WWP1, TGF β and KLF5 gene expression levels as new possible factors involved in cervical oncogenesis

Nivelele de expresie a genelor WWP1, TGFβ şi KLF5 - noi posibili factori implicaţi în oncogeneza cervicală

Anca Botezatu[1*], Cristina D. Goia-Ruşanu[1], Anca D. Stănescu[2], Iulia V. Iancu[1], Elena Popa[3], Elena Nistor[4], Irina Huica[1], Gabriela Anton[1], Adriana Pleşa[1]

1. "Ştefan S. Nicolau" Institute of Virology, Viral Genetic Engineering Department, Romania
2. "Carol Davila" University of Medicine and Pharmacy, Gynecology Department, Romania
3. "Bucur" Hospital, Anatomic pathology Department, Bucharest, Romania
4. "Bucur" Hospital, Gynecology Department, Bucharest, Romania

Abstract

Infection with human papilloma virus (HPV) is the main cause of cervical cancer. Host genetic alterations, like modifications of gene expression levels can be induced as a response to the viral infection. Kruppel-like factor 5 (KLF5) is a transcription factor that has been implicated in pathways critical to carcinogenesis. Controversy persists as to whether it functions as a tumor suppressor or as an oncogene. WWP1 was amplified and overexpressed in some cancer cell lines and negatively regulated the function of KLF5. WWP1 negatively regulates also the TGFβ signaling by interacting with and degrading multiple components. The present study aims to establish the possible changes of TGFβ, KLF5 and WWP1 gene expression during cervical cancer development and the possible relationship with HPV infection. Quantitative Real Time PCR was used to evaluate the expression level of target genes. HPV DNA was detected and genotyped using Linear Array HPV Genotyping Test. Data were statistically analyzed using the Kruskal-Wallis test. The genotypes most present were high-risk HPV 66, 31, 33, 16 and 18 single or in co-infections. TGFβ expression level increased in CIN II-III lesions and tumors. KLF5 gene expression was down-regulated especially in SCC. Expression of WWP1 gene was increased in SCC and CIN II-III and was significant higher in HPV negative samples, suggesting that the carcinogenesis mediated by this factor is independent of HPV infection (p=0.0023). All three investigated genes suffer changes in gene expression level suggesting the possibility to use them in the future for disease management.

Keywords: *cervical cancer, HPV, tumor growth factor-beta, KLF5, WWP1.*

Rezumat

Infecţia cu virusul papilloma uman (HPV) este principala cauză a cancerului de col uterin. Modificările survenite în genomul gazdei, precum modificări ale nivelului de expresie genică pot fi rezultantele infecţiei vira-

[*]**Corresponding author:** Anca Botezatu "Ştefan S. Nicolau" Institute of Virology, Viral Genetic Engineering Department, 285, Mihai Bravu Ave, 030304, Bucharest, Romania.
Tel. 021 / 324 25 90, e-mail: gnanka30@yahoo.com

le. KLF5 este un factor transcripţional implicat în căi critice ale oncogenezei. Persistă însă o controversă privind rolul său de supresor tumoral sau de oncogenă. Gena WWP1 este amplificată şi supaexprimată în unele linii celulare tumorale şi reglează negativ funcţia proteinei KLF5. WWP1 reglează negativ de asemenea semnalizarea mediată de TGFβ prin interacţia şi degradarea multiplelor componente ale căii. Acest studiu şi-a propus să evalueze posibilele schimbări în nivelele de expresie a genelor TGFβ, KLF5 şi WWP1 în timpul oncogenezei cervicale şi posibila relaţie cu infecţia HPV. Tehnica qRT-PCR a fost utilizată pentru a cuantifica nivelele de expresie ale genelor investigate. Genotipurile virale au fost detectate şi identificate folosind Linear Array HPV Genotyping Test. Datele au fost analizate statistic utilizând testul Kruskal-Wallis. Genotipurile HPV de risc înalt frecvente au fost 66, 31, 33, 16 şi 18 singure sau în co-infecţii. Nivelul expresiei genei TGFβ creşte în stadiile CIN II-III şi în tumori. Expresia genei KLF5 este scăzută în special în carcinoamele scuamoase. Expresia genei WWP1 creşte în carcinoamele scuamoase şi în CIN II-III, fiind semnificativ mai crescută în probele HPV negative, astfel că oncogeneza mediată de acest factor este independentă de infecţia cu HPV (p=0.0023). Toate genele investigate suferă modificări în nivelul de expresie conducând la posibilitatea utilizării acestor teste în viitor pentru o bună conduită în diagnosticul şi tratamentul cancerului cervical.

Cuvinte-cheie: *cancer cervical, HPV, TGFβ, KLF5, WWP1.*

Introduction

Cervical cancer is one of the most frequent cancers affecting women in Romania. Infection with human papilloma virus (HPV) is the main cause for cervical neoplasia, and the remodeling effects of this virus on cellular regulatory functions (apoptosis, cell cycle, senescence) are known.

Accumulating evidence suggests that E3 ubiquitin ligases play important roles in cancer development. It has been shown that WWP1 (E3 ubiquitin ligase) gene is amplified and overexpressed in prostate and breast cancers and is a potential oncogene (1, 2). The chromosomal region of WWP1 localization 8q21 was frequently found to be amplified in human prostate and breast cancers. WWP1 gene is overexpressed in 58–60% of prostate and breast cancer samples and about 31–51% of cancer samples show gene copy amplification. By blocking WWP1 gene expression in prostate and breast cancer cell lines, cell proliferation was suppressed and/or apoptosis was induced. Therefore WWP1 is a key factor of the oncogenesis process, interacting with multiple important proteins. Its oncogenic function may represent a potential therapeutic target in cancer treatment.

WWP1 protein interferes with regulation of the transforming growth factor-β signaling pathway, which is known to suppress epithelial proliferation and induce apoptosis in normal cells, but promote tumor development at later stage of oncogenesis. Several reports asserted that WWP1 negatively regulates the TGFβ signaling by interacting with multiple components, including TGFβ receptor 1 (TbR1), Smad2, and Smad4. Following interaction, the target proteins are ubiquitinated via proteasome degrading pathway (3-5).

TGFβ actions are mediated through a cell membrane receptor, TGFβ receptor II (TbRII). After the ligand binding, TbRII forms a heterodimer receptor with TGFβ receptor (TbRI). The heterodimer activates serine/threonine kinase which in turn phosphorylates Smad2 or Smad3. The phosphorylated Smad2/3 forms a complex with Smad4 which moves to the nucleus and binds to DNA targets initiating complicated TGF-b effects (*Figure 1*) (6-9).

WWP1 interacts with TGF-b receptor complex via Smad7, and induced ubiquitination and degradation of the TGF-b type I receptor (10, 5).

Other factors involved in oncogenesis that are targeted for ubiquitin-mediated proteolysis by WWP1 are Notch, Runx2 and KLF5 (11 - 13). The oncogenic function of WWP1 has been sustained in a study conducted by Laine et al; the authors showed that WWP1 inhibits p53

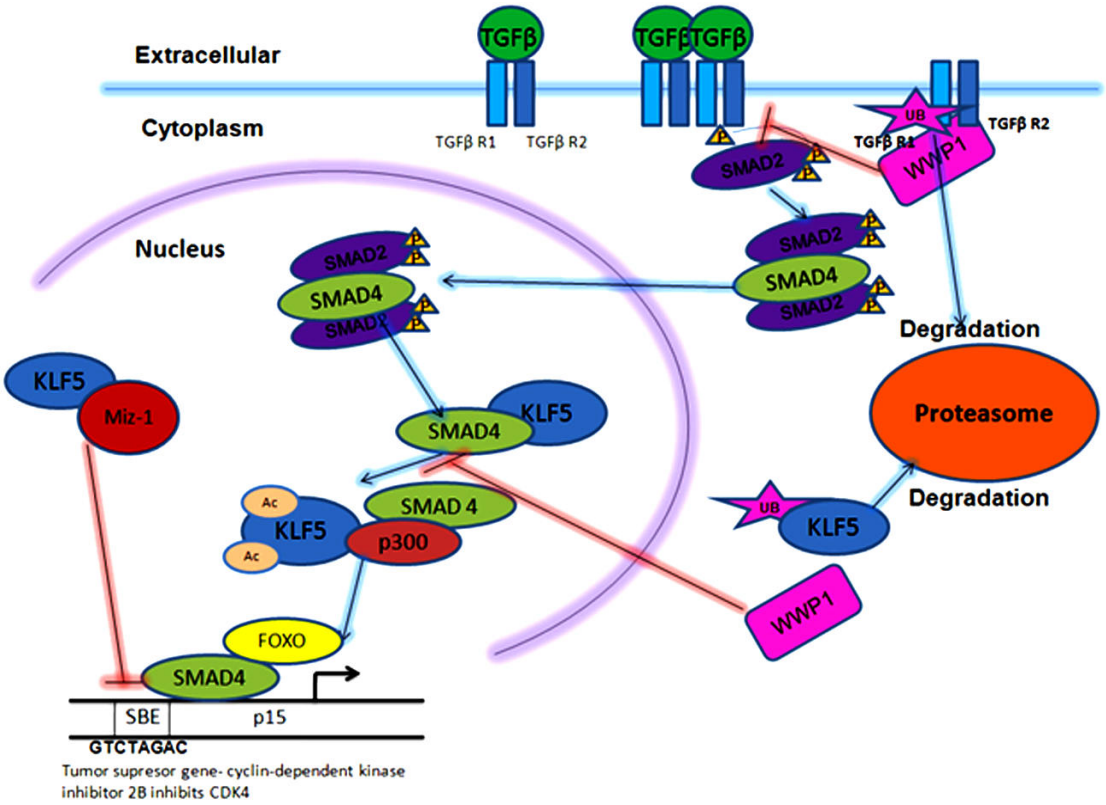

Figure 1. Molecular and cellular pathways of p15 tumor suppressor gene activation. TGFβ ligands bind their heteromeric receptors. After the interaction, the receptors dimerize and become activated, phosphorilating SMAD2. SMAD2 forms a complex with SMAD4 and this complex is translocated in the nucleus, where SMAD4 is released and interacts with p300 (histone acetyltransferase) and KLF5. FOXO transcriptional factor is activated and toghether with SMAD4 interact with a specific sequence (SBE- Smad Binding Element) of p15 gene promotor activating the transcription of this gene. The p15 gene transcription can be blocked in two ways: Miz-1 protein may sequestrate KLF5, and the second way is represented by the the overexpression of WWP1, which can degrade excessively TGFβ receptors 1, 2 or KLF5 via proteasome.

activity through exporting p53 from the nucleus by ubiquitination. The results in this field are at beginning, therefore the role of WWP1 in tumorigenesis remains to be elucidated (14).

Kruppel-like factor 5 (KLF5) is a transcription factor implicated in pathways critical to carcinogenesis. A controversy persists regarding the KLF5 function as a tumor suppressor or as an oncogene, but its role seems to be context-dependent (15, 16). Even if the expression of KLF5 enhances cell proliferation in untransformed cells or transforms normal fibroblasts, KLF5 expression suppresses cell growth in cancer cell. On the other hand, the KLF5 gene suffers frequent genomic deletion in human cancer, being haplo-insufficient. This indicates a loss of expression of the KLF5 gene during carcinogenesis. Several studies showed that KLF5 has an important role in the control of cell proliferation, differentiation, cell cycle regulation, and angiogenesis. (17 - 20).

The overexpression of WWP1 gene in cancer cell lines determines a negative regulation of the KLF5 gene. Active degradation of the KLF5 protein by ubiquitin-proteasome pathway is an important mechanism which characterizes the cancer cell, rather than a normal cell (13).

The present study aims to establish the possible changes of TGFβ, KLF5 and WWP1 gene expression during the cervical cancer development and the possible relationship with HPV infection.

Materials and methods

Cervical samples (n=59) were obtained from patients who self-referred for gynecological investigations at Obstetrics and Gynecology Hospital Bucur and underwent cervical biopsy. The samples originated from women (age 18 – 63 years) with cervix neoplasia according to histological exam: CIN I, II, III (cervical intraepithelial neoplasia). All samples were collected in a sterile tube and formalin fixed before embedded in paraffin.

Tumor tissue specimens (n=31) were obtained from patients that underwent surgery for tumor mass removal. Tumors were prepared according to the same protocol of CIN biopsy fragments. Tumor samples were classified according to the histological exam in squamous cell carcinomas (SCC) and adenocarcinomas (AC).

Normal tissue samples (n=25) were obtained from HPV negative patients with normal PAP tests who underwent surgical interventions for other conditions.

Nucleic acids isolation

DNA was isolated from cervical samples using High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals, Mannheim, Germany). In order to isolate the nucleic acids from paraffin, tissue sections were immersed in xylene to deparaffinize for approximate 30 min. The quantity of each sample was established according to the manufacturer recommendations. After isolation, DNA samples were stored at -20°C. DNA concentration for all samples was evaluated with NanoDrop spectrophotometer (NanoDropTechnologies, Montchanin, DE) as well as the purity.

The total RNA was isolated using TriZol reagent (Sigma). The purity and concentration of the total RNA isolated was measured using NanoDrop spectrophotometer, and the samples were stored at -80ºC. Total RNA was reverse transcribed starting from a quantity of 2.5µg using AccessQuick™ RT-PCR System (Promega, Madison USA) according to the manufacturer instructions. For genes expression quantification TaqMan primers and probes were used for each target gene and for endogen control beta – actin. The Real – Time PCR quantification was performed in 7300 Applied Biosystems (standard PCR program) (Life Technologies Corporation, Carlsbad, California). Gene expression levels were calculated for each gene using double normalization method (ΔΔCt). After the normalization, the expression ratio (folds) = $2^{-\Delta\Delta CT}$ was calculated.

HPV detection and genotyping

HPV genotyping was performed with Linear Array HPV Genotyping Test (Roche Molecular Biochemicals, Mannheim, Germany), according to the manufacturer's instructions. This test uses biotinylated primers to define a sequence of nucleotides within the polymorphic L1 region of the HPV genome of approximately 450 base pairs long. A pool of HPV primers present in the Master Mix is designed to amplify HPV DNA from 37 HPV genotypes including 13 high risk genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68). An additional primer pair targets the human β-globin gene to provide a control for cell adequacy, extraction and amplification.

Statistical analysis was performed using a non-parametric test Kruskal-Wallis in order to evaluate the differences between group medians of target genes expression in different histological categories and in HPV positives and negatives patients.

Results

HPV genotyping analysis

The genotypes most present were high-risk HPV 16 and 18 single or in co-infection, along with other genotypes like: 66, 31, 33, 68, 58, 53 and 45. In CIN I the HPV co-infection is dominant (40%), and slightly decreases with the severity of neoplasia (CIN II-III -31.58%, SCC-26.32, AC – 9.68%) (*Figure 2*). The selection of

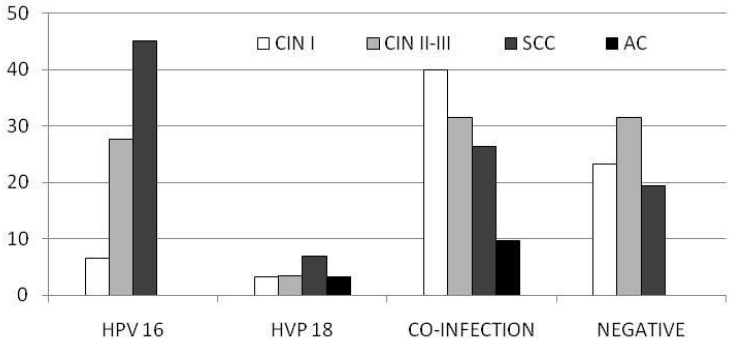

Figure 2. Graphic representation of HPV 16 and 18 genotypes (single or co-infection) prevalence (%) in different groups of study

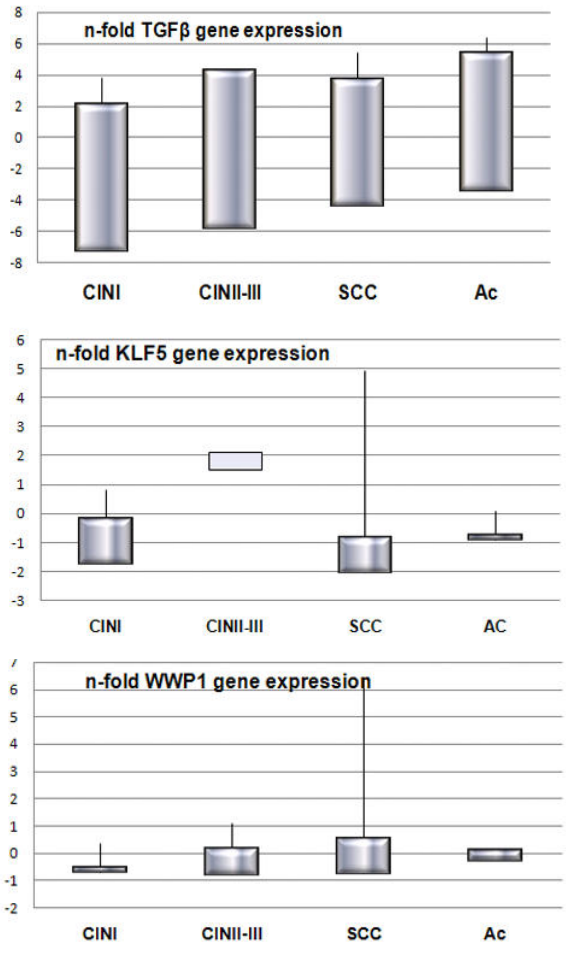

Figure 3. Graphic representation of N-fold gene expression levels for target gene investigated (TGFβ, KLF5, and WWP1)

one genotype is evident for advanced neoplasia stages and for SCC, especially for HPV16 (CINI – 6.66%, CINII-III- 27.6%, SCC – 45.16%). HPV 18 as single genotype was present in less percentage than HPV16 (CINI – 3.33%, CINII-III – 3.45%, SCC – 6.99%). On the other hand an important number of CINII-III and tumor samples were negative (31.58% CINII-III, 19.35 % SCC).

In CIN I, one of most common genotype found was 66 (5/30–22.58%), followed by 68 (4/30-13.33%), 31 (3/30-10%). CIN II-III showed other genotypes besides HPV16 and 18, but the number of concurrent genotypes in co-infection is less than CIN I cases and were represented by 68, 31, 58 (3/29 – 10.35%), and 33 (2/29-6.99%).

Target gene expression quantification and its correlation with HPV infection

We assessed the changes of genes expression levels (n-fold) in patients with different stages of disease (*Figure 3*). TGF-β gene expression level is increased in CINII-III samples (range between: -5.80563, 4.290086; median = 0.480714), in SCC (range: -4.37165, 5.39196; median= 3.69055), and in AC samples (range:-3.4324, 6.37165; median= 5.37463), whereas in CINI samples it is lower (range:-7.2062, 3.79596; median = 2.09568). KLF5 gene expression pattern is different and a decrease of expression level in SCC can be observed (range between:-2.047, 4.934; median= -0.85222) and AC samples (range: -0.91445, 0.090024; median = -0.76614), in contrast with CINI (range: -1.74597, 0.7899; median = -0.19241) and CINII-III (range:-0.80563, 2.091857, median = 1.508417) samples. Regarding the fold changes in expression of the third target gene investigated (WWP1), an important increase of the expression level can be observed especially in SCC (range: -0.74267, 6.43358, median = 0.523257), but also in CINII-III (range:-0.80563, 1.07524; median = 0.134939) cases. Adenocarcinomas seemed to follow a dif-

ferent oncogenesis pathway, presenting a decreased level of WWP1 (range: -0.26942, 0.090024; median = 0.090012).

The assessment of gene expression levels between the HPV positive and negative cases showed significant differences. The WWP1 gene expression levels increase significantly in CIN I and AC in HPV negative samples (p=0.0022). Also for KLF5 gene in AC HPV negative samples the expression level significantly increases (p=0.002). TGFβ gene expression level is decreased in HPV negative samples, a borderline result can be noticed for CINI group (p=0.0405) (*Table 1*).

No significant differences can be observed between SCC HPV positive and negative groups for all target genes investigated, and between AC HPV positive and negative groups for TGF-β gene.

Discussion

During tumor initiation and early progression TGFβ is thought to be a tumor suppressor, whereas late in tumor progression TGFβ signaling promotes tumorigenesis. This role of TGFβ is mediated through its overexpression effect on tumor cell invasion, angiogenesis, changes in the tumor microenvironment and the cells of the immune system. TGFβ can induce an invasive phenotype in cancer cells by its overexpression. The research conducted on breast cancer revealed an apparently opposite relationship between TGFβ and disease progression suggesting that TGFβ may have different effects on breast cancer progression depending on the stage of the disease. Previous studies evaluated the effect of TGFβ on breast cancer survival, but the findings are quite inconsistent (21). The present study showed that the level of TGFβ expression increased in CINII-III and tumor samples. No differences in the expression levels between HPV positive and negative group could be established suggesting that the implication of HPV cannot be associated with TGFβ overexpression.

Down-regulation of KLF5 gene has also been observed in intestinal, bladder tumorigenesis and the gene was deleted in prostate and breast cancer. Thus, KLF5 appears to undergo haplo-insufficiency during breast carcinogenesis. The study of Chen et al. showed that 8/30 (27%) of breast cancer cell lines exhibit loss of expression and more often neither hemizygous deletion nor promoter methylation is detected (15). Our findings related to KLF5 gene expression in cervical

Table1. Changes in gene expression of WWP1, KLF5 and TGFβ in CIN I, SCC and AC relative to controls

Gene	Lesion type	Range n- fold log_{10} expression		Average n- fold log_{10} expression		p-value
		HPV POS	HPV NEG	HPV POS	HPV NEG	
WWP1	CIN I	-0.70687, -0.33405	0.00237, 0.52789	-0.52091	0.285446	p=0.0022
	SCC	-0.74267, 1.496224	-0.65235, 1.243794	0.40788	0.38678	p>0.05
	AC	-1.3421, 0.0327	0.090012, 1.5623	-0.52627	0.743504	p=0.0023
KLF5	CIN I	-2.047, -1.5218	-3.7543, -0.0237	-1.7673	-1.84132	p>0.05
	SCC	-1.59263, 2.091857	-0.58216, 0.44855	-0.3473	-0.49617	p>0.05
	AC	-1.2376, 0.0327	0.063727, 0.5982	-0.70645	0.262886	p=0.002
TGFβ	CIN I	-1.5218, 3.206202	-7.08165, 1.654	1.398222	-2.96222	p=0.0405
	SCC	3.784099, 5.84244	4.44855, 6.110772	5.030398	5.209752	p>0.05
	AC	1.2376, 8.0327	2.12673, 10.5982	5.601461	6.753004	p>0.05

cancer showed that this gene is also down-regulated especially in SCC. For AC HPV infection seemed to have a protective effect on down-regulation of gene expression, HPV positive samples having a higher level of expression than negative samples. Therefore, the decrease of KLF5 gene expression might be an important event in carcinogenesis. In cervical cancer the down-regulated expression may be a result of a deleterious effect or of an epigenetic change, but that remains to be investigated.

One of the most common causes of carcinogenesis is the hyper-degradation of proteins with tumor suppressor role. This action is realized by the overexpression of ubiquitin ligase proteins such as Mdm2 and Skp2 (22, 23). E3 ubiquitin ligase (WWP1) degrades excessively its protein targets by gene amplification, supporting the concept that WWP1 functions like an oncogene in human cancers. Therefore, E3 ubiquitin ligases may be potential therapeutic targets, and it is necessary to identify their relationship and contribution to cancer development.

Our study showed that the expression of WWP1 gene is increased in SCC and CINII-III cases, as also shown by the results of Chen *et. al* on breast carcinogenesis (13). The expression of WWP1 gene is significantly higher in HPV negative samples, suggesting that the carcinogenesis mediated by this factor is independent of HPV infection.

All three genes investigated (TGFβ, KLF5 and WWP1) suffer changes in gene expression levels making them possible tools in cervical cancer screening and treatment. Future studies are needed in order to establish which of these genes is more suited for prediction of cervical disease outcome. Finding new biomarkers for early diagnosis and treatment is an important target and is necessary in order to achieve a better patient management.

Acknowledgements: This study was funded by PNII 41-081 research grant. No conflict of interest declared.

References

1. Chen C, Sun X, Guo P, Dong XY, Sethi P, Zhou W. Ubiquitin E3 ligase WWP1 as an oncogenic factor in human prostate cancer. Oncogene 2007 Apr; 26: 2386–94.
2. Chen C, Zhou Z, Ross JS, Zhou W, Dong JT. The amplified WWP1 gene is a potential molecular target in breast cancer. Int J Cancer 2007 Jul; 121: 2834–41.
3. Komuro A, Imamura T, Saitoh M, Yoshida Y, Yamori T, Miyazono K. Negative regulation of transforming growth factor-beta (TGF-beta) signaling by WW domain-containing protein 1 (WWP1). Oncogene 2004 Jun; 23: 6914–23.
4. Seo SR, Lallemand F, Ferrand N, Pessah M, L'Hoste S, Camonis J. The novel E3 ubiquitin ligase Tiul1 associates with TGIF to target Smad2 for degradation. EMBO J 2004 Sep; 23: 3780–92.
5. Moren A, Imamura T, Miyazono K, Heldin CH, Moustakas A. Degradation of the tumor suppressor Smad4 by WW and HECT domain ubiquitin ligases. J Biol Chem 2005 Aug; 280: 22115–123.
6. Buck MB, Knabbe C. TGFβ signaling in breast cancer. Ann N Y Acad Sci 2006 Nov; 1089: 119–26.
7. Chang CF, Westbrook R, Ma J, Cao D. Transforming growth factor beta signaling in breast cancer. Front Biosci 2007 May; 12: 4393–401.
8. Dumont N, Arteaga CL. Transforming growth factor-beta and breast cancer: tumor-promoting effects of transforming growth factor-beta. Breast Cancer Res 2000 Feb; 2: 125–32.
9. Wakefield LM, Piek E, Bottinger EP. TGF-beta signaling in mammary gland development and tumorigenesis. J Mammary Gland Biol Neoplasia 2001 Jan; 6(1): 67–82.
10. Komuro A, Imamura T, Saitoh M, Yoshida Y, Yamori T, Miyazono K, Miyazawa K. Negative regulation of transforming growth factor-beta (TGF-beta) signaling by WW domain-containing protein 1 (WWP1). Oncogene 2004; 23(41): 6914–23.
11. Shaye DD, Greenwald I. LIN-12/Notch trafficking and regulation of DSL ligand activity during vulval induction in Caenorhabditis elegans. Development 2005 Nov; 132: 5081–92.
12. Shen R, Chen M, Wang YJ, Kaneki H, Xing L, O'Keefe R J. Smad6 interacts with Runx2 and mediates Smad ubiquitin regulatory factor 1-induced Runx2 degradation. J Biol Chem 2006 Feb; 281(6): 3569–76.
13. Chen C, Sun X, Guo P, Dong XY, Sethi P, Cheng X. Human Kruppel-like factor 5 is a target of the E3 ubiquitin ligase WWP1 for proteolysis in epithelial cells. J Biol Chem 2005 Dec; 280: 41553–561.
14. Laine A, Ronai Z. Regulation of p53 localization and transcription by the HECT domain E3 ligase WWP1. Oncogene 2007 Aug; 26: 1477–83.
15. Chen C, Bhalala HV, Qiao H, Dong JT. A possible tumor suppressor role of the KLF5 transcription factor in

human breast cancer. Oncogene 2002 Sep; 21(43):6567-72.
16. Bateman NW, Tan D, Pestell RG, Black JD, Black AR. Intestinal tumor progression is associated with altered function of KLF5. J. Biol. Chem. 2004 Mar; 279:12093-101.
17. Sun R, Chen X, Yang VW. Intestinal-enriched Krüppel-like factor (Krüppel-like factor 5) is a positive regulator of cellular proliferation. J. Biol. Chem. 2001 Mar; 276:6897–900.
18. Oishi Y, Manabe I, Tobe K, Tsushima K, Shindo T, Fujiu, K**.** Krüppel-like transcription factor KLF5 is a key regulator of adipocyte differentiation. CellMetabolism 2005 Jan; 1(1): 27–39.
19. Nandan MO, Yang VW. The role of Kruppel-like factors in the reprogramming of somatic cells to induced pluripotent stem cells. Histol Histopathol 2009 Oct; 24(10):1343-55.
20. Shindo T, Manabe I, Fukushima Y, Tobe K, Aizawa K, Miyamoto S, at al. Krüppel-like zinc-finger transcription factor KLF5/BTEB2 is a target for angiotensin II signaling and an essential regulator of cardiovascular remodeling. Nat. Med 2002. 8:856–63.
21. Mu L, Katsaros D, Lu L, Preti M, Durando A, Arisio R, Yu H. TGF-b1 genotype and phenotype in breast cancer and their associations with IGFs and patient survival British Journal of Cancer 2008 Oct; 99(8): 1357 – 63.
22. Lu L, Schulz H, Wolf DA. The F-box protein SKP2 mediates androgen control of p27 stability in LNCaP human prostate cancer cells. BMC Cell Biol 2002 Aug; 3:22.
23. Leite KR, Franco MF, Srougi M, Nesrallah LJ, Nesrallah A, Bevilacqua RG, et al. Abnormal expression of MDM2 in prostate carcinoma. Mod Pathol. 2001 May; 14(5):428-36.