RESEARCH ARTICLE

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# Epigenetic Status of *FBXW7* Gene and Its Role in Ovarian Cancer Pathogenesis

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## Abstract

**Background:** Chromatin immunoprecipitation (ChIP) analysis revealed that the *FBXW7* gene and the long non-coding RNA (LINC01588) are potential candidates in epithelial ovarian cancer (EOC) pathogenesis. However, their exact role in EOC is not yet known. Thus, the present study sheds light on the impact of the mutations/ methylation status of the *FBXW7* gene. **Materials and Methods:** We used public databases to assess the correlation between mutations/ methylation status and the *FBXW7* expression. Furthermore, we performed Pearson's correlation analysis between the *FBXW7* gene and LINC01588. We performed gene panel exome sequencing and Methylation-specific PCR (MSP) in HOSE 6-3, MCAS, OVSAHO, and eight EOC patients' samples to validate the bioinformatics results. **Results:** The *FBXW7* gene was less expressed in EOC, particularly in stages III and IV, compared to healthy tissues. Furthermore, bioinformatics analysis, gene panel exome sequencing, and MSP revealed that the *FBXW7* gene is neither mutated nor methylated in EOC cell lines and tissues, suggesting alternative mechanisms for *FBXW7* gene regulation. Interestingly, Pearson's correlation analysis showed an inverse, significant correlation between the *FBXW7* gene and LINC01588 expression, suggesting a potential regulatory role of LINC01588. **Conclusion:** Neither mutations nor methylation is the causative mechanism for the *FBXW7* downregulation in EOC, suggesting alternative means involving the lncRNA LINC01588.

**Keywords:** Downregulation- *FBXW7*- mutation- promoter hypermethylation- lncRNA- Ovarian Cancer

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## Introduction

Epithelial ovarian cancer (EOC) is a lethal gynecological malignancy (Sung et al., 2021) and the fifth leading cause of cancer-related deaths among women (Siegel et al., 2018). Due to the delayed detection caused by the asymptomatic nature of the disease, the majority of EOC cases (80%) are diagnosed at advanced stages (IIb to IV) (Jelovac and Armstrong, 2011), resulting in poor survival outcomes (Doubeni et al., 2016; Ovarian Cancer - Cancer Stat Facts; Vaughan et al., 2011). Hence, elucidating the mechanism of EOC pathogenesis and identifying a panel of tumour markers is crucial for screening, diagnosis, and treatment of EOC (Alharbi et al., 2018).

*FBXW7* is a tumor suppressor gene encoding three different isoforms (*FBXW7*α, *FBXW7* β, *FBXW7*$_\gamma$) (Akhoondi et al., 2010). *FBXW7* has been implicated in the pathogenesis of several cancers, including breast, colon, and gastric malignancies(Calcagno et al., 2013; Iwatsuki et al., 2010; Strohmaier et al., 2001). During human tumorigenesis, *FBXW7* inactivation seems to occur through either mutation, deletion, or hypermethylation (Sailo et al., 2019; Yumimoto et al., 2015; Yumimoto & Nakayama, 2015). In addition, such inactivation could lead to an upregulation of growth-promoting genes, including cyclin-E(Koepp et al., 2001; Strohmaier et al., 2001) and c-myc (Koepp et al., 2001; Welcker et al., 2004). Earlier studies reported an association between the loss of *FBXW7* expression and poor clinic-pathological features and prognosis in lung and hepatocellular carcinomas(Yokobori et al., 2014). Moreover, the *FBXW7* β-isoform is silenced by promoter hypermethylation in breast cancer and thymomas (Akhoondi et al., 2007; Gu et al., 2008).

*FBXW7* expression was significantly lower in serous EOC than in the benign and borderline tissues. Furthermore, the *FBXW7* expression in the p53 mutant

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group was downregulated than in the p53 wild-type EOC tissues (Kitade et al., 2016). Although missense mutations in the *FBXW7* gene were associated with cancer progression in several cancer types (Agrawal et al., 2011; Jardim et al., 2014), they are not frequent in EOC (Kwak EL et al.). Despite the advancements in cancer research on *FBXW7*, the exact mechanism of *FBXW7* inactivation in EOC is not known yet.

This study screened for mutation/deletion in EOC samples using a gene panel exome-sequencing and compared the results to normal ovarian tissue samples. Furthermore, we assessed the frequency of *FBWX7* promoter hypermethylation and analysed the biological significance of this gene in EOC.

## Materials and Methods

*Bioinformatics analysis*

A flowchart displaying the analysis performed in this study is shown in Figure 1. We investigated the alteration frequency and the expression of *FBXW7* gene across the different stages of EOC patients (585 tumor samples) using cbioportal and CSIOVDB databases, respectively (Cerami et al., 2012; Gao et al., 2013; Tan et al., 2015). The correlation of the *FBXW7* expression with the methylation status using ß-values (ß is the degree of methylation indicator) was performed using cbioportal. Furthermore, we used the SMART App and Wanderer database to assess the *FBXW7* gene methylation (Díez-Villanueva et al., 2015; Li et al., 2019). In order to determine the correlation between the *FBXW7* gene and the lncRNA (LINC01588), we performed Pearson's correlation analysis using the GEPIA database (Tang et al., 2017).

*Patients recruitment*

The study was authorized by the Sultan Qaboos University Medical Research Ethics Committee (No.1168), and written consent from all patients was obtained. According to a predefined protocol, sixteen controls and twenty-seven patients diagnosed with EOC were recruited from April 2016-December. EOC cases were confirmed using different tests (e.g., imaging, biopsy reports, and molecular tests). The patients below 18 and above 90 years and those who had undergone previous chemotherapy, radiotherapy, or mastectomy and had chronic use of corticosteroids or non-steroidal anti-inflammatory drugs were excluded from this study, resulting in the inclusion of 4 controls and 8 EOC samples. Tissue samples were classified into normal, benign, borderline, and high-grade tumors and were stored in liquid nitrogen until use.

*Cell line propagation*

The OVSAHO, and MCAS, human EOC cell lines were obtained from the JCRB cell bank, Osaka, Japan (catalog no. JCRB1046 and JCRB0240), respectively. While the OVSAHO line represents high-grade serous EOC, MCAS is a low-grade mucinous serous EOC model. The HOSE6-3 cell line, a kind donation from Prof. GSW Tsao's laboratory School of Biomedical Sciences, The University of Hong Kong, represents the normal epithelial ovarian cell line. MCAS, HOSE 6-3, and OVSAHO cell lines were propagated in DMEM medium (Gibco, Life Technologies). In addition, 10% fetal bovine serum (FBS) and 1% of penicillin and streptomycin were supplemented to the media before incubation at 37°C in a 5% CO2 atmosphere.

*Quantative Real-time polymerase chain reactions (qRT-PCR)*

We used the PureLink RNA mini kit (Invitrogen, CA, USA) to extract the total RNA from the EOC cell lines according to the manufacturer's protocol. We reverse-transcribed RNA to complementary DNA using a high-capacity reverse transcription kit (Invitrogen, CA, USA). The qRT-PCR was performed on EOC cells using the TaqMan Universal PCR master mix and primer-probe mix (Thermo Scientific Fisher, USA). We normalized data to GAPDH expression used as a reference and computed relative expression using the 2-ΔΔCt method (Livak & Schmittgen, 2001 ).

*Proteins Analysis*

To investigate *FBXW7* expression in EOC cells, we performed Western Blotting. Cells were lysed with RIPA buffer supplemented with proteases and phosphatase cocktail inhibitors (Santa Cruz Biotechnology Inc., CA). Cell lysates were centrifuged, the supernatant was collected, and protein concentrations were determined using the Bradford Protein Assay Reagent (Pierce, Rockford, IL). ~50 µg of proteins were resolved on 8% polyacrylamide gels and electroblotted onto nitrocellulose membranes. We subsequently probed membranes with a polyclonal rabbit anti-*FBXW7* (Abcam: abID#171961) and anti-β-actin antibody (Abcam: abID#8229) at a dilution of (1:200), followed by a mouse anti-rabbit IgG secondary antibody (Cell Signaling) at a dilution of (1/4000), and was revealed using ECL western blotting detection reagent (Pierce Biotechnology, Inc., Rockford, IL). Finally, we quantified the protein bands using ImageJ software.

*Methylation-specific PCR (MSP)*

To investigate the methylation status of the *FBXW7*, we extracted DNA from the OVSAHO cells and eight human ovarian tissue samples using the DNeasy Mini Kit (Qiagen, USA). In addition, HOSE 6-3 and four normal ovarian samples (N1-N4) were used as controls.

We performed bisulfite treatment using EpiTect® Fast Bisulphite Conversion Kit (Qiagen, USA). Subsequently, we carried out amplification reactions as follows: 100 ng bisulfite modified DNA was added to 10µM specific primer mix and 9µl of True Allele® PCR Premix (Thermo Fisher Scientific, USA) in a total volume of 17 µl. The amplification reaction was carried out in a thermal cycler (Applied Biosystems, Ontario, Canada) under the following cycling conditions: Initial denaturation at 95°C for 7 minutes, followed by 35 cycles of denaturation at 96°C for 30 seconds, specific annealing for 30 seconds, and elongation at 72°C for 30 seconds. Finally, the reaction mixtures were held at 72°C for 10 minutes for the final elongation and then incubated at 4°C until storage. The sequences of primers used for MSP were localized in the

CpG-rich island spanning the promotor of the *FBXW7* β isoform (Table 1).

*Gene Panel Exome Sequencing*

Based on the list of genes identified, using previously performed ChIP as actual targets regulated by E2F5 (Malgundkar et al. 2022) (Supplementary file, S1: Tables 1-4), a gene panel including *FBXW7* was designed using the online custom panel Ion AmpliSeq Designer (ThermoFisher Scientific, USA). Pre-capture libraries were constructed using Ion AmpliSeq Library Kit 2.0-96V for the Ion Torrent system (ThermoFisher Scientific, USA), following the manufacturer's guidelines. The fragmented genomic DNA (10ng) was purified with Agencourt and quantified using Real-time PCR by Ion Library TaqMan™ Quantitation Kit (ThermoFisher Scientific, USA).

After equalizing the library to 100pM, the pooled library was amplified by emulsion PCR using Ion PI™ Hi-Q™ OT2 Chemistry 200 Kit (ThermoFisher Scientific, USA) using Ion OneTouch 2 Emulsion PCR Machine. Finally, Templated Ion spear particles were enriched using the Ion Enrichment System (ThermoFisher Scientific, USA) as per the manufacturer's guidelines.

Enriched samples were sequenced using the Ion PI™ Hi-Q™ Sequencing 200 Kit (ThermoFisher Scientific, USA). The Ion Proton was used to sequence the ion spear particles. Multiplex samples were loaded on the Ion PI™ Chip Kit v3 (ThermoFisher Scientific, USA). Sequencing data were processed on the instrument server using the Torrent Suite v5.0.4, and BAM files were generated.

*Statistical Data Analysis*

The data represent the mean and the standard deviation from at least three independent experiments performed in triplicates. GraphPad prism program (version 8.0.2) was used for data analysis. Differences among the two groups were analysed using unpaired t-test/Mann-Whitney test and the statistical significance was set at a p-value <0.05 .

## Results

*Public database analysis*

We utilized publicly available databases to analyse the *FBXW7* gene expression and methylation status. The cbioportal database revealed *FBXW7* gene alterations in only 15 of 584 EOC cases (2.6%; Figure 2A). The CSIOVDB database indicated *FBXW7* downregulation in ovarian tumor samples (Figure 2B), with significant downregulation in stages III and IV (Figure 2C). Furthermore, a low negative correlation (r = -0.25, p=7.668x10-6) was observed between *FBXW7* expression and the methylation status. Most EOC samples have no mutations in *FBXW7*, and the beta values observed were less than 0.2 (hypomethylated) (Figure 3A). Furthermore, five EOC subtypes displayed *FBXW7* hypomethylation (beta values <0.1), Figure 3B. The majority of the *FBXW7* methylation probes (65%) showed low *FBXW7* beta values (<0.5), with the average beta value corresponding to 0.3 in EOC (Figure 4).

Previously, ChIP analysis revealed the *FBXW7* gene and the lncRNA (LINC01588) as E2F5 targets with a potential role in EOC pathogenesis. Hence, we performed Pearson's correlation analysis between the

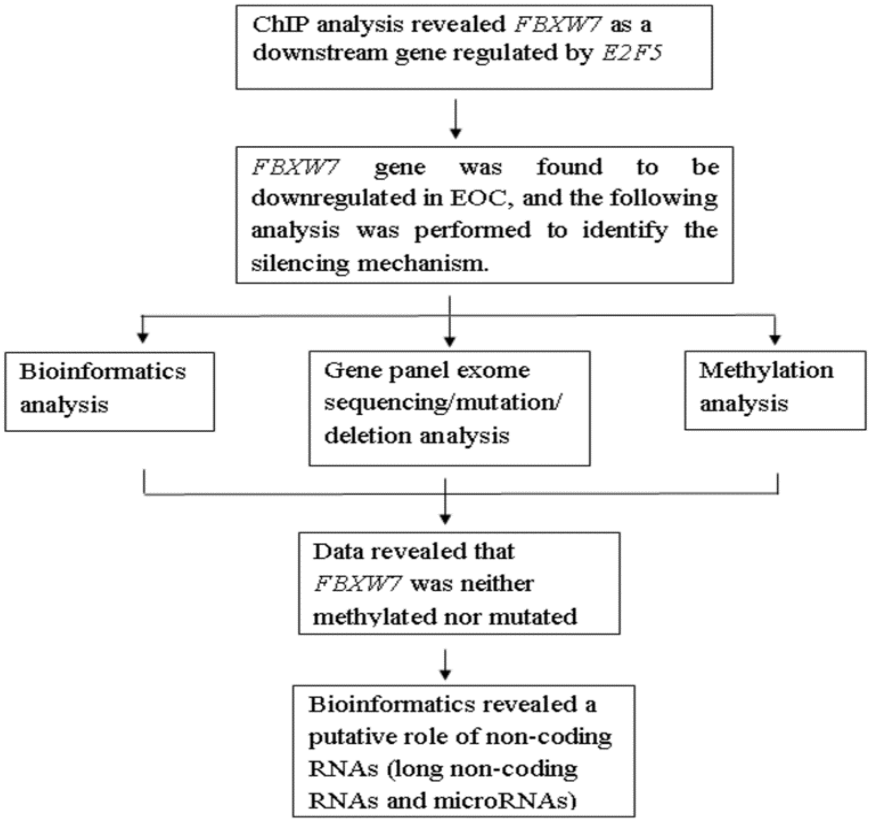



Figure 1. Flowchart Illustrating the Study Design.

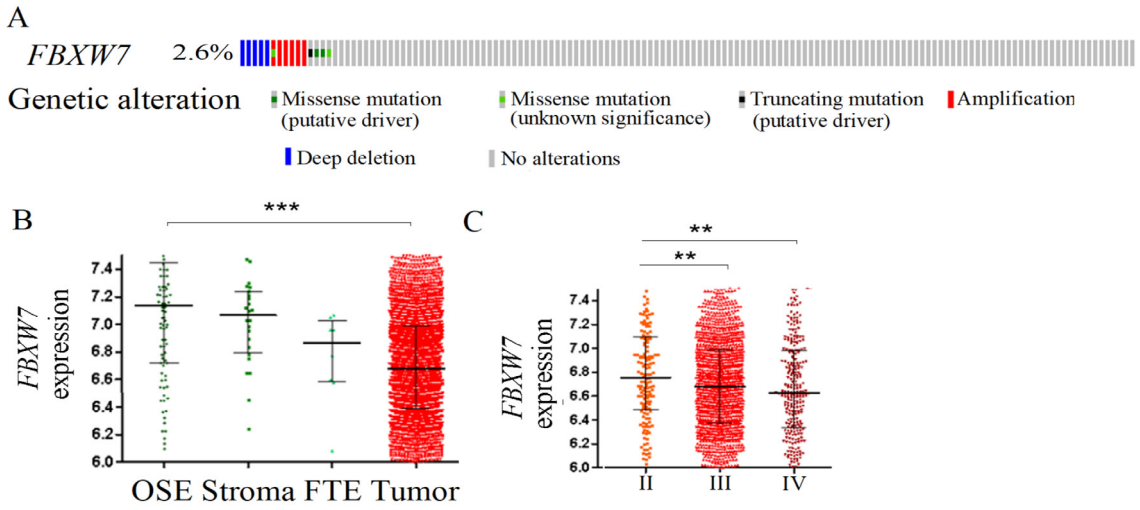



Figure 2. Bioinformatics Analysis of *FBXW7* Expression in EOC Samples. (A). Bioinformatics analysis of EOC samples from the cbioportal database revealed low *FBXW7* alteration frequency compared to other cancer types (2.6%) (B). *FBXW7* was downregulated in ovarian tumor samples compared to normal ovarian surface epithelial tissues ($p<0.0001$). (C) The late stages EOC samples (stages III and IV) displayed lower *FBXW7* expression ($p<0.001$). Abbreviations; OSE, Ovarian surface epithelium; FTE, Fallopian tube epithelium.

Table 1. Primers and Annealing Temperatures (Ta) for MSP and NMSP for *FBXW7*

| Promotor of FBXW7 β isoform | | | | |
|---|---|---|---|---|
| | Primer Sequence (5'--> 3') | | T (°C) | Product Size (bp) |
| Primer | Forward | Reverse | | |
| MSP | AGACCCAGGAAGAGGAAAAGAGGA | TGGGTTGGTTCCCTTCCTCCTTC | 58 | 179 |
| NMSP # | TTTTGTTTTGGAGTGTATGAATT | CACCCTCAAAACTAAAACAA | 60 | 118 |

#NMSP, Non-methylation specific primer

*FBXW7* gene and LINC01588. The analysis revealed a significant inverse correlation ($P=1.9e^{-13}$, R=-0.32), suggesting a potential regulation of the *FBXW7* gene by the LINC01588 (Figure 5).

*Expression of FBXW7 in EOC Cell Lines*

Consistent with the results obtained through bioinformatics analysis, qRT-PCR revealed *FBXW7* expression in MCAS but not in OVSAHO cells ($P<0.01$). Obtained results were confirmed by Western Blotting ($P<0.0001$). Furthermore, *FBXW7* expression was significantly downregulated in eight EOC patient samples as compared to healthy individuals ($P<0.0001$) (Figures 6A-D).

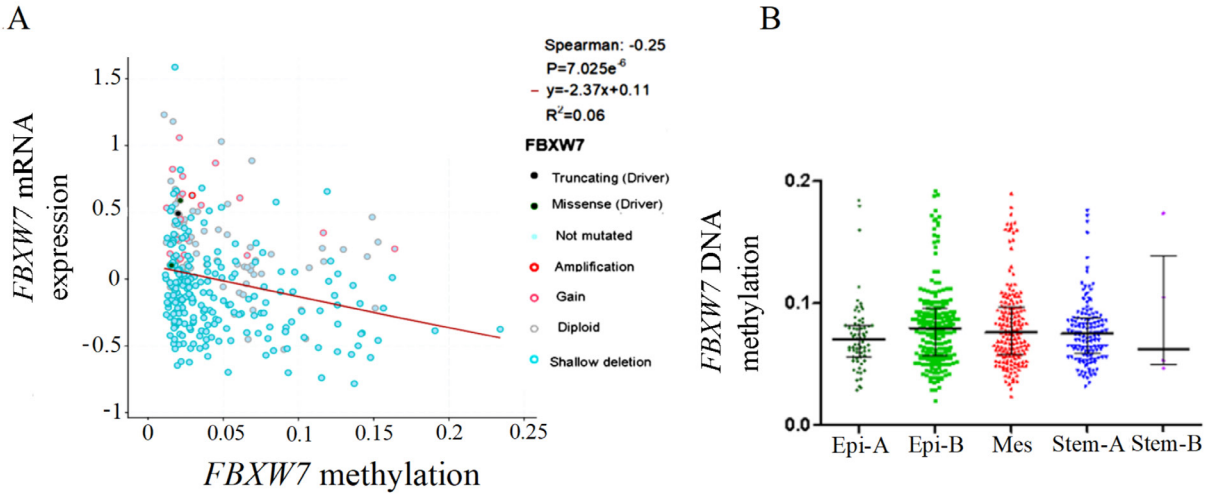



Figure 3. (A) Analysis of 316 EOC samples from the cbioportal database revealed a low negative correlation between the degree of methylation (beta value) and the FBXW7 expression ($r=-0.25$, $p=7.668e^{-6}$); beta value< 0.2. (B). *FBXW7* gene was hypo-methylated in five different subtypes of ovarian cancer (beta values <0.1). Epi-A epithelial A, Epi-B, epithelial B; Mes, Mesenchymal; Stem-A, Stem-like A; Stem-B, Stem-like B

Table 2. Summary of Data Obtained by Gene Panel Exome Sequencing Analysis of *FBXW7* Gene in EOC Patients

| Exon | Region | rs# | Nucleotide Change[a] | Effect on Protein | Frequency in Patients | Frequency in Controls |
|---|---|---|---|---|---|---|
| Mutation | | | | | | |
| 10 | Exon | | c.1435C>G | p.Arg479Gly | 2/16 (8%) | 0/8 (0%) |
| Deletion | | | | | | |
| 9 | Exon | - | c.1386_1386delC | p.Thr463fs | 1/16 (6.25%) | 0/8 (0%) |

[a]Numbering based on RefSeq NM_032043 (for all the variants within the coding region). For exonic variants, The numbering starts at codon 1

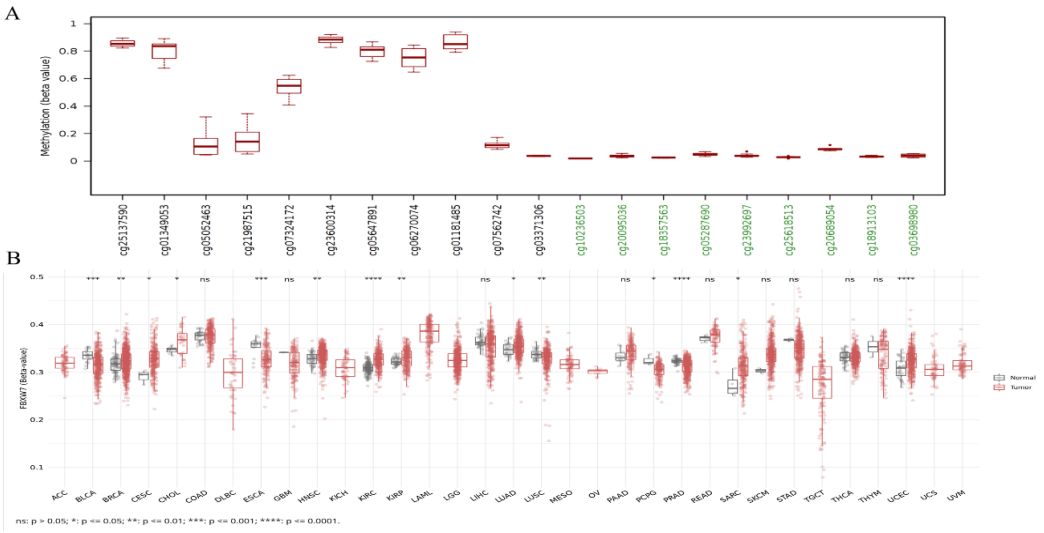



Figure 4. (A). Methylation of *FBXW7* in tumor ovarian serous cystadenocarcinoma (n=10). The majority of the FBXW7 methylation probes (65%) showed beta values <0.5, with the average beta value corresponding to 0.3. (B). CpG-aggregated methylated values across all samples. The beta value for the *FBXW7* gene was very low (0.3) in EOC samples as compared to other cancer types.

*FBXW7 silencing in EOC does not occur through methylation*

MSP was performed to support further the idea that the *FBXW7* gene is not silenced by methylation. Consistent with the bioinformatic analysis, the *FBXW7* gene was not methylated in HOSE 6-3, and MCAS cells. Furthermore, OVSAHO cells and 4 of the 8 epithelial ovarian tumor samples showed *FBXW7* methylation. However, the intensity of the bands was weak, indicating partial or low methylation status (Figure 7).

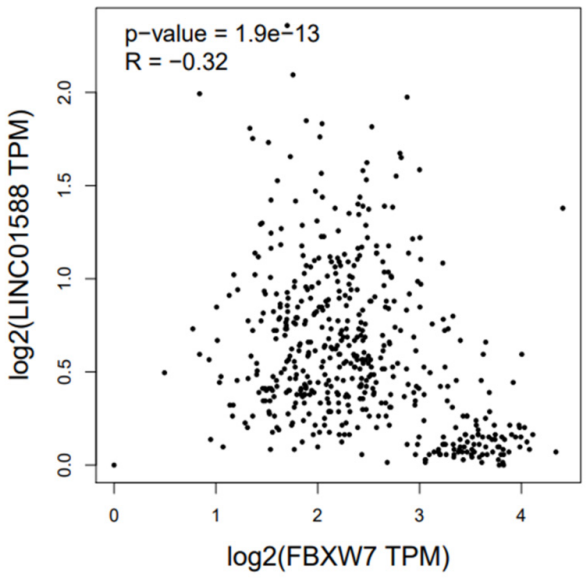



Figure 5. Pearson's Correlation Analysis Displays an Inverse Correlation between *FBXW7* and *LINC01588* ($P=1.9e^{-13}$, R=-0.32).

*Gene-Panel-Exome-Sequencing*

To further validate the *FBXW7* methylation status in EOC pathogenesis, gene-panel-exome-sequencing was performed on six EOC samples and five normal/benign ovarian tissue samples. No significant mutations and deletions (Table 2; Supplementary file S2) were recorded, supporting our hypothesis that other mechanisms involving the lncRNA (*LINC01588*) in regulating the *FBXW7* gene in EOC.

## Discussion

In a previous study using ChIP to study downstream genes regulated by E2F5, a transcription factor overexpressed during the early EOC stages (Malgundkar et al., 2022, Kothandaraman et al., 2010), we identified 145 E2F5 targets. These included *FBXW7* and a few ncRNAs (four miRNAs and twenty lncRNAs, including LINC01588), which might themselves have a role as a biological marker in EOC. Determining *FBXW7* expression and its promoter methylation status may contribute to relevant clarification about the mechanism responsible for its downregulation, EOC prognosis, aggressiveness, and response to treatment.

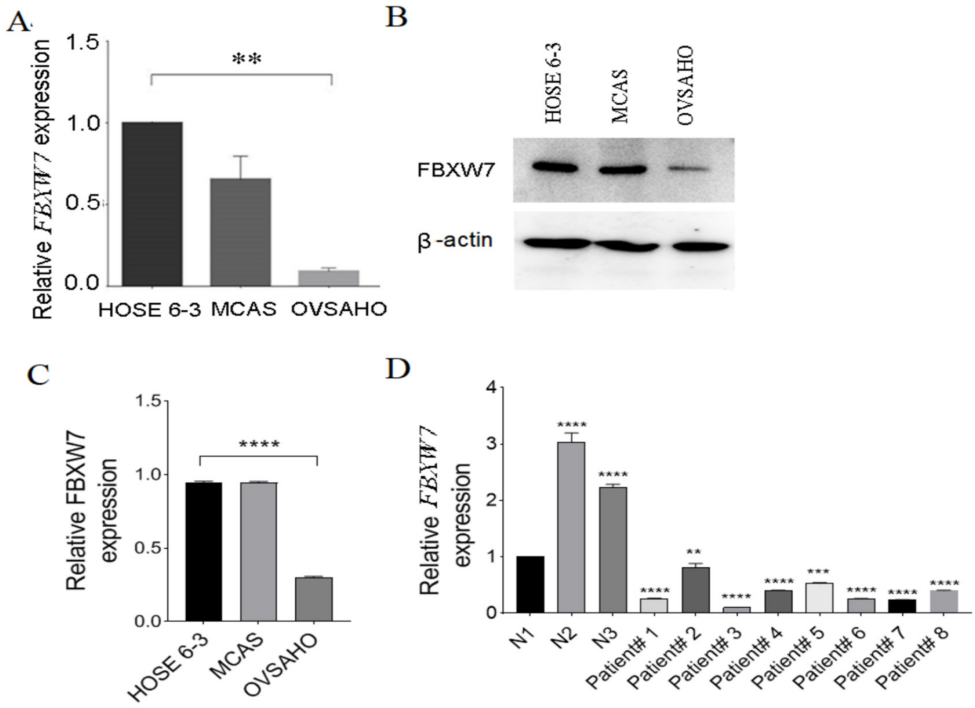
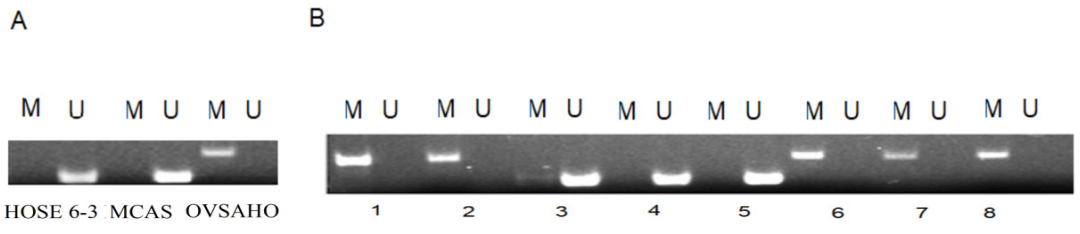



Figure 6. *FBXW7* Expression Analysis Using qRT-PCR and Western Blotting. (A). MCAS cells and HOSE 6-3 displayed a relatively higher *FBXW7* expression than OVSAHO cells. GAPDH was used as the housekeeping gene, and HOSE 6-3 normal ovarian cell line was used as control (B). Western blot protein expression analysis showed high *FBXW7* expression in MCAS and no expression in OVSAHO cell lines; β-actin was used as a loading control (C). Quantification of *FBXW7* expression at the protein level was performed by Image J software. (D). *FBXW7* expression was significantly downregulated in eight EOC patient samples as compared to healthy individuals (P<0.0001)

This study examined the mutation and methylation status of *FBXW7* in a large cohort of EOC patients using publicly available databases (cbioportal and CSIOVDB). The cbioportal revealed that *FBXW7* was not frequently mutated in EOC; only 15 out of 584 samples (2.6%) showed mutation (Figure 2A). In terms of expression, *FBXW7* down-regulation was observed in EOC samples compared to healthy tissues (Figure 2B), with lower expression in the late stages of EOC (III and IV), Figure 2C. Moreover, a low negative correlation was observed between the degree of methylation (beta value) and the *FBXW7* expression (Figure 3A). The beta values were found to be low among the five EOC subtypes as observed using the CSIOVDB database (Figure 3B), where Epi-A, Epi-B, Stem-A, Stem-B, Mes represents the EOC subtypes, which exhibit characteristic clinicopathological features, deregulated pathways, and prognosis (Tan et al., 2013). ß-values are the degree of methylation at a given locus, and its value ranges from 0 (unmethylated CpG sites) to 1 (methylated CpG sites) (Emes and Wessely, 2012). A previous study reported a β-value cut-off ranging from 0.2-0.3 as a gene hypo-methylation indicator (Men et al., 2017). Hence, bioinformatics analysis revealed that, in most of the EOC patients, the ß-values for *FBXW7* were




| Patient # | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | % |
|---|---|---|---|---|---|---|---|---|---|
| FBXW7 methylation | M | M | U | U | U | M | U | M | 50 |

Figure 7. *FBXW7* Gene Methylation Status in Cell Lines and EOC Patients. (A). Methylation was observed in *OVSAHO* cells but not in HOSE 6-3 and MCAS cells (B). MSP analysis of the *FBXW7* gene promoter was performed using DNA extracted from EOC tumor samples. Four out of eight EOC patient samples (50%) showed evidence of *FBXW7* methylation/silencing. Weak methylation bands were observed in the majority of the samples indicating partial or low methylation status with an estimated β-value <0.3. M, methylated; U, unmethylated.

less than 0.2 (hypomethylated), indicating that *FBXW7* downregulation is not likely to occur through methylation. Also, the majority of the *FBXW7* methylation probes (65%) displayed low *FBXW7* beta values (0<0.5), with average beta value in EOC samples corresponding to 0.3, further confirming the presence of weak or no *FBXW7* gene methylation (Figure 4).

Gene silencing is a complex process involving more mechanisms in addition to promoter methylation. Numerous studies have emphasized the function of ncRNAs in chromatin modifications as a key regulator of epigenetic control (Wei et al., 2017), and the aberrant expression of these ncRNAs has been linked with the development of several diseases, such as cancer (Wery et al., 2011). To identify *FBXW7* regulation by alternative mechanisms involving lncRNAs, we performed Pearson's correlation analysis. An inverse correlation was observed between the *FBXW7* gene and LINC01588, suggesting a potential regulation of the *FBXW7* gene by LINC01588 through a competing endogenous RNA network (Figure 5).

To validate the results obtained through bioinformatic analysis, we performed qRT-PCR and Western blotting. Based on our previous ChIP analysis, *FBXW7* interacted with E2F5 only in MCAS and OVSAHO cells, low and high-grade EOC cell lines, respectively. (Supplementary file S1). Hence, we examined the *FBXW7* gene expression in HOSE 6-3, MCAS, and OVSAHO cells, and our analysis revealed *FBXW7* expression at RNA and protein levels in HOSE 6-3 and MCAS cells (Figure 6). Since *FBXW7* expression is dependent on TP53, a TP53 mutation may be directly responsible for *FBXW7* silencing (Wang et al., 2014). The binding sites of TP53, a direct transcriptional regulator of *FBXW7*, were identified on the *FBXW7* gene. The OVSAHO cell line is a model for high-grade serous EOC, harboring P53 mutations (Domcke et al., 2013), and lacks *FBXW7* expression (Figure 6). In support of this, a study on EOC found a statistically significant correlation between low *FBXW7* expression and p53 mutation (Kitade et al., 2016).

*FBXW7*, a well-characterized tumor suppressor gene (TSG), is involved in G1-S checkpoint regulation in the cell cycle. *FBXW7* downregulation and accumulation of Cyclin E, c-Myc, and other oncoproteins are observed in gastric (Calcagno et al., 2013), colon (Iwatsuki et al., 2010), ovarian (Corney et al., 2008), and breast (Donnellan and Chetty, 1999) cancer.

Several studies, including whole-exome sequencing, have confirmed that *FBXW7* mutations are not frequent in EOC (Despierre et al., 2014; Jardim et al., 2014; Kwak EL et al., n.d.; Pereira et al., 2015). For example, genotyping 262 primary EOC samples found no mutations in the *FBXW7* gene(Despierre E et al., 2014). Recently, a study on 36 EOC samples (Pereira et al., 2015) demonstrated consistent results, while TCGA has only listed two EOC having *FBXW7* mutations in the COSMIC database, both of which are low-grade EOC(Cerami et al., 2012; Gao et al., 2013). Consistent with these findings, our results from gene-panel-exome-sequencing (Table 2) revealed the presence of a previously identified mutation (c.1435C>G) and a novel deletion (c.1386_1386delc), indicating that *FBXW7* is not frequently mutated in EOC, suggesting alternative mechanisms for the inactivation of the *FBXW7* gene. Furthermore, consistent with the results from the bioinformatic analysis, MSP revealed no evidence of *FBXW7* methylation in the HOSE 6-3 and MCAS cells. Moreover, the majority of the EOC patient's samples displayed faint bands indicating partial/ low *FBXW7* methylation status (Figure 7).

In conclusion, this study provides evidence that *FBXW7* is not mutated or methylated in EOC; hence it is likely to be silenced by alternative mechanisms involving the lncRNA (LINC01588) potentially via the competing endogenous RNA network.

## Author Contribution Statement

MH and IG performed the experiments and interpreted the data. MH, IG, and SH wrote the first draft of the manuscript under the supervision of YT. AO, IB, MK, and RL contributed to constructive critiques and discussions related to the clinical section of the project. They also contributed to the manuscript review. All the authors approved the final version of this manuscript..

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