# Direct Identification of Neo-Epitopes in Tumor Tissue

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## Experimental Workflow and Patient Selection

Antigen presentation by MHC is central to the development of adaptive immunity. While traditional modeling approaches yield large numbers of candidates and high attrition rates, direct identification of naturally-processed peptides by mass spectrometry has the potential to significantly streamline the identification of neo-epitopes. To demonstrate the feasibility and advantages of this approach, a pilot study was conducted in clear cell renal carcinoma patients with the same tumor type and grade but heterogeneous HLA haplotypes. The process, outlined in Figure 1, included isolation of MHC I-peptide complexes from tumor and matched adjacent normal tissue followed by mass spectrometric analysis of presented peptides. Tumor-associated antigen presentation reflected protein expression changes previously reported in renal cell carcinoma, and identified multiple novel candidates. In addition, in many cases multiple HLA allele-specific peptides derived from the same protein were identified, thereby increasing target protein coverage across different haplotypes. Direct identification of naturally processed peptides generated a small but high quality list of candidates for further investigation.

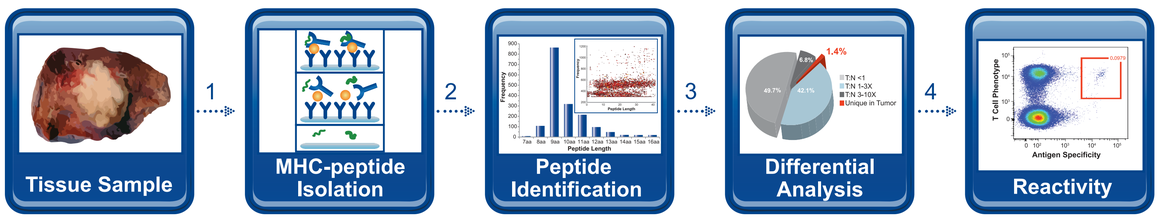



**Figure 1. Neo-Epitope Identification and Characterization Workflow**

- Tissue samples were obtained from 5 treatment-naïve clear cell renal carcinoma patients with the same tumor type and grade (**Table 1**)
- Patients exhibited heterogeneous HLA haplotypes with moderate overlap (Table 1)

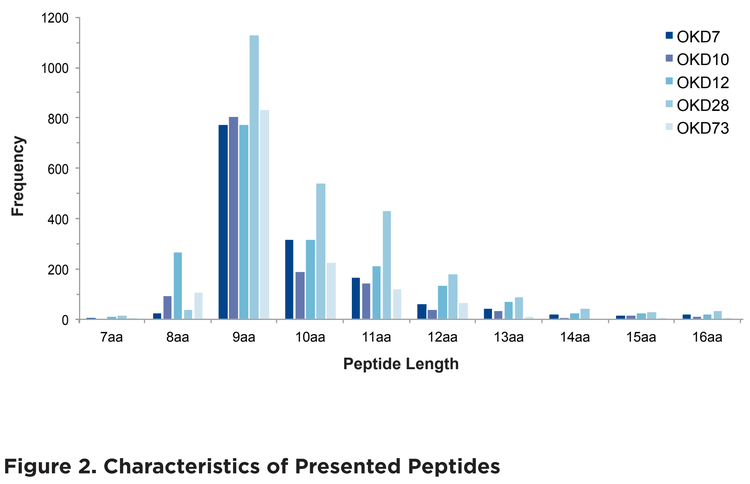
**Table 1: Patient Characteristics**

| Patient ID | Gender | Age | Tumor Grade | HLA-A | | HLA-B | | HLA-C | |
|---|---|---|---|---|---|---|---|---|---|
| OKD7 | F | 64 | Fuhrman 2/4 - pT3a | 03 | 29 | 40 | 44 | w10 | w16 |
| OKD10 | M | 47 | Fuhrman 2-3/4 - pT3a | 26 | 32 | 14 | 38 | w8 | w12 |
| OKD12 | F | 47 | Fuhrman 2/4 - pT3a | 01 | 01 | 08 | 44 | w5 | w7 |
| OKD28 | M | 62 | Fuhrman 2/4 - pT3a | 01 | 68 | 13 | 27 | w2 | w6 |
| OKD73 | M | 60 | Fuhrman 2-3/4 - pT3a | 01 | 02 | 40 | 08 | w10 | w7 |

- Tissue samples (1.3 – 1.6 g each) were micro-dissected and homogenized (**Figure 1, Step 1**)
- MHC-peptide complexes were isolated by antibody affinity chromatography
- Peptides were released from the MHC complexes and analyzed by mass spectrometry (**Figure 1, Step 2**), yielding the peptide sequence and its relative quantity in each sample
- Peptide sequences that were unique or highly up-regulated in the tumor compared to normal tissue (**Figure 1, Step 3**) were identified

## Results

2A




2B

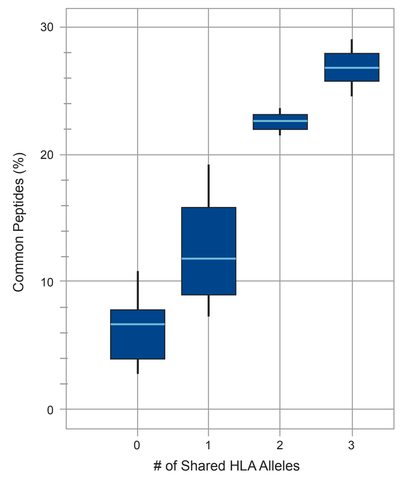



**Figure 2. Characteristics of Presented Peptides**

- An average of 1873 peptides were detected in each sample
- Peptides exhibited the expected size distribution for MHC I presented peptides (**Figure 2A**) with 48% of peptides 9 amino acids in length
- Overlap between presented peptides was related to the number of common HLA alleles (**Figure 2B**)
  - » Patients with no HLA alleles in common shared approximately 6% of all presented peptides
  - » Number of presented peptides in common increased approximately 7% for every shared allele

- The vast majority of peptides were not highly up-regulated in cancer
  - » Approximately 50% were under-expressed in tumor
  - » Approximately 40% were modestly over-expressed (1-3X) in tumor
- A small minority of peptides (1-4%) were unique in tumor
- Despite different subject haplotypes, a consistent frequency of differentially expressed MHC peptides was observed

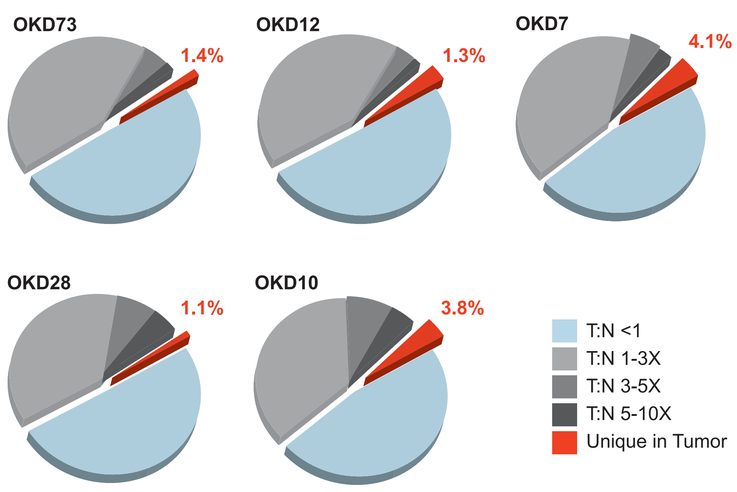



**Figure 3. Identification of Cancer-Associated Peptides**

- Clear cell renal carcinoma is known to induce a strong response to hypoxia and to induce angiogenesis
- Multiple proteins associated with both hypoxia and angiogenesis were significantly over-represented in tumor compared to normal tissue

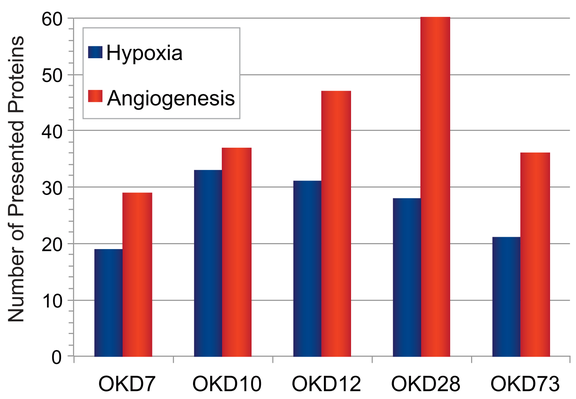



**Figure 4. Presented Peptides Reflected Changes in Hypoxia and Angiogenesis**

5A

| PEPTIDE | OKD7 | OKD10 | OKD12 | OKD28 | OKD73 |
|---|---|---|---|---|---|
| APLLRWVL | – | – | X | – | – |
| AENAEFMRNF | X | – | X | – | – |
| EVIPYTPAMQRY | – | X | – | – | – |
| TRYLGDLSGGQVL | – | X | – | – | – |
| KIAQKALDL | X | – | X | – | -- |
| IEEAKTAFL | X | – | – | – | – |
| EVIPYTPAM | – | X | X | – | – |
| EVIPYTPAMQR | – | – | – | X | – |

5B

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MERPQPDSMP QDLSEALKEA TKEVHTQAEN AEFMRNFQKG QVTRDGFRLV
MASLYHIYVA LEEEIERNKE SPVFAPVYFP EELHRKAALE QDLAFWYGPR
WQEVIPYTPA MQRYVKRLHE VGRTEPELLV AHAYTRYLGD LSGGQVLKKI
AQKALDLPSS GEGLAFFTFP NIASATKFKQ LYRSRMNSLE MTPAVRQRVI
EEAKTAFLLN IQLFEELQEL LTHDTKDQSP SRAPGLRQRA SNKVQDSAPV
ETPRGKPPLN TRSQAPLLRW VLTLSFLVAT VAVGLYAM
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5C

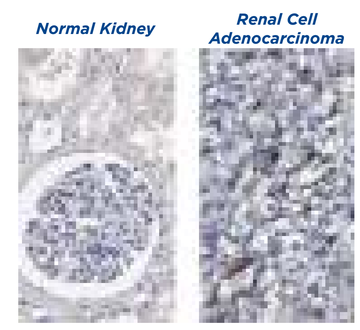


**Figure 5. Identification of Known and Novel Epitopes from Heme Oxygenase I (HMOX1)**

6A

| PEPTIDE | OKD7 | OKD10 | OKD12 | OKD28 | OKD73 |
|---|---|---|---|---|---|
| HIESRTLAI | – | – | X | – | X |
| KSELLVEQY | – | – | X | – | – |
| SLLTSSKGQLQK | X | – | – | – | – |
| TSALPIIQK | X | – | – | – | – |
| MAGDIYSVFR | – | – | – | X | -- |
| MTSALPIIQK | – | – | – | X | – |
| STVHLIEFAR | – | – | – | X | – |
| STKDQYPYLK | – | – | – | X | – |
| GVMAGDIYSVFR | – | – | – | X | – |

6B

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MASVAVDPQP SVVTRVVNLP LVSSTYDLMS SAYLSTKDQY PYLKSVCEMA
ENGVKTITSV AMTSALPIIQ KLEPQIAVAN TYACKGLDRI EERLPILNQP
STQIVANAKG AVTGAKDAVT TTVTGAKDSV ASTITGVMDK TKGAVTGSVE
KTSKVVSGSI NTVLGSRMMQ LVSSGVENAL TKSELLVEQY LPLTEEELEK
EAKKVEGFDL VQKPSYYVRL GSLSTKLHSR AYQQALSRVK EAKQKSQQTI
SQLHSTVHLI EFARKNVYSA NQKIQDAQDK LYLSWVEWKR SIGYDDTDES
HCAEHIESRT LAIARNLTQQ LQTTCHTLLS NIQGVPQNIQ DQAKHMGVMA
GDIYSVFRNA ASFKEVSDSL LTSSKGQLQK MKESLDDVMD YLVNNTPLNW
LVGPFYPQLT ESQNAQDQGA EMDKSSQETQ RSEHKTH
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6C

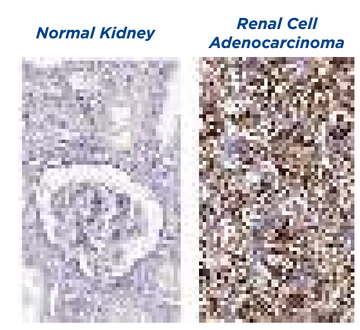


**Figure 6. Identification of Multiple Presented Peptides Derived from Perilipin-2 (PLIN2)**

- Peptide APLLRWVL (highlighted in yellow in **Figure 5A & 5B**) was previously reported to be presented by HLA-B08 in renal cancer tissue[1]
- The same peptide was identified in patient OKD-12, who has the same HLA allele
- Furthermore, 7 previously unreported peptides derived from HMOX1 were identified (highlighted in blue), thereby expanding coverage across different HLA types

- Multiple PLIN2 peptides presented by MHC I were substantially over-expressed (>30-fold) in tumor tissue (**Figure 6A & 6B**)
- PLIN2 is a putative biomarker of renal cell carcinoma; Increases in urinary PLIN2 have been shown to correlate with tumor size and stage[2] and to differentiate RCC from other cancers and kidney masses[3]
- Identified 9 novel epitopes derived from PLIN2 that reflected known protein expression changes in renal cell carcinoma

## Conclusions

→ This study demonstrates the feasibility of direct identification of naturally presented peptides from MHC I complexes.

→ HMOX1 and PLIN2 were detected in normal tissue but substantially over-expressed (> 30-fold) in tumor tissue, results which were consistent with previous reports.

→ Multiple novel epitopes were also identified, including an average of over 40 peptides per patient that were unique to tumor.

→ Compared to modeling approaches, this workflow yields fewer and higher quality candidates, facilitating subsequent characterization of immune response and improving overall efficiency.

### References

1. Flad T, Mueller L, Dihazi H, Grigorova V, Bogumil R, Beck A, Thedieck C, Mueller GA, Kalbacher H, Mueller CA. (2006) "T cell epitope definition by differential mass spectrometry: identification of a novel, immunogenic HLA-B8 ligand directly from renal cancer tissue" Proteomics 6:364-74.
2. Morrissey JJ, Mobley J, Song J, Vetter J, Luo J, Bhayani S, Figenshau RS, Kharasch ED. (2014) "Urinary concentrations of aquaporin-1 and perilipin-2 in patients with renal cell carcinoma correlate with tumor size and stage but not grade" Urology 83(1):256 e9-14.
3. Morrissey JJ, Mobley J, Figenshau RS, Vetter J, Bhayani S, Kharasch ED. (2015) "Urine aquaporin 1 and perilipin 2 differentiate renal carcinomas from other imaged renal masses and bladder and prostate cancer" Mayo Clin Proc. 90:35-42.