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Placenta specific protein 1 (PLAC1) expression in HPV 16/18 positive cervical cancers is associated with tumor histology

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Abstract

Objective—Expression of the trophoblast-specific gene Placenta-specific Protein 1 (PLAC1) has been detected in a wide variety of cancers. However, to date, PLAC1 expression has not been shown in cervical cancer. We have carried out a preliminary study that shows for the first time that PLAC1 is expressed in cervical cancers.

Methods—Sixteen primary cervical tumors were obtained from patients shown to be Human Papilloma Virus (HPV) 16/18 positive. Total cellular RNA, genomic DNA and total protein was purified from each tumor. These materials were then used to determine PLAC1 expression, TP53 mutation status, and p53 expression.

Results—PLAC1 expression was demonstrated in all sixteen primary cervical tumors. The highest levels of expression were found in the more aggressive squamous and adenosquamous histologic types compared with adenocarcinomas. Moreover, the proportion of total PLAC1 message coming from the P1 promoter, also termed the distal or cancer promoter, was significantly greater in the more aggressive squamous and adenosquamous histologic types compared with adenocarcinomas. Finally, in spite of all sixteen tumors being HPV 16/18 positive, three of eight squamous cell cancers and two of five adenocarcinomas expressed wild-type p53 protein. Consistent with the recently shown suppression of the PLAC1P1 promoter by wild-type p53, these p53 positive tumors displayed among the lowest P1-specific PLAC1 expression levels.

Conclusions—Placenta-specific protein 1 (PLAC1) expression has been demonstrated for the first time in cervical cancers. This preliminary study has further revealed a complex relationship

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between PLAC1 expression, cervical cancer histologic type, p53 and HPV type that requires further investigation.

Keywords

Cervical cancer histology; PLAC1 transcription dynamics; p53; Human papilloma virus

Placenta-specific protein 1 (PLAC1) is a small, secreted cell-adhesion protein first discovered nearly two decades ago.¹ Located at chromosome Xq26 in a region containing several other genes known to be associated with gestational disorders, PLAC1 is normally expressed only in placental trophoblasts.² Beginning only a few years after its discovery, reports began to appear indicating that PLAC1 expression is induced in a variety of human cancers.³ It was also shown that the majority of PLAC1 transcripts in cancer originate from a different promoter than do transcripts in placental tissues.^{4,5} In the placenta, PLAC1 is primarily transcribed from a promoter called P2 or “proximal” and in tumors PLAC1 is primarily transcribed from a promoter called P1 or “distal.” Additional studies have demonstrated that the placental P2 promoter is regulated by the MED1/TRAP complex⁶ while the cancer P2 promoter is largely regulated by the presence of wild-type p53 protein.⁷

It has been shown that PLAC1 is one of a number of genes whose expression can be induced by the Epstein-Barr virus.⁸ We sought to determine if PLAC1 expression is induced in cervical cancer, which is well known to have a causal viral component- human papilloma virus (HPV).⁹ Moreover, we wanted to determine if the P1, or “cancer” promoter, would drive PLAC1 expression in a virally induced cancer. We are able to report here for the first time that PLAC1 expression is in fact induced in human cervical cancers. Further, we report that induction of PLAC1 expression from the P1 promoter is significantly associated with cervical cancer histology with the more aggressive squamous cell and adenosquamous tumors producing not only more PLAC1 message but a much greater percentage of P1-driven PLAC1 message.

Subjects and Methods

Tumor procurement

Primary cervical cancer tumor tissues were obtained under informed consent (IRB#200209010) from patients undergoing surgical procedures in the Department of Obstetrics and Gynecology of the University of Iowa Hospitals and Clinics between 1991 and 2004. These tissues were identified as histologically confirmed primary cervical cancers archived in the Gynecologic Tumor Bank that is part of the Department of Obstetrics and Gynecology Women's Health Tissue Repository.¹⁰ We were able to identify 16 such tissues. All 16 samples were originally obtained as primary surgical specimens frozen following histologic examination. Tumor characteristics are shown in Table 1.

Nucleic acid purification

Total cellular RNA was purified from 0.1g of tumor specimen using the mirVana miRNA isolation kit following manufacturer's recommendations (Thermo Fisher Scientific). RNA

concentration and purity were determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific).

High molecular weight genomic DNA (gDNA) was also purified from 0.1g pieces of each tumor using the DNeasy Blood and Tissue Kit following manufacturer's recommendations (Qiagen). DNA concentration and purity were determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). In addition, DNA integrity was assessed by visualization of 100ng aliquots run on a 1.0% horizontal agarose gel.

PLAC1 and PLAC1P1 qPCR assay

Total PLAC1 expression and the proportion of that expression accounted for by the P1 promoter were determined via SYBR Green (Power SYBR Green, Thermo Fisher Scientific) qPCR assay. Fixed RNA mass aliquots (500ng) were reverse transcribed in the presence of SuperScript III reverse transcriptase (Thermo Fisher Scientific). The resulting cDNAs were then assayed in the presence of Power SYBR Green Master Mix using primers specifically designed to assess both total PLAC1 message and PLAC1P1-specific message (Table 2). PLAC1P1 primers were designed to detect both P1-specific isoforms. These assays were carried out on an Applied Biosystems Model 7900HT Real-Time PCR System in the Genomics Division of the University of Iowa Institute of Human Genetics (IHG).

Total PLAC1 copy number and percent P1-specific message (% P1) were determined for each tumor based upon linear regressions from total PLAC1- and PLAC1P1-specific standard curves. The standard curves were run in the same assays and consisted of seven serial dilutions of PLAC1- and PLAC1P1-specific target clones ranging from 10^{12} to 10^6 copies. Total PLAC1- and PLAC1P1-specific copy numbers were calculated as $PLAC1_T = 10^{(13.912 - 0.293(CtT))}$ and $PLAC1_{P1} = 10^{(14.148 - 0.306(CtP1))}$. Primer efficiencies are 0.968 for PLAC1_T and 0.946 for PLAC1_{P1}.

HPV genotyping

HPV detection was performed using the COBAS HPV test (Roche, Inc), a real-time PCR assay that uses Taqman primers and probes for the detection of 14 High Risk (HR) HPV types, including the specific identification of HPV16 and HPV18. Amplification was performed according to the manufacturers' instruction. Cycle threshold (CT) values were reported for HR HPV, HPV 16 or 18 positive specimens per instrument readout. Positive results were confirmed and genotyped using Linear Array HPV Genotyping (Roche, Inc) on a GeneAmp PCR System 9700 (Applied Biosystems) using the following conditions: 2 minutes at 50°C; 9 minutes at 95°C; (42-45 cycles) of 30 seconds at 95°C, 1 minute at 55°C, 1 minute at 72°C; 5 minutes at 72°C; 72°C indefinitely, with a ramp rate of 50%. Amplicons were detected after denaturation and hybridization to Linear Array HPV Genotyping Test Strip containing capture probe sequences complementary to the L1 gene of 37 HPV genotypes. The Linear Array HPV Genotyping Test Strip results were read blinded to confirm results.

TP53 sequencing

Tumor-specific p53 sequencing was carried out on an Applied Biosystems Model 3730xl 96-capillary sequencer in the Genomics Division of the University of Iowa Institute of Human Genetics (IIHG). 100ng gDNA aliquots were PCR amplified in four blocks using primer sequences shown in Table 2. Each block amplicon was verified in a 1.3% horizontal agarose gel and then processed for sequencing using the QIAquick PCR purification kit following manufacturer's recommendations (QIAGEN). Each amplicon was completely sequenced in both directions.

p53 Western blotting

Total protein was purified by homogenizing tumor tissue with a Fisher PowerGen 125 homogenizer in the presence of protease and phosphatase inhibitors. Final protein concentration was determined by BCA assay (Thermo Fisher Scientific)¹¹ read in duplicates on a BioRad xMark microplate spectrophotometer. Protein concentration was calculated against within-plate standards.

p53 protein expression was assessed via Western blot. Fixed protein mass (40µg) for each tumor as determined by the BCA concentrations were run on a 10% non-denaturing acrylamide gel and then transferred to a nitrocellulose membrane. p53 expression was detected with the Santa Cruz antibody sc-126 and the β-actin loading control with Sigma-Aldrich antibody A1978.

Statistical analyses

Univariate and multivariate association analyses with linear regression were carried out for both total PLAC1 expression and % P1 contribution against patient age at diagnosis and tumor variables including histologic type, stage, grade, and p53 expression. Significant variables in the univariate analyses were then included in a multivariate analysis to assess independent association. Statistical significance was accepted at $p < 0.05$.

The relationship between total PLAC1 expression and percent due to the P1 promoter was assessed using a Spearman Rank Order Correlation (www.wessa.net/rankcorr.wasp).¹² This method was chosen as the copy number value range relative to the % P1 value range caused inflated conventional correlation coefficients (see Table 3).

Results

PLAC1 expression in HPV positive cervical tumors

Standard curve-derived PLAC1 copy numbers and % P1 promoter contributions in the cervical tumor panel are shown in Table 3 and Figure 1A. There was a statistically significant positive correlation between total PLAC1 and the proportion of transcripts originating from the P1 promoter by Spearman Rank Correlation ($\sigma = 0.68$, $p < 0.01$, $df = 14$), confirming that PLAC1 expression in these tumors is driven primarily by the P1, or cancer, promoter.

Cervical cancers, and other HPV-associated cancers, are predominantly squamous cell histology with adenocarcinoma and adenosquamous types accounting for the majority of the remaining cases.¹³ We therefore examined whether PLAC1 expression is more abundant in a particular histologic subtype of cervical cancer. We observed differential PLAC1 expression by histologic type, especially with respect to % P1 promoter-driven expression (Figure 1B). Specifically, % P1 promoter-driven expression was significantly higher among squamous and adenosquamous histology versus adenocarcinomas (Figure 1B, $p < 0.01$). It should also be noted that small cell tumors are quite rare (<3%) and have the worst prognosis among all cervical cancers.¹⁴

A previous study reported on induction of PLAC1 expression in some cancers by the Epstein-Barr virus⁸ yet to date no studies have sought to examine the potential contribution of other viruses. Since the vast majority of cervical cancers are attributed to high risk HPV 16/18, we performed HPV genotyping on our tumor panel. High molecular weight genomic DNA purifications were carried out in order to test for HPV genotype. All 16 tumors were positive for the high risk HPV 16/18 types (Table 1). We eliminated the possibility of cross-contamination of these gDNAs by PCR amplification and direct sequencing of the hypervariable 1 region (HVR1) of the mitochondrial genome.¹⁵ We chose this target as it is not from the nuclear genome and is roughly the same size as HPV. Our mtDNA PCR primers are shown in Table 2. The mtDNA HVR1 sequences were unique to each individual in the panel. Thus, we are confident that the HPV typing results we obtained are not due to cross-contamination.

Inverse relationship between PLAC1 and p53 in cervical tumors

Many cancers are characterized by *TP53* mutations that result in changes in protein function or loss of protein expression altogether. Since p53 protein has been shown to suppress PLAC1 transcription at the P1 promoter⁷, we examined both *TP53* mutation status and p53 protein expression in our cervical tumor panel. Using the same gDNAs as were used for HPV genotyping, we sequenced *TP53* genomic sequence in each tumor. *TP53* mutations were found in two of the 16 tumors (Table 3). One of these was a same sense mutant (CX07; CCG→CCA; P36P) and the other was a missense mutant of as yet unknown functional significance (CX04; GAA→AAA; E286K). This result is consistent with previous reports indicating that *TP53* mutations are uncommon in cervical cancers.¹⁶

We next examined p53 protein levels and were able to obtain sufficient usable protein from 14 of the 16 tumors. Whereas *TP53* mutation was detected in only two of the tumors, nine of the 14 tumors were p53 null by Western blotting (Figure 2). Additionally, two of the 14 tumors (CX03 and CX06) expressed a very low level of protein, two more (CX04 and CX12) produced a moderate level of expression and one (CX02) had a high level of expression even after accounting for a relatively high amount of total protein loaded for that tumor. Of note, one of the tumors showing a moderate level of p53 protein expression was one with the sole missense mutation (CX04). Relative p53 expression levels were scored for signal intensity (Table 3). Using this scoring, we found that % P1 expression was negatively associated with p53 protein expression by both univariate and multivariate analyses ($p <$

0.01). Importantly, these data are in line with p53 suppression of P1 promoter-derived PLAC1 transcription.⁷

Finally, the well-known p53 P72R polymorphism has been linked with differential susceptibility to HPV 16/18 e6 protein-driven p53 protein ubiquitination and degradation.^{17,18} Consistent with what has been reported previously¹⁷, we found a significant deviation of P72R genotype frequencies from expectation based upon relevant frequencies reported in dbSNP for rs1042522 (Table 3; $p < 0.05$).

Discussion

PLAC1 expression in human solid tumors was first reported more than a decade ago.¹⁹ Since that time, induction of PLAC1 expression has been demonstrated in a wide range of cancers including hepato-cellular, colorectal, gastric, breast, prostate, ovarian and uterine tumors.³ Here, we report for the first time that PLAC1 is also expressed in HPV-positive cervical cancers encompassing four histologic types.

In addition to demonstrating that PLAC1 expression is induced, we have shown that there is a significant relationship between cervical tumor histology and the proportion of the PLAC1 transcripts originating from the P1 promoter. Tumors of the squamous cell or adenosquamous histologic type were significantly more likely to produce a high percentage of PLAC1 transcript from the P1 promoter than cervical adenocarcinomas. We also observed a trend toward a histologic association with total PLAC1 though this failed to reach statistical significance despite a significant correlation between total PLAC1 message and % P1 promoter. However, taking both total PLAC1 message and % P1 promoter into account, we conclude that squamous cell and adenosquamous cervical tumors are more likely to have both higher total PLAC1 expression and a higher percentage of that expression coming from the P1 promoter as compared to cervical adenocarcinomas. It is well known that the squamous cell and adenosquamous histologic subtypes usually have a worse prognosis than do adenocarcinomas.²⁰ Moreover, several studies of PLAC1 in human cancers have noted an association between higher relative PLAC1 expression and a poor prognosis.³ The data presented here are consistent with those observations.

We also observed a significant negative association between % P1 PLAC1 expression and the presence of p53 protein. p53 is a major component of P1-driven PLAC1 expression.⁷ Specifically, wild-type p53 suppresses transcription from the P1 promoter whereas mutant p53 de-suppresses P1-driven PLAC1 transcription.⁷ It is also well-established that the HPV 16/18 e6 protein complexes with cellular ubiquitin ligase E6AP which, in turn, binds to p53 to induce its ubiquitination and degradation.²¹⁻²³ It appears that, at least for some of the tumors analyzed here, p53 protein was absent despite wild-type *TP53* sequence. Thus, our data are consistent with published reports of p53 degradation by the HPV 16/18 e6/E6AP complex. Other studies have suggested that none of the common p53 mutants affect HPV-driven degradation.²³ However, one p53 mutant (R290E) does appear to block the binding complex.²³ Possibly relevant to this observation is that the tumor in our panel, CX04, containing an E286K mutation, has moderate p53 protein levels, suggestive of sustained p53 expression. Further study is required to determine why four cervical tumors are both *TP53*

wild-type and p53 protein positive in spite of being HPV 16/18-positive. Also of interest is the high proportion of cervical tumors with the P72R polymorphism in *TP53*, which we detected at a higher frequency than was predicted based on the dbSNP frequency of this polymorphism in the general population. The role of the P72R polymorphism in PLAC1 expression and HPV 16/18 e6/E6AP complex association remains unclear and warrants further study.

Stimulated by the report that Epstein-Barr Virus can induce PLAC1 expression in some cancers, we carried out an exploratory study of PLAC1 expression in another virus connected cancer- cancer of the cervix. PLAC1 expression was present in all 16 tumors spanning the four major cervical cancer histologic subtypes comprising our panel. Further, the proportion of total PLAC1 expression transcribed from the P1, or cancer, promoter was significantly associated with tumor histologies that carry a poorer prognosis. There was also a significant negative association between P1-driven PLAC1 transcription and the presence of wild-type p53 protein. Finally, our data suggest that P1-driven PLAC1 expression in cervical cancers may be governed, at least in part, by HPV 16/18 e6/E6AP complex-mediated p53 degradation.

The results presented here, while clearly limited by the small sample size available, point to a PLAC1/p53/HPV axis in cervical cancer. The mechanistic details of the suggested PLAC1/p53/HPV relationship should be explored both in an expanded panel of cervical cancers as well as *in vitro* using relevant cervical cancer cell lines. Elucidation of these details could eventually inform treatment decisions not only for cervical cancer patients but for all patients afflicted with cancers known to be associated with HPV exposure, including oropharynx and other genital cancers.¹³

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Figure 1.

Analysis of PLAC1 expression in cervical tumors. A. PLAC1 and % P1 promoter-driven expression by cervical cancer histology. Green: squamous cell tumors; Purple: adenosquamous subtype; Yellow: adenocarcinomas; Blue: small cell subtype. B. Average PLAC1 and % P1 promoter-driven expression by cervical cancer histology. Green: squamous cell tumors; Yellow: adenocarcinomas; Striped: combined squamous cell and adenosquamous tumors. ** $p < 0.01$.

Figure 2.

Analysis of p53 protein levels in cervical tumors. Expression of p53 was assessed by Western blot. TP53 mutation status and P72R genotype are also shown; GG: P72; CC: R72; CG: heterozygous for P72R polymorphism. β -actin, loading controls.

Table 1

Tumor characteristics.

Tumor ID	Pt. Age	Histology	Stage	Grade	HPV Type
CX01	44	Squamous Cell	IB	---	16/18
CX02	47	Squamous Cell	---	---	16/18
CX03	42	Squamous Cell	IB	3	16/18
CX04	60	Adenocarcinoma	IB1	---	16/18
CX05	50	Squamous Cell	IB	---	16/18
CX06	70	Squamous Cell	IB1	---	16/18
CX07	32	Small Cell	IB	---	16/18
CX08	82	Adenocarcinoma	IB1	1	16/18
CX09	48	Squamous Cell	IB2	3	16/18
CX10	50	Squamous Cell	IB2	3	16/18
CX11	86	Adenocarcinoma	IIB	---	16/18
CX12	63	Adenocarcinoma	IB1	3	16/18
CX13	57	Adenocarcinoma	IIA	3	16/18
CX14	38	Adenosquamous	IB2	1	16/18
CX15	34	Adenosquamous	IB2	3	16/18
CX16	39	Adenocarcinoma	IB2	3	16/18

Table 2

Primers used for PCR, qPCR and sequencing.

Amplicon	Size	Sequence ^a
PLAC1	232bp	For: 5'-CACCAGTGAGCACAAAGCCACATT-3' Rev: 5'-CCATGAACCAGTCTATGGAG-3'
PLAC1P1	370bp	For: 5'-AAACTTACACGAGGAGTCTGTC-3' Rev: 5'-CTGTGACCATGAACCAGTCTAT-3'
p53 Block1 ^b	908bp	For: 5'-CAGACACTGGCATGGTGTT-3' Rev: 5'-ATATTCAACTTTGGGACAGGAGT-3'
p53 Block2 ^b	661bp	For: 5'-TGTAGACGCCAACTCTCTCTA-3' Rev: 5'-AACCCATTACTTTGCACATCTC-3'
p53 Block3 ^b	1002bp	For: 5'-TCATCTTGGGCCTGTGTTAT-3' Rev: 5'-AAAGCTGGTCTGGTCCTTIA
p53 Block4 ^b	1412bp	For: 5'-GGTACTTGAAGTGCAGTTTCTACT-3' Rev: 5'-GTGCAGGCCAACTTGTTC-3'
mtDNA HVR1 ^c	447bp	For: 5'-TCCACCATTAGCACCCAAAGCTA-3' Rev: 5'-ATTGATTTCACGGAGGATGG-3'

^a All primers were designed using PrimerQuest software available on-line at Integrated DNA Technologies (www.idtdna.com). All primers were synthesized by IDT as well.

^b Block 1 contains Exons 2-4, Block 2 contains Exons 5-6, Block 3 contains Exons 7-9, Block 4 contains Exons 10-11.

^c Based upon the Revised Cambridge Reference Sequence.

Table 3
PLAC1 and p53 expression and sequencing data for the cervical tumor panel

Tumor ID	PLAC1 Copies ^a	% P1	TP53 Status	p53 Expression ^b	P72R ^c
CX01	48977882	0.99	wt	nd	GG
CX02	724436	0.00	wt	+++	GG
CX03	4365158	0.48	wt	++	GG
CX04	16982437	0.12	E286K	++	CC
CX05	8912509	0.86	wt	0	GC
CX06	5888437	0.76	wt	+	GG
CX07	3548134	0.63	P36P	0	CC
CX08	1548817	0.25	wt	0	GC
CX09	31622777	0.88	wt	0	GC
CX10	112201845	0.98	wt	0	GG
CX11	691831	0.00	wt	0	GG
CX12	22908677	0.15	wt	++	GG
CX13	5128614	0.58	wt	0	GG
CX14	933254	0.75	wt	0	GG
CX15	6760830	0.69	wt	0	CC
CX16	1621810	0.00	wt	nd	GG

^aCopy number per 500ng of total cellular RNA.

^bp53 protein levels were scored as negative (0), low (+), moderate (++) or high expression (+++); nd: not determined based on insufficient total protein from tumor lysate.

^cExpected genotype frequencies based upon HapMap Caucasian allele frequencies C = 0.23; G = 0.77 as reported in dbSNP for rs1042522. Chi-square (2) = 7.4; p = 0.05.