate peptide profiles, whereas the differences between white North American and Kenyan specimens also showed a strong yet intermediate separation independent of histology.

To sequence and identify proteins from these peptide signatures, we first needed to establish and compare the statistically significant peptides of each race group within either histology (Table 4). Unique peptide signatures were noted to specify a given race relative to the others for both blastema (Figure 2) and stroma (Figure 3).

Variations in peptide profiles among WT patient and disease characteristics

The COG 2013 Blueprint for Research of Renal Tumors calls for the identification of new targets that confer treatment resistance to FH WT. From this proteomic analysis, we identified peptide signatures arising in either cellular compartment (i.e., blastema or stroma) that associate with both treatment success and failure (Table 4; Figure 4). A number of peptide signatures also associated with each stage of disease, and those discriminating Stages I and IV are shown as an example (Figure 5). Distinct peptides from either histology were also noted to increase or decrease with age at diagnosis and to associate with gender (Figure 6).

Sequencing and identification of proteins from peptides of interest

To sequence and identify candidate protein targets from the peptide signatures associating with these specific patient and disease characteristics of WT, we selected two of the COG specimens having ample blastema and stroma for hydrogel trypsin digestion and LC-MS/MS. Although the sequenced proteins from many of the statistically significant peptide signatures appeared novel and intriguing to pursue as biologic markers and candidate drug targets, we chose to validate, as proof of concept, gene transcription of several sequenced proteins in an independent set of WT (n=20) first, that distinguished race and treatment groups and second, that have documented and unique roles in WT pathogenesis (Table 5). For example, KIF2A (kinesin heavy chain member 2-A; m/z signature 1103), which most distinguished black from Kenyan WT specimens ($p=0.00001$), and PPIA (peptidyl-prolyl cis-trans isomerase A; m/z signature 1055), which most distinguished black from white WT ($p=0.0059$), were significantly upregulated in WT relative to adjacent kidney specimens (Figure 7). Exemplifying proteins of interest in WT biology that were sequenced from these peptide signatures, further validating the specificity of our approach, TOP2A (DNA topoisomerase 2- α), a marker of poor prognosis WT and a direct target of etoposide (a stalwart agent in the treatment of disease-relapse), was identified in the blastema compartment.(22, 23) Several components of the retinoic acid synthesis and signaling pathway were also sequenced from our list of peptides, including ALDH1A2 (retinal dehydrogenase 2, formerly RALDH2), RBP1 (retinol binding protein-1), and CRABP2 (cellular retinoic acid binding protein-2), although we attempted to validate only the former as upregulated in WT relative to adjacent kidneys. Of additional interest, HDGF (hepatoma-derived growth factor) was also sequenced from the list of peptide signatures, the gene locus for which is located at 1q22–23, as is *CRABP2*, a region that if a gain in copy number occurs in FH WT, both event-free and overall survival are reduced.(24, 25) Interestingly, *CRABP2*

and *HDGF* have been previously shown to be upregulated at the mRNA level in WT blastema relative to embryonic kidneys.

DISCUSSION

To our knowledge, this study applying innovative proteomic methodologies is the first to investigate and identify peptide profiles, which discriminate WT specimens arising from different race groups of both North America and Kenya and from which the parent proteins were sequenced. Despite the long-known predisposition of black African-Americans and sub-Saharan Africans to develop WT, the search for a molecular etiology of this disparity seemingly has been neglected, given the absence of published reports in the literature to date. To initiate discovery of the biological basis for the disparate incidences of WT between race groups, we previously conducted two pilot analyses of WT specimens originating from Tennessean and Kenyan patients, and those prior efforts suggested WT peptide profiles might indeed differ according to race group.(5, 20) In the current studies, we aimed to build on those concepts and to examine, using MALDI-TOF-IMS and subsequent LC-MS/MS, a much larger cohort of WT specimens for peptide signatures that could lead to the discovery of new race-specific targets, while incorporating molecular fingerprints of other patient and disease characteristics, as an initial means to personalize WT therapy.

As expected from these current studies, peptide profiles of WT specimens from North American and Kenyan patients differed significantly in composition. However, unexpectedly, the signatures arising from North American black children differed more from the Kenyan WTs than did those of white patients. Interestingly, among all race group comparisons, NAWT specimens of black and white patients were most similar in peptide make-up, which was true for both blastema and stroma compartments. The capability of the peptide signatures to recognize WT specimens originating from disparate race groups was overall good and improved when controlling for cellular compartment (i.e., blastema or stroma). Principal component analysis showed that both clustered and paired peptides could reliably distinguish WT specimens of different race groups. Moreover, using a univariate approach, statistically significant peptides for each race group comparison could be determined and from which the parent protein could be sequenced and identified with high certainty (>99%). This combined proteomic approach, to identify relevant peptide spectra and then to sequence the actual proteins, revealed candidate targets that can be easily validated (e.g., KIF2A, PPIA, components of the retinoic acid synthesis pathway, and TOP2A). In an effort to lay the groundwork to personalize WT therapy in the future, race-specific protein targets may well provide the necessary initial clues for the development of new target-specific agents. Because WTs are often histologically heterogeneous (i.e., vary in content of blastema, epithelia, and stroma), we were able to identify discriminating peptides and clustered signatures from both blastemal and stromal cell compartments that were independent of one another, further validating this approach. This strategy might be applicable to other disparate cancers of children and adults to discover race-specific biologic processes and novel therapeutic targets.

Because of the high incidence of this childhood cancer in sub-Saharan African nations, where WT is gravely lethal, Kenyan WT specimens were included in this analysis to identify candidate targets as a surrogate of these at-risk black populations for the discovery of new and more efficacious drugs. We had postulated that these KWTs would harbor unique peptide signatures of black African ancestry and treatment resistant, advanced stage disease. Although black American WT peptide profiles were more distinct from Kenyan than were white American, the identification of unique targets in these KWT specimens (e.g., FXR1) from a resource-constrained nation may lend to future drug development specific for the biology of this and other genetically isolated and predisposed populations.

The COG 2013 Blueprint for Research of Renal Tumors urges investigators to identify molecular pathways that confer treatment resistance to favorable histology WT and from which new strategies can be devised for the development of more efficacious and less toxic drugs.(19) In this comprehensive study, we enriched our cohort to include 38 NAWT patients of either black or white race that had failed standard therapy (i.e., disease relapse or progression to death). Peptide profiles from either blastema or stroma histologies were indeed significantly different between WT specimens responding to or failing treatment, and specific peptides were observed to associate with one or the other outcome. Peptide signatures from either cellular compartment also were found to associate with increasing or decreasing stages of disease overall and specifically with individual stages. Finally, unique peptide signatures, detected in either blastema or stroma histology, were observed to correlate with age and gender. Taken together, whether analyzing blastema or stroma cell compartments, unique peptide signatures can be defined that associate with race, patient, and disease characteristics. Of note, we attempted to examine peptide profiles in WT specimens having loss of heterozygosity (LOH) at 1p and 16q, although the numbers of affected specimens (n=3) were too low to make statistically meaningful observations from this cohort.

Therapy decisions for WT patients currently depend on the COG risk stratification schema, which are determined by age of child, size of specimen, stage of disease, LOH at 1p and 16q, and histology (i.e., favorable or unfavorable).(26) Although this treatment algorithm offers reduction or intensification of therapies according to specific patient and disease characteristics, it does not truly personalize WT therapy according to tumor-specific biology and targets. Applying both MALDI-TOF-IMS and LC-MS/MS to identify proteins from the various WT features (i.e., race, treatment failure, stage of disease, age, gender, and LOH) is a sound initial strategy in the mission to begin to personalize therapy for these children.

Here, we used an innovative approach (i.e., trypsin impregnated hydrogel digest, followed by LC-MS/MS) to sequence and identify proteins from the various peptide signatures of both blastema and stroma cell compartments that associated with race and other WT characteristics. This methodology appears highly valid, as multiple WT-specific and novel proteins were sequenced from our analysis. As proof of concept, we chose to validate at the transcriptional level expression of several of these gene products. While we sequenced many statistically significant peptides from each analysis of patient and disease characteristics, the task to *validate* each protein in WT would be monumental, not only from a pure volume of work but also from the identification of numerous relatively new or understudied proteins, which landed as statistically significant peptides of, or had high correlation with, certain comparisons, and that would require significant resources and time to develop both qPCR conditions and antibodies.

Several limitations of this work are acknowledged. First, WTs are characteristically large, and yet we analyzed only a tiny area of each, which brings in to question whether the results from an individual WT tissue section are representative of the entire specimen. However, we believe that an adequate sample size of NAWT specimens (n=80) was analyzed and especially by controlling for specific cellular compartments. Second, when using specimens archived in a national tissue bank for high-throughput molecular discovery, the quality of fixation may vary between contributing institutions. Nevertheless, our approach overcomes this potential problem in molecular integrity of each specimen by choosing to analyze *proteins* that are more stable than mRNA (e.g., in contrast to using microarray gene expression analyses). Moreover, employing imaging guidance confers a high precision to the mass spectrometry analysis, as only viable regions of the various cell compartments are selected (i.e., WTs notoriously harbor large regions of necrosis), and cellular heterogeneity can be controlled for within and between WT specimens. Third, although members of our

investigative team reside in or have traveled to Kenya to abstract patient data from the four contributing institutions, the available paper medical records were often incomplete or missing, and disease-specific mortality and loss to follow up rates were high, which makes associating peptide signatures to outcome in this underserved group limited. Unfortunately, the number of viable KWT specimens (18%) that we were able to analyze with MALDI-TOF-IMS was only a fraction of the patient blocks we collected, as most affected Kenyan children are pretreated before resection. The KWT specimens we chose for analysis showed no microscopic evidence of treatment effect, and documentation of a child having received neoadjuvant therapy was not reliably verified from the medical record. Also, three of the 18 KWT showed features of unfavorable histology, although we believe the tight clustering within this group of specimens discounts introduction of “histologic” bias. Finally, although the innovative hydrogel tissue digestion and peptide extraction technique used in these studies enhanced the sequencing yield of proteins from the statistically significant peptides for each comparison, numerous m/z signatures were not identified applying this methodology. For those proteins successfully sequenced, it was further not feasible to validate each in our independent set of WT specimens. Although planned, we have yet to test the statistically significant proteins as functionally relevant to Wilms tumorigenesis or disease progression, or as mechanistically targetable, of which many appear intriguing for such future investigation.

CONCLUSIONS

Unique peptides and signature clusters can distinguish WT specimens according to race group and to patient- and disease-specific characteristics. From these peptide profiles, parent proteins can be sequenced and validated with excellent reliability, thereby revealing abundant candidate targets for the development of new drugs in the evolution of WT therapy. This innovative strategy, to explore the molecular basis of disparate WT incidences between race groups, advances our knowledge in the collective effort to begin to personalize therapy for these children, including those in resource-constrained regions of the world, such as Kenya, where little is understood about the population-specific biology of this otherwise treatable childhood kidney cancer. Lessons learned from the present studies might be applied as a strategy to gain greater knowledge about and to optimize outcomes from other disparate pediatric and adult cancers.

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Abbreviations

WT	Wilms tumor
NAWT	North American Wilms tumor
KWT	Kenyan Wilms tumor
FH	favorable histology
MALDI-TOF	matrix-assisted laser desorption ionization time of flight

IMS	imaging mass spectrometry
m/z	mass-to-charge ratio
LC-MS/MS	liquid chromatography mass spectrometry
COG	Children's Oncology Group
LOH	loss of heterozygosity

APPENDIX

Members of the Kenyan Wilms Tumor Consortium:

James N'Dungu, Oliver Oruko, Jessie Githanga, Fatmah Abdullah, Kenyatta National Hospital, University of Nairobi, Nairobi, Kenya; Joyce Musimbi, Festus Njuguna, Kirtika Patel, Moi University Teaching and Referral Hospital, Eldoret, Kenya; Gabriel Ellsworth, Michael Mwachiro, Russell White, Tenwek Mission Hospital, Kenya.

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Figure 1.

Principal component analyses show good recognition and separation of WT specimens according to race group status that is maintained in the different cellular compartments (triphasic: A, B; blastema: C, D; and stroma: E, F). Black NAWT specimens are shown in black, white (“Caucasian”) NAWT in cyan, and KWT in Kenyan copper. (A, C, E) The top three clusters of multiple peptide signatures (labeled PC1, PC2, and PC3) that recognize each race group are shown for each WT cellular compartment. (B, D, F) Pairs of peptides (m/z: mass to charge ratio) are also capable of recognizing the race group of origin of WT specimens.

Figure 2.

(A–F) Several of the statistically significant peptides, detected from blastemal compartments, for each race group comparison are shown. The fold change difference in peak intensity (vertical axes) for each peptide specifying a race group is provided, as are the p-values. All data are displayed using standard box plots. Each peptide signature represents a mass-to-charge ratio (m/z).

Figure 3.

(A–F) Several of the statistically significant peptides, detected from stromal compartments, for each race group comparison are shown. The fold change difference in peak intensity (vertical axes) for each peptide specifying a race group is provided, as are the p-values. All data are displayed using standard box plots. Note that the only common peptide between the blastema and stroma compartments is m/z 816, which appears to recognize black NAWT specimens most specifically. Each peptide signature represents a mass-to-charge ratio (m/z).

Figure 4.

Among NAWT specimens only, several of the specific peptides detected in blastema (A, B) or stroma (C, D) that associates with either treatment failure (A, C) or success (B, D) are shown. Fold change differences and p-values are provided. Each peptide signature represents a mass-to-charge ratio (m/z).

Figure 5.

Among NAWT specimens only, specific peptides, detected in blastema (A, B) or stroma (C, D), were observed to change with increasing or decreasing stages of disease. Because stage I and IV disease change COG risk stratification criteria, we focused our analysis to identify peptide peaks that associate with either end of the stage spectrum. Higher intensity of peptide detection in early stage (A, C) or stage IV (B, D) WT is shown (fold changes and p-values are displayed). Each peptide signature represents a mass-to-charge ratio (m/z).

Figure 6.

For either NAWT cellular compartment (blastema: A–D; stroma: E–H), specific peptides were observed to increase or decrease with age at disease presentation (A, B, E, F) and to associate with gender (female = pink; male = blue). Each peptide signature represents a mass-to-charge ratio (m/z).

Figure 7.

Validation of gene transcription of specific proteins, which either were sequenced from the most statistically significant peptides identified in a given race group comparison (A, B) or represent proteins of interest to WT biology (C, D), using an independent set of WT (n=20) and adjacent kidney (n=12) specimens. Comparing WT to adjacent kidney specimens: KIF2A $p=0.004$; PPIA $p=0.0004$; TOP2A $p<0.0001$; ALDH1A2 $p=0.078$.

Table 1

Descriptive Statistics for Each Wilms Tumor Patient and Disease Characteristic According to Race Group

Patient or disease characteristic	Black (n=40)	White (n=40)	p Value*	Kenyan (n=17) [†]
Age, mo, median range)	47.0 (25.5–61.2)	51.0 (34.8–63.5)	0.361 [‡]	38.0 (9–108)
Treatment failure, n (%)			0.654 [§]	4 LTFU
No	22 (55)	20 (50)		9 (69)
Yes	18 (45)	20 (50)		4 (31)
LOH, n (%)			0.499 [§]	
1p–/16q–	35 (88)	33 (82)		N/A
1p+/16q+	2 (5)	1 (2)		N/A
Sex, n (%)			0.648 [§]	
Female	23 (57)	25 (62)		9 (53)
Male	17 (42)	15 (38)		8 (47)
Stage, n (%)			0.455 [§]	
I	9 (22)	7 (18)		0 (0)
II	13 (32)	9 (22)		5 (29)
III	8 (20)	14 (35)		8 (47)
IV	10 (25)	10 (25)		4 (24)

* Statistical analysis of Children's Oncology Group Wilms tumor patients only to show equivalency of patient selection in each race group; Kenyan demographics shown for reference.

[†] The medical record for one of the 18 Kenyan patients did not contain these specific patient and disease characteristics; 3 Kenyan WT specimens showed features of anaplasia.

[‡] Wilcoxon.

[§] Pearson.

LOH, loss of heterozygosity; LTFU: lost to follow up; WT, Wilms tumor.

Table 2

Primer Sequences

Primer sequence	Anneal temperature
ALDH1A2 Forward 5'- TTGCTGATGCTGACTTGGAC-3' ALDH1A2 Reverse 5'- GCAGCACTGACCTTGATTGA-3'	56°C
KIF2A Forward 5'-GAACGGGAGGTCAAATGTGT-3' KIF2A Reverse 5'-CCGAGATGAATGTGCATTTG-3'	54°C
PPIA Forward 5'-TTCATCTGCACTGCCAAGAC-3' PPIA Reverse 5'-TTTCACTTTGCCAACACCA-3'	54°C
TOP2A Forward 5'- GATGGTTCCCATCAAAGG-3' TOP2A Reverse 5'- CAGAAAACGATGTCGCAGAA-3'	55°C

Table 3

Capability of the Classification Algorithm to Recognize Different Race Groups from the WT Peptide Profiles

Recognition capability	Triphasic, %	Blastema, %	Stroma, %
Overall	87.9	90.4	91.6
Black	90.4	93.7	95.9
White	84.3	88.2	91.8
Kenyan	88.9	89.3	87.2
Cross validation*			
Overall	70.9	75.5	81.3
Black	77.1	82.8	85.8
White	63.2	72.1	76.8
Kenyan	72.3	71.4	81.4

* The support vector machine algorithm used a leave-20%-out cross validation over 10 iterations to estimate the accuracy of the models.

Table 4

Number of Unique Peptides Achieving Statistically Significant Differences ($p < 0.05$) for Each Comparison Between Patient and Disease Characteristics

Patient or disease characteristic	Blastema (722 total peptides)	Stroma (778 total peptides)
Race, n (%)		
Black versus white	11 (1.5)	83 (10.7)
Black versus Kenyan	320 (44.3)	329 (42.3)
White versus Kenyan	216 (29.9)	149 (19.2)
North American versus Kenyan	307 (42.5)	296 (38.0)
Treatment, n (%)		
Failure	13 (1.8)	17 (2.2)
Success	11 (1.5)	5 (0.6)
Stage, n (%)		
Overall	50 (6.9)	41 (5.3)
I versus IV	7 (1.0)	11 (1.4)
Age, mo (%)	23 (3.2)	50 (6.4)
Sex, n (%)	33 (4.6)	26 (3.3)

The total number of *monoisotopic* peptide signatures identified from each histology is shown. The number of statistically significant peptide signatures that discriminate groups for each comparison is also shown, followed in brackets by the fraction of the total. The greater the number of peptides achieving a statistically significant difference between like groups suggests that these groups differ more regarding molecular composition. Note the greatest variability in peptide profiles was evident in the race group comparisons.

Table 5

Proteins Sequenced from the Most Statistically Significant Peptides for the Various Race Group Comparisons

Protein/gene, statistically significant	Comparison	Fold change	Identification probability, %	p Value	Signature (m/z)
<i>KIF2A</i>	Black vs Kenyan	1.31	99.7	0.00001	1103
<i>PPIA</i>	Black vs White	1.22	99.7	0.0059	1055
<i>YWHAQ (14-3-3)</i>	White vs Kenyan	2.21	61.5*	0.0002	816
<i>FXR1</i>	NAWT vs Kenyan	0.52	99.3	0.00006	1948
<i>EMILIN3</i>	Failure vs success	1.15	99.7	0.013	1140

* Lower identification probability reflects multiple isoforms of this protein.

KIF2A - kinesin heavy chain member 2-A; PPIA - peptidyl-prolyl cis-trans isomerase A; YWHAQ (14-3-3) - tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide; FXR1 - fragile X mental retardation, autosomal homolog 1; EMILIN3 - elastin microfibril interfacer 3.