RESEARCH ARTICLE

Downregulation of Tumor Promotor Genes in *Oryza Sativa* Linn.-Induced Antiproliferative Activity of Human Squamous Carcinoma Cells

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Abstract

   Objectives: Oral cancer represents the third leading cause of death in Southeast Asia and targeted therapy could prevent or delay disease etymology. *Oryza sativa* Linn. (OS) extract has been implicated as an antitumor agent in many cancer types, however none has been investigated in human squamous carcinoma-2 (HSC-2) cells, thus we aim to investigate the effects of OS on HSC-2 cells. **Methods:** Our study investigated the growth inhibitory effects of an ethanolic extract of OS on HSC-2 cells by BrdU ELISA and MTT assays, as well as changes in tumor promoter genes using RT-qPCR and western blotting. **Results:** We found that OS was able to induce cell cytotoxicity and inhibit HSC-2 proliferation. OS also decreased the expression of genes involved in the TGF-β/Smads signaling pathway and genes involved in cell motility such as *GPNMB, ITGB6*, and E2F1 by RT-qPCR. Western blotting confirmed the downregulation of TGF-β1 by OS. Co-treatment of OS and 5-Flurouracil also reversed Snail and Slug overexpression caused by HSC-2 exposure to 5-Flurouracil. **Conclusion:** Together, these results indicate that OS can inhibit HSC-2 cell proliferation and this may involve TGF-β1 downregulation. Thus, this study shows OS could be useful for the treatment of patients with squamous carcinoma.

   Keywords: *Oryza sativa* Linn, oral squamous cell carcinoma, anthocyanins, antiproliferation, TGF-β signaling

Introduction

   Oral squamous cell carcinoma (OSCC) accounts for up to 3.6% of cancer-related deaths and is the most common cancer of the head and neck (Shield et al., 2017; Bray et al., 2018). Despite the lack of progress in early detection, current research focuses on costly therapeutic procedures, such as chemotherapy combined with reconstructive surgery. However, a significant number of patients still experience tumor recurrence and metastasis during the course of therapy (Mignogna et al., 2002). Research has revealed protein targets involved in epithelial mesenchymal transition (EMT), responsible for developmental processes such as mesoderm formation, as well as those involved in cell adhesion and motility (Janiszewska et al., 2020). Molecular pathways involved in EMT include those of the Snail family (Nieto, 2002) and transforming growth factor-β (TGF-β), while integrins and cadherins are established markers for cell adhesion and play important roles in cancer malignancy. Natural compounds for cancer treatment remain of interest due to resistance to current chemotherapy drugs such as 5-fluorouracil (5-

FU) and cisplatin in OSCC, with resistance frequencies of up to 40.2% and 33%, respectively (Longley et al., 2003; Atashi et al., 2021; Sethy and Kundu, 2021). High doses of 5-FU can cause myelosuppression and other toxic effects (Macdonald, 1999). Thus, research into less invasive, preventive, and alternative methods of treatment to counteract chemotherapy resistance is needed to reduce oral cancer incidence and fatalities.

   Oryza sativa (OS), a rice species containing tocopherols and anthocyanins, has shown antioxidant properties and can inhibit tumor growth (Pintha et al., 2014). The extract has been found to inhibit proliferation and induce apoptosis in colon (Forster et al., 2013; Wongjaikam et al., 2014) and breast cancer cell lines (Pintha et al., 2014), and also to have antimetastatic properties against human oral CAL27 cells by inhibiting metalloproteinases-1 (MMP-1) (Phetpornpaisan et al., 2014; Haryanto et al., 2020). OS contains many bioactive compounds (Table 1). Anthocyanins from OS have also been implicated in preventing NF-κB activation by 5-FU and blocking various cell cycle regulatory proteins (Wang and Stoner, 2008; Tancharoen et al., 2018), with selective growth

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inhibitory effects in cancer cells while leaving normal cells intact (Hakimuddin et al., 2004). The crude extract of OS has been shown to inhibit colon cancer cell promotion in vivo and LPS-induced activation of RAW 264.7 cells by suppressing tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) (Phannasorn et al., 2017), as well as to decrease cancer cell motility in the PC3 prostate cancer cell line by reducing Smads and Snail expression (Jongsomchai et al., 2020). However, the effects of the crude extract of OS on human oral squamous carcinoma cells remain unstudied.

It is well-known that cancer is a disease propagated by many signaling pathways that integrate, resulting in carcinogenesis and metastasis. Therefore, the verification of a compound that targets many pathways involved in the hallmarks of cancer is beneficial. This study thus aims to analyze the effects of OS on human Squamous carcinoma cell proliferation, in comparison to the well-known chemotherapy drug, 5-FU.

Materials and Methods

Cell culture

The human squamous carcinoma (HSC-2) cell line was purchased from the National Institute of Biomedical Innovation, Health and Nutrition (Japanese Collection of Research Bioresources Cell Bank (JCRB); Osaka, Japan) and cultured in 1X Eagle's minimum essential medium (EMEM) with Earle's salts, without L-glutamine and phenol red (Corning, USA), containing 10% fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin streptomycin solution (Hyclone, UK). Cell culture was kept at 37°C in an incubator containing 5% CO_2. Media was changed every three days.

Plant extract preparation

Grains of *Oryza sativa* Linn. (OS) were obtained from Chachoengsao province, Thailand, and identification of specimen verified by the Organic Agriculture Certification Thailand (ACT). OS grains were extracted in 60% ethanol, concentrated using a Büchi B-490 rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland) and lyophilized using a freeze-dryer (Labconco Corp., Kansas City, MO, USA) as previously reported (Palungwachira et al., 2019). Extraction yields 3.5% of dry weight. Extract was dissolved in deionized water to obtain stock solutions of 500 mg/ml and stored at 4°C. Solutions of OS were prepared fresh and filtered through a 0.45 μm sterile- and endotoxin-free filter (Whatman, UK) prior to treatment.

MTT cytotoxicity assay

3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Promega, USA) colorimetric assay was used to determine cell viability as previously reported (Leenutaphong et al., 2022). Cells were seeded at a density of 1x10⁴ with fresh medium before treatment at concentrations of 2.5, 5 and 10 mg/ml OS, and 5 mg/ml 5-FU (Sandoz, Switzerland). After 6, 24, 48, and 72 h of treatment with the extract and 24 h treatment with 5-FU, 0.5 mg/ml MTT was added and the cells were incubated for 4 h at 37°C before DMSO was added. The

absorbance was measured at 570 nm and 690 nm (blank) using a microplate reader (BioTek, Vermont, USA). The cell viability was calculated according to: OD sample/OD control x 100%. The assay was performed in triplicate.

Cell proliferation assay

Cell proliferation was determined using Bromodeoxyuridine (BrdU) ELISA kit (Abcam; cat. no. 126556, UK) according to the manufacturer's instructions. Briefly, cells were seeded in a 96-well plate at a density of 5x10³ cells/100 μl/well and treated with OS at 0.3125, 0.625, 1.25, 2.5, 5, and 10 mg/ml for 24 h, as well as 5 mg/ml 5-FU for 24 h. 20 μl of 1X BrdU was added to each well and incubated along with the treatment. Cells were fixed for 30 min before incubated with anti-BrdU primary antibody for 1 h, and then with peroxidase goat anti-mouse IgG. After washing, TMB peroxidase was added for 30 min before the addition of stop solution. Cell proliferation was quantified by measuring the absorbance at 450 nm and 595 nm using a microplate reader (BioTek, Vermont, USA).

Real-time quantitative reverse transcription-PCR analysis

HSC-2 cells were plated at a density of 2x10⁶ cells/cm² and exposed to either 5 mg/ml OS and 5 mg/ml 5-FU, or both, for 24 h before transcriptomics analysis by real-time quantitative reverse transcription-PCR (RT-qPCR) with selected target genes. Total RNAs of the cells were harvested by trypsinization and RNA was extracted using RNeasy kit (Qiagen, China) according to the manufacturer's instructions. RNA concentration was measured using NanoDrop ND-1000 (NanoDrop Technologies, Delaware, USA). RNA was reversely transcribed to cDNA using oligo(dT) primers and SuperScript II reverse transcriptase kit (Qiagen, China). The primers were designed using PrimerBank (Massachusetts, USA) and are shown in Table 1. Real-time RT-qPCR reactions were performed in a total of 25 μl of reaction mixture using KAPA SYBR Green (Bio-Rad Laboratories., CA, USA). Data were analyzed using the comparative Ct method and was normalized by GAPDH expression in each sample.

Western blot analysis

HSC-2 cells were seeded at a density of 7x10⁵ cells and treated with 5 mg/ml OS and 5 mg/ml 5-FU for 24 h. Protein samples were lysed using mammalian protein extraction reagent (M-PER) (Thermo-Fisher Scientific, USA) supplemented with protease inhibitor cocktail (Sigma-Aldrich, USA). Protein concentration was determined using PierceTM BCA protein assay (Thermo-Fisher Scientific, USA). Western blotting was used to detect a change in protein expression as per methods previously reported (Leenutaphong et al., 2022). Protein was loaded into 10% SDS-polyacrylamide gels and electrophoretically transferred onto polyvinylidene difluoride membranes and blocked with bovine serum albumin. The membrane was exposed to primary antibodies against TGF-β1 (1:1,000) (Abcam, cat. no. ab215715, Cambridge, UK) and then washed before incubation with secondary antibodies (horseradish

peroxidase (HRP)-conjugated polyclonal goat anti-rabbit IgG; 1:1,000) (Dako, cat. no. P0447, Glostrup, Denmark). Proteins were visualized using a chemiluminescence kit and X-ray films (Bio-Rad Laboratories., CA, USA). Protein band densitometric analysis was done by a Scion Image Software (Scion Corp., Frederick, MD, USA).

Statistical analysis

All experiments were performed in triplicate. Results were expressed as mean ± SD. The differences between multiple groups were assessed using one-way analysis of variance followed by Tukey-Kramer method. The Student's t-test was used to determine the significance between two groups of treated and untreated controls. The level of significance was set at $p < 0.05$. All analyses were performed using SPSS 20.0 (SPSS Institute, Cary, NC, USA).

Results

The effects of Oryza sativa on HSC-2 cell cytotoxicity

Water-soluble *O. sativa* Linn. (OS) was tested for cytotoxic effects on HSC-2 cell lines by MTT assay at concentrations of 2.5, 5, and 10 mg/ml between 0 – 72 h (Figure. 1A). The IC_{50} values were 4.25 h and 34.33 h for cells treated with 10 mg/ml and 5 mg/ml OS, respectively. At 24 h, 10 mg/ml OS caused an approximate 25-fold decrease, compared to ~1.5-fold decrease after treatment with 5 mg/ml. Treatment with 2.5 mg/ml OS saw significant cytotoxicity after cell exposure to the extract for 72 h (p-value=0.041). Peak HSC-2 cytotoxicity effects were at 24 h for 10 mg/ml OS, and at 48 h for 5 mg/ml OS. Taken together, these results suggest that crude extracts of OS can attenuate cancer cell growth in a time- and dose-dependent manner. Figure. 1B shows that at 5 mg/ml, both OS and 5-FU caused significant cell death, however exposure to 5-FU caused a higher decrease in cell viability compared to when exposed to the same concentration of OS (p-value=0.0006).

Table 1. The Primer Used for Real-Time RT-qPCR Analysis

Genes	Primer sequence
TGFβ1	GGCCAGATCCTGTCCAAG
	GTGGGTTTCCACCATTAG
TGFβR II	GTAGCTCTGATGAGTGCAATGAC
	CAGATATGGCAACTCCCAGTG
SMAD3	TGGACGCAGGTTCTCCAAAC
	CCGGCTCGCAGTAGGTAAC
SMAD4	CTCATGTGATCTATGCCCGTC
	AGGTGATACAACTCGTTCGTAGT
SMAD7	TTCCTCCGCTGAAACAGGG
	CCTCCCAGTATGCCACCAC
SNAI1	TCGGAAGCCTAACTACAGCGA
	AGATGAGCATTGGCAGCGAG
SNAI2	CGAACTGGACACACATACAGTG
	CTGAGGATCTCTGGTTGTGGT
E2F1	ACGCTATGAGACCTCACTGAA
	TCCTGGGTCAACCCCTCAAG
ITGB6	TCCATCTGGAGTTGGCGAAAG
	TCTGTCTGCCTACACTGAGAG
GPNMB	CTTCTGCTTACATGAGGGAGC
	GGCTGGTGAGTCACTGGTC
GAPDH	GGAGCGAGATCCCTCCAAAAT
	GGCTGTTGTCATACTTCTCATGG

The effects of Oryza sativa Linn. (OS) on cell proliferation in HSC-2 cells

The inhibitory effects of OS compared to 5-FU on cell proliferation in HSC-2 cells was assessed by bromodeoxyuridine (BrdU) assay. We found that percentage cell proliferation decreased at a dose-dependent manner Figure 2A. Furthermore, treatment with OS and 5-FU at the same concentration (5 mg/ml)
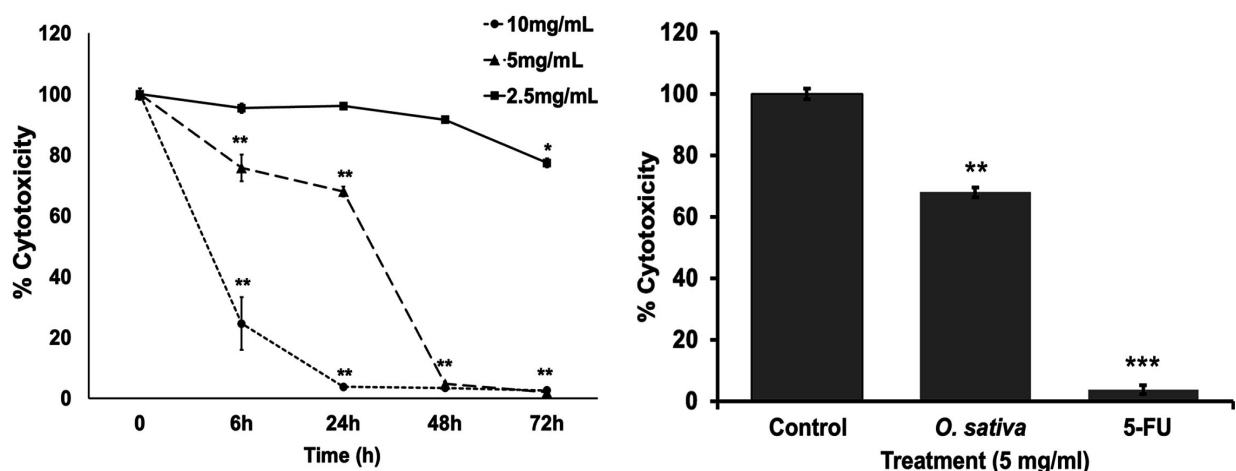

Figure 1. HSC-2 Cytotoxicity was Measured Using MTT Assay. Cell viability in response to (A) varying concentrations of OS (2.5, 5, and 10 mg/ml) and treatment time (0-72h) are displayed as a percentage of viable cells compared to control, and (B) 24 h treatment with 5 mg/ml of OS compared with 5-FU. Data expressed as means±SD; n=3 *p<0.05, **p<0.01, ***p<0.001.
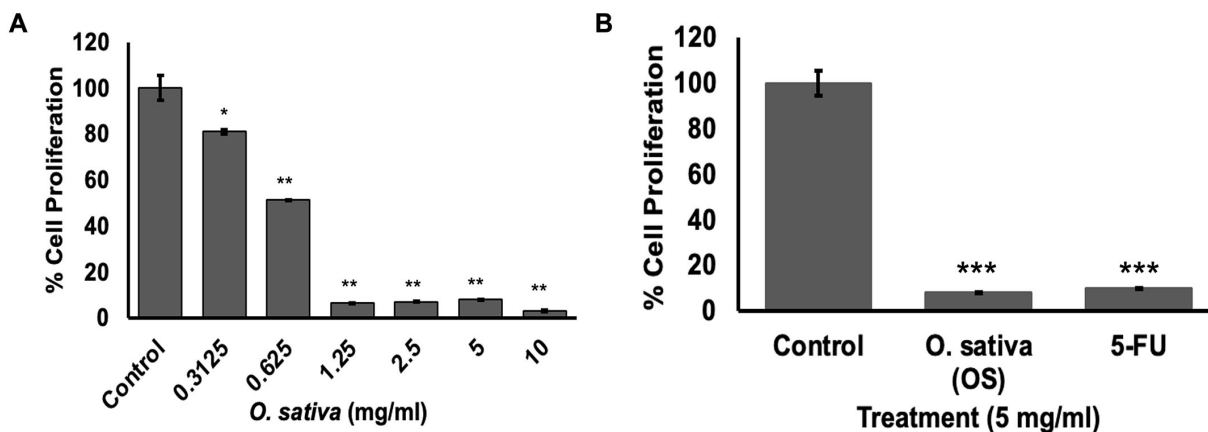
Figure 2. Cell Proliferation Inhibition after Treatment with *O. sativa* Linn. (OS). (A) Graph representing the percentage of cell proliferation with BrdU staining, after HSC-2 treatment with 0.31–10 mg/ml OS. (B) Comparison of percentage cell proliferation at 5 mg/ml OS and 5 mg/ml 5-FU. Data expressed as means±SD; n=3 *p<0.05, **p<0.01, ***p<0.001

caused similar inhibition of cell proliferation and there were no significant differences between the two treatment groups (Figure 2B).

Detection of Oryza sativa (OS)-responsive genes by real-time PCR

We examined changes in RNA in HSC-2 cells after treatment with OS and 5-FU by real-time PCR (RT-qPCR) analysis. We confirmed the changes of a subset of 12 genes that are involved in TGF-β/Smads signaling and tumor markers. Primers used to examine genes by RT-qPCR are listed in Table 1, while Table 2 shows fold change of each gene expressed in HSC-2 cells after treatment with 5 mg/ml OS and 5-FU for 24 h. Six genes out of ten were
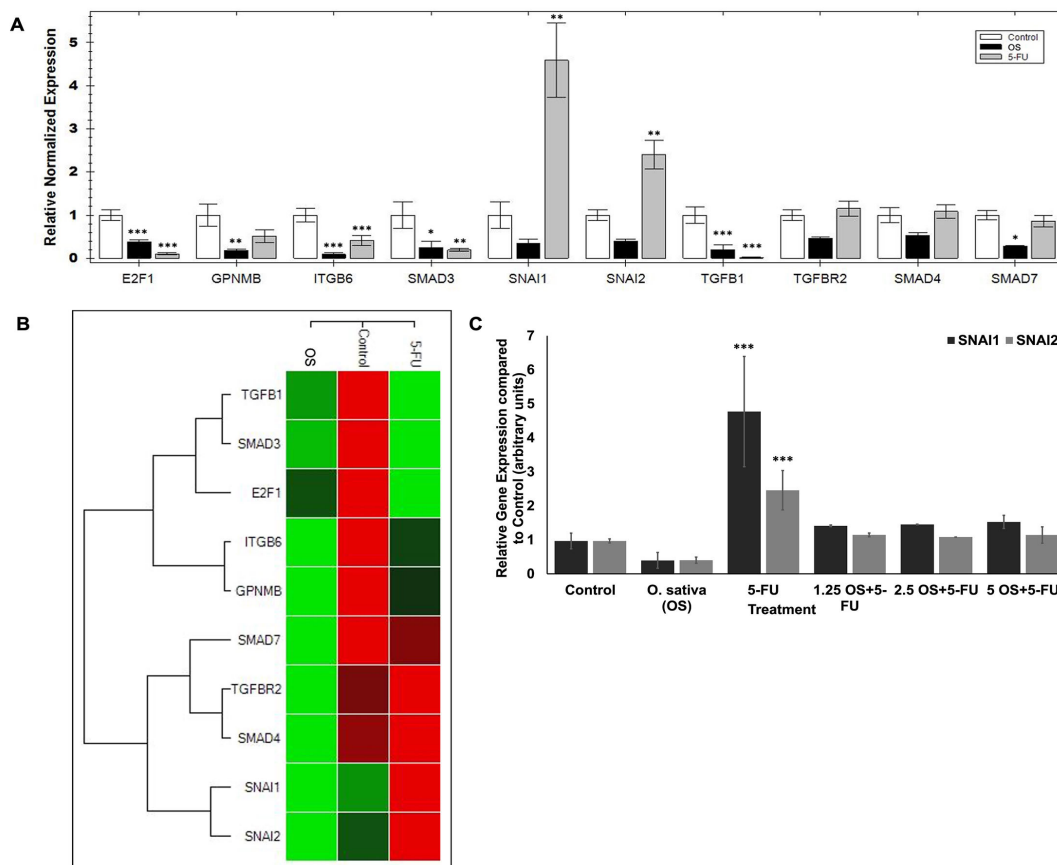

Figure 3. Differential Gene Expression by RT-qPCR Analysis. (A) Changes in gene expression in HSC-2 cells in response to 24 h treatment with either 5 mg/ml *O. sativa* Linn. (OS) or 5-FU by real-time RT-qPCR normalized to the reference gene, *GAPDH*, and control. (B) Heatmap and clustering of differentially expressed genes in the two treatment groups compared to the control group. (C) Changes in gene expression of *SNAI1* and *SNAI2* following combined treatment with 1.25, 2.5 and 5 mg/ml OS and 5 mg/ml 5-FU compared to treatment with 5 mg/ml plant extract alone, 5 mg/ml 5-FU alone, and control. Data expressed as means±SD; n=3 *p<0.05, **p<0.01, ***p<0.001.

Table 2. Fold Changes of Genes by RT-qPCR

Genes	HSC-2	
	OS	5-FU
Transforming growth factor β1 (*TGFβ1*)	-4.96	-13.952
Transforming growth factor β receptor II (*TGFβR II*)	-2.195	1.151
Smad family member 3 (*SMAD3*)	-4.016	-5.237
Smad family member 4 (*SMAD4*)	-1.871	1.085
Smad family member 7 (*SMAD7*)	-3.577	-1.156
Snail family transcriptional repressor 1 (*SNAI1*)	-2.864	4.586
Snail family transcriptional repressor 2 (*SNAI2*)	-2.541	2.41
E2F transcriptional factor 1 (*E2F1*)	-2.591	-9.759
Integrin subunit β6 (*ITGB6*)	-11.262	-2.431
Transmembrane glycoprotein NMB (*GPNMB*)	-5.368	-1.957
Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)	-	-

Figure 4. (A) Western blotting of TGF-β1 (*TGFB1*) following treatment with 5 mg/ml *O. sativa* Linn. (OS), 5 mg/ml 5-FU and co-treatment with equal amounts of plant extract and chemotherapy drug at 5 mg/ml compared to control (no treatment). (B) Quantification of electrophoresis bands showing TGF-β1 downregulation after 24 h treatment with various conditions. Data expressed as means±SD; n=3 *$p<0.05$, **$p<0.01$.

verified to be differentially expressed. Treatment with OS resulted in significant downregulation of *E2F1, GPNMB, ITGB6, SMAD3, TGFB1,* and *SMAD7*, while treatment with 5-FU at the same concentration resulted in significant downregulation of *E2F1, ITGB6, SMAD3,* and *TGFB1* (Figure 3A). The heatmap of differential expression shows clustering of genes involved in TGF-β/Smads signaling, as well as genes involved in tumor antigen presentation and intermembrane receptors involved in cellular regulation such as *GPNMB* and *ITGB6* (Figure 3B) (Taya and Hammes, 2018). Two genes, *SNAI1* and *SNAI2* encoding for Snail and Slug proteins, were found to be significantly upregulated in response to 5-FU, which may be a result of HSC-2 resistance to 5-FU (Zhang et al., 2012; Nakamura
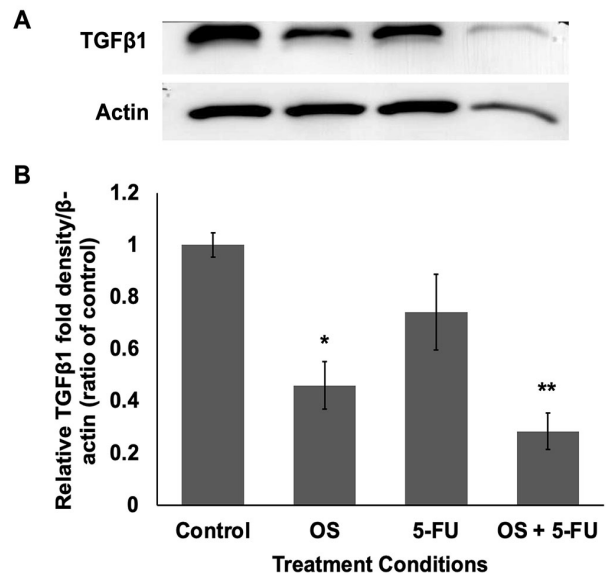
et al., 2018). Consequently, we further investigated the effects of combining OS and 5-FU on HSC-2 cells.

Reversal of SNAI1 and SNAI2 upregulation by co-treatment with Oryza Sativa Linn (OS) and 5-FU

PCR analysis revealed an upregulation in Snail (SNAI1) and Slug (SNAI2) genes, which suggests 5-FU
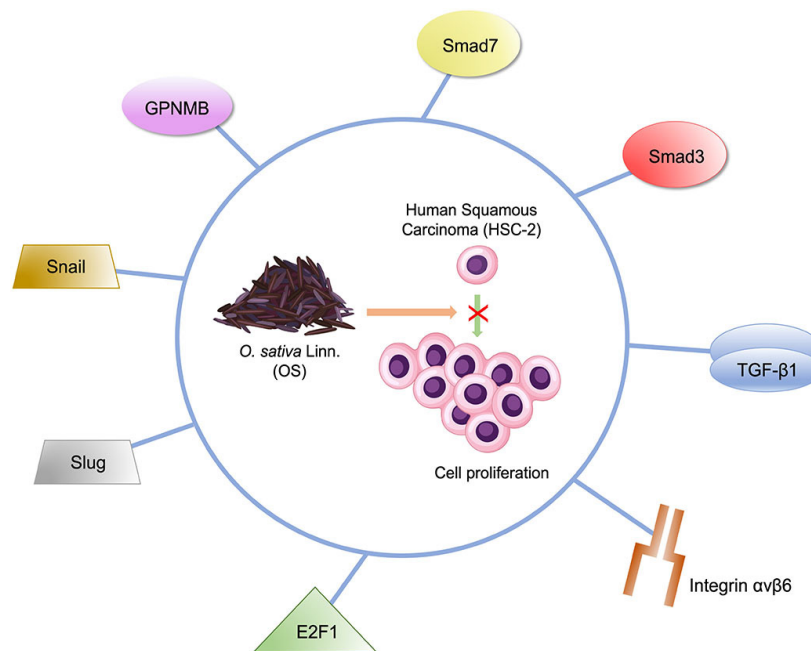

Figure 5. Summary of Selected Proteins that are Potentially Involved in Antiproliferative Effects of *O. sativa* Linn. (OS) on HSC-2 cells via modulating cell cycle and epithelial mesenchymal transition (EMT) pathways in the tumor microenvironment. GPNMB: glycoprotein non-metastatic melanoma protein B; TGF-β1: transforming growth factor β1; E2F1: E2F transcription factor 1.

drug resistance (Zhang et al., 2012; Nakamura et al., 2018). We found approximately 5-fold upregulation of Snail after cell exposure to 5-FU for 24 h (p-value=0.0007) and approximately 3-fold upregulation of *SNAI2* (p-value<0.0001). Combined treatment with 5 mg/ml 5-FU and 1.25, 2,5 and 5 mg/ml OS reversed both Snail and Slug upregulation caused by 5-FU (Figure 3C) to levels similarly seen in the control group (no difference in p-values).

Western blotting of TGF-β1 gene following treatment with Oryza sativa Linn and 5-FU

To further investigate the role of TGF-β1 in extract-induced antiproliferative effects in HSC-2 carcinoma, gel electrophoresis was performed to observe fold changes of the protein, TGF-β1 (Figure 4A). Density of bands were normalized to β-actin, before treatment groups were normalized to control and compared. We found significant downregulation of the protein in HSC-2 cells following treatment with 5 mg/ml OS and co-treatment with OS (p-value=0.043) and 5-FU for 24 h (p-value=0.0014, respectively) (Figure. 4B). This confirms our PCR results which also showed downregulation of the *TGFB1* gene, following 24 h treatment with the extract. However, unlike RT-qPCR results obtained with 5-FU treatment, western blotting showed no change in protein expression following HSC-2 exposure to 5-FU.

Discussion

Oral squamous carcinoma incidence remains high and immunotherapy provide a means of non-invasive treatment by targeting pathways regulating cell proliferation to prevent cancer metastasis. Our study shows *Oryza sativa* Linn. (OS) extract has antiproliferative effects against human squamous carcinoma cells (HSC-2), potentially involving proteins associated with TGF-β/Smads and GPNMB. Our results showed that OS extract inhibited HSC-2 cytotoxicity in a dose and time-dependent manner, with significant cytotoxicity compared to 5-FU at the same concentration. Similar findings have been reported in various cancer cells and tissues (Hakimuddin et al., 2004; Phetpornpaisan et al., 2014; Wongjaikam et al., 2014), and that anthocyanin action on cell cycle regulation proteins may be responsible (Wang and Stoner, 2008). Our study adds to the knowledge of OS's antiproliferative activity in oral squamous carcinoma *in vitro.*

To further understand the extract's effects on cell proliferation, we examined changes in the expression of a subset of genes involved in epithelial mesenchymal transition (EMT) and cell motility, such as those of the Snail family (Nieto, 2002) and TGF-β signaling (Hao et al., 2019). The TGF-β signaling pathway has been hugely implicated in cancer promotion (de Caestecker et al., 2000; Ikushima and Miyazono, 2010). We found that *ITGB6, E2F1, GPNMB, SMAD3, SMAD7* and *TGFB1* were downregulated in OS-treated HSC-2 cells after 24 h. In comparison, treatment with 5-FU induced downregulation of *E2F1, ITGB6, SMAD3* and *TGFB1*, as well as upregulation of Snail (*SNAI1*) and Slug (*SNAI2*). Integrins are transmembrane receptors involved in various signaling

processes, and integrin αvβ6 *(ITGB6)* is exclusively expressed in squamous carcinoma (Bandyopadhyay and Raghavan, 2009). In OSCC, *ITGB6* is elevated and aids cancer proliferation by binding to the TGF-β1 latency associated protein (Munger et al., 1999; Thomas et al., 2002). ITGB6 plays a role in TGF-β1 activation, and because TGF-β1 modulates EMT, integrin-TGFβ1 interaction likely has an important role in tumor metastasis (Sheppard, 2005). 5 mg/ml OS reduced both ITGB6 and TGF-β1 expression. Gel electrophoresis confirmed significant downregulation of TGF-β1. Co-treatment with OS and 5-FU also showed significant downregulation of TGF-β1, suggesting the potential of using OS extract to enhance 5-FU efficacy. Dysregulation of TGF-β1 signaling via Smads promotes fibrosis, and elevated levels of Smad7 are observed in TGF-β1 dysregulation (Stolfi et al., 2020). OSCC shows overexpression of Smad7, leading to accelerated conversion from malignant epithelial cells to the carcinoma stage (He et al., 2001; Liu et al., 2003). OS's ability to downregulate *ITGB6*, Smad7, Smad3, and TGF-β1 suggests a possible inhibition of cell proliferation through an integrin-TGFβ1-Smads interaction.

Our study also found E2 promoter binding factor 1 (*E2F1*) to be significantly downregulated following treatment with OS. A potent cell cycle regulator, *E2F1* can drive the cell's transmission from G1 to S phase and promote cell proliferation (Wu et al., 2001; Kent and Leone, 2019). Our results found downregulation of the gene, which was in line with our findings of antiproliferative activity. There is also evidence in the literature of interaction between Smad3 and members of the E2F family through signaling via the microRNA, miR-618, in head and neck squamous cell carcinoma (HNSCC) (Hui et al., 2016), and through troponin C-1 in gastric cancer (Fang et al., 2022). Smad3 is highly associated with TGF-β signaling (Derynck and Zhang, 2003) and when downregulated, is associated with higher patient survival rate in HNSCC (Xie et al., 2013). Our study reports significant downregulation of Smad3 following treatment with both OS and 5-FU at the same concentration, suggesting potential OS targeting of the TGF-β/Smads signaling pathway via this gene.

Glycoprotein non-metastatic melanoma protein B (GPNMB) is expressed in various aggressive cancers including glioblastoma, melanomas and breast cancer and promotes tumor growth through interaction with integrins (Taya and Hammes, 2018). It sheds it's extracellular domain protein, which binds to integrins α and β, triggering expression and secretion of tumorigenic factors (Taya and Hammes, 2018). CDX-011, which consists of the human-GPNMB- specific monoclonal antibody CR011 conjugated to a highly potent anti-mitotic agent called monomethyl auristatin E (MMAE), has shown effectiveness as an antitumor agent (Tse et al., 2006). Suppression or downregulation of GPNMB may be beneficial in targeted cancer therapy, and our study provides evidence of GPNMB downregulation by OS.

Our results revealed abnormal overexpression of two genes: *SNAI1* and *SNAI2* following treatment with 5-FU which is associated with drug resistance (Zhang et al., 2012; Liu et al., 2013), and our RT-qPCR results confirmed

that co-treatment of the extract and chemotherapy drug reversed Snail and Slug overexpression. Western blotting also showed that this co-treatment was able to reduce TGF-β1 expression. Together, these results suggest potential use for the extract combined with 5-FU administration to increase drug efficacy.

There are many pathways that contribute to carcinogenesis and the ideal immunotherapeutic substance must be able to target these pathways, while maintaining its specificity to cancerous pathways and not disrupting normal cellular functions. By choosing different genes involved in pathways that regulate cell motility and EMT, we found that many of them were differentially expressed after exposure to OS. Those genes may play a role in the extract's effects on HSC-2 proliferation. In conclusion, OS was able to modulate HSC-2 proliferation and proteins involved in cell adhesion, cell motility and EMT may play a role in the extract's antiproliferative effects on HSC-2, as summarized in Figure 5. There is also potential use for equal combination of OS and 5-FU administration to improve the efficacy of cancer drug treatment and the mechanism may involve modulation of the proteins Snail and Slug.

Limitations

Additional studies are needed to determine biochemical markers and investigate the exact mechanism of OS on HSC-2 cell cytotoxicity. Targeted phytochemical profiling of bioactive compounds in OS should also be conducted. This study is the first to report changes in gene regulation through TGFβ/Smads signaling, GPNMB, and integrin αvβ6 induced by OS. These findings are preliminary, and further testing is necessary to fully understand the mechanism of OS on HSC-2 cell proliferation.

Author Contribution Statement

Petchpailin Leenutaphong: Methodology, Data curation, Formal analysis, Visualization, Writing-Original draft preparation, Software analysis, Funding acquisition. Salunya Tancharoen: Supervision, Project administration Reviewing manuscript and provided access to crucial research components, Validation, Resources. Funding acquisition. Kiyoshi Kikuchi: Resources. Thamthiwat Nararatwanchai: Supervision, Validation. Chareerut Phruksaniyom: Methodology, Investigation, Software, Formal analysis. Sirintip Chaichalotornkul: Supervision, Validation.

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Ethical Approval

Not applicable. All research methods in this study was performed to the quality accepted by Mahidol University, Bangkok, and Mae Fah Luang University, Chiang Rai, Thailand.

Availability of Data

Data are available from corresponding author upon reasonable request. The present study follows the guidance of Critical Appraisal Tool (AIMRDA) developed for the Peer-Review of Studies Assessing the Anticancer Activity of Natural Products (Ahmad et al., 2022).

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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