Peptide spectra in Wilms tumor that associate with adverse outcomes

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Abstract

Background: The 2013 Children's Oncology Group (COG) blueprint for renal tumor research challenges investigators to develop new, risk-specific biological therapies for unfavorable histology and higher-risk Wilms tumor (WT) in an effort to close a persistent survival gap and to reduce treatment toxicities. As an initial response to this call from the COG, we used imaging mass spectrometry (IMS) to determine peptide profiles of WT associated with adverse outcomes.

Materials and Methods: We created a WT tissue microarray containing 2 mm punches of formalin-fixed, paraffin-embedded specimens archived from 48 sequentially treated WT patients at our institutions. IMS was performed to compare peptide spectra between three patient groups: unfavorable versus favorable histology, treatment success versus failure, and COG higher- versus lower-risk disease. Statistically significant peptide peaks differentiating groups were identified and incorporated into a predictive model using a Genetic algorithm.

Results: 131 peptide peaks were differentially expressed in unfavorable vs. favorable histology WT ($p < 0.05$). 203 peaks differentiated treatment failure from success ($p < 0.05$). 71 peaks differentiated COG higher-risk disease from the very-low, low, and standard risk groups ($p < 0.05$). These peaks were used to develop predictive models that could differentiate between patient groups 98.49%,

94.46%, and 98.55% of the time respectively. Spectral patterns were internally cross-validated using a leave-20%-out model.

Conclusions: Peptide spectra can discriminate adverse behavior of WT. Following future external validation and refinement, these models could be used to predict WT behavior and to stratify intensity of chemotherapy regimens. Furthermore, peptides discovered in the model could be sequenced to identify potential risk-specific drug targets.

Key Words: Wilms tumor, imaging mass spectrometry, proteomics, tissue microarray

1.1 Introduction

Wilms tumor (WT) is the most common childhood kidney cancer worldwide, with overall 5-year survival currently exceeding 90% in developed nations. Despite the remarkable successes in treatment achieved through large-scale cooperative trials conducted over the last 40 years, there remain patient populations who experience suboptimal outcomes. The most significant survival gap remains for patients whose tumors exhibit anaplasia (unfavorable histology), which is defined by nuclear gigantism, hyperchromasia, and bizarre multipolar mitoses.(1) Although anaplasia is found in only 6% of WT cases, it is associated with significant treatment resistance and accounts for 50% of WT deaths.(2) Therefore, the Children's Oncology Group (COG) 2013 Blueprint for renal tumors calls on the scientific community to identify biomarkers that associate with treatment resistance and also to seek novel risk-specific therapies.(3) The current COG risk-stratification algorithm, which has been extensively validated and refined by decades of cooperative trials, now stratifies patients according to high-risk molecular features including loss of heterozygosity (LOH) at the chromosomal loci 1p and 16q. These genetic alterations are accurate surrogates for aggressive biologic behavior and are useful to assign more or less intensive therapies, but currently are not themselves *targetable* moieties.(2, 4)

In an effort to eventually identify cell-specific, potentially targetable molecular signatures of high-risk disease, we established a WT tissue microarray (TMA) containing specimens from 48 sequentially treated WT patients at our respective institutions. We hypothesized that three patient groups with disparate outcomes would have unique underlying peptide signatures: unfavorable vs. favorable histology, treatment failure (death or disease relapse) vs. success, and COG higher-risk tumors vs. very-low, low, and standard-risk tumors. Tissues were analyzed using matrix absorption laser desorption ionization time of flight (MALDI/TOF) imaging mass spectrometry (IMS) to derive a peptide signature that differentiates samples grouped according to adverse or standard risk disease features.

1.2 Materials and Methods

1.2.1 Selection of WT patient groups for comparison

We hypothesized that three patient group comparisons could have underlying biologic differences associated with outcome discrepancies. The first comparison was conducted between 6 patients having unfavorable histology (diffuse anaplasia) and 39 patients having favorable histology tumors (Table 1). Two tumors containing focal anaplasia were excluded from this analysis. The second comparison was conducted between 12 patients who experienced treatment failure, defined as death or disease relapse, and 22 patients with treatment success, defined as at least 18 months without death or disease

relapse post-treatment (Table 1). Patients having fewer than 18 months of follow-up were excluded from this comparison. The third comparison was conducted between 6 patients having COG higher-risk tumors (defined as stage IV or LOH at 1p or 16q) and 24 patients having COG very low, low, or standard risk tumors (stage I-III WT without loss of heterozygosity at 1p or 16q; Table 1).(5) Patients with bilateral or unfavorable histology tumors were excluded from this analysis.

1.2.2 Creation of a Wilms Tumor tissue microarray (TMA)

Formalin-fixed paraffin-embedded (FFPE) tissue blocks contained within our laboratory's Wilms tumor tissue repository originated from 14 sequentially treated WT at the Children's Hospital of Philadelphia from 1996-98 and 33 sequentially treated at the Monroe Carrell Jr. Children's Hospital at Vanderbilt from 2002 to 2012. Tissue procurement was supervised by the same primary investigator (HNL) and identical protocols used between institutions. 2 mm circular areas containing representative, viable regions of tumor were selected and marked for inclusion in the TMA. These areas were subsequently cored out from the original tissue blocks and subsequently re-embedded into a common FFPE tissue block for high-throughput analysis (Figure 1). Additional microarrays containing metastatic, pre-treated, and post-treated tumors were assembled for proof-of-concept, preliminary experiments.

1.2.3 Imaging Mass Spectrometry (IMS)

IMS was used as previously described.(6) Briefly, two serial sections from each TMA block were cut at 5 μm thickness, and one section of each pair was stained with hematoxylin and eosin (H & E) (Figure 1). Photomicrographs of these serial TMA sections were marked for 300 μm diameter histologic regions of interest within blastemal and stromal compartments. The current analysis is limited to the blastemal compartment to ensure that unique histologic regions did not confound results. Tissue sections for mass spectrometry analysis were deparaffinized and subjected to heat-induced antigen retrieval. Digital images of the stained sections were matched to the corresponding unstained serial sections using Photoshop (Adobe, San Jose, CA). Coordinates of the histological annotations were determined and transferred to an acoustic robotic microspotter for deposition of trypsin and α -cyano-4-hydroxycinnamic acid matrix (10 mg/ml in 50:49.9:0.1 acetonitrile:water:trifluoroacetic acid). Spectra were collected using a Bruker UltrafleXtreme 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. Ion density maps were used to display the intensity of peptide expression in a given specimen (Figure 1). The data were preprocessed (baseline subtraction, noise level estimation, and alignment to common peaks), and classifiers were determined using a support vector machine with optimization by genetic algorithms.

1.2.4 Statistical Analysis

For IMS data, statistical analyses between selected groups were accomplished by feeding data into ClinPro Tools statistical analysis software package (Bruker Daltonics). Peak picking was performed manually to assure proper peak boundaries and selection of only the monoisotopic peak. Principal component analyses and receiver operating characteristics (ROC) were performed to classify peptide spectra according to patient group. Peptides with an area under the curve from the ROC plots of greater than 0.7 were considered significant.

1.3 Results

1.3.1 Unfavorable vs. Favorable Histology

To determine a molecular signature of unfavorable histology and therefore treatment resistance, we compared the peptide spectra obtained from the blastemal compartment of 6 tumors with unfavorable histology (defined as diffuse anaplasia) and 39 with favorable histology. Analysis of peptide spectra revealed 131 differentiating peptide peaks with p-values less than 0.05. Principal component analysis was used to plot two peptide peaks (6612 Da and 1263 Da) that differentiated most between the favorable and unfavorable histology groups (Figure 2). Receiver operating characteristics analysis determined 33 peaks with areas under the curve (AOC) > 0.7 (Figure 2). A genetic algorithm identified 15 peptide peak classifiers to create a model that could identify favorable histology tumors 99.67% of the time and unfavorable histology tumors 97.3% of the time, resulting in an overall recognition capability of 98.49%. This model was derived

from 80% of peptide spectra collected. The remaining 20% of peptide spectra were used for cross-validation of this model. Upon cross-validation, the model could identify favorable histology tumors 97.39% of the time and unfavorable histology tumors 75% of the time, with an overall recognition capability of 86.2%.

1.3.2 Treatment Failure vs. Success

We compared the peptide spectra obtained from the blastemal compartment of 12 patients who experienced treatment failure (defined as death or disease relapse within 18 months of treatment conclusion) and 22 patients with treatment success (no death or disease relapse) out to a minimum of 18 months from completion of therapy. Analysis of peptide spectra revealed 203 differentiating peptide peaks with p-values less than 0.05. Principal component analysis was used to plot two peptide peaks (1263 Da and 2916 Da) that differentiated most between treatment failure and treatment success groups (Figure 3). Receiver operating characteristics analysis determined 4 peaks with AOC > 0.7 (Figure 3). A genetic algorithm identified 15 peptide peak classifiers that could identify a tumor from a patient with treatment failure 90.54% of the time and treatment success 98.38% of the time, for an overall recognition capability of 94.46%. Using the 20% of spectra that were left out of the model for cross validation, the model correctly identified spectra from a patient with treatment failure 72.73% of the time and from treatment success 91.53% of the time, for an overall recognition capability of 82.13 %.

1.3.4 Higher-risk vs. Very-low, Low, and Standard-risk tumors

We compared the peptide spectra obtained from the blastemal compartment of 6 patients with COG higher-risk tumors and 24 patients with COG very-low, low, and standard-risk tumors. Analysis of peptide spectra revealed 71 differentiating peptide peaks with p-values less than 0.05. Principal component analysis was used to plot two peptide peaks (1045 Da and 1336 Da) that differentiated most between these two groups (Figure 4). Receiver operating characteristics analysis determined 20 peaks with AOC > 0.7 (Figure 4). A genetic algorithm was used to create a model incorporating 15 peptide peaks could identify a higher-risk tumor 97.1% of the time and a very-low, low, and standard-risk tumor 100% of the time, for an overall recognition capability of 98.55%. Using the 20% of peptide spectra left out of this model for cross validation, the model could correctly identify a higher risk tumor 81.88% of the time and a VLLS tumor 98.3% of the time, for an overall recognition capability of 90.09%.

1.3.5 Ion density maps

As a proof-of-concept experiment, ion density maps were created to explore the intensity of expression of a given peptide peak across the tissue microarray. A microarray containing specimens from metastatic, pre-treated, and post-treated tumors was created. Peptides with m/z of 1515.6, 1790.6, and 2215.5 corresponded with tumor-specific histologic regions of four separate pulmonary metastases in the same patient (Figure 1). In contrast, within these same pulmonary metastases, and in an additional pulmonary metastasis from a

different patient, peptides with m/z of 1032.8, 1326.1, and 2190.0 showed decreased expression relative to other samples on the TMA.

1.4 Discussion

1.4.1 This study is the first to use imaging mass spectrometry (IMS) to identify peptide spectra associated with adverse outcomes in WT. We sought spectra that reliably differentiated between unfavorable and favorable histology tumors, treatment failure (death or disease relapse) and success, and higher-risk and very-low, low, and standard-risk tumors. For each of these three comparisons, an internally validated model was created to assign spectra from a given tumor to one group or the other. For example, one model created in our study could reliably pick whether a primary tumor was associated with higher risk (metastatic or LOH 1p, 16q) disease or not approximately 90% of the time.

These studies are preliminary in nature. Although the models created herein were internally validated, external validation will require application to larger, independent sets of WT. Peptides used to create these models can then be sequenced, their parent proteins identified, and queried for targetable moieties.(7) Once validated by an external tumor set, these data could be used to not only identify tumors with known outcomes, but also to potentially predict the likelihood of treatment failure or metastasis and thereby identify patients that would benefit from more intensive chemotherapy at the time of diagnosis.

Previous studies have also confirmed the ability to utilize FFPE WT tissues for proteomic, mass spectrometry analysis in efforts to determine biomarkers that associate with WT.(8, 9) Prior work from our laboratory has delineated proteomic, tumor-specific differences between tumors from race groups that may explain the increased incidence and adverse WT behavior observed in minority groups.(7, 10, 11)

The 2013 COG blueprint for research of renal tumors sets the goal of closing a persistent survival gap in WT and reducing treatment-associated toxicities by identifying risk-specific molecular targets that may better address patients with COG high-risk (unfavorable histology) or higher-risk (metastatic disease or LOH 1p or 16q) disease.(3) Current treatments for these patient populations involve 4 drug chemotherapy regimens and radiation which are accompanied by significant long-term side effects including cardiotoxicity, neurotoxicity, ototoxicity, and the late risk for secondary malignancies.(12) The current study confirms the underlying biologic differences between COG risk groups on the protein level and is an initial step in identifying unique molecular signatures that may contain targetable moieties in high-risk and higher-risk disease.

1.4.2 Conclusions

This preliminary study identifies peptide spectra that differentiate WT based on COG risk-stratification criteria and adverse outcomes.

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Figure Legends

Figure 1. WT tissue microarray with ion density map. (A) A photomicrograph of a WT TMA depicts 2mm tissue cores assembled onto common slide for high-throughput analysis. (B) After IMS analysis, ion-density-maps are utilized to display the relative intensity of peptide expression at a given mass-to-charge (m/z) ratio (blue = low expression, pink = high expression). The five tissue cores outlined (white box) are from four separate pulmonary metastases in the same patient. The graphic demonstrates relatively high expression of peptides with m/z 1790.6 and 2215.5 in tumor-specific histologic regions within the core, whereas peptides with m/z 1326.1 and 2190 exhibit a relative paucity of expression.

Figure 2. Unfavorable vs. favorable histology. (A) Graphic display of principal component analysis used to detect peptide peak 23 (661 Da) and peak 224 (1263 Da), which differentiate between unfavorable histology WT (green circles) and favorable histology WT (red X). (B) Receiver operating characteristics (ROC) curve demonstrating the binary classification capacity of peak 268 (1504.37 Da) with area under curve (AUC) of 0.82. (C) The intensity of peptide expression at Peak 268 (1504.37 Da) is higher in unfavorable histology WT (green line) when compared favorable histology WT (red line) $p < 0.05$. This is the peak used for ROC analysis in (B).

Figure 3. Treatment failure vs. success. (A) Graphic display of principal component analysis used to detect peptide peak 380 (2916 Da) and peak 174 (1263 Da), which differentiate between WT with treatment failure (death or disease relapse, red x) and treatment success (green circles). (B) ROC curve demonstrating binary classification capacity of peak 377 (2843.31 Da) with area under curve (AUC) of 0.70. (C) The intensity of peptide expression at peak 377 (2843.31 Da) is higher in WT with treatment failure (red line) when compared to WT with treatment success (green line) $p < 0.05$. This is the peak used for ROC analysis in (B).

Figure 4. Higher-risk WT vs. very-low, low, and low-risk WT (A) Graphic display of principal component analysis used to detect peptide peak 240 (1336 Da) and peak 152 (1045 Da) which differentiate between higher risk WT (red x) and very-low, low, and low-risk WT (green circles). (B) ROC curve demonstrating binary classification capacity of peak 240 (1336 Da) with area under curve (AUC) of 0.799. Note: this peak is also used in principal component analysis in (A). (C) The intensity of peptide expression at peak 240 (1336 Da) is higher in very-low, low, and low-risk WT when compared to higher risk WT ($p < 0.05$). An adjacent peak at 1338.19 Da was also found to follow a similar pattern ($p < 0.05$, ROC AUC=0.74).

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Table 1

		Comparison 1		Comparison 2		Comparison 3	
		Unfavorable histology ¹	Favorable Histology ²	Treatment Failure ³	Treatment Success ⁴	Higher Risk ⁵	Very low, low, or standard risk ⁶
Number		6	39	12	22	6	24
Race	Black	1	3	1	2	1	2
	White	5	32	10	17	5	20
	Latino	0	3	1	2	0	1
	Asian	0	0	0	0	0	0
	Other	0	1	0	1	0	1
Gender	Male	1	14	9	11	1	12
	Female	5	25	3	11	5	12
Mean age at diagnosis	Months	46.5	40.3	39.8	36.2	73.2	36.9
Stage	I	0	8	0	7	0	7
	II	1	7	1	5	0	7
	III	2	10	3	6	0	9
	IV	0	7	1	3	6	1
	V	3	7	7	1	0	0
Disease Relapse		5	8	11	0	1	3
Mortality		4	3	4	0	0	0

¹Absence of diffuse anaplasia; ²Presence of diffuse anaplasia; ³Disease-specific death or disease relapse; ⁴No death or disease relapse with a minimum 18 months of post-treatment follow-up; ⁵COG higher risk disease includes Stage IV and LOH at 1p or 16q. No tumors had LOH at 1p or 16q in this study; ⁶COG very low, low, or standard risk disease. One patient with stage IV disease had early disappearance of pulmonary mets and therefore qualified for standard risk treatment according to COG risk stratification.

Figure 1

A

B

Figure 2

Figure 3

Figure 4