RESEARCH ARTICLE

Decreased expression of EFS is correlated with the advanced prostate cancer

Selda Sertkaya · Syed Muhammad Hamid · Nihat Dilsiz · Lokman Varisli

Abstract Prostate cancer is the most frequently diagnosed malignant neoplasm in men in the developed countries. Although the progression of prostate cancer and the processes of invasion and metastasis by tumor cells are comparatively well understood, the genes involved in these processes are not fully determined. Therefore, a common area of research interest is the identification of novel molecules that are involved in these processes. In the present study, we have used in silico and experimental approaches to compare the expression of embryonal Fyn-associated substrate (EFS) between normal prostate and prostate cancer. We showed that EFS expression is remarkably downregulated in prostate cancer cells, compared to normal prostate cells. We also found that decreased expression of EFS in prostate cancer cells is due to DNA methylation. In addition, we showed that high EFS expression is important to suppress a malignant behavior of prostate cancer cells. Therefore, we suggest that EFS should be considered as a novel tumor suppressor gene in prostate cancer.

Keywords EFS · Prostate cancer · DNA methylation

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Abbreviations

PCa	Prostate cancer
EFS	Embryonal Fyn-associated substrate
CAS	Crk-associated substrate
BCAR1	Breast cancer resistance 1
NEDD9	Neural precursor cell expressed, developmentally downregulated 9
HEPL	HEF1-EFS-P130 Cas-like
FBS	Fetal bovine serum
5-Aza	5′-Aza-2′-deoxycytidine
TSA	Trichostatin A
TBS-T	Tris-buffered saline containing 0.1 % Tween 20
PVDF	Polyvinyledine fluoride
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

Introduction

Prostate cancer (PCa) is the most common form of cancer, excluding skin cancer, in men in the developed countries with increasing rates in the developing world [1]. Although the progression of PCa and the processes of invasion and metastasis by tumor cells are comparatively well understood, the genes involved in these processes are not fully determined. Therefore, various approaches have been used to fully understand the molecular basis of these processes. Differential expression of some genes is accepted as a marker in both development and progression of cancer. Therefore, the gene expression profiling has been extensively used to identify novel diagnostic and prognostic biomarkers [2, 3]. In several studies, correlations between tumor expression alterations and clinical outcome have been identified in PCa [4, 5]. Consistently, some genes reported as differentially expressed in PCa may reflect the differences between metastatic and organ-confined tumors [6, 7]. Recently, using database-based

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transcriptomics approaches, we reported that several genes are downregulated and may be involved in progression and metastasis of the disease in PCa [8]. In the present study, we have used the same approach as a starting point to show embryonal Fyn-associated substrate (EFS) as a differentially expressed gene in PCa.

Crk-associated substrate (CAS) family is composed of breast cancer resistance 1 (BCAR1) [9]; neural precursor cell expressed, developmentally downregulated 9 (NEDD9) [10]; HEF1-EFS-P130 Cas-like (HEPL) [11]; and EFS [12] which have important roles in both normal cellular physiology and malignant transformation [13]. Although EFS (UniProt number=043281) was identified as a CAS family member, there is no any study on the function of EFS, up to date. However, it was shown that EFS interacts with Src, Fyn, and Yes which are Src family kinases, via an SH3 domain, like other CAS family members [12, 14]. Therefore, it was supposed that EFS may be an adapter molecule, like other CAS family proteins that are regulated by phosphorylation-dependent mechanisms [15]. Although there are several reports on the relationship between other CAS family members and cancer, there is only one report on the EFS expression in carcinogenesis. In that report, Neumann et al. have shown that bi-allelic methylation of EFS promoter is a common event in melanoma and which has been associated with poor prognosis [16].

In the present study, we have used in silico and experimental approaches to investigate EFS expression in prostate cells. We demonstrate that EFS expression is downregulated in PCa cells compared to normal prostate. Our results also reveal that the ectopic EFS expression decreases cell proliferation, cell migration, and colony formation abilities of PCa cells. In summary, our findings provide further insight for the anti-oncogenic role of EFS in mediating PCa tumorigenesis.

Material and methods

In silico analysis

We used Oncomine Cancer Microarray database (http://www.oncomine.org/) [17] in order to compare the gene expression of EFS in a tumor sample to its normal counterpart, and gene expression data from the same study, performed with the same methodology, were used as described previously [18, 19]. The gene expression data were log transformed and median centered per array, and the standard deviation was normalized to one per array [17]. A gene was considered as underexpressed when its mean value in tumor samples was significantly lower to its mean value in the normal tissue counterpart using a t test (≤ 0.001), and the fold of induction was ≤ -2.

Cell lines and culture conditions

PC-3, DU145, and LNCaP PCa cells were kindly provided by Dr. Kemal S. Korkmaz from Ege University, Turkey. PNT1a cells were kindly provided by Dr. Ö. Faruk Bayraktar from Yeditepe University, Turkey. The cells were propagated in RPMI 1640 or DMEM/F12 (Gibco, USA) supplemented with 5–10 % fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) and were cultured in 5 % CO_2 environment in a humidified incubator at 37 °C.

Primer design, plasmid construct, and transfections

The full-length open reading frame of EFS was amplified using a forward primer (GAAGGAGATACCACCATGGCC ATTGCCACGTCGAC) and a reverse primer (GGGCACGT CATACGGTGGAGCCAGGCTAGTGAG), which were designed using Primer 3 software. The PCR product was cloned into a linearized pME-HA (Lucigen, USA) vector according to the manufacturer's recommendations. The inserts were verified by PCR amplification. All transfections were performed using FuGENE HD (Promega, USA) transfection reagent according to the manufacturer's recommendations. Briefly, cells were seeded in 60-mm plates at 60 % confluency 1 day prior to transfection. In the following day, the transfection solution was prepared in a 1.5-ml microcentrifuge tube by adding 3 μl of FuGENE HD transfection reagent into 100 μl of pre-warmed DMEM/F12 or RPMI 1640 and was incubated for 5 min, and 1 μg of pME-HA-EFS plasmid DNA was added. The mixture was added to the cells drop-wise after incubating for 15 min at room temperature.

Antibodies and immunoblotting

Cells were lysed with ice-cold RIPA buffer (1 % Nonidet P-40, 50 mM Tris–Cl, pH 7.4, 0.25 % Na deoxycholate, 150 mM NaCl), including 1 mM NaF, 1 mM EDTA, and 1 mM Na_3VO_4, complete protease and phosphatase inhibitor cocktails (BioVision, USA), unless otherwise indicated. Ten percent of SDS–polyacrylamide gels were used for separation, and the proteins were immobilized onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad, USA) using a wet transfer blotter. Briefly, membranes were blocked using Tris-buffered saline containing 0.1 % Tween 20 (TBS-T) containing 5 % skim milk (w/v). Antibody incubations were performed using TBS-T containing 0.5 % skim milk at room temperature (RT) for 1 h or at 4 °C overnight. Membranes were developed using Clarity Western ECL Substrate (Bio-Rad, USA) for 5 min and were photographed using Kodak X-ray films in a dark room. EFS and β-actin antibodies were purchased from Bios (Gräfelfing, Germany) and Santa Cruz Biotechnology, Inc. (Bergheimer, Germany), respectively.

Total RNA isolation and RT-PCR

Total RNA was isolated from cultured PC-3, DU145, and LNCaP cells using the RNeasy kit (Qiagen, CA), and yields were calculated by absorbance readings at 260 nm. Using anchored oligo-dT primers, 2 μg of total RNA was reversely transcribed to make complementary DNA (cDNA) using the Omniscript kit (Qiagen, CA). The resultant cDNA was then used in PCR reactions and analyzed by agarose gel electrophoresis. The following primers were used: EFS forward: TGGCCGAGGAGTATGAC, EFS reverse: TCTCCGGTGG ACACAAC; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward: CATTGCCCTCAACGACCACTTT, and GAPDH reverse: GGTGGTCCAGGGGTCTTACTCC. PCR conditions were as follows: 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 2 min. The final extension was at 72 °C for 2 min.

Proliferation, migration, and colony formation assays

PC-3, DU145, and LNCaP cells were plated in 60-mm plates. Twenty-four hours after plating, cells were transfected with an empty vector or a full-length EFS using FuGENE HD as suggested by the manufacturer. On the next day (day 1), cells were trypsinized and plated in triplicates in 96-well dishes at a concentration of 4×10^3 cells/well. Cell proliferation was measured using WST-1 assay kit (BioVision, USA) at designated time points, and relative proliferation was calculated by normalizing to day 1 values. Colony formation assays were performed as described previously [20]. Briefly, PC-3, DU145, and LNCaP cells were transfected with an empty vector or an EFS. After 24 h of transfection, cells were trypsinized and seeded on new six-well plates (5×10^3 cells for PC-3 and DU145 and 1×10^4 cells for LNCaP) and cultured for 2 weeks in DMEM/F12 or RPMI 1640, supplemented with 5–10 % FBS. Cells were then fixed with −20 °C cold methanol for 30 min. Colonies were stained with crystal violet (0.2 %), and the area covered on each plate by the colonies was measured using a colony counter software program [21]. An in vitro wound-healing model (streak assay) was used to detect migration differences between vector and EFS-transfected PC-3 cells, as described previously [20]. In this assay, sub-confluent PC-3 cells were transfected with vector or EFS, and 24 h later, confluent cells were scarped with a blue pipette tip and rinsed with PBS to remove any floating cells. Then, fresh medium was added and images of multiple regions were taken 0 and 24 h after scarping.

5′-Aza-2′-deoxycytidine and trichostatin A treatment

Treatments were performed as described previously [22]. Briefly, PC-3 cells were seeded at a density of 1×10^6 cells/60-mm dish and allowed to attach for 24 h. In the following day, 10 μM 5-aza (Sigma, St. Louis, MO) was added to the medium for 72 h. At every 24-h interval, fresh medium containing the drug was added. For the synergistic study, cells were first incubated with 10 μM 5′-aza-2′- deoxycytidine (5-aza) for 56 h at 37 °C and then 50 nM trichostatin A (TSA) (Sigma, St. Louis, MO) was added for an additional 16 h (total 72 h).

Results

Loss of EFS expression is associated with advanced PCa

To investigate how EFS expression is altered in PCa, we used Oncomine to analyze published microarray data, as described previously [8, 18, 19]. The results showed that the levels of EFS messenger RNAs (mRNAs) are lower in PCa tissues when compared with normal tissues, in six independent studies [4, 23–27] (Table 1). Furthermore, EFS mRNA levels decrease in the higher Gleason score samples compared to lower score samples [28] (Fig. 1a). Oncomine data also revealed a decrease in EFS mRNAs in metastatic tumors when compared with the primary tumors [29] (Fig. 1b).

EFS expression in prostate cells

Since in silico results revealed that EFS expression is downregulated in PCa, we examined the expression of the EFS by Western blotting using three cancer cell lines and one normal prostate cell line. We found that EFS expression is remarkably high in normal prostate at both mRNA (Fig. 2a) and protein levels (Fig. 2b). However, the EFS level is very low in PC-3 and LNCaP PCa cells (Fig. 2a, b). Although EFS protein levels were readily detectable in DU145 cells compared to PC-3 and LNCaP cells, it was remarkably lower than normal prostate cell line PNT1a (Fig. 2a, b). Because both DNA

Table 1 EFS is downregulated in prostate cancer. Analysis of the PCa datasets deposited in Oncomine database showed decreased expression of EFS in PCa compared to normal prostate. Underexpression of EFS was considered when its mean expression value in the tumor samples was significantly lower than that of the normal tissue counterpart using a t test ($P<0.001$), and the fold of induction was ≤-2

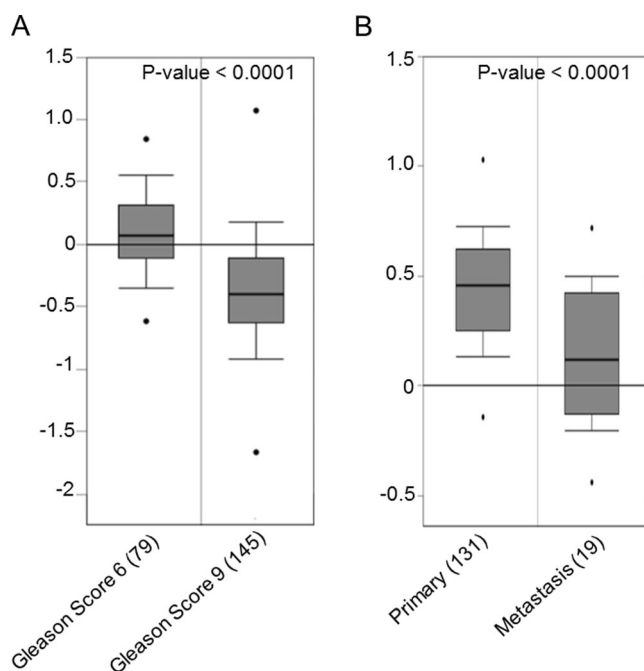| Dataset	Fold change (cancer/normal)	P value	References
LaTulippe Prostate	−4.645	8.74E−11	[23]
Tomlins Prostate	−3.499	2.59E−08	[24]
Arredouani Prostate	−3.044	1.20E−05	[25]
Luo Prostate 2	−2.747	4.78E−04	[26]
Singh Prostate	−2.337	2.56E−04	[4]
Grasso Prostate	−2.109	9.14E−15	[27]

Fig. 1 EFS expression is further decreased in advanced PCa. **a** The level of EFS is lower in Gleason score 9 compared to Gleason score 6. **b** EFS mRNA expression is lower in metastatic PCa compared to primary tumor. Oncomine was used for the analysis of expression data. Student's *t* test was used for differential expression analysis, and *P* values are shown on the figures. *Y axis* of the graphs represents log2 median-centered ratios, *dots* are maximum and minimum values, *boxes* are 75th and 25th percentiles, and *whiskers* are the 90th and 10th percentiles. Sample sizes are shown in *parentheses on the figures*

methylation and histone deacetylation are common epigenetic events in cancers that regulate gene expression at the promoter level, we checked whether inhibition of the activities of DNA methyltransferases, using 5-aza, and/or histone deacetylases, using TSA, could restore the expression of EFS in PCa cell
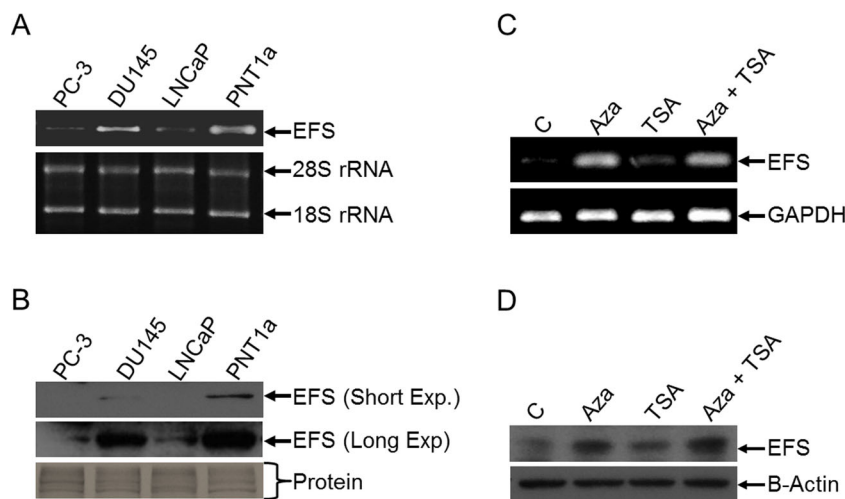
line PC-3 which has a very low EFS level. The results showed that the treatment of the cells with the deacetylase inhibitor TSA had little effect; however, when 5-aza was used alone or in combination with TSA, there was a dramatic increase in EFS mRNA (Fig. 2c) and protein (Fig. 2d) levels.

Increased expression of EFS repressed malignant behavior of PCa cells

Our in silico results showed that decreased expression of EFS is associated with aggressive PCa. Therefore, we assumed that increased expression of EFS in the PCa cells may suppress the malignant phenotype. Firstly, we cloned full-length human EFS to a mammalian expression vector and then transiently transfected PC-3, DU145, and LNCaP PCa cells with full-length EFS or empty vector to evaluate the potential role of EFS as a tumor suppressor in these cells. We first noticed a change in the growth rates between EFS and vector-transfected cells. EFS overexpression caused a decrease in cell proliferation in all of the cancer cells which were used in this study (Fig. 3b). On the other hand, it is known that for a cancer cell to metastasize, the cells must migrate from the primary tumor and enter the circulation system [30]. Therefore, we investigated the effect of EFS overexpression on cell migration by wound-healing assay. The assay was performed within 24 h to minimize secondary effects of cell proliferation. The results showed a dramatic decrease in cell migration when EFS was overexpressed, compared to the vector-transfected cells, in concordance with in silico results. Furthermore, the frequency of the colony formation in the cells that were transfected with full-length EFS was significantly lower than that of the cells with an empty vector, in agreement with the above results.

Fig. 2 EFS expression in prostate cells. **a** The EFS mRNA and **b** EFS protein levels are downregulated in PC-3, DU145, and LNCaP PCa cells as compared to PNT1, a normal prostate cell line. Since different cells were used in A and B experiments which have different expression levels of GAPDH and β-actin, total mRNA and protein were also used as

loading control, respectively. 5-Aza and TSA treatment restored EFS mRNA (**c**) and protein (**d**) levels in PC-3 PCa cells. GAPDH was used as loading control for RT-PCR and β-actin for Western blotting. The expression of EFS mRNA (**a** and **c**) and EFS protein (**b** and **d**) were analyzed by RT-PCR and Western blotting, respectively
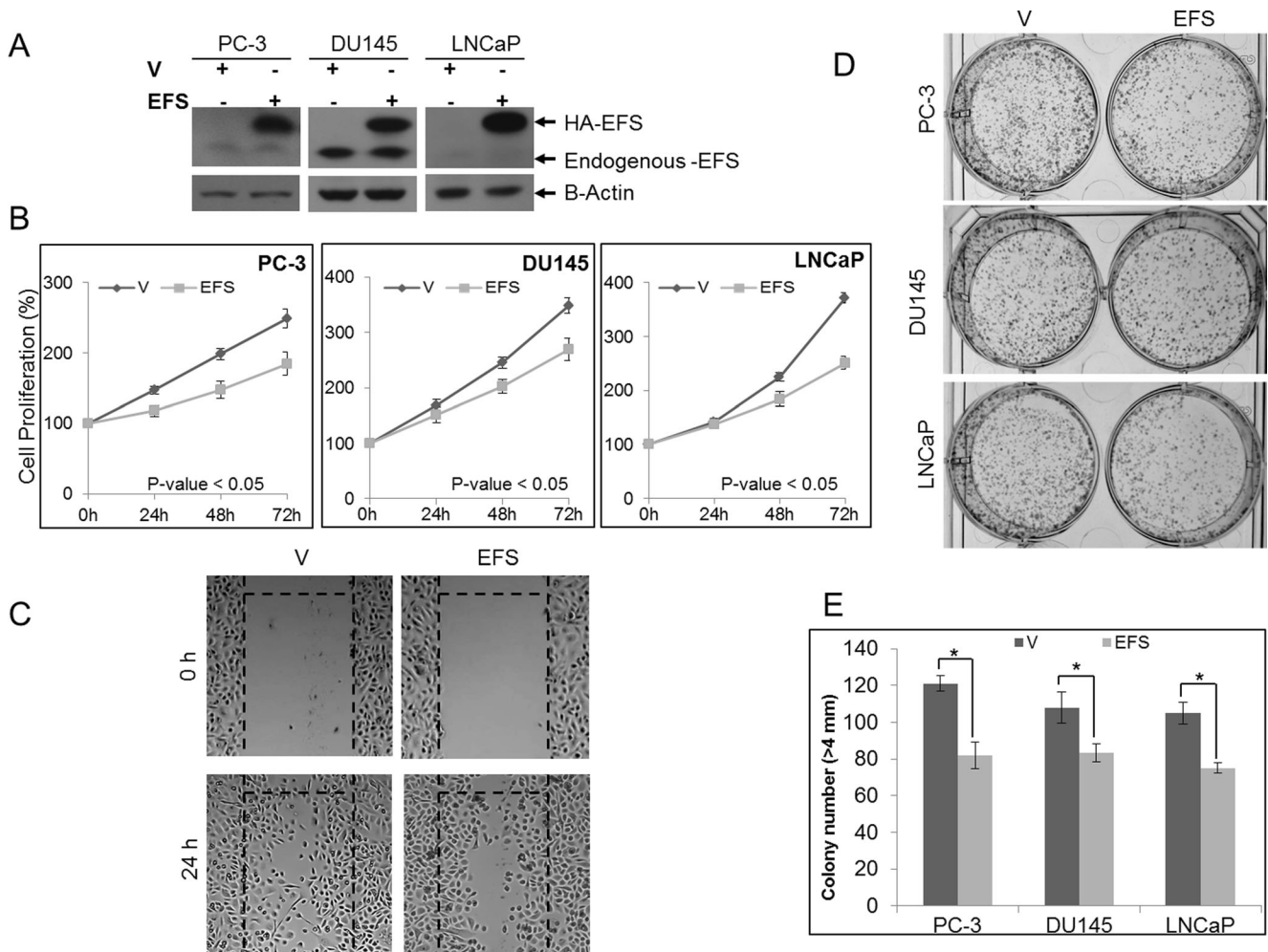
Fig. 3 Overexpression of EFS represses malignant behavior of PCa cells. **a** Transfection of EFS construct lead to increased EFS protein in PC-3, DU145, and LNCaP cells. **b** The effect of EFS overexpression on proliferation of PCa cells. The cell growth of vector or EFS-transfected PC-3, DU145, and LNCaP cells was examined by WST-1 assay over 3 days. The results represent the mean±SD of three independent experiments. $P<0.05$ as determined by Student's t test. **c** Effect of EFS overexpression on wound healing in PC-3 prostate cancer cells. Sub- confluent PC-3 cells were transfected with vector or EFS, and 24 h later, confluent cells were scarped with a blue pipette tip. The wounded area was imaged at 0 time and 24 h after injury. **d**, **e** The effect of EFS overexpression on colony formation abilities of PCa cells. Vector or EFS-transfected PC-3, DU145, and LNCaP cells were incubated in a six-well plate and cultured for 2 weeks and then photographed after staining by crystal violet. The results represent the mean±SD of three independent experiments (*$P<0.05$)

Discussion

In the present study, we have provided the first evidence that decreased expression of EFS is associated with the development and progression of PCa. The analysis of public data deposited in Oncomine cancer transcriptomics database revealed that EFS expression is significantly downregulated in PCa, compared to normal prostate tissues. In concordance, we have seen that EFS is downregulated in PCa cell lines at both mRNA and protein levels, compared with normal prostate epithelial cells. Since Neumann et al. showed that the promoter of EFS is methylated in melanoma cells and this event is associated with poor prognosis, we investigated putative promoter methylation of EFS in PC-3 PCa cells using DNA methyltransferase and histone deacetylase inhibitors. Indeed,

we have seen that inhibition of DNA methyl transferases and histone deacetylases resulted to increased expression of EFS at both mRNA and protein levels. Therefore, we suggest that promoter methylation may cause downregulation of EFS expression in the PCa cells.

The analysis of Oncomine also revealed that decreased expression of EFS is associated with advanced and aggressive PCa. To asses this idea, we have cloned EFS to a mammalian expression vector and transiently transfected PCa cell lines to investigate whether high EFS level will change a malignant behavior of PCa cells. Indeed, we have seen that high EFS level repressed cell proliferation, migration, and colony formation abilities of all the investigated PCa cells.

Donlin et al. reported that EFS is an important regulator in T lymphocyte development and that EFS deficiency leads to

aberrant T cell activation and, consequently, inflammation [31, 32]. It is known that the inflammation contributes to cancer development and progression [33]. Therefore, EFS deficiency-dependent malignant behavior changes may be linked to inflammation-related processes. In addition, it was shown that BCAR1 and NEDD9 which are the other members of CAS family regulate membrane localization of E-cadherin and its interaction with catenin molecules [34]. Therefore, another possible mechanism to explain the role of EFS may be via the regulation of E-cadherin/β-catenin signalling. Although these hypotheses may open new doors to better understand the relationship between inflammation and/or E-cadherin/β-catenin and cancer, it needs further investigation.

In summary, we have successfully combined the in silico analysis and experimental approaches to find a new gene involved in the development and progression of PCa. We also provide ample evidence that EFS should be categorized as a tumor suppressive in PCa. We also suggest that restoration of EFS expression may be a potential therapeutic strategy for the treatment of prostate cancer. Molecular mechanism by which EFS exerts its tumor-suppressive role needs to be further investigated.

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