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Abstract

Steroid hormones, estrogen and progesterone, and their intracellular receptors play an important role in the development and progression of breast cancer. Coactivator proteins modulate the biological activity of these hormone receptors. We have cloned and E3 ubiquitin-protein ligand-activated enzyme, E6-associated protein (E6-AP) as coactivators of steroid hormone receptors. The purpose of this research is to explore the possibility that the altered expression of E6-AP may contribute to the development of breast cancer. We have examined this possibility by studying the expression patterns of E6-AP and estrogen receptor-alpha (ERa) in various human breast cancer cell lines breast tumor biopsy samples. Additionally, we have correlated the expression profile of E6-AP as that of ER in breast tumor biopsies. To date, we have examined 13 samples of invasive breast cancer (IBC), 12 samples of ductal carcinoma in situ (DCIS) and a tissue array with 36 different stages breast cancer samples by immunhistochemistry, and 19 samples by immunpfluorescence. We found inverse correlation between the expression of E6-AP and the expression of ER in these tumors. Furthermore, E6-AP is down regulated in invasive breast tumors compared with their adjacent tissues, whereas the downregulation of E6-AP was not seen in DCIS. The downregulation of E6-AP is stage-dependent. These data suggest a possible role of E6-AP in mammary gland development and tumorigenesis. We also proposed to generate stable cell lines which overexpress either wild-type or liagse-mutated E6-AP and test its tumorigenecity both in vitro and in vivo. However, we weren't able to finish the task on time due to technical problems.

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Introduction

Breast cancer is the most prevalent form of cancer (excluding skin cancer) among females in the United States (US). It is anticipated that one out of ten women will present with breast cancer at some point during her lifetime (1). The predominant treatment for patients with breast cancer is endocrine therapy, but these therapies are ineffective in some patients. Moreover, many patients, who initially respond to endocrine therapy, develop resistance later (2-6). Therefore, it is critical to identify the molecular mechanisms associated with breast cancer and with the development of endocrine-resistant tumors.

The steroid hormones, estrogen and progesterone, play a major role in the development of normal mammary gland and in breast tumor development (7-9). These molecules mediate their signaling through intracellular receptors called estrogen (ER) and progesterone (PR) receptors. ER and PR are members of a family of structurally related ligand-activated transcription factors (10, 11). These factors contain common structural motifs, which include a less well-conserved amino-terminal activation function (AF-1) that affects transcription efficiency, a central DNA-binding domain, which mediates receptor binding to specific DNA enhancer sequences and determines target gene specificity, and a carboxy-terminal hormone-binding domain (HBD). The HBD contains activation function-2 (AF-2), the region, which mediates the hormone-dependent activation function of receptors (11). In order to activate gene transcription, ER and PR undergo a series of well-defined steps. When bound to hormone, these receptors undergo a conformational change, they dissociate from cellular chaperones, dimerize with each other, phosphorylate, interact with coactivators, bind to the promoter region of the target gene, and subsequently recruite basal transcription factors to form a stable preinitiation complex (PIC). These steps are followed by up- or down-regulation of target gene expression (12).

Coactivators represent a growing class of proteins, which interact with receptors in a ligand-specific manner and serve to enhance their transcriptional activity. A number of coactivators have been cloned to date, including SRC-family members (13, 14), TIF2 (GRIP1) (15-18), p/CIP (ACTR/RAC3/AIB1/TRAM-1) (19-22), PGCs (23), SRA (24, 25), CBP (26, 27) and **E6-associated protein (E6-AP),** etc. and this list is still growing rapidly. Coactivators were originally envisioned to serve a bridging role, linking the receptor to the basal transcription machinery (28, 29). Recently, the functional role of coactivators has expanded by the observation that they have been shown to possess enzymatic activities, which contribute to their ability to enhance receptor-mediated transcription. SRC-1, p300/CBP, and RAC3/ACTR/AIB1 possess histone acetyl transferase activity (HAT) (19, 20, 30-32); E6-AP and RPF1/RSP5 contain ubiquitin-protein ligase activity (33, 34); and SUG1/TRIP1 contains ATPase activity (35). Ligand-activated receptors are thought to bring these activities to the promoter region of the target genes and presumably manifest part of their coactivation functions through these enzymatic activities. Because of their ability to enhance receptor mediated gene expression, coactivators are thought to play an important role in regulating the magnitude of the biological response to steroids, vitamin D, and retinoids in different tissues. The level of coactivator expression may contribute to variations in hormone responsiveness

seen in the population and disruption in coactivator expression could lead to the pathologically hyper- or hypo-sensitivity to steroid hormones. The finding that disruption of the SRC-1 locus in mice resulted in an attenuated response to steroid hormones is consistent with this hypothesis (14).

Recently, our laboratory has identified ubiquitin pathway enzymes as coactivators of the nuclear hormone receptor superfamily. **We have cloned an E3 ubiquitin-protein ligase, E6-AP, as steroid hormone receptor interacting protein using a yeast two-hybrid screening assay (34). E6-AP enhances the hormone-dependent transcriptional activity of steroid hormone receptors, PR, ER, androgen (AR) and glucocorticoid receptors (GR).** E6-AP was previously identified as a protein of 100 kDa (36), present both in the cytoplasm and the nucleus. E6-AP mediates the interaction of human papillomaviruses type 16 and 18 E6 proteins with p53, a growth-suppressive and tumor-suppressive protein. The E6/E6-AP complex specifically interacts with p53 and promotes the degradation of p53 via the ubiquitin-proteasome protein degradation pathway (37). E3 enzymes have been proposed to play a major role in defining the substrate specificity of ubiquitin system (36, 38-41). Protein ubiquitination also involves two other classes of enzymes, namely the E1 ubiquitin activating enzyme (UBA) and E2 ubiquitin conjugating enzymes, UBCs. Firstly, ubiquitin is activated by UBA in an ATP-dependent manner, then the activated ubiquitin forms a thioester bond between the carboxyl-terminal glycine residue of ubiquitin and a cysteine residue of the UBA. Next, ubiquitin is transferred from the E1 to one of the several E2s (UBCs), preserving the high-energy thioester bond (1, 42-45). In some cases, ubiquitin is transferred directly from E2 to the target protein through an isopeptide bond between the Ú-amino group of lysine residues of the target protein and the carboxyl-terminus of ubiquitin. In other instances, the transfer of ubiquitin from UBCs to target proteins proceeds through an E3 ubiquitin-protein ligase intermediate such as E6-AP (41). The carboxyl-terminal 350 amino acids (aa) of E6-AP contains a "*hect*" (homologous to the E6-AP carboxy terminus) domain, which is conserved among all E3 ubiquitin protein-ligases and E6-AP related proteins characterized to date (46) 48). The extreme carboxyl-terminal 100 aa of E6-AP contains the catalytic region, which transfers ubiquitin to the protein targeted for degradation. We have shown that the ubiquitin-ligase activity of E6-AP is not required for the coactivation function of E6-AP (34). It has been shown that the conserved cysteine (C) 833 residue in E6-AP forms a thioester bond with ubiquitin and is necessary for the transfer of ubiquitin to the proteins targeted for ubiquitination. The mutation of C833 to alanine (A) or serine (S) has been shown to eliminate the ubiquitin-protein ligase activity of E6-AP (41). **In cotransfection studies, we showed that an E6-AP bearing a C-to-S mutation at the critical site was still able to coactivate steroid hormone receptors.** These findings indicate that E6-AP possesses two independent, separable functions, coactivation and ubiquitin-protein ligase activity.

The role of E6-AP in mammary gland functions has not been studied yet. Considering, the influence of E6-AP as a coactivator on transactivation of target genes by ER and PR and also as an E3 ubiquitin-protein ligase, we are interested in studying the role of E6-AP in the development and progression of breast cancer. A large amount of evidence suggests that breast tumor development may involve coactivators of steroid hormone receptors, especially those of ER and PR. It has been shown that altered expression of one

nuclear receptor coactivator, AIB1, contributes to the development of hormone-dependent breast and ovarian cancers (19), while HER-2 *neu*(47) and Cyclin D are involved in breast cancer development(48, 49). Interaction of AIB1, SRC-1, TIF2, and p/CIP with CBP/ p300 is important for the coactivation function (19). Thus, overexpression or loss of expression of any of these coactivators could potentially perturb signal integration by CBP/ p300 and affect multiple transduction pathways. It has also been shown that another steroid receptor coactivator, SRA is also elevated in breast tumors (25, 50). **Furthermore, we have recently shown that E6-AP is overexpressed in 90-95% of tumors using a mouse model of multistage mammary tumorigenesis developed by Medina et al (51, 52). Additionally, our data from human breast cancer biopsy samples shows that the majority of the advanced stage human breast tumors express high levels of E6-AP protein.** Since E6-AP is an E3 ubiquitin-protein ligase and recently, we have shown that ER is degraded through the ubiquitin-proteasome pathway (53), we also analyzed the expression profile of ER in human advanced stage breast cancers and compared it with that of E6-AP. **We found an inverse correlation between the expression of E6-AP and the expression of ER in these tumors. The Spearman Rank Correlation Coefficient is 0.38 and the p value is 0.004, indicating that this correlation is statistically significant.**

Body

In this original proposal, we hypothesized that the E3 ubiquitin-protein ligase, E6-AP, is an important modulator of the steroid hormone receptor-mediated signal transduction pathway, cell growth, and cell cycle control in the context of breast cancer development. In order to test this hypothesis we propose following objectives:

Aim 1. Expression analysis of endogenous E6-AP in human breast cancer samples and in human breast cancer cell lines, and comparison of the expression pattern of E6-AP with that of endogenous ER.
Aim 2. Generation of stably transfected breast cancer cell lines that overexpress wild-type and ubiquitin-protein ligase defective mutant E6-AP.
Aim 3. Analysis of the growth properties of stably transfected cell lines and in vivo analysis of tumorigenicity of these stably transfected cell lines in athymic nude mice.

Task 1. Expression analysis ER and E6-AP in different breast cancer cell lines.

Since we want to compare the expression profile of E6-AP with that of ERα, we performed dual fluorescent immunocytochemistry for MCF-7, T47-D, ZR75-1 and MDA-MB-231 cell lines. HeLa cell line was used as a negative control. Positive signal for ER is seen as green staining, whereas E6-AP is seen as red. As shown in Figure 1, ERα is expressed in MCF-7, T47D and ZR-75-1 breast cancer cell lines, which are known as ER positive, but it is negative in the HeLa cells and MDA-MB-231 cell line, which is known as ERα negative. The ER expression is nuclear in these cell lines. On the other hand, E6-AP is expressed in all the four breast cancer cell lines as well as in Hela cells. The E6-AP expression is both cytoplasmic and nuclear in MCF-7, ZR75-1 and MDA-MB-231 cell lines. The MDA-MB-231 cell line expresses more E6-AP in nucleus than in the

cytoplasm. The expression of E6-AP in T47-D cells is mainly nuclear. In this case HeLa cells were used as a positive control for E6-AP expression.

Task 2. Effect of steroids on the expression of E6-AP.

It is possible that steroid hormones (estrogens/progesterones) may regulate endogenous expression of E6-AP in breast cancer cell lines. To test this possibility, MCF-7, a hormone-dependent breast cancer cell line, was grown in the medium containing stripped serum for a week. Afterward, cells were grown either in the absence or presence of steroid hormones for 48 hours and the expression patterns of E6-AP were determined by fluorescent immunocytochemistry. Figure 2 suggests that the estrogen treatment have no significant effect on the expression of E6-AP. The E6-AP expression levels are identical both in the presence and absence of hormone. This data suggests that E6-AP regulation is not under the control of steroids.

As a control for these experiments, we also analyzed the effect of estrogen on the expression of PR and ER. It has been established that estrogen upregulates the expression of PR protein and it downregulates the levels of ER in MCF-7 cells (54). As expected, Figure 3 demonstrated that estrogen treatment increases the expression of PR protein. In contrast, estrogen down regulates ER expression.

Task 3. Expression analysis of E6-AP and ER in breast tumor samples.

As mentioned above, the ubiquitin pathway enzyme, E6-AP acts as a coactivator of steroid hormone receptors. Furthermore, we have also demonstrated that the ER protein, which is a major modulator of normal mammary gland development and breast tumor development, is rapidly degraded in mammalian cells in an estrogen-dependent manner via the ubiquitin-proteasome pathway. Additionally, our *in vitro* studies suggest that ER degradation observed in mammalian cells is dependent on the ubiquitin-proteasome pathway (53). Besides, Western blot analysis of advanced stage human breast cancer samples found varied levels of expression of E6-AP and an inverse correlation between the expression of E6-AP with that of ER. To further explore the possibility that the altered expression of E6-AP may contribute to the development of breast cancer, we analyzed immunohistochemically the expression of E6-AP in invasive breast cancer (IBC) and ductal carcinoma in situ (DCIS) samples and compared their expression with their adjacent normal breast tissues. We also analyzed the expression levels of E6-AP in different stages breast cancer samples by immunohistochemistry. To compare the expression of E6-AP with that of ERα, dual immunofluorescent staining was applied.

A. Immunohistochemistry

In order to study the expression profile of E6-AP in breast tumors and in normal breast tissues, we performed immunohistochemical analysis. Figure 4 shows a representative breast cancer case. The normal tissue, DCIS and IBC were all found in one slide, making them ideal controls for each other. In normal human breast tissues, E6-AP is highly expressed in the cytoplasm of the ductal epithelial cells. Compared with the normal

tissue, the immunostaining of E6-AP is greatly decreased in the IBC, whereas there was no significant change of E6-AP expression in the DCIS. We analyzed 13 cases of IBC and 12 cases of DCIS with their adjacent normal tissues. To compare the expression of E6-AP in tumors with that in normal tissues, the immunostaining results were evaluated using automated cellular imaging system (ACIS, Chroma Vision Medical Systems, Inc., San Juan Capistrano, CA). This system combines color based imaging technology with automated microscopy to provide quantitative information on intensity of staining (and if desired the percent of positively stained cells). **Figure 5** summarizes the scanning results of E6-AP immunostainig in IBC and their adjacent normal tissues. "**A**" is a table showing the intensity of E6-AP immunostaining in both IBC and normal tissues from each of the 13 breast cancer cases. "**B**" is a bar chart comparing the intensity of E6-AP immunostaining in normal and tumor tissues side by side. All of the 13 IBC samples express reduced level of E6-AP compared with their adjacent normal tissues. In average, there is a 25% decrease of E6-AP expression in tumor than in the normal tissues. Student paired *t*-test indicates that the difference is statistically significant (p=0.000001). **Figure 6** summarizes the scanning results of E6-AP immunosaining in DCIS and their adjacent normal tissues. "**A**" is a table showing the intensity of E6-AP immunostaining in both IDCIS and normal tissues from each of the 12 breast cancer cases. "**B**" is a bar chart comparing the intensity of E6-AP immunostaining in normal and tumor tissues side by side. Unlike in IBC, the expression level of E6-AP is not significantly different from that of their normal tissues (p=0.9397). Taken together, these results indicate that the downregulation of E6-AP in breast cancers is a relatively late event.

Next, we performed immunohistochemical staining of E6-AP on a microarray breast cancer slide which contains 36 different stages breast cancer samples. As shown in **Figure 7**, the immunostaining of E6-AP was graded manually according to the overall density of brown color in each sample, with the highest level as 4, the lowest as 0 and the intermediate as 1, 2 and 3. In comparison to the normal breast tissues, the levels of E6-AP in cancers were mostly downregulated. However, E6-AP expression goes down at Stage I, and then goes up a bit at Stage IIA, after that, its expression goes down again. To analyze the differences between different stages of breast cancers, Wilcoxon rank-sum test was used, as shown in **Figure 8**. Combined with Table 1, it is observed that the expression of E6-AP is decreased gradually from stage I to stage IIB, which is the lowest point, suggesting a possible role of E6-AP in the progression of breast tumors. These results suggested that the expression of E6-AP is stage-dependent and the changes of its expression levels might be involved in the progression of breast carcinomas.

B. Immunofluorescence

Combining the data from Western blot and Immunohistochemistry analysis, it is suggested that E6-AP is down regulated in breast tumors and the expression of E6-AP is correlated with that of ER alpha. To confirm this, we further performed dual color immunofluorescence to analyze the expression of E6-AP with that of ER. As shown in Figure 9, E6-AP and ER is differently expressed in tumors and in normal tissues: (1) ER is expressed in the nucleus, whereas E6-AP is expressed in the cytoplasm; (2) In normal tissues, ER is discontinuously expressed in the epithelial cells, whereas E6-AP is

ubiquitiously expressed in the epithelial cells; (3) In tumor tissues, ER is highly and ubiquitiously expressed in the epithelial cells, whereas the expression of E6-AP is low. Negative control was included in the experiment by omitting the primary antibody. This result further indicated that the inverse correlation of E6-AP with ER in breast tumors does exist. A total of 19 human breast cancer samples were analyzed by dual immunofluorescence using antibodies aganist E6-AP and ER. The expression levels of E6-AP and ER were artificially graded, which is shown in **Figure 10**. Wilcoxon Rank Correlation Coefficient is 0.503, $p<0.05$, indicating an inverse correlation between the expression of E6-AP and ER.

Task 4. Generation of the expression plasmids for overexpression of E6-AP.

One of the goals of this proposal is to construct expression plasmids for either wild-type E6-AP or ubiquitin-protein ligase defective, C833S mutant E6-AP in order to make stable cell lines, which will overexpress wild-type or C833S mutant E6-AP proteins in breast cancer cell lines. As suggested in the proposal, we first cloned the relevant cDNAs of wild-type or mutant E6-AP into the mammalian expression vector pcDNA3.1 and attempted several times in transfecting the plasmids into MCF-7 cells. However, the transfection was not successful. We therefore switched to a regulable gene expression system by using Tet-Off-IN system (Clontech Laboratories). This system is composed of two critical components. The first component of the system is the **regulatory protein,** based on TetR (Tet repressor protein). In the pRevTet-Off-IN System, the amino acids 1–207 of TetR and the C-terminal 127 a.a. of the Herpes simplex virus VP16 activation domain were fused, forming a 37-kDa fusion protein. Addition of the VP16 domain converts the TetR from a transcriptional repressor to a transcriptional activator, and the resulting hybrid protein is known as the tetracycline-controlled transactivator (tTA). tTA is encoded by the pRevTet-Off-In regulator plasmid (**Figure 11**), which also includes a neomycin-resistance gene to permit selection of stably transfected cells. The second component of the system is the **response plasmid (Figure 11),** which expresses a **gene of interest (Gene X)** under the control of the tetracycline-response element, or TRE. The TRE, which consists of seven direct repeats of a 42-bp sequence containing the tetO (tet operator sequences), located just upstream of the minimal CMV promoter (P_{minCMV}), which lacks the strong enhancer elements normally associated with the CMV immediate early promoter. Because these enhancer elements are missing, there is extremely low background expression of Gene X from the TRE in the absence of binding by the TetR domain of tTA. When cells contain both the regulatory (pRevTet-Off-In) and the response (pRevTRE) vectors, Gene X is only expressed upon binding of the tTA protein to the TRE (**Figure 12**). In the Tet-Off system, tTA binds the TRE and activates transcription in the absence of Tc (Tetracycline) or Dox (Doxycyclin). Therefore, the transcription of Gene X is turned on or off in response to Dox in a precise and dose dependent manner.

One possible reason that we were not successful in generating stable cell lines using pcDNA3.1 vector is that constant overexpression of E6-AP is harmful to the cells so that those cells which were transfected could not survive eventually. The Tet-Off-In system allows us to turn on and off the transcription of our genes according to our needs,

therefore, it is a better expression system than the previous one. We cloned the wild-type and C833S mutant E6-AP cDNAs into the multiple cloning site of pRevTRE response plasmid. The E6-APs were tagged with four repeats of flag sequences at the N-termini, therefore the recombinant proteins can be easily detected with an anti-flag antibody. In order to confirm whether the system works properly, we transiently transfected both regulator and response plasmids into Hela cells. Briefly, Hela cells were maintained in DMEM supplemented with 10% fetal bovine serum. Twenty-four hours before transfection, $1x10^6$ cells were plated in three 10cm Falcon dishes. Cells in two of the three dishes were transiently transfected with 5μg of pRevTet-Off-In and pRevTRE/E6AP-WT by using Fugene 6 transfection reagent (Roche). The third dish of cells were not transfected and used as a control. Dox was added to one of the two transfected dishes 4 hrs later. After 24 hr, cells were harvested and lysed and equal amounts of proteins were resolved by a 10% SDS-PAGE then transfered to nitrocellulose membrane. The expression of recombinant E6-AP was detected using an anti-Flag antibody. As shown in **Figure 12**, in the untransfected Hela cells (-), no Flag was detected. When both pRevTet-Off-IN and pRevTRE/E6AP were introduced to the cells, a very strong signal of Flag was detected in the absence of Dox (-Dox), which represents the expression of recombinant E6-AP protein. The expression of this protein was inhibited when Dox was added to the culture (+Dox). This result indicated that the constructed plasmids were working as expected.

In order to introduce the controllable expression system into MCF-7 cells, firstly, the regulator plasmid, pRevTet-Off-IN, need to be transfected into MCF-7 cells. After selection with neomycin, the neomycin-resistant cells are expanded and used for the second step transfection with the response plasmid, pRevTRE/E6AP(WT) or pRevTRE/E6AP(Mu). The cells expressing both pRevTet-Off-IN and pRevTRE/E6AP(WT) or pRevTRE/E6AP(Mu) will survive Hygromycin selection.

For selection of stably transfected cells, the sensitivity of MCF-7 cells to G418 and Hygromycin were determined first. MCF-7 cells, which were maintained in DMEM supplemented with 10% fetal bovine serum, were plated at $1x10^5$/well in 12-well tissue culture plates. After 24 hours, either G418 (0, 50ug/ml, 100ug/ml, 200ug/ml, 400ug/ml, 600/mlug, 800ug/ml, 1mg/ml) or Hygromycin (0, 25ug/ml, 50ug/ml, 100ug/ml, 200ug/ml, 400ug/ml, 600ug/ml, 800ug/ml) were added to the culture medium respectively. Cells were cultured for 2 weeks with medium change every 3-4 days and the optimal concentration of the antibiotics was determined as the lowest concentration which kills most of the cells at 9-10 days and completely deleted cells in two weeks. For G418, it was determined as 400ug/ml, and for Hygromycin, it was 50ug/ml.

For the transfection of the pRevTet-Off-In plasmid, MCF-7 cells were cultured in 150mm Falcon tissue culture dishes. Cells were trypsinized and washed. The cell pellet of $1x10^7$ was resuspended in 400ul of DMEM containing 20% FBS. Cells were kept on ice and electroporated at 250 mF and 500 V using a GenePulser II (BioRad). The cells were plated into 10cm Falcon plate and cultured at 37°C. G418 was added to the medium the nest day after transfection. Every 3 to 4 days, the cell culture medium was changed with fresh medium containing 400ug/ml of G418. G418-resistant cells forming colonies were picked up and expanded in culture and used for the further study.

For the past few months, I had been working on introducing the response plasmid, pRevTRE/E6AP(WT) or pRevTRE/E6AP(Mu) into the regulator plasmid stably expressed

cells. Unexpectedly, I had encountered some difficulties in getting hygromycin-resistant cells after electroporation. As explained above, this pRevTet-OFF-In system needs the presence of Dox to inhibit the expression of exogeneous gene expression (E6-AP, in this case). It seems the timing of adding Dox to culture is critical. If Dox is added too early, it will kill the cells that were already injured by the electroporation; if added too late, the cells may not survive the pressure of overexpressed E6-AP as it does in the constantly expression system. I am currently trying to find the best time in adding Dox in order to obtain double stable cell lines. Because of these technical problems, I was not able to finish our proposed tasks as scheduled.

Statement of work accomplished/in progress

Task 1. Expression analysis of ER and E6-AP in different breast cancer cell lines. **Accomplished.**
Task 2. Effect of steroids on the expression of E6-AP. **Accomplished.**
Task 3. Expression analysis of ER-alpha and E6-AP in breast tumor samples. **Accomplished.**
Task 4. Generation of the expression plasmids for overexpression E6-AP. **Accomplished.**
Task 5. Development of stable cell lines. **In progress.**
Task 6. Characterization of stable cell lines. **Not Attempted Yet.**
Task 7. Determination of growth properties of stable cell lines. **Not Attempted Yet.**
Task 8. Determine the tumorigenicity of stably transfected cell lines in athymic nude mice. **Not Attempted Yet.**

Key Research Accomplishments

- Expression analysis of ER and E6-AP in different breast cancer cell lines has been completed.
- Effect of steroids on the expression of UbcH7 and E6-AP has been studied.
- Expression of ER and E6-AP has been analyzed.
- Expression profile of E6-AP has been compared with that of ER expression.
- Generation of the expression plasmids for overexpression of UbcH7 and E6-AP has been completed.
- Development of stable cell lines in progress.

Reportable Outcomes

1. An article regarding the roles of coactivators, including E6-AP, in cancers, has been published in Molecular Cancer in November, 2002 (see appendix 2).
2. A poster entitled "E6-associated protein, E6-AP is involved in the carcinogenesis of human breast and prostate" was posted at the 95th AACR annual meeting (see appendix 3).
3. An article entitled 'Decreased expression of E6-AP in breast and prostate carcinomas' has been published in Endocrinology (see Appendix 4).

Conclusions

We have successfully analyzed the expression of E6-AP and ER in different breast cancer cell lines. Additionally, we have also examined the effects of steroids on the expression profile of E6-AP and ER. In order to study the expression profile of E6-AP and ER in human breast tumors, we have examined 13 samples of invasive breast cancer (IBC), 12 samples of ductal carcinoma in situ (DCIS) and a tissue array of 36 breast cancer samples by immunhistochemistry, and 19 samples of invasive breast carcinoma by immunofluorescence. We found an inverse correlation between the expression of E6-AP and the expression of ER in these tumors. Furthermore, E6-AP is down regulated in IBC compared with their adjacent normal tissues, whereas the downregulation of E6-AP was not seen in DCIS. The downregulation of E6-AP in breast cancers is stage-dependent. These data suggest a possible role of E6-AP in mammary gland development and tumorigenesis. We have attempted to create novel in vitro models in stable cell lines, which will overexpress E6-AP in a controllable manner. Although we succeeded in the construction of the expression plasmid and the inducible expression system worked well in the Hela cells, we encountered technical difficulties in obtaining double stable MCF-7 cells, which prevented us from completing the tasks as proposed. We are still trying everything possible to solve the problem.

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Appendix I

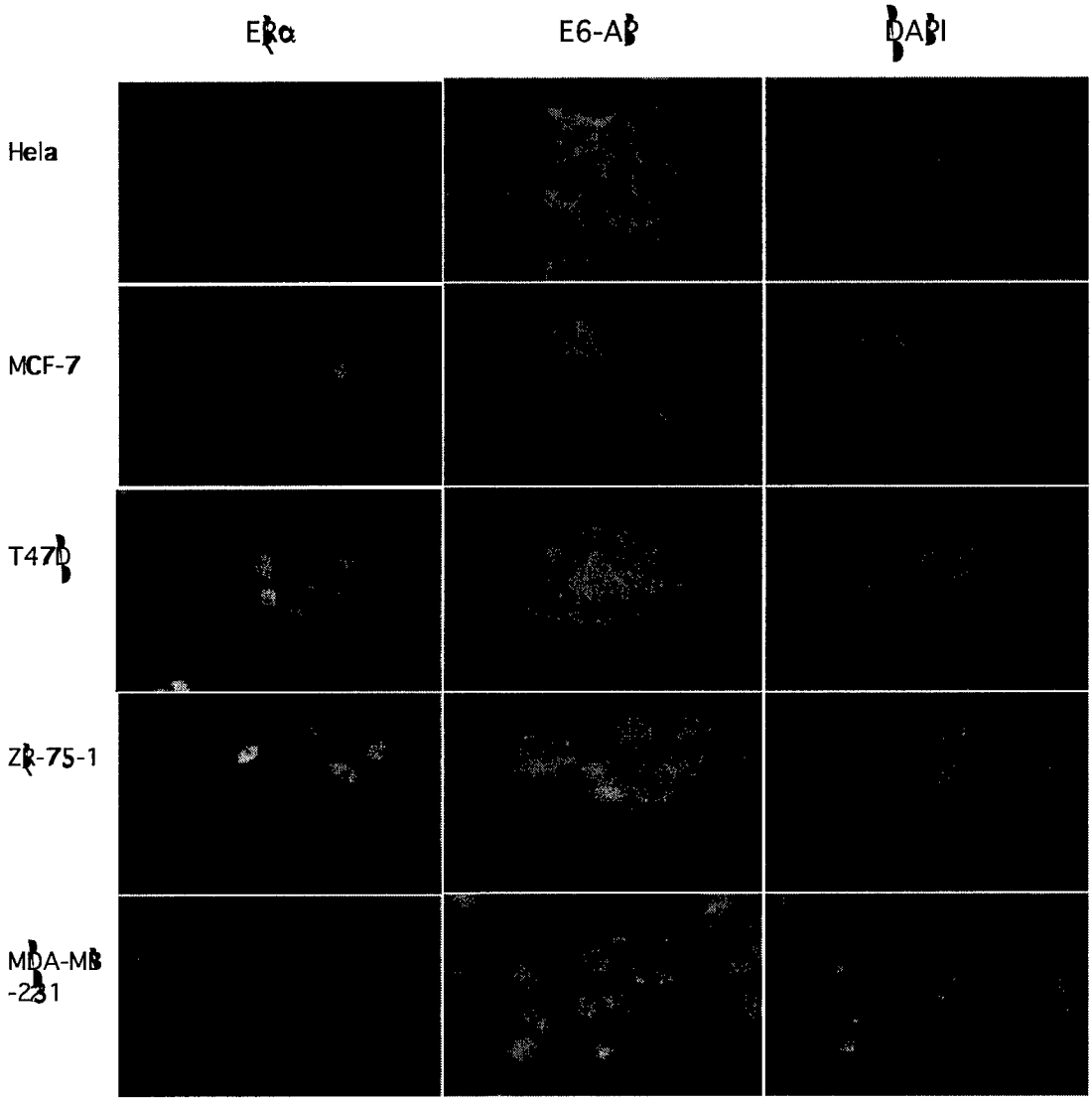


Figure 1: Expression analysis of ERα and E6-AP in different cell lines (7, T47D, ZR-75-1, and MDA-MB-231). Cells were grown on a chamber slid 24 hours and expression of ERα and E6-AP was analysed by immunocytochemistry using an anti-ERα antibody (6F11 from an antibody against E6-AP. Positive signal for ER is seen as green spot and positive signal for E6-AP is seen as red spot. DAPI staining was used to localization of nucleus. ERα, ERα expression profile; E6-AP, profile; DAPI, DAPI staining for nucleus.

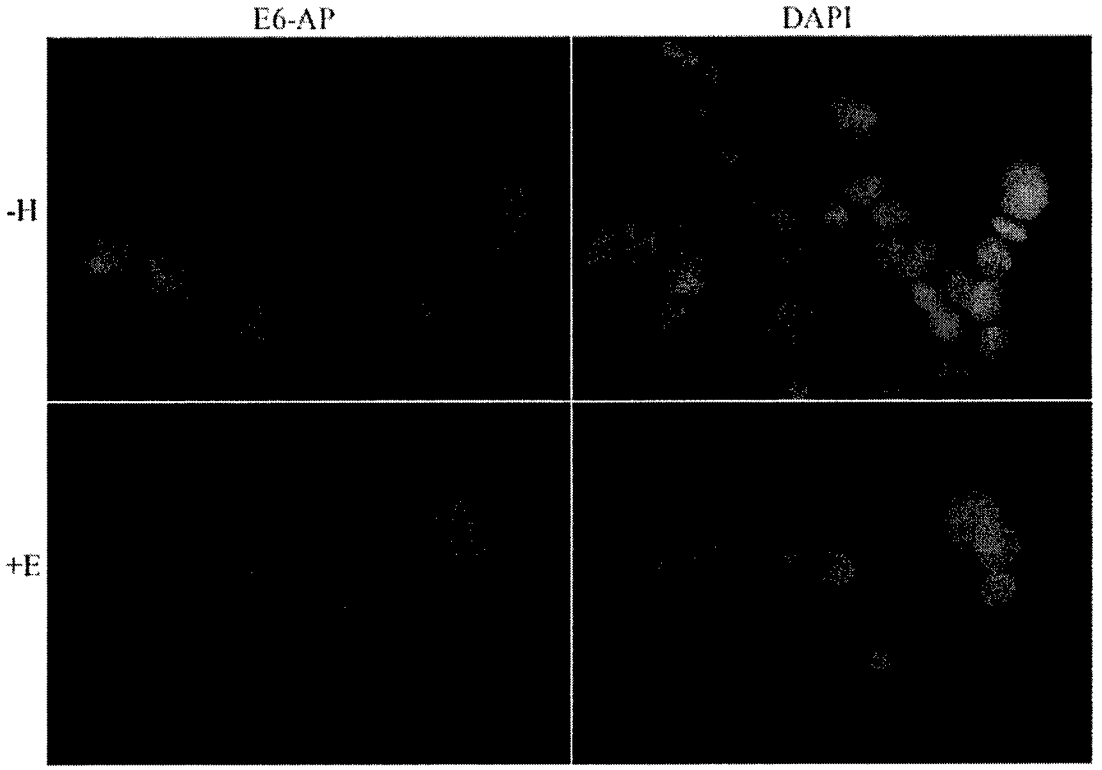


Figure 2: Effect of estrogen on the expression of E6-AP in MCF7 cells. Cells were grown on a chamber slide either in the absence (-H) or in the presence of estradiol (+E). 24 hrs after hormone treatment, the expression of endogenous E6-AP was analyzed by fluorescent immunocytochemistry using an anti-E6-AP antibody. Positive signal for E6-AP is seen as (red) spots and nucleus is seen as (blue) spots in DAPI staining. E6-AP, E6-AP expression profile; DAPI, DAPI staining for nucleus.

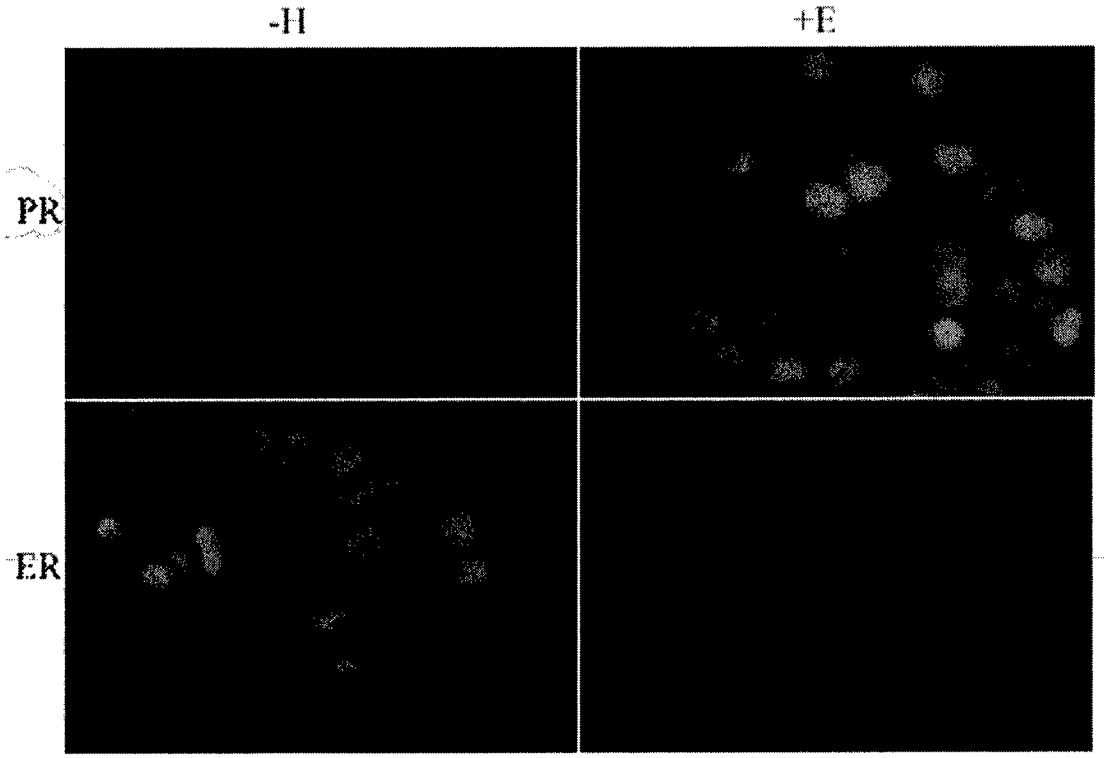


Figure 3: Effect of estrogen on the expression of PR and ER-alpha in MCF7 cells. Cells were grown on a chamber slide either in the absence (-H) or in the presence of estradiol (+E). 24 hrs after hormone treatment, the expression of endogenous PR and ER-alpha was analyzed by fluorescent immunocytochemistry using anti-PR and anti-ER-alpha antibodies. Positive signal for PR and ER-alpha is seen as (green) spots and nucleus is seen as (blue) spots in DAP1 staining. PR, PR expression profile; ER, ER-alpha expression profile; DAP1, DAP1 staining for nucleus.

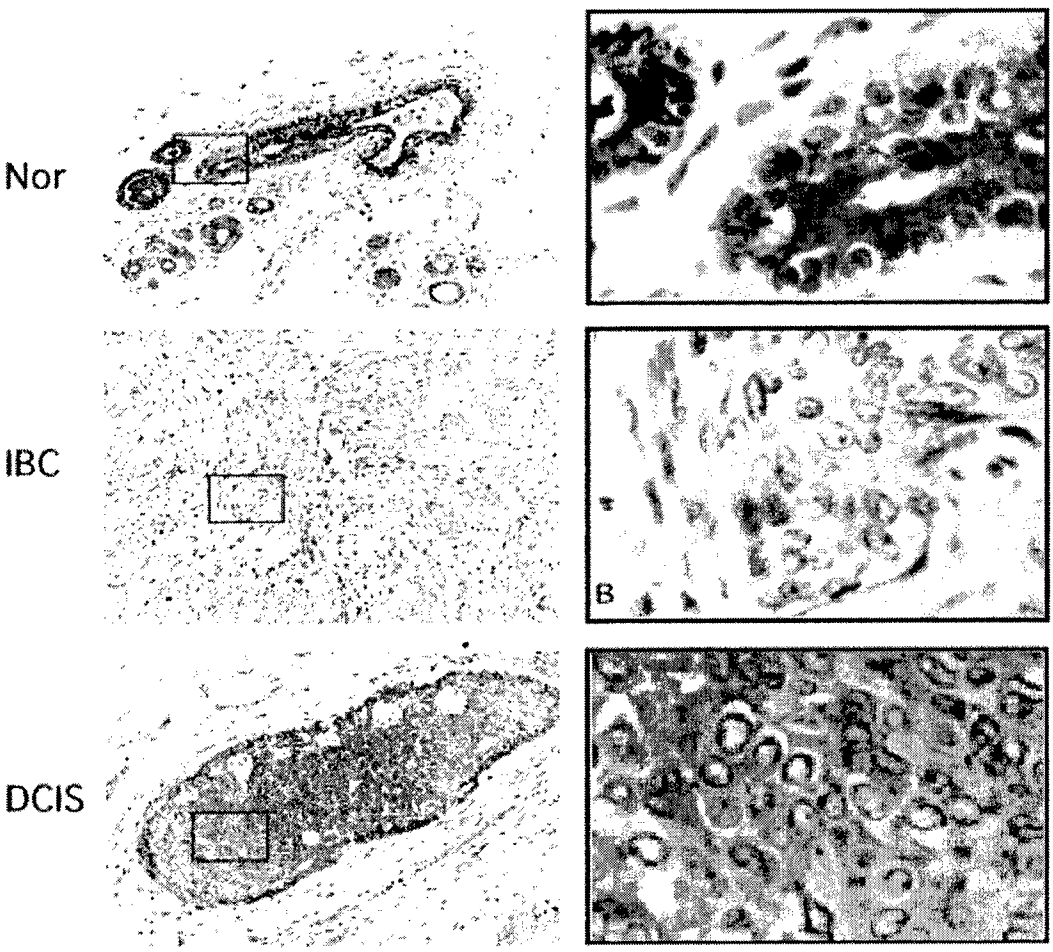


Figure 4: Immunohistochemical analysis of E6-AP in normal and malignant breast tissues-a representative case that include normal area, DCIS and IBC in one section. Paraffin-embedded human breast cancer biopsy samples were sectioned, deparaffinized, blocked, and incubated with an anti-E6-AP antibody. This was followed by incubation in a biotinylated anti-rabbit IgG and then the Vectastain ABC reagent (Vector Laboratories, Inc.). DAB kit (Vector Laboratories Inc.) was used to detect the bound antibody. After countersaining with Hematoxylin, the slides were dehydrated and mounted. Positive signal for E6-AP is seen as brown staining. Blue spots indicate the negative stained nuclei. a. Nor, normal area; b. IBC, invasive breast carcinoma; c. DCIS, ductal carcinoa in situ. "A", "B", and "C" are the enlarged image of "a", "b" and "c", respectively.

A. Immunostaining Intensity in DCIS and Normal Breast Tissues

Patient	Normal	IBC	T/N (%)
1	131	124	94.66
2	162	121	74.69
3	161	114	70.80
4	129	112	86.82
5	137	86	62.77
6	130	79	60.77
7	135	103	76.30
8	120	99	82.50
9	129	103	79.84
10	131	101	77.10
11	143	108	75.52
12	154	103	66.88
13	139	104	74.82
Average	138.54	104.38	75.34

(paired student *t*-test, p=0.000001)

B.

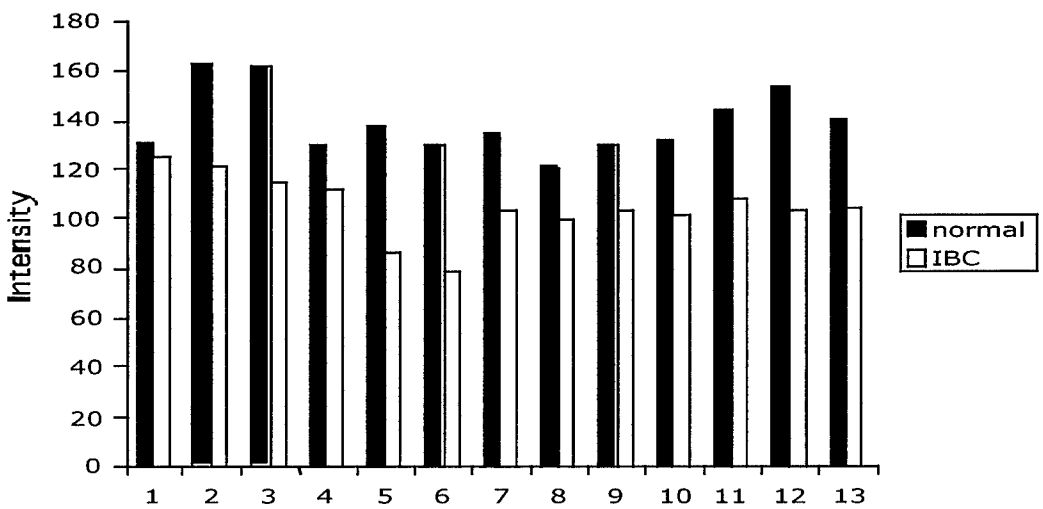


Figure 5: Intensity (mean brown value) of E6-AP immunostaining in normal and malignant human breast tissues. Thirteen paraffin-embedded human breast cancer biopsy samples including adjacent normal tissues were analyzed by Immunohistochemistry as mentioned above. The immunostaining results were evaluated using automated cellular imaging system. **A.** A table showing the intensity of E6-AP immunostaining in both normal and tumor tissues from each of the 13 breast cancer cases. **B.** A bar chart comparing the intensity of E6-AP immunostaining in normal and tumor tissues side by side.

A. Immunostaining Intensity in DCIS and Normal Breast Tissues

Sample No.	Normal	DCIS	T/N (%)
1	116	127	91.3
2	114	118	103.5
3	111	108	97.3
4	102	124	121.6
5	112	89	79.5
6	130	144	110.8
7	98	86	87.7
8	118	120	101.7
9	140	118	84.3
10	126	110	87.3
11	118	131	111.0
12	96	102	106.2
Average	115.08	114.75	98.52

(paired student *t*-test, p=0.9397)

B.

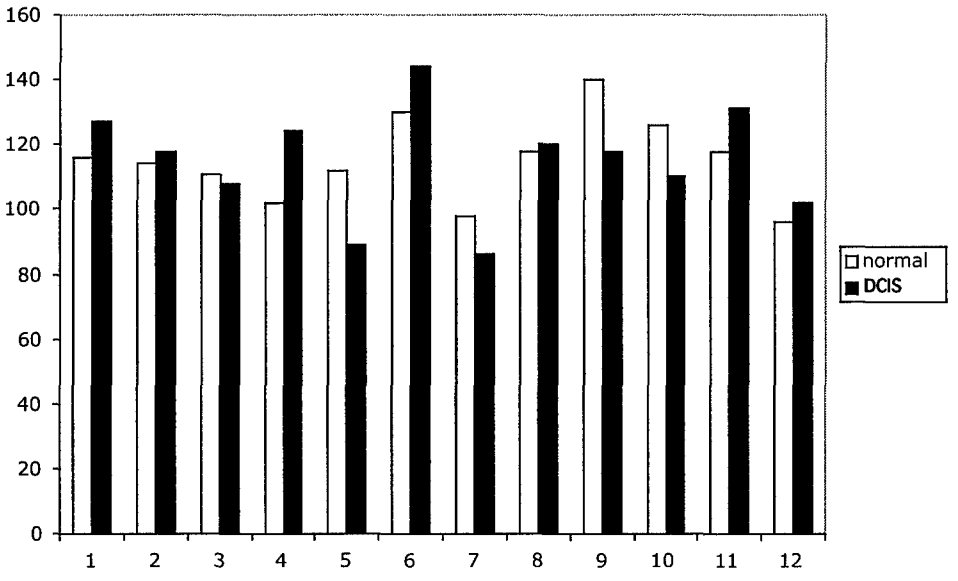


Figure 6: Intensity (mean brown value) of E6-AP immunostaining in DCIS and normal breast tissues adjacent to the tumors. Twelve paraffin-embedded human DCIS biopsy samples including adjacent normal tissues were analyzed by Immunohistochemistry as mentioned above. The immunostaining results were evaluated using automated cellular imaging system. **A.** A table showing the intensity of E6-AP immunostaining in both normal and tumor tissues from each of the 12 breast cancer cases. **B.** A bar chart comparing the intensity of E6-AP immunostaining in normal and tumor tissues side by side.

The Expression of E6-AP in Different Stages of Human Breast Cancers

Stage I	Stage IIA	Stage IIB	Stage IIIA	Stage IIIB
1	0.5	0.5	1.5	
1.5	1	1	2	
1.5	1	1	2	
2	1	1	2	
2	1.5	1	2	
2	1.5	1.5	2.5	
2	2	2		
2.5	2			
3	2			
	2			
	2			
	2.5			
	3			
	3			
N1=9	N2=14	N3=7	N4=6	
$\bar{X}1=1.94$	$\bar{X}2=1.78$	$\bar{X}3=1.14$	$\bar{X}4=2.00$	

Figure 7. Expression analysis of E6-AP in different stages of breast ca
tumor array with 36 breast cancer samples including tumors from stage I to stage IIIA in one slide was used for this study. Immunohistochemistry was carried out as mentioned above. The levels of expression were graded manually according to the overall density of brown color in each sample, with the highest level as 4, the lowest as 0 and the intermediate as 1, 2 and 3.

Comparison of The Expression Level of E6-AP between Different Stages of Breast Cancers

(Wilcoxon rank-sum test)

Stages Compared	Rank-sum T	n1,n2-n1	*P* value
I & IIB	32	7, 2	<0.01 *
IIA & IIB	52.5	7, 7	>0.05
IIIA & IIB	59.5	6, 1	<0.01 *
I & IIA	115	9, 5	>0.1
I & IIIA	50.5	6, 3	>0.1

Figure 8. Comparison of the levels of E6-AP in different stages of breast cancers. The immunostaining result of the tissue array slide, as mentioned in Figure 7, was statistically analyzed using Wilcoxon rank-sum test. The difference between different stages was compared. Combined with Figure 7, it is observed that the expression of E6-AP is decreased gradually from stage I to stage IIB which is the lowest point, suggesting a possible role of E6-AP in the progression of breast tumors.

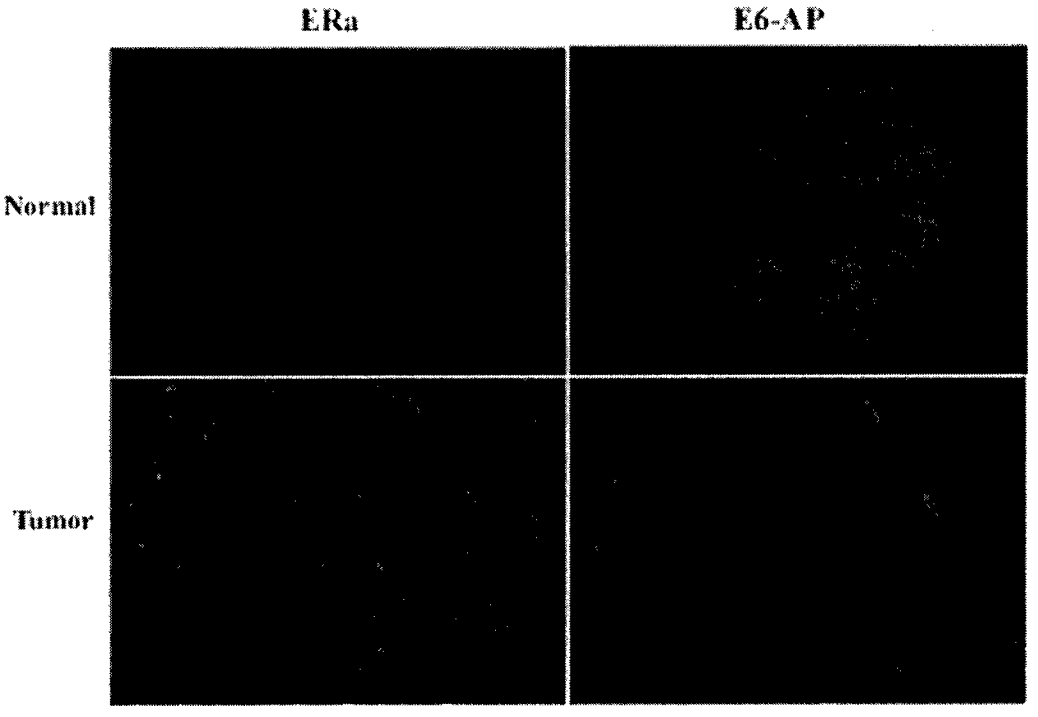


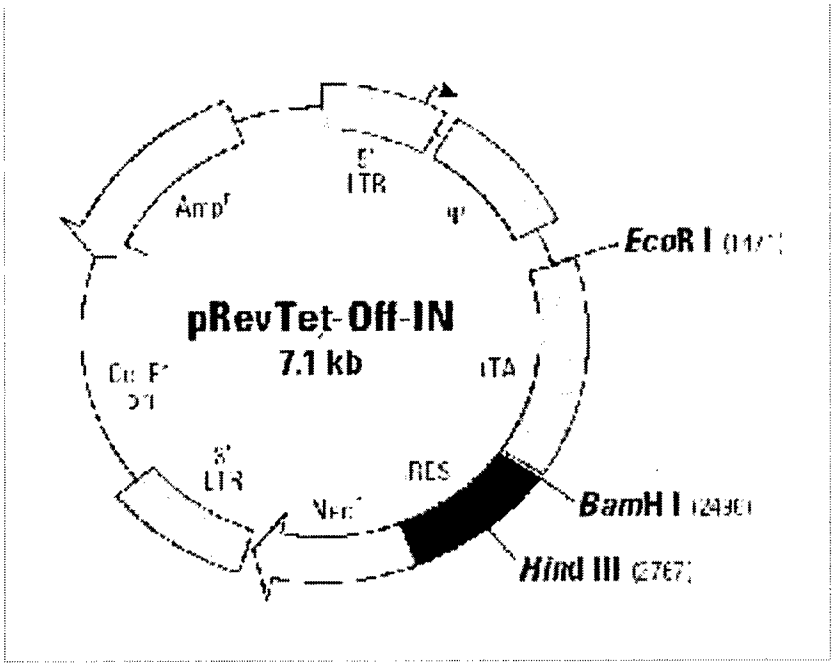
Figure 9. The expression of E6-AP is inversely correlated with that of ERα. The expression patterns of E6-AP and ERα in breast tumors and normal tissues were studied by means of dual immunofluorescent staining using antibodies against E6-AP and ER alpha. This picture is a typical example from the 8 pairs of samples studied. E6-AP is seen as red spot, while ERα is seen as green spot. In the normal breast tissues, ERα is expressed in the nuclei of epithelial cells in a discontinuous manner, whereas E6-AP is highly and broadly expressed in the epithelial cells, mostly in the cytoplasm. In comparison with its normal controls, the expression level of E6-AP is lower in tumors, while the expression of ERα is higher. Altogether, 5 out of 8 tumors that have lower levels of E6-AP express higher levels of ERα.

#	E6-AP	ERα	#	E6-AP	
1	2	4	11	0	3
2	3	3	12	2	1
3	0.5	3	13	0	3
4	1	2	14	0	0
5	1.5	3	15	2	2
6	1	3	16	2	3.5
7	0	0	17	2	4
8	0.5	3	18	0.5	1
9	1	3	19	0.5	0
10	1	3			

(Spearman Rank Correlation Coefficient r=0.503, $p<0.05$)

Figure 10. Correlation of the expression of E6-AP with that of ER-α in breast tumors. Expression levels of E6-AP and ERα from fluorescent immunohisto-chemical analysis were artificially graded according to the intensity of the respective colors; red for E6-AP and green for ERα. ERα is expressed in the nucleus, whereas E6-AP is expressed mostly in the cytoplasm. "0" represents negative expression and "0.5" represents very low expression. From "1" to "4" represent the gradually increasing levels of expression from low to high. Spearman Rank Correlation Coefficient for the expression of E6-AP with that of ERα is 0.503, $p<0.05$.

A.



B.

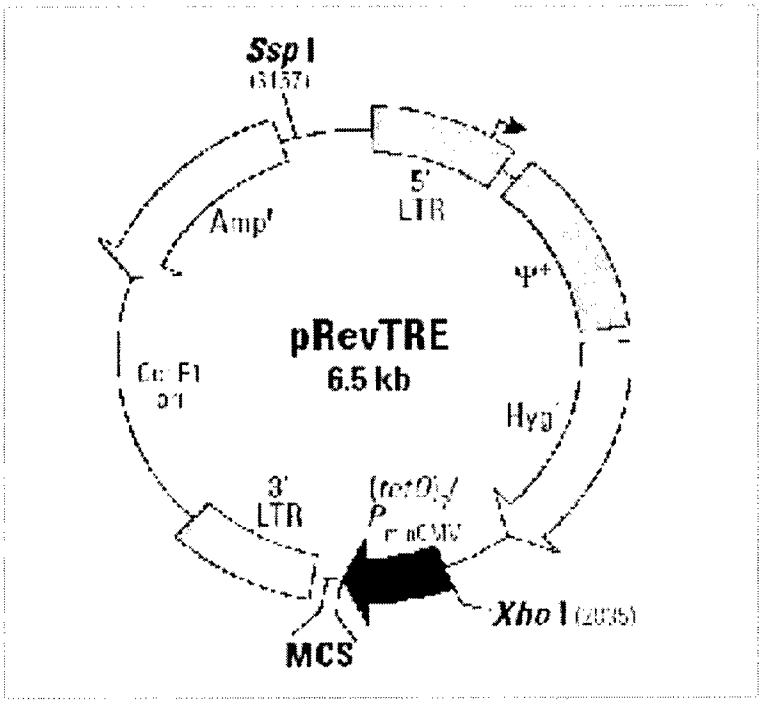


Figure 11. Schemastic structures of pRevTet-Off-In vector and pRevTRE vector. A. pRevTet-Off-In; B. pRevTRE.

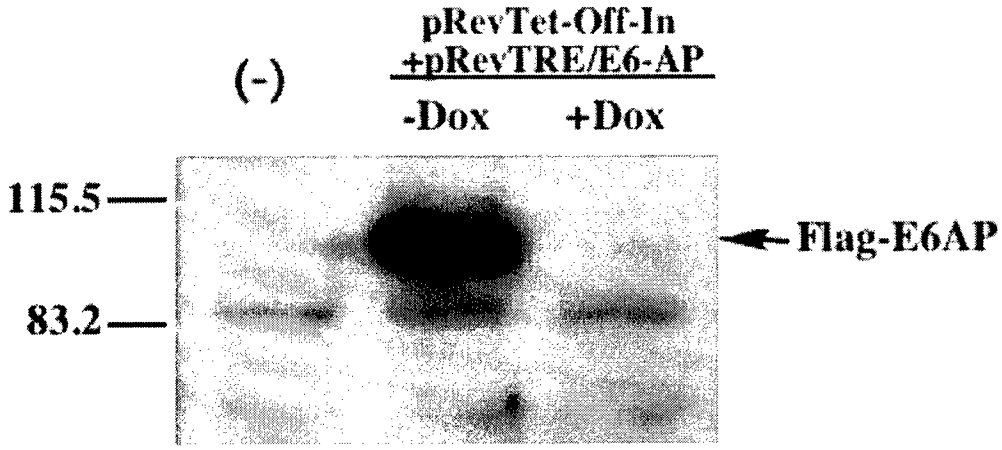


Figure 12. Cotranfection of pRevTet-Off-In and pRevTRE /E6AP plasmids into Hela cells were maintained in DMEM supplemented with 10% fetal bovine serum. Twenty-fo hours before transfection, $1x10^6$ cells were plated in three 10 cm Falcon dishes. Cells the three dishes were transfected with 5 µg of pRevTet-Off-In and pRevTRE using Fugene 6 transfection reagent (Roche). The third dish of cells were untransfected and used as a control. Dox was added to one of the two transfected dishes 4 hrs later. After 2 were harvested and lysed and equal amounts of proteins were resolved by a 10% then transfered to nitrocellulose membrane. The expression of recombinant E6-AP was c using an anti-Flag antibody. a. (-), untransfected Hela cells, b. Hela cells pRevTet-Off-In and pRevTRE /E6AP and no Dox was added to the culture. c. transfected with the same plasmids but Dox was added to the culture.

Appendix II: Article published in Endocrinology
Decreased expression of e6-associated protein in breast and prostate carcinomas.
Gao X, Mohsin SK, Gatalica Z, Fu G, Sharma P, Nawaz Z.
Endocrinology. 2005 Apr;146(4):1707-12.

Appendix III: Abstract posted at 2004 AACR annual meeting

Title: E6-associated protein, E6-AP is involved in the carcinogenesis of human breast and prostate

Shazia Zafar1, X Gao 1, S K Mohsin 2, Z Gatalica 1and Z Nawaz 1. 1Creighton University Medical Center, Omaha, NE and 2Baylor College of Medicine, Houston, TX

Aim: The E6-associated protein, E6-AP, is a dual function protein. It acts as an E3 ubiquitin-protein ligase as well as a steroid hormone receptor coactivator. Considering the importance of steroid hormone receptors and their coactivators in the normal development and tumorigenesis of reproductive organs of both genders, the roles of E6-AP in the tumorigenesis of female breast and male prostate tissues need to be investigated.

Methods: 1. Immunohistochemistry. a. Formalin-fixed paraffin-embedded tumor samples from 13 human invasive breast carcinomas, 20 in-situ carcinomas, 7 human prostate carcinomas, and the corresponding normal glands were evaluated immunohistochemically for the expression of E6-AP, estrogen (ERα) and androgen (AR) receptors. The intensity of expression and the proportion of positive cells were measured using Automated Cell Imaging System (ACIS, ChromaVision). H-score of expression was obtained by multiplying the intensity with the proportion of positive cells (H=I x P). b. Breast tissues from E6-AP knockout mice were fixed, embedded, sectioned and immunohistochemically analyzed similarly as we did for human samples. 2. Protein degradation and ubiquitination assay. Human ERα protein was synthesized in vitro and radiolabeled by ^{35}S using a transcription and translation kit. Then the ER protein was subjected to protein degradation either in the presence or absence of purified E6-AP.

Results: 1. Normal mammary and prostate luminal epithelium strongly expressed E6-AP. A 25% and 27% decrease in expression intensity were observed for invasive breast carcinomas and prostate carcinoma, respectively, in comparison to their paired normal glands ($p<0.001$). Analysis of E6-AP expression in *in-situ* breast carcinomas indicated no difference when compared with their adjacent normal tissue. 2. In contrast to E6-AP, ERα expression is higher in invasive breast carcinomas than in *in-situ* breast carcinomas, indicating an inverse correlation between the expression of E6-AP and that of ERα. 3. The levels of ERα are higher in E6-AP knockout mammary glands compared with that of normal wild-type mammary glands. 4. E6-AP is required for the degradation of ERα through the ubiquitin-proteasome pathway.

Conclusion: The expression of E6-AP is down regulated in advanced stage human breast and prostate cancers. There is an inversely correlation between the expression of E6-AP and ERα. E6-AP modulates the expression levels of ERα by promoting its degradation via the ubiquitin-proteasome pathway.

Appendix IV: Article published in Molecular Cancer

The roles of sex steroid receptor coregulators in cancer.
Gao X, Loggie BW, Nawaz Z.
Mol Cancer. 2002 Nov 14;1(1):7.