Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Yuan H, Myers S, Wang J, et al. Use of reprogrammed cells to identify therapy for respiratory papillomatosis. N Engl J Med 2012;367:1220-7. DOI: 10.1056/NEJMoa1203055

Supplementary Appendix

Conditionally reprogrammed cells from a patient with progressive respiratory papillomatosis identify a mutant HPV-11 genome and an effective therapy

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Clinical History

Juvenile-onset recurrent respiratory papillomatosisis characterized by numerous squamous papillomasof the respiratory tract caused by infectionwith HPV types 6 and11. HPV is thought to be acquired at the timeof vaginal delivery in the presence of genital lesions,and patients typically present with symptoms ofhoarseness or respiratory obstruction in childhoodor adolescence. In the majority of cases, the papillomasare confined to the larynx; only 3-5% of patientsexhibit more distal involvement of the trachea,and involvement of lung parenchyma occursin fewer than 1% of cases.Patients that have extension below the larynxfrequently require tracheostomyand oftendevelop distal extension into the lower respiratory tract. The unfortunate patients who develop tumors within the lung parenchyma usually die from respiratoryinsufficiency or infections.

In the current study, the patient is a 24 year old African-American male non-smoker with a 20-year history of virally-induced RRP. Please see the diagram of the patient's progressive clinical disease (Supplementary Figure 1). He was diagnosed with RRP at the age of three and one-half years old in 1991. Soon after diagnosis, tracheotomy was performed to bypass laryngeal obstruction. He has had over 350 procedures to manage his upper airway disease including numerous surgical debridements and CO_2 laser ablations, and two additional tracheostomies at ages 9 and 11.Intralesionalcidofovir (10 to 15 mg each time, 2001-2011) was injected when the laryngeal papilloma excision was performed between 2001 and 2011 (age 13 to 23). Additionally, Intralesional bevacizumab (10-12.5 mg, 2010-2011) was used. The patient was also treated with interferon alpha (Intron A) from 1998 to 2010 (age 10 to 22) and methotrexate between 2001 and 2003 (age 13 to 15). No significant clinical responses were noted to any of these therapies. In recent years, the patient has required 3 to 4 surgeries to control the laryngeal papilloma growth. Lung lesions were first detected in 1993 at the age of 5, and the first thoracotomy (left lung wedge resection), was performed in the same year at

Children's National Medical Center (Washington, DC). The second thoracotomy (also left lung wedge resection) was performed at age of 12 (2000). The patient's care then was transferred to Georgetown University Hospital in 2001. In 2007, the third thoracotomy (wedge resections of the right upper and middle lobes) was performed at the age of 19. At age 22 (2010) the patient had worsening dyspnea and occasional hemoptysis, and CT scans revealed that there were multiple bilateral pulmonary nodules that had accelerated in growth, with 3 index nodules increasing in size by 21%, 72% and 130% in 11 months. The patient was classified clinically as having chemo-resistant, progressive disease.

The fourth thoracotomy (an upper lobe anterior segmentectomy) had to be performed only three yearslater due toaccelerated tumor growth in lung, the concern for malignant transformation,and the proximity of tumor adjacent to a pulmonary arterial branch. Samples of the surgically-excised lung tumor were examined histologically and shown to consist of papillomatosis with focal koilocytoticatypia, consistent with spread from the preceding laryngeal lesions. No malignant transformation was found. Normal and tumor samples were obtained with the informed consent of the patient according to a Georgetown University Hospital IRB protocol (1992-048). Cells from the tumor and adjacent normal lung were propagated using the CRC culture technique. The cultured tumor cells (and primary tumor)containedan HPV-11 genome with duplication of the promoter and E6/E7 oncogenes), which may have contributed to the observed aggressive clinical behavior. In vitro drug testing identified vorinostat as a potential new therapeutic approach for treating advanced pulmonary extension of RRP.Based on the in vitro sensitivity data, the patient was placed on vorinostat therapy (400 mg/daily). The vorinostat dose of 400mg daily for 21 days was given for 12 cycles of 28 days (7 days off each cycle), consistent with published dose ranges[1,2,3], with the plan to test one year of treatment but to reduce the dose if indicated by toxicity. This course was tolerated for one year with observed toxicities of mild elevation of creatinine and blood pressure, CTCAE grade 2 (v4.0), and transient leg muscle pain, CTCAE grade 1.The patient had some nausea, vomiting, bloating,

and diarrhea on initiation of vorinostat, but these symptoms resolved within 1 week, although the patient needed occasional anti-nausea medications during future treatment cycles.

The chest CT scan after 3 months of treatment revealed highly encouraging results. There was shrinkage of both small and large tumors and no new lesions were identified. As illustrated in Fig 3D, the mass in the right lower lobe showed a 13% decreased in size between January 2011 and April 2011. Two lung tumors and 1 right hilar lymph node were considered "measurable target lesions" by RECIST criteria in January 2011. The sum of the long and short measurements was 7.9 cm. In April 2011, the sum of target lesions was 6.6 cm, a 16% decrease from baseline. After a 12-month treatment, the tumor sizes have remained stable (a 14% decrease from baseline in April 2012).

Reference

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2. Kelly WK, O'Connor OA, Krug LM, et al: Phase I study of an oral histone deacetylase inhibitor, suberoylanilidehydroxamic acid, in patients with advanced cancer. J ClinOncol 23:3923-3931, 2005

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Methods:

Cell isolation and propagation

The methods for this new cell culture technique were recently published [5].Tissues were minced and digested with a mixture of dispase and collagenase 1A (StemCell Technologies Inc, Vancouver, BC, Canada). Epithelial cells were cocultivated with irradiated (3000 rad) Swiss 3T3 fibroblasts (J2 strain) in F medium [3:1 (v/v) F-12 Nutrient Mixture (Ham)–Dulbecco's modified Eagle's medium (Invitrogen), 5% fetal bovine serum, 0.4 μg/mL hydrocortisone (Sigma-Aldrich), 5 μg/mL insulin (Sigma-Aldrich), 8.4 ng/mL cholera toxin (Sigma-Aldrich), 10 ng/mL epidermal growth factor (Invitrogen), and 24 μg/mL adenine (Sigma-Aldrich)] with addition of 5 to 10 μmol/L Y-27632 (Enzo Life Sciences). All cells were maintained at 37°C in a humidified incubator, with 5% CO2, and passaged at a 1:4 to 1:16 split ratio when 80% to 90% confluent. Differential trypsinization was used to separate feeder and epithelial cells during passaging. Briefly, feeder/epithelial co-cultures were rinsed with PBS and incubated with 0.05% trypsin solution at room temperature for 30 seconds to 1 minute, with close monitoring by phase microscopy. When the feeder cells rounded up and began to detach from the substrate, the cultures were gently tapped and the detached cells were removed by aspiration. The epithelial colonies remained tightly adherent. The epithelial cells were rinsed again with PBS and then retrypsinized at 37°C for 3 to 5 minutes. The cells were pelleted through PBS containing 10% serum (to neutralize trypsin). After centrifugation at 500 × g, the cell pellets were resuspended in F medium for passaging.

Rolling Circle Amplification of HPV genome

Multiply primed RCA was performed with the TempliPhi 100 amplification kit (GE Healthcare) according to the manufacturer's instructions. Two microliters of extracted DNA from cells (containing 0.2 to 0.5 μg of total DNA) or from tissue (0.5 to 1 μg of total DNA), or water (negative control), was transferred into a 0.5-ml tube with 10μl of TempliPhi sample buffer, containing exonuclease-protected random hexamers. The samples were denatured at 95°C for 3 min and afterwards were cooled to room temperature. A premix was prepared on ice by mixing, for each sample, 10μl of TempliPhi reaction buffer, containing salts and deoxynucleotides (dNTPs), and 0.4μl of TempliPhi enzyme mix, containing the φ29 DNA polymerase and exonuclease-protected random hexamers in 50% glycerol. The reaction mixtures were incubated 18 hours at 30°C. Afterwards, the reaction mixtures were cool to 4°C, and stored at −20°C until further analysis.

PCR detection of genome duplication

General HPV-11 primer set: forward: 5'-GACTGCCCCCCGTTGGAACTTA-3'; reverse: 5'-CCACATGGCGCATGTATTCC-3'. Duplication specific primer set 2: forward: 5'-GACAGGCATATTACACAAAA-3'; reverse: 5'-AGTGTCTAGCACTTTCGTTT-3'. Duplication specific primer set 5: forward: 5'-AGGAAGAGGAGGTGGAGGAC-3'; reverse: 5'-ACCAATGAGCCACTAGGTGT-3'. Duplication specific primer set 6: forward: 5'-GCAGGAGGCGGATGCTCATT-3'; reverse: 5'-TCATCAGGCACAGGTTCCCC-3'. Reaction mixtures with a total volume of 25 μl contained 2 × PCR MasterMix (Promega). The amplification was performed: 95°C 3 min, (95°C 30 sec, 52°C 30 sec, 72°C 1 min) X 25 cycles,

75°C 5 min. Ten µl of PCR product were run in 1% agarose gel with ethidium bromide at 100 V for 30 min.

Quantitative PCR

TaqMan real-time quantitative PCR was performed on a Bio-Rad iCyclerMyiQ, using primers and probes for the quantitation of HPV11 L2 (sense primer, 5'-TCTTCCTCGTGCTTTTCCTC-3'; anti-sense primer, 5-CTTCTCCTTCATAGACAGGGTTG-3'; and TaqMan probe, 5'-/56-FAM/TGGGTTTGT/ZEN/ATAGTCGTGCCTTACAGC/3IABkFQ/-3'). Human ß-Globin Gene (sense primer, 5'-GGGCCTCACCACCAACTTC-3'; anti-sense primer, 5-TGCATTTGACTCCTGAGGAGAA-3'; and TaqMan probe, 5'-//56-FAM/CTG CCG TTA /ZEN/CTG CCC T/3IABkFQ/-3') was used as an endogenous reference in each reaction. Real-time PCR reactions were done in triplicate for all samples. The levels of HPV DNA were analyzed using iQ5 software with the normalized expression ({Delta}{Delta}CT) method according to the manufacturer's (Bio-Rad's) guidelines.

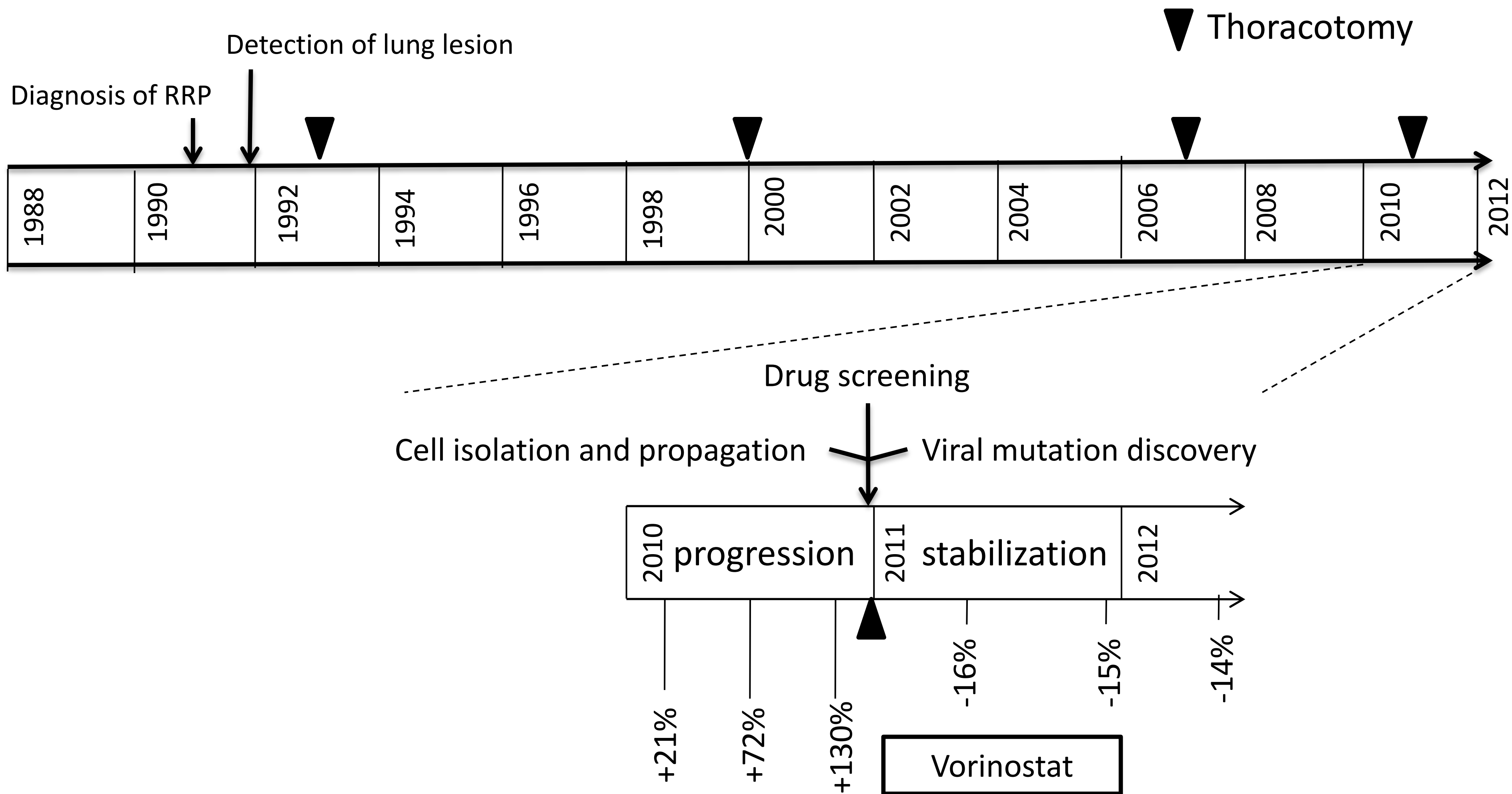

Figure S1: The timeline indicates the patient's clinical history, therapy and disease status. In 2010, CT scans revealed multiple pulmonary tumors with accelerated growth. Based on the in vitro sensitivity data, the patient was placed on vorinostat therapy from January 2011 until January 2012. Using the RECIST criteria, the overall response was categorized as "stable disease“ between January 2011 and April 2012.

Table S1

	Lung 10.5 kb	Larynx 8 kb	
LCR(B)	3 substitutions, 1 deletion, 2 two-nucletide insertions	3 substitutions, 1 deletion, 1 insertion, 1 two-nucletide insertion	LCR
E6(B)	2 substitutions, 1 deletion	2 substitutions	E6
E7(B)	1 substitution	1 substitution	E7
E1(B)	6 substitutions	6 substitutions	E1
E2	7 substitutions	7 substitutions	E2
E4	3 substitutions	3 substitutions	E4
E5A	3 substitutions	3 substitutions	E5A
E5B	0	0	E5B
L2	2 substitutions	2 substitutions	L2
L1(A)	1 substitution	1 substitution	L1
LCR(A)	3 substitutions, 1 deletion, 1 insertion, 1 two-nucletide insertion		
E6(A)	2 substitutions		
E7(A)	1 substitution		
E1(A)	0		
L1(B)	0		

Table S1. DNA sequence variations of viral genomes isolated from lung or larynx compared to the prototype HPV-11.