Detection of Novel HPV Mutations and Chromosomal Number Imbalance (CNI) in Laryngeal Cancer Using Next Generation Sequencing (NGS)

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Abstract

Background: We selected HPV-associated laryngeal papillomas and squamous cell carcinomas for genomic analysis of viral induced benign and malignant neoplasms. The nearly universal presence of HPV in Recurrent Respiratory Papillomatosis (RRP) and in certain squamous cell carcinomas provides an important model for the study of biomarkers and clinical outcomes as well as the generation of robust panels of viral and genomic mutation biomarkers that can be used to follow disease progression.

Methods: DNA was isolated from biopsies of three patients and sequenced on a NGS Illumina HiSeq platform. The sequences were compartmentalized in 250kbp bins, normalized, and compared to the mean (+/- 3SD) of Chronix controls to identify regions of chromosomal number imbalance (CNI). Sequences were also compared to databases with known viral genomes.

Results: Sample 1: an exophytic, focally invasive, HPV positive (PCR), squamous cell carcinoma from a never-smoker. The full genome of HVP16 was detected in low amount (~ 500 sequences/10 million sequences, average coverage=42 fold). Mismatches to the closest reference HPV16 strain isolate were found in protein (number of mismatches) E7 (1), E1 (2), E2 (4), E4 (1), L2 (3) and L1 (4). Sample 2: from RRP patient, contained the full genome of HPV6, also in low amount (~300 sequences/ 10 million sequences, average coverage = 24 fold). Interestingly, the HPV long control region was a variant as yet undescribed. Sample 3: from RRP patient whose papillomas slowly and spontaneously regressed, did not contain any detectable viral DNA. Only the HPV16 squamous cell carcinoma contained CNI with gains at chromosomes 3q and 8p, and losses at chromosomes 3p, 11q, 12p, and 21q consistent with a malignant transformation.

Conclusion: NGS reveals the identity and copy number of viral genes directly from clinical samples. This approach has revealed new HPV mutations not described by current viral analytical procedures. The detection of novel HPV mutations and CNI analysis in a single NGS run on HPV-related diseases provides important information into viral-host dynamics while generating biomarkers that can be correlated with treatment outcomes.

Patients and Samples

DNA was extracted from formalin fixed paraffin embedded (FFPE) biopsy tissue from twelve patients, 3 females and 9 males under informed consent and IRB approval. 2 patients presented with Recurrent Respiratory Papillomatosis (RRP). 10 patients presented with squamous cell carcinomas (SCC) of the throat (2 laryngeal, 8 pharyngeal). Details are listed in the Table 1.

Materials and Methods

DNA was extracted from FFPE tissue and PBMCs from EDTA blood samples. Shotgun NGS libraries were prepared using the NEBNext Ultra Library Preparation Kit and sequenced on an Illumina HiSeq2000. Sequencing reads were mapped to (i) the human genome (HG19) and (ii) a database containing the complete genomes of 59 different HPV genotypes.

Depth of Coverage analyses was performed using the program ReadDepth (Miller CA et al. (2011). PLoS ONE 6(1): e16327) and resulting log2 read depth ratios were smoothed by applying a circular binary segmentation algorithm (Olshen, AB et al. (2004) Biostatistics 5 (4): 557-572). The Circos software package was used for copy-number data visualization (Krzywinski, M. et al. (2009) Genome Res 19:1639-1645). Chr19 was excluded from copy-number analyses due to its high GC-content and resulting sequencing biases.

Reads mapping to the HPV database were counted and tissue samples with >10 reads were called positive for the respective HPV type. HPV reads were extracted and aligned to the genome of the respective type using Sequencher Software, an consensus by plurality sequence was created and used to compare the HPV of each patient against the NCBI virus database.

HPV PCR

HPV genotyping for samples T123, T124 and T125 were performed by Esoterix, Inc. For the rest of the samples, RNA and DNA were extracted from tumor samples using Tri-reagent standard protocol (Life Technologies). PCR was then preformed using HPV16 E6 forward primer 5'-CGCGGATCCATGCACCAAAAGAGAACTG-3' and HPV16 E6 reverse primer 5'-CGAGATCTTTACAGCTGGGTTTCTCT-3'. Cycling conditions were 94°C for 10 min, 94°C for 40 sec, 55°C for 40 sec, and 72°C for 1 min for a total of 30 cycles using an Eppendorf Mastercycler gradient thermocycler (Fisher Scientific, Pittsburgh, PA).

Results: Patient Information and Chromosomal Gains and Losses

	Sex	Age	Comment	HPV DNA	HPV RNA	Clinical Outcome	Gains	Losses
T161	F	57	Tonsil SCC	+	+	Metastases	**3q(122-198Mb)**, 8q(34-146Mb), 22q(0-51Mb)	8p(0-1Mb), 10pq(0-135Mb), **11pq(0-134Mb)**, 17p(0-21Mb), 2q(130-132Mb)
T163	F	71	Anterior Tongue/floor of mouth SCC	-		Remission	**3q(97-198Mb)**, 4q(136-191Mb), 8p(31-34Mb), 11q(68-71Mb), 16p(3-5Mb), 21pq(0-48Mb), 22pq(0-51Mb)	**3p(0-90Mb)**, 4p(0-48Mb), 8p(0-31Mb), 9p(0-39Mb), **11q(71-134Mb)**, 18q(23-77Mb)
T165	M	66	Floor of mouth SCC	-	-	Remission	1q(145-248Mb), 2q(142-162Mb), **3q(90-198Mb)**, 5pq(0-180Mb), 6p(29-58Mb), 6q(122-142Mb), 6q(167-170Mb), 7p(0-55Mb), 7q(76-116Mb), 8q(75-146Mb), 9pq(0-141Mb), 11p(30-40Mb), 12pq(0-133Mb), 13q(24-114Mb), 14(0-107Mb), 15pq(0-102Mb), 16pq(0-90Mb), 17pq(0-80Mb), 20pq(0-63Mb)	1p(0-121Mb), 2q(124-142Mb), 2q(207-243Mb), **3p(0-90Mb)**, 4pq(8-189Mb), 5pq(0-180Mb), 6p(8-10Mb), 6p(20-26Mb), 6p(26-29Mb), 6q(85-122Mb), 6q(142-160Mb), 8p(0-75Mb), 10pq(0-135Mb), 11p(0-30Mb), 18pq(0-77Mb), 21pq(0-48Mb), 22pq(0-50Mb)
T170	M	62	Left Tongue SCC	-		Remission	1p(39-55Mb), 1q(145-248Mb), 2p(0-89Mb), **3q(90-198Mb)**, 4q(71-79Mb), 4q(179-191Mb), 5p(0-48Mb), 6p(42-58Mb), 7p(0-58Mb), 8q(0-29Mb), 10q(38-84Mb), 11q(43-45Mb), 11q(68-71Mb), 12p(0-7Mb), 12q(9-31Mb), 18q(21-23Mb), 22q(35-51Mb)	1p(0-29Mb), 1p(55-121Mb), **3p(0-90Mb)**, 5p(46-180Mb), 7q(97-102Mb), 8p(0-29Mb), 9pq(0-141Mb), 10p(0-38Mb), 10q(84-135Mb), 11p(0-43Mb), 11p(45-50Mb), 13pq(0-114Mb), 16pq(0-90Mb), 18q(23-77Mb), 21pq(0-48Mb)
T172	M	35	Left tonsil SCC	faint +	+	Remission	**3q(90-198Mb)**, 9pq(0-141Mb)	2q(0-29Mb), 4p(0-53Mb),**11q(80-132Mb)**, 13pq(0-114Mb)
T173	F	52	Supraglottis SCC		-	Remission	2pq(0-243Mb), **3q(148-198Mb)**, 5p(0-45Mb), 6p(0-58Mb), 7p(12-23Mb), 7p(36-53Mb), 7q(76-97Mb), 7q(102-159Mb), 8pq(0-146Mb), 10p(0-38Mb), 11q(55-134Mb), 17q(21-80Mb), 18p(0-14Mb), 22p(0-26Mb)	**3p(0-88Mb)**, 3p(90-121Mb), 4pq(0-191Mb), 5q(45-180Mb), 6q(151-170Mb), 7p(0-12Mb), 7p(23-36Mb), 7q(97-102Mb), 9pq(0-116Mb), 11p(0-47Mb), 12pq(0-133Mb), 14pq(0-107Mb), 17p(0-21Mb), 18q(14-77Mb), 22q(26-51Mb)
T177	M	53	Tongue SCC		-	Remission	1q(145-248Mb), 6p(49-58Mb), 8q(43-146Mb), 21q(0-18Mb)	1p(113-120Mb), 8p(0-43Mb), 13q(19-27Mb), 18q(23-77Mb)
T178	M	69	SCC of oral cavity		-	Persistent Disease	1p(0-8Mb), **3q(0-47Mb)**, 10q(123-135Mb), 13pq(0-114Mb), 18p(0-19Mb), 20q(55-63Mb), 21pq(0-48Mb)	none
T179	M	66	Tonsil SCC		-	Remission	3p(0-47Mb), **3pq(51-198Mb)**, 7p(7-55Mb), 15pq(0-102Mb), 18pq(0-77Mb), 20pq(0-63Mb), 21pq(0-48Mb), 22pq(0-51Mb)	2q(130-132Mb), **3p(47-51Mb)**
T123	M	**54**	**Laryngeal SCC, in situ**	**+**		Persistent Disease	**3q(133-198Mb)**, 8p(4-13Mb)	3p(56-90Mb), **11q(59-134Mb)**, 12p(0.3-33Mp), 21(27-33Mb)
T124	M	**33**	**Laryngeal RRP**	**+**		Minimal residual papillomas	None	None
T125	M	**78**	**Laryngeal RRP**	**-**		Disease free	None	None

Table 1: Patients samples identified for age, gender, clinical presentation, HPV testing and chromosome gains and losses. Bolded regions in gains and losses indicate genomic changes found in previous publications.

Number of aligned sequencing reads

Sequence reads	Tumor (n=12)	PBMC (n=9)
Average	43,319,255	44,860,019
STDEV	8,161,066	6,551,889

Number of HPV Reads per Neoplasm by NGS Analysis

	T161	T163	T165	T170	T172	T173	T177	T178	T179	T123	T124	T125
HPV type 16	**1,752**	**2**	**-**	**18**	**3,658**	**5**	**-**	**-**	**1**	**1,672**	**-**	**-**
HPV type 18	-	-	-	-	-	-	-	-	**42**	-	-	-
HPV type 6	-	-	-	-	-	-	-	-	-	-	**914**	-

Integrated Chromosomal Gains and Losses in Tumors as a Function of HPV Status (5 HPV+/5 HPV-)

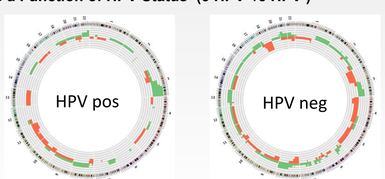

Figure 3: Circos plots of the sum of 5 HPV positive and 5 HPV negative patients.

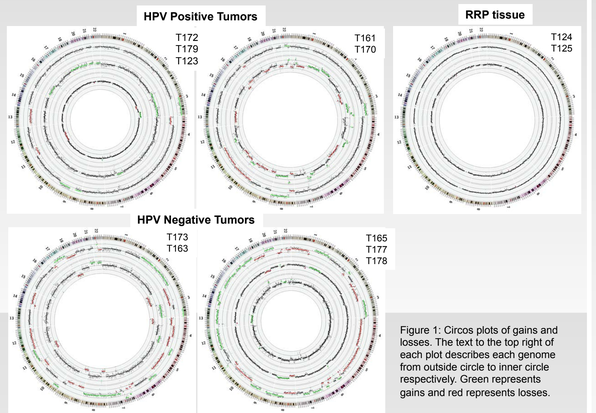

Figure 1: Circos plots of gains and losses. The text to the top right of each plot describes each genome from outside circle to inner circle respectively. Green represents gains and red represents losses.

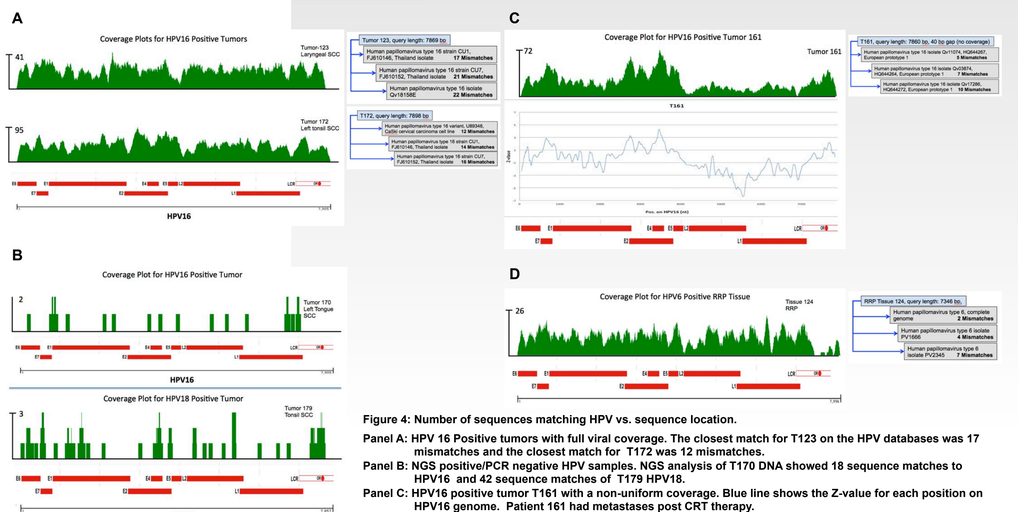

Figure 4: Number of sequences matching HPV vs. sequence location.

Panel A: HPV 16 Positive tumors with full viral coverage. The closest match for T123 on the HPV databases was 17 mismatches and the closest match for T172 was 12 mismatches.

Panel B: NGS positive/PCR negative HPV samples. NGS analysis of T170 DNA showed 18 sequence matches to HPV16 and 42 sequence matches of T179 HPV18.

Panel C: HPV16 positive tumor T161 with a non-uniform coverage. Blue line shows the Z-value for each position on HPV16 genome. Patient 161 had metastases post CRT therapy.

Panel D: HPV6 coverage from Patient 124 with laryngeal RRP. Note, the long control region has a variant as yet undescribed.

Genome Change Index and Clinical Response

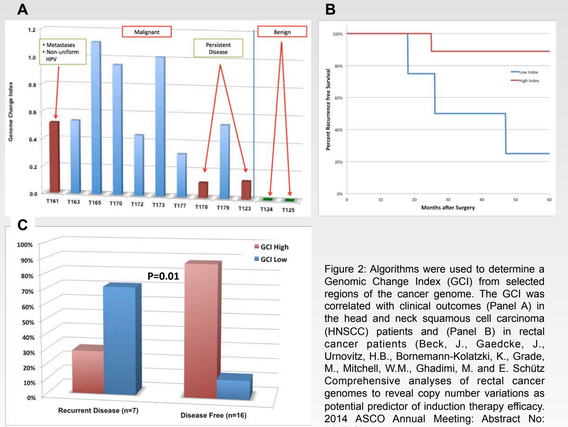

Figure 2: Algorithms were used to determine a Genomic Change Index (GCI) from selected regions of the cancer genome. The GCI was correlated with clinical outcomes (Panel A) in the head and neck squamous cell carcinoma (HNSCC) patients and (Panel B) in rectal cancer patients (Beck, J., Gaedcke, J., Urnovitz, H.B., Bornemann-Kolatzki, K., Grade, M., Mitchell, W.M., Ghadimi, M. and E. Schütz Comprehensive analyses of rectal cancer genomes to reveal copy number variations as potential predictor of induction therapy efficacy. 2014 ASCO Annual Meeting: Abstract No: e14549).

Panel A: The clinical outcomes associated with the 10 HNSCC were the responses to chemo-radiotherapy (CRT) as either complete remission, metastasis, or persistent disease as compared to the GCI. The two patients with the lowest GCI continued to have persistent disease. The patient with tumor T161 had a medium GCI score and exhibited metastases. As seen in Figure 4, a non-uniform HPV coverage was revealed in T161. Panel B: The survival outcomes (months after surgery) associated with 13 rectal cancer patients are plotted against GCI. Panel C: The combined data from HNSCC and rectal cancer (N=23) showed a p-value = 0.01 (Fisher's exact test) for the prediction of tumor recurrence vs. disease free by GCI with a accuracy of 84% (relative risk = 5.7).

Summary and Conclusions

1. **NGS detected HPV sequences in cancer genomic DNA that conventional PCR failed to detect. The 2 examples of NGS positive/PCR negative HPV detection revealed incomplete HPV genomic sequences.**
2. **NGS detected non-uniform HPV sequence coverage in T161 with metastatic disease suggesting clinical value in the combination of GCI and HPV analysis.**
3. **NGS provides a GCI that distinguishes between benign and malignant tumors.**
4. **HPV found in all cancer DNA samples contained SNPs that were not found on current databases. This suggests potential clinical value in the formation of a genotyping database for cancer DNA HPVs with correlation with clinical outcomes.**
5. **GCI appears to have clinical utility in predicting treatment outcomes for chemo-radiotherapy in two different tumor types. If this observation bears out in larger clinical trials and more types of cancers, treatment decision making can be tailored for non-DNA targeting (e.g. EGFR inhibitors, etc) when the GCI index is low.**
6. **NGS provides personalized cancer mutation panels that include unique fusions and query more than a limited number of cancer related exon SNPs to provide additional treatment options.**
7. **Personalized NGS cancer mutation panels allow real time, low cost treatment efficacy evaluation using a liquid biopsy blood test for cell-free DNA of the newly discovered cancer specific mutations (Beck, J. et. al. Mol. Cancer Res. 2010; 8: 385-92)**

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