DEVELOPMENT OF A DIAGNOSTIC ASSAY BASED ON THE BINDING OF HIGH-RISK HPV-E6 ONCOPROTEINS TO PDZ

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BACKGROUND

PDZs are conserved protein domains involved in protein-protein interaction. The name "PDZ" relates to the first three proteins discovered to contain PDZs: **P**SD-95, **D**LG-1, and **Z**O-1. More than 230 different PDZ domains have been identified to date in humans, widespread biological functions that include cell-to-cell contact, intercellular signaling, and cell polarity. PDZ domains extend over approximately 100 amino acids, but the basic PDZ binding motif, herein referred to as "PL" (for PDZ ligand) consists of the four most C-terminal amino acids on proteins that bind to PDZ. An example for a classical PL reads X-T/S-X-V. Numerous viral proteins have PLs that can bind to cellular PDZs. For example, all high-risk HPV-E6 proteins can bind PDZs.

INTRODUCTION

High-risk HPV types cause over 99% of cervical cancers. Accumulating evidence suggests that expression of high-risk HPV oncoproteins E6 and E7 leads to cervical cancer progression and is necessary to maintain the transformed state. HPV16-E6 and HPV18-E6 proteins have been shown previously to bind several PDZ domain-containing proteins via a HPV-E6-C-terminal PL. Our studies indicate that E6 proteins of **all** high-risk HPV types contain a PL and bind PDZ domains, and that low risk HPV-E6 proteins do **not** contain a PL (Fig. 1). Further tests on the binding of a C-terminal peptide of HPV16-E6 to the nearly complete set (>230) of human PDZ domains identified the PDZ domain (AVC PDZ88) that binds E6 with the highest affinity (Fig. 2). Specific binding of all high-risk HPV-E6 to PDZ88 is the basis of a novel rapid immunoassay (developed by Arbor Vita Corporation) for detection of E6 protein from human cells (Fig. 4).The assay promises to be highly predictive of malignant transformation because it measures E6 oncoprotein, a primary cause of transformation, whereas infection with the HPV virus causes neoplasia in only a small fraction of those infected.

Within a collaborative agreement between Arbor Vita and PATH, a lateral flow prototype of this novel assay is currently under development. The assay is targeted especially for use in developing countries, and will be designed to detect E6 of seven of the most highly prevalent HPV types (16, 18, 31, 33, 45, 52 and 58). The criteria for the assay include that it be accurate, reproducible, affordable, simple, rapid, portable, and of moderate throughput.

RESULTS

Components of a novel cervical neoplasia diagnostic assay: "PDZ oncogenic E6 detector" and anti-E6 monoclonal antibodies (anti E6 mAbs).

The basis of the novel assay is the specific capture of **all** high-risk HPV-E6 via a PDZ domain, the "PDZ oncogenic E6 detector" (AVC PDZ88; Fig. 2 and 3), and detection via HPV-E6-specific monoclonal antibodies (Fig. 4). Arbor Vita has generated several high-affinity anti-E6 mAbs that recognize recombinant HPV-E6 at a sensitivity better than 80 pg in the sandwich ELISA format as shown in Fig. 5A. The mAbs were confirmed to detect E6 protein from cervical cancer cell lines in the presence of total cell lysate at a sensitivity of approximately 60,000 cells (Fig.5B).

Binding of all high-risk HPV-E6 recombinant proteins to the PDZ oncogenic E6 detector was examined. All high-risk HPV-E6 proteins bind to the PDZ; E6 proteins of those HPV types that are more prevalent in cervical cancer bind the PDZ with high strength, whereas low-prevalence HPV-E6 bind the PDZ with lower strength (Fig. 7).

Detection and quantification of E6 protein in cervical cancer cell lines and clinical samples.

Detection and quantification of high-risk HPV16-E6 protein was performed via Western Blot technology. E6 was detected in cervical cancer cell lines and a HPV16 positive cervical tumor. E6-specific signal intensities from cervical cell lines and the tumor were compared to defined quantities of recombinant MBP-E6; roughly, 10^6 cervical cancer cells contain 1 ng E6 protein (Fig. 6B). Maximal sensitivity for E6 detection from cervical cancer cell lines by Western Blot technology is shown in Fig. 6C, where E6 from approximately 17,000 CasKi cells can be detected. Detection of E6 from a clinical cervical scrape from a woman diagnosed with CIN3 is shown in fig. 6D.

Development of a lateral flow based cervical neoplasia assay.

The PDZ capture/anti E6 mAb detection-based sandwich principle of the ELISA-based format (Fig. 4 and 5A, B) has been applied by PATH to develop a lateral flow-based assay ("strip test"; Fig. 5C). Here, the anti-E6 mAb is conjugated to colloidal gold, thus allowing visual detection. Current sensitivity of the lateral flow assay is approximately 1.3 ng recombinant E6 (3.9 ng recombinant MBP-E6 protein corresponds to 1.3 ng untagged E6 protein).

SUMMARY AND CONCLUSIONS

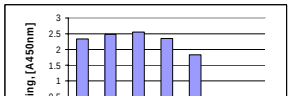
A prototype of a novel diagnostic assay for cervical precancer and cancer has been developed in both a sandwich ELISA-based and a lateral flow-based format ("strip test"). The test specifically detects presence of high-risk HPV-E6, and therefore promises to be of higher predictive value for cervical precancer and cancer than other assays detecting merely presence of HPV. The lateral flow assay format is simple and less costly and particularly suited for point-of-care testing, allowing immediate results and follow-up.

The ELISA-based assay format has a sensitivity of approximately 60,000 cervical cancer cells (SiHa, CasKi), and, in its current form, sensitivity of the lateral flow test is at 1.3 ng E6 protein. Quantification of E6 protein in cervical cancer cell lines was show that E6 protein of HPV16 is present at approximately 1 ng/10^6 cells (Figs. 5B and 6B). Target sensitivity of the assay was determined at detection of E6 from 10,000 to 50,000 cervical cancer cells. Consequently, sensitivity of the lateral flow assay needs to be increased at minimum by a factor of 25. Steps towards this goal will include optimization of the capture molecule (PDZ oncogenic E6 detector) and the readout technology of the lateral flow signal.

Prospective cohort studies are underway in women with histologically confirmed CIN1 lesions to confirm the predictive validity of this biomarker. Cross-sectional laboratory and field validation studies will be conducted to evaluate the performance of this biomarker in women with normal findings and cervical neoplasia.

Figure 3

Binding of High-Risk HPV-E6 but Not Low-Risk HPV-E6 to the PDZ Oncogenic E6 Detector



Binding of the PDZ oncogenic E6 detector to high-risk HPV-E6 but not to low-risk HPV-E6. Binding of recombinant HPV-E6 proteins representing five high-risk HPV (types 16, 18, 35, 45, 58) and one low-risk HPV (type 11) was tested via direct ELISA.

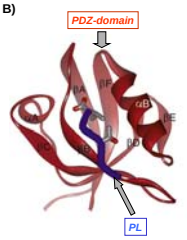
Figure 1

All High-Risk HPV-E6 Have a C-Terminal PDZ Domain-Binding Motif

A)

HPV strain	E6 C-terminal sequence	PL	Oncogenic
		YES / NO	
HPV 4	GYCRNCRRKQ	No	No
HPV 11	WTTCMEDLLF	No	No
HPV 20	GICRLCKHFQ	No	No
HPV 24	KGLCRQCKQI	No	No
HPV 28	WLRCTVRIPQ	No	No
HPV 36	RQCKHFYNDW	No	No
HPV 48	CRNCSHEGFR	No	No
HPV 50	CCRNCYEHEG	No	No
HPV 16	SSRTTRETQL	Yes	Yes
HPV 18	RLQRRRETQV	Yes	Yes
HPV 31	WRRPRTETQV	Yes	Yes
HPV 35	WKPTRRETEV	Yes	Yes
HPV 30	RRTLRRETAV	Yes	Yes
HPV 39	RRLTRRETQV	Yes	Yes
HPV 45	RLRRRRETQV	Yes	Yes
HPV 51	RLQRRNETQV	Yes	Yes
HPV 52	RLQRRRVTQV	Yes	Yes
HPV 56	TSREPRESTV	Yes	Yes
HPV 59	QRQARSETLV	Yes	Yes
HPV 58	RLQRRRQTQV	Yes	Yes
HPV 33	RLQRRRETAL	Yes	Yes
HPV 66	TSRQATESTV	Yes	Yes
HPV 68	RRRTRQETQV	Yes	Yes
HPV 69	RRREATETQV	Yes	Yes
HPV 26	RPRRQTETQV	Yes	Yes
HPV 53	RHTTATESAV	Yes	Yes
HPV 73	RCWRPSATVV	Yes	Yes
HPV 82	PPRORSETQV	Yes	Yes

B)



PL motif: X-S/T-X-V/L

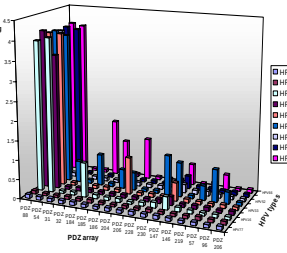
100% Structural Specificity

A) E6 oncoprotein of **all** known high-risk HPV types has a C-terminal PDZ binding motif (PL) for PDZ ligand; none of the low-risk HPV have a PL. This correlation is 100% complete. B) Ribbon rendering of a PL bound to a PDZ domain.

(Picture from: HARRIS & LIM. (2001) J CELL SCI 114:3219-31)

Figure 2

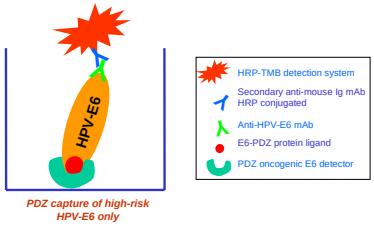
Selection of the "PDZ Oncogenic E6 Detector" via Screening of All Human PDZ's for Binding to HPV-E6 C-Termini



Binding of seven high-risk and two low-risk HPV-E6 C-terminal peptides to the Arbor Vita "human-PDZ domain reference set" was tested via ELISA. The PDZ reference set represents more than 95% of all known human PDZ domain proteins (>230 different PDZ domains). Representative binding data for 17 different PDZs are shown. Only peptides representing high-risk E6, but not those representing low-risk E6, showed binding to PDZ domains. PDZ88 bound **all** oncogenic E6 tested. Arbor Vita determined the relative binding affinity as measured by EC_{50} values of HPV16-E6 for 12 candidate PDZ domains. PDZ88 binds HPV 16-E6 with the highest affinity (<100 nM) and constitutes the "PDZ oncogenic E6 detector" for a novel diagnostic cervical cancer assay.

Figure 4

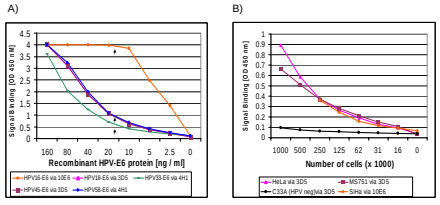
Concept of a Novel Cervical Neoplasia Diagnostic Assay Based on Specific Detection of High-Risk HPV-E6 via PDZ Oncogenic E6 Detector



Principle of a novel E6 protein-based cervical cancer assay. Specific capture of high-risk HPV-E6 oncoprotein occurs via the PDZ oncogenic E6 detector. Detection occurs via a (mouse) anti-E6 mAb, an anti-mouse Ig antibody conjugated to HRP and the HRP-TMB-based detection system.

Figure 5

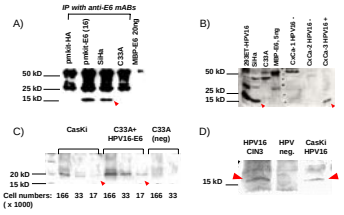
Specific Detection of High-Risk HPV-E6 by Sandwich ELISA and Lateral Flow Cervical Cancer Diagnostic Assay Prototypes



A) Recognition of recombinant E6 of HPV types 16, 18, 33, 45, and 58. The colorimetric signal obtained for detection of recombinant HPV-E6 corresponding to 1 ng E6 is indicated by arrows.
B) Recognition of HPV-E6 in cell lysates from HPV positive cervical cancer cell lines SiHa (HPV16), HeLa (HPV18), and MS751 (HPV45). C33A is a HPV negative cervical cancer cell line.
C) Lateral flow strips of titrations of recombinant HPV16-E6 (rE6). PDZ oncogenic E6 detector was immobilized on the membrane. Anti-E6 mAb (10E6) was gold conjugated and preincubated with rE6. The premixture (0.1 ml) was then migrated up the strip via capillary action.
Titration of recombinant HPV16-E6 and detection via mAb 10E6. At the threshold of detection, 3.9 ng rE6 (= ~1.3 ng E6 without tag) is detected (arrow head).
All anti-E6 mAbs used in the above experiments have been generated by Arbor Vita Corporation.

Figure 6

Detection of HPV16-E6 From Cervical Cancer Cell Lines, a Cervical Tumor and a Cervical Scrape Diagnosed CIN3

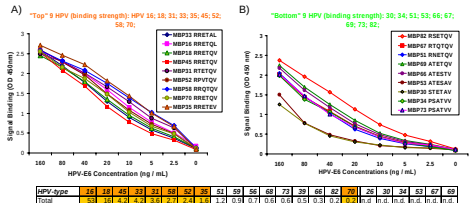


Detection and quantification of E6 protein via Western and an anti-E6 mAbs. Arrowheads point to bands representing E6 protein. A) HPV16-E6 protein was detected by immunoprecipitation from HPV16 cervical cancer cells (SiHa) and from 293 EBNA-T cells transfected with the E6 expressing construct pmkit-E6. Samples were run side by side for E6 protein size confirmation. C33-A is a HPV negative cervical cancer cell line. B) Cell lysates prepared from different cell lines and cervical tumors were analyzed by Western. Lysates corresponding to approximately $1x10^6$ cells were loaded. Quantity of E6 protein in various cervical cancer cell lines was determined at approximately 1 ng/10^6 cells. C) Determination of maximal detection sensitivity of HPV16-E6 in the cervical cancer cell line CasKi and HPV16-E6 stable transfected cells (C33A+HPV16-E6) via Western blotting. D) Detection of HPV16-E6 from a cervical scrape; cytology of this sample revealed a CIN3 pre-cancerous stage.

The anti-E6 mAbs (6F4 and 4C6) used in the above experiments were generously provided by Dr. E. Weiss, University Louis Pasteur of Strasbourg.

Figure 7

HPV-E6 Binding Strength to PDZ Oncogenic E6 Detector Correlates to HPV-Type Prevalence in Cervical Cancer



HPV-type	16	18	45	33	31	58	52	35	51	59	56	68	73	39	66	82	70	26	30	34	53	67	69
Total prevalence*	53	16	4.2	4.2	3.6	2.7	2.4	1.6	1.2	0.9	0.7	0.6	0.6	0.5	0.3	0.2	0.2	n.d	n.d	n.d	n.d	n.d	n.d
HPV-type	16	18	45	33	31	58	52	35	51	59	56	68	73	39	66	82	70	26	30	34	53	67	69
Total prevalence*	53	16	4.2	4.2	3.6	2.7	2.4	1.6	1.2	0.9	0.7	0.6	0.6	0.5	0.3	0.2	0.2	n.d	n.d	n.d	n.d	n.d	n.d

* Worldwide (includes: Africa, Asia, Europe, North- and South-America, Australia)

Binding strength of 23 recombinant high-risk HPV-E6 proteins with a maltose binding protein tag (MBP-E6) to the PDZ oncogenic E6 detector was tested via direct ELISA. PDZ oncogenic E6 detector was immobilized on the ELISA plate, and binding of MBP-E6 was detected via an anti-MBP-mAb recognizing the MBP tag of the recombinant E6 protein. Binding signal intensities roughly fall into two distinct groups. The nine recombinant E6 proteins that bind strongest to the PDZ oncogenic E6 detector (A) are also most prevalent in cervical cancers, and the nine weakest binding E6 proteins (B) are less prevalent in cervical cancer.

cervical cancer group
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