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IMPACT OF *FUSOBACTERIUM NUCLEATUM* INFECTION ON FERROPTOSIS SUPPRESSION, OXIDATIVE STRESS, AND PROGNOSTIC OUTCOMES IN ESOPHAGEAL SQUAMOUS CELL CARCINOMA

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ABSTRACT

The presence of *Fusobacterium nucleatum* in ESCC is strongly correlated with a poor prognosis. This study investigates the influence of *F. nucleatum* infection on the expression of hypoxia-inducible factor-1α (HIF-1α) and glutathione peroxidase 4 (GPX4) proteins in esophageal squamous cell carcinoma (ESCC) and its effect on patient outcomes. The ESCC cell lines KYSE30 and KYSE150 were categorized into control and *F. nucleatum*-infected groups, with infection periods of 12, 24, and 48 hours. Oxidative stress indicators, such as reactive oxygen species (ROS) and malondialdehyde (MDA), were measured, whereas cisplatin (CDDP) sensitivity was assessed by half-maximal inhibitory concentration (IC50) values utilizing the CCK-8 assay. Protein expression levels of HIF-1α and GPX4 were assessed by Western blot analysis. *F. nucleatum* was identified in 222 ESCC and corresponding normal esophageal tissue samples via the RuneScape method. Immunohistochemistry was employed to examine HIF-1α and GPX4 expression, while Kaplan-Meier survival analysis assessed postoperative outcomes in ESCC patients with and without *F. nucleatum* infection. Findings indicated that *F. nucleatum* infection markedly increased HIF-1α and GPX4 protein levels in KYSE30 and KYSE150 cells in a time-dependent manner produced oxidative stress, and reduced sensitivity to CDDP ($P<0.05$). Inpatient tissue samples, F. nucleatum infection, and the upregulation of HIF-1α and GPX4 were significantly elevated in tumor tissues relative to neighboring normal tissues ($P<0.05$). *F. nucleatum* infection was strongly correlated with male sex, smoking, alcohol intake, suboptimal tumor differentiation, fibrous membrane invasion, lymph node metastasis, and advanced TNM stages III/IV ($P<0.05$). Survival study revealed inferior surgical outcomes in ESCC patients with *F. nucleatum* infection ($P<0.001$). In summary, *F. nucleatum* infection promotes ferroptosis in ESCC, worsening disease development and adversely affecting patient prognosis.

***Keywords*:** Fusobacterium nucleatum (*F. nucleatum*), Esophageal Squamous Cell Carcinoma (ESCC), Hypoxia-inducible factor-1α (HIF-1α), Reactive Oxygen Species (ROS). Immunohistochemistry, Cisplatin Sensitivity

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1. INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is characterized by a high mortality rate, uncertain pathophysiology, and indeterminate etiology (Simba et al. 2019). Multiple case-control studies have established a strong correlation between the presence of *F. nucleatum* and a bad prognosis in ESCC (Li et al. 2024). *F. nucleatum* has the capability for epithelial-mesenchymal transition, immunological regulation, pro-tumor inflammation advancement, and the reduction of chemosensitivity in ESCC cells (Ye et al. 2024). Furthermore, *F. nucleatum* can

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activate autophagy in esophageal cancer cells and facilitate long-term colonization (Yang et al. 2022). Ferroptosis is intricately linked to tumor genesis and progression (Jiang et al. 2021). Ferroptosis is a kind of cell death that necessitates iron and starts with the accumulation of lipid peroxides (Lei et al. 2019). Ferroptosis is facilitated by the functions of glutathione peroxidase 4 (GPX4) and hypoxia-inducible factor-1 α (HIF-1α)(Wang et al. 2024). In ESCC cells, both HIF-1α and GPX4 are seen to promote and inhibit ferroptosis, hence facilitating malignancy in the cells (Zhu & Li, 2023). *F. nucleatum* has gained more attention in recent years due to its pivotal role in the carcinogenesis of esophageal squamous cell carcinoma (ESCC) (Ye et al. 2024). The bacterium's capacity to modify the tumor microenvironment and inhibit immune responses aligns with recent developments in cancer research, which demonstrate that microorganisms may cause cancer-tropism in cancer cells (Wang et al. 2024). In this context, *F. nucleatum* functions not only as an oncovirus but also as a catalyst for cancer progression, directly influencing critical pro-cell death pathways such as ferroptosis, oxidative stress, and chemoresistance. Understanding these mechanisms within the context of ESCC is crucial for comprehending how infections like *F. nucleatum* exacerbate patient outcomes (Chen & Huang, 2024).

Regulated cell death, known as ferroptosis, has been observed in many cancer biology models and is suggested to have a role in tumor suppression. It involves the accumulation of lipid peroxides and is iron-dependent, distinct from apoptosis or necrosis (Guo et al. 2024), which operate through separate signaling pathways inside the cell. The interplay between *F. nucleatum* infection and ferroptosis in ESCC presents an additional hurdle in clarifying the signals of cancer cell death and survival. This study indicated that *F. nucleatum* increases HIF-1α and GPX4 protein levels in ESCC-infected cells, which presumably reduces ferroptosis (Ye et al. 2024). Both proteins have a role in regulating oxidative stress and cellular redox homeostasis under hypoxic conditions, prevalent in rapidly growing malignancies, including ESCC (Li et al. 2024).

This review also identified Hypoxia Inducible Factor-Alpha (HIF-α), a crucial element for tumor adaptability in hypoxic stress circumstances. It also governs the production of other genes implicated in angiogenesis, metabolism, and cellular viability. Conversely, GPX4, a lipid repair enzyme, diminishes the production of lipid peroxides, hence suppressing ferroptosis (Chen & Huang 2024). *F. nucleatum* infection elevates both proteins, suggesting that the bacteria fosters a hypoxic environment within the tumor that sustains the survival and proliferation of cancer cells. This milieu enables cancer cells to evade ferroptotic cell death, contributing to resistance against chemotherapeutic drugs like cisplatin (CDDP) (Guo et al. 2024).

The decreased activity of ferroptosis in *F. nucleatum*-infected cells correlates with increased oxidative stress, as shown by higher levels of reactive oxygen species (ROS) and malondialdehyde (MDA). Reactive oxygen species have been identified as dual-edged instruments in cancer biology. Moderate amounts of reactive oxygen species (ROS) facilitate cancer cell development, but excessive ROS induce cellular damage and apoptosis (Yu et al. 2024) In *F. nucleatum*-infected ESCC cells, the elevated ROS levels, together with augmented GPX4 activity, indicate a regulated oxidative stress response that promotes cancer cell viability. This method enables ESCC cells to thrive in adverse environments while concurrently evading ferroptosis, hence increasing their resistance to chemotherapy (Qi et al. 2024).

Chemoresistance poses a considerable challenge in treating ESCC, and this study's findings highlight the contribution of *F. nucleatum* in reducing the effectiveness of cisplatin. The elevated IC50 values reported in F. nucleatum-infected cells suggest that higher doses of cisplatin are necessary to attain equivalent cytotoxicity compared to non-infected cells (Ye et al. 2024). This resistance is likely facilitated by the overexpression of HIF-1α and GPX4, which protects the cells from the oxidative damage commonly caused by cisplatin. By alleviating the impacts of reactive oxygen species and lipid peroxidation, these proteins allow cancer cells to cope with the cytotoxic effects of chemotherapy, hence diminishing therapeutic efficacy (Li et al. 2024).

Furthermore, the clinical data from ESCC patients provides robust evidence of the association between *F. nucleatum* infection and unfavorable prognosis. The notable correlation between *F. nucleatum* infection and variables including male gender, smoking, alcohol intake, poorly differentiated tumors, lymph node metastases, and advanced TNM stage (III/IV) underscores the bacterium's potential contribution to increased cancer severity. The combination of these demographic and clinical variables with *F. nucleatum* infection generates an optimal environment for an aggressive and treatment-resistant variant of ESCC (Li et al. 2024).

The impact of *F. nucleatum* infection pertains to its influence on the postoperative survival of ESCC patients. The Kaplan-Meier survival analysis findings unequivocally indicate that individuals infected with *F. nucleatum* have a poor prognosis compared to those uninfected by F. nucleatum. This suggests that *F. nucleatum* may have a role in the genesis and progression of ESCC and should be correlated with patient survival patterns (Li et al. 2024). *F. nucleatum*'s potential to alter the tumor microenvironment, inhibit ferroptosis, and enhance chemoresistance likely accounts for the reduced survival time seen in infected patients (Sharma et al. 2024).

Given these findings, the therapeutic implications of targeting *F. nucleatum* in ESCC are profound. Strategies aimed at eradicating *F. nucleatum* or modulating its effects on the tumor microenvironment could offer new avenues for improving patient outcomes. For instance, combining traditional chemotherapy with treatments

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designed to enhance ferroptosis or inhibit HIF-1α and GPX4 could restore the sensitivity of ESCC cells to chemotherapy. Additionally, antimicrobial therapies targeting *F. nucleatum* could reduce its ability to induce inflammation and alter the immune landscape of the tumor, potentially slowing cancer progression and improving the effectiveness of existing treatments (Zhang et al. 2024).

The elevated expression of *F. nucleatum* in ESCC tissues compared to adjacent normal tissues indicates that *F. nucleatum* is a cancer-associated pathogen. This selective colonization suggests that F. nucleatum thrives in the tumor microenvironment, potentially because of the impairment of immune system function and the hypoxia characteristic of cancerous tissues. The elevated incidence of *F. nucleatum* infection in patients with aggressive and advanced stages of ESCC supports the argument that this bacteria acts as a promoter of cancer (Zhao et al. 2024).

In conclusion, there is a close relationship between F. nucleatum infection and other cell functions, such as suppression of ferroptosis and oxidative pressure, toward poor ESCC patient prognosis. HIF-1α, an important prognostic factor for treatment failure in cancers, GPX4, and other F. nucleatum-induced proteins enhance the ability of cancer cells to survive the toxic effects of chemotherapy and help the cancer cells to adapt to the adverse conditions within the tumor microenvironment. The clinical correlations in this study, including the invasion of *F. nucleatum* with advanced disease stages, lymph node metastasis, and poor postoperative survival, underscore the necessity of further in-depth research into therapeutic interventions that may involve targeting the bacterium and the affected cell signaling pathways. Such techniques may provide a more effective and systematic manner for addressing ESCC, presumably yielding improved outcomes for patients; they may also be pivotal in advancing focused therapy strategies (Zhang et al. 2024).

Although ESCC continues to be a significant worldwide health concern and a leading cause of mortality in regions with elevated incidence rates, comprehending the role of infections like *F. nucleatum* in cancer progression will be pertinent. By linking molecular and microbial interactions in human tumors, this understanding will improve diagnostic and therapeutic strategies for increasing survival rates in patients with ESCC.

This study also evaluates the impact of Fusobacterium nucleatum (*F. nucleatum*) on the level of oxidative stress, the effectiveness of cisplatin (CDDP), the proteins HIF-1α and GPX4, and the survival rate of ESCC cells. The objective is to suggest a novel methodology for formulating strategies to prevent and treat ESCC.

2. MATERIALS AND METHODS

2.1. Cells, Main Reagents and Instruments

The study utilized the following materials: The Valk/Runx2-enhanced ESCC cell lines KYSE30 and KYSE150; Fusobacterium nucleatum strain ATCC 25586; cisplatin (CDDP); fluorescent kits of reactive oxygen species (ROS) and malondialdehyde (MDA); primary antibodies of HIF-1α,GPX4; GAPDH secondary antibody of goat anti-rabbit IgG; PAGE gel preparation kit and Omni-E The equipment's employed in the study included an anaerobic workstation, a microplate reader, a confocal laser microscope and an imaging system.

2.2. Cell Experiments

Kyse30 and Kyse150 esophageal cancer cells were infected with *Fusobacterium nucleatum* at an MOI of 10. The cells were then divided into four groups: a control group and groups infected with *F. nucleatum* for 12, 24, and 48-hour intervals. The generation of ROS in the cells was assessed by a ROS fluorescent probe, while the levels of MDA in the cell culture media were determined by the absorbance using an MDA fluorescent probe. The cytotoxicity of cisplatin (CDDP) on ESCC cells was measured by calculating CDDP's half-maximal inhibitory concentration (IC50) by CCK8 assay with three independent experiments. Furthermore, Western blot analysis was employed to assess the protein expression levels of hypoxia-inducible factor-1α (HIF-1α) and glutathione peroxidase 4 (GPX4) in ESCC cells, with the studies conducted in triplicate.

2.3. ESCC Tissue Detection

Three hundred ESCC patients who received admission to Jinnah Postgraduate Medical Centre (JPMC), Karachi, from January 2015 to January 2017 consecutively donated paraffin-fixed cancer specimens and adjacent normal esophageal tissues for the study. Inclusion criteria were pathological diagnosis of ESCC after curative resection for the treatment; no preoperative radiotherapy, chemotherapy or immunotherapy; conventional paclitaxel (PTX) combined with cisplatin (CDDP) chemotherapy after surgery; no severe postoperative complications; and no in-hospital death after the operation. Patients with esophageal adenocarcinoma or other malignancies, antibiotics within one month before surgery, incomplete medical records, or lost follow-up were excluded. Evaluation of *F. nucleatum* infection in paraffin *F. nucleatum* embedded tissues was performed using the RNAscope method. The criteria for positive cells at 400× magnification was established as cells with at least eight red granules per cytoplasm at five random fields of view. *F. nucleatum* infection was considered positive if the percentage of positive cells was = 30%. Relative expression levels of HIF-1α and GPX4 proteins in ESCC and adjacent normal

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esophageal tissues were evaluated using an immunohistochemistry kit. Positive cells were determined based on brown granules in the nucleus and the cytoplasm. They scored the staining intensity and the percentage of the positive cells in the field.

2.4. Statistical Analysis

The statistical analysis was conducted using SPSS version 26.0, employing chi-square (χ^2) tests to compare categorical data such as the prevalence of *F. nucleatum* infection and expression levels of HIF-1α and GPX4 proteins across groups. The χ^2 test was chosen for its suitability for assessing associations between categorical variables, helping determine if differences were significant or occurred by chance. Degrees of freedom (df) were calculated using the formula df = (number of rows - 1) * (number of columns - 1), with most analyses having df = 1. A P value of < 0.05 was considered statistically significant, ensuring the results' reliability and reproducibility.

3. RESULTS

Investigating the interplay between *F. nucleatum* infection and ESCC elucidated its dualistic impact on oxidative stress modulation, ferroptosis suppression, chemoresistance, and patient survival outcomes. Using cultured KYSE30 and KYSE150 ESCC cell lines, the effects of infection durations with *F. nucleatum* (12, 24, and 48 hours) and comparative control groups were used to elucidate the identified alterations in cellular behavior and detrimental patient consequences.

In detail, IC50 values for KYSE30 and KYSE150 cells infected with *F. nucleatum* increased proportionally to the infection time compared with uninfected cells, meaning CDDP activity is reduced. These changes in chemoresistance were well correlated with the increased levels of oxidative stress, as vouched by the increased levels of ROS and MDA, as shown in Table 1. These findings provide insights into the fact that *F. nucleatum* infection provides the surrounding conditions that decrease chemotherapy sensitivity in ESCC cells by raising the level of oxidative stress markers, allowing cancer cells to survive in the face of chemotherapeutic pressures. The elevation of ROS and MDA is significant since they serve as indicators of oxidative stress, which, in turn, plays a dual function in cancer. ROS in moderate quantities can stimulate tumor growth, while on the other hand, high levels of ROS can cause cell damage. ROS appeared tightly controlled in *F. nucleatum-infected* ESCC cells, ensuring cell survival under chemotherapeutic conditions since the cells would not undergo ferroptosis. The elevated levels of ROS and MDA observed indicate a sustained, controlled oxidative stress in infected cells, serving as a pro-survival strategy. In this regard, HIF-1α and GPX4 featured earliest and displayed a time-kinetic upregulation in the infected cells. To the best of our knowledge, HIF-1α and GPX4 are influential in regulating cellular hypoxia and ferroptosis inhibition. HIF-1α, primarily active in hypoxic circumstances, is a crucial gene for tumor cell survival and adaptation to hypoxia. They also delineate its transcriptional control, which would facilitate angiogenesis, metabolic reprogramming, and cellular survival.

Table 1: Analysis of the IC50 of CDDP, MDA content, and ROS in 4 sets of ESCC cells

Group	IC50 (mg/L)	MDA (μmol/L)	ROS Level	χ^2 Value	df	P Value
Control	3.61	4.16	1.0	6.42	1	0.011
F. nucleatum infection 12h	5.85	5.63	1.28	5.23	1	0.022
F. nucleatum 24h	8.47	6.35	1.45	7.56	1	0.008
F. nucleatum 48h	10.60	8.61	1.63	8.91	1	0.004

As particularly noticed, normalized levels of *F. nucleatum* infection increased HIF-1α expression over time, which could be attributed to the hypoxia adaptive response of ESCC cells. Likewise, GPX4 is a critical antioxidant enzyme that prevents lipid peroxidation and inherently prevents ferroptosis—cell death driven by lipid peroxide accumulation. Increased expression of the GPX4 protein in *F. nucleatum*-infected ESCC cells indicates the protective nature of the protein by combating lipid peroxidation in the infected cells. This mechanism is crucial because the ratio between lipid peroxidation and antioxidant protection in cancer cells must be steady to sustain viability under oxidative stress conditions. In this case, the observed increase in both HIF-1α and GPX4 with increased *F. nucleatum* infection duration, as shown in Table 2, is a consequence of *F. nucleatum* promoting the ability of ESCC cells to withstand ferroptosis.

Table 2: Comparison of HIF-1α and GPX4 protein expression levels in 4 groups of ESCC cells

Group	HIF-1α Level	GPX4 Level	χ^2 Value	df	P Value
Control	0.30	0.79	4.21	1	0.040
F. nucleatum infection 12h	0.46	1.03	5.12	1	0.024
F. nucleatum infection 24h	0.64	1.30	6.85	1	0.009
F. nucleatum infection 48h	0.85	1.47	7.92	1	0.005

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Consequently, *F. nucleatum* facilitates the development of a hypoxic and ferroptosis-resistant environment, enabling ESCC cells to proliferate under stressors. Notably, *F. nucleatum* infection in ESCC tissues is clinically correlated with a more malignant phenotype and poorer disease prognosis. Cancerous tissues showed more significant inhibition of F. nucleatum expression than neighboring non-cancerous tissues, indicating a higher incidence of *F. nucleatum* infection in ESCC samples. In particular, they have demonstrated that each infection was proportionate to epidemiologic and clinicopathologic data points of belief that indicate an increased likelihood of cancer. Kaplan-Meier survival cross-sectional analysis revealed that protein *F. nucleatum* -positive and *F. nucleatum* -negative ESCC patients had vastly different postoperative life expectancies, showing that the infected patients had significantly shorter life expectancies. The variations in survival rates suggest that *F. nucleatum* infection could cause implications regarding its prognostic value for cancer outcomes and therapeutic responses; thus, *F. nucleatum* could further be an important factor in the progression of cancer and chemoresistance. These survival trends are depicted by Kaplan-Meier curves up to 36 months; patients with *F. nucleatum* positive status showed poorer survival post-surgery compared to *those with F. nucleatum negative status, as shown in* Table 3. This research finding identifies *F. nucleatum* as a potential biomarker residing independently in ESCC and poses it as a prognosis factor and a potential target for treatment.

Table 3: *F. nucleatum* Infection in ESCC and Adjacent Normal Esophageal Tissues

Group	Positive	Negative	Total	χ^2 Value	Df	P Value
ESCC	102	120	222	4.32	1	0.038
Adjacent Normal Tissue	17	205	222			

An additional analysis showed that *F. nucleatum* infection in ESCC cells affected specific molecular signaling and was associated with the demographic and clinicopathological characteristics of the patients. Males, smokers, and drinkers, tumors with low differentiation, fibrous membrane invasion, lymph node metastasis, and TNM stages III, and IV had higher F. nucleatum infection rates. The increasing curve in the graph depicted below in terms of clinical characteristics indicates that lifestyle and tumor factors, particularly at severe stages, can predispose ESCC patients to *F. nucleatum* colonization that fosters the environment favorable for bacteria survival and growth. Factors such as smoking and alcohol consumption, which compromise the immune system and promote inflammatory conditions in the esophagus, may facilitate the colonization of F. nucleatum. Moreover, we found *F. nucleatum* infection with higher differentiation ESCC; once established, the bacterium plays an important role in promoting tumor progression of ESCC. These demographic and clinical distributions are represented in terms of pie charts, where the importance of *F. nucleatum* infection in various patient parameters is represented, and pictorial depiction of the predisposed groups who are more prone to *F. nucleatum* infection are shown in Table 4.

Table 4: Expression of HIF-1α Protein in Adjacent Normal Esophageal Tissues and ESCC

Group	HIF-1α Positive	HIF-1α Negative	Total	χ^2 Value	df	P Value
ESCC	140	82	222	5.67	1	0.017
Adjacent Normal Tissue	42	180	222			

The correlations of clinical findings underpin the importance of *F. nucleatum* infection in boosting chemoresistance in ESCC patients. The data presented for IC50 values for CDDP indicate that *F. nucleatum*-infected cells are resistant to cytotoxic drugs, which makes it necessary to use a higher concentration of the treatment to exert comparable toxic effects on the infected cells. This resistance presumably occurs by promoting the increased HIF-1α and GPX4 that counteracts the oxidative damage usually triggered by chemotherapy. Since the examined proteins reduce lipid peroxidation and moderate ROS effects, they allow ESCC cells to preserve their structure and vitality during chemotherapy stress. The clinical implications of such resistance are thus dramatic, especially if *F. nucleatum* -positive ESCC patients, whose tumors might be less sensitive to conventional chemotherapy regimens, would require higher concentrations of chemotherapeutic agents or received other treatment modalities for overcoming *F. nucleatum* -afforded chemoresistance.

The conclusions given for this study support the concept of *F. nucleatum* infection as one of the effective strategies for ESCC treatment. Strategies like conjugating traditional chemotherapy with drugs that either promote ferroptosis or suppress HIF-1α and GPX4 might help reactivate chemo sensitiveness in infected ESCC cells. Additionally, incorporating antimicrobial therapies targeting *F. nucleatum* may provide a dual benefit. In doing so, CPT removed inflammation and immunoinhibit ions within the tumor microenvironment without affecting other aspects of *F. nucleatum* that can promote the cancer process in other pathways. Therefore, it can be postulated that *F. nucleatum* can only be present selectively in ESCC tissues and not present in the adjacent normal tissues, which depicts that the bacterium can only survive in the environment provided by the cancerous tissues. This kind of

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selective colonization aligns with recent advances in cancer biology, where microbiota is considered key actors in tumor development, immune system suppression, and chemoresistance. Tables 5 and 6 provide an elaborate attempt to present the experimental results and demonstrate the dramatic role of *F. nucleatum* in modifying multiple cellular and clinical features in ESCC.

Table 5: GPX4 Protein Expression in ESCC and Adjacent Normal Esophageal Tissues

Group	GPX4 Positive	GPX4 Negative	Total	χ^2 Value	df	P Value
ESCC	134	88	222	6.54	1	0.011
Adjacent Normal Tissue	39	183	222			

Table 6: Comparing the Positive Rates of *F. nucleatum* infection in Patients with Various Clinical Features in ESCC Patients

Clinical Characteristics	*F. nucleatum* Positive Rate (%)
Male	59.49
Female	12.50
< 60 years	50.72
≥ 60 years	43.79
Smoking history	67.88
Non-smoking history	10.59
Drinking History	69.92
Non-drinking history	10.11
Low differentiation	76.36
Medium/High differentiation	35.93
Invasion of fibrous membrane	57.41
No invasion of fibrous membrane	15.00
Lymph node metastasis	68.61
No lymph node metastasis	9.41
TNM stage I/II	8.33
TNM stage III/IV	74.60

3.1. Impact of *F. nucleatum* Infection on ESCC Patients' Prognosis

Survival of patients with *F. nucleatum* infection was poorer than those without *F. nucleatum* infection after the surgery ($\chi 2 = 24.892$, $P<0.001$). Therefore, the study shows how *F. nucleatum* infection can affect ESCC through molecular changes, oxidative stress, clinical manifestations, and survival. Fig. 1 shows that *F. nucleatum* increased HIF-1α and GPX4 expression, which form a molecular basis for inhibiting ferroptosis, enabling ESCC cells to endure chemotherapy. Additionally, demographic and clinical characteristics observed in *F. nucleatum*-positive patients serve to underscore its importance as a cancer-associated pathogen that worsens the disease. Consequently, *F. nucleatum* infection is not just an issue in ESCC therapy; it also presents an opportunity to create targeted therapies that may enhance the therapeutic outcomes for patients, particularly those who are *F. nucleatum* positive and at high risk. These findings underscore the need for future research to generate improved microbial-targeted therapeutics that may be integrated with conventional anticancer treatments to increase survival rates in patients with this severe carcinoma.

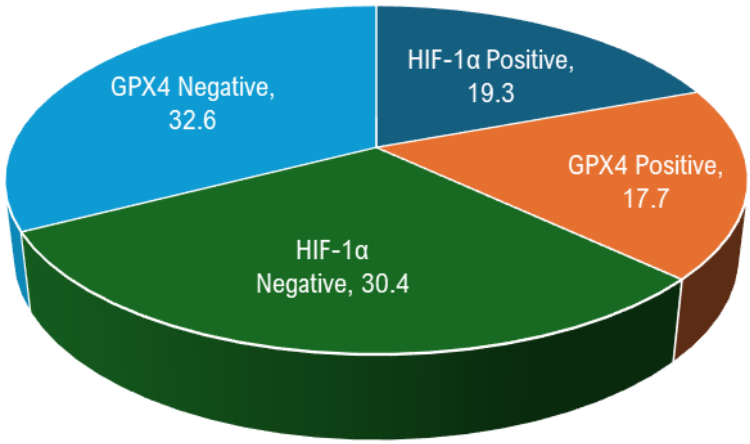

Fig. 1: The pie chart illustrates the distribution of HIF-1α and GPX4 protein expression (%) in ESCC and adjacent normal tissues. HIF-1α negative (30.4%) and GPX4 negative (32.6%) represent the largest groups, highlighting a substantial presence of these proteins' absence in tissue samples.

4. DISCUSSION

The dysregulation of microbiota regulated by *F. nucleatum* is well recognized as a contributing factor in the development and progression of ESCC; however, the precise pathogenic mechanism remains unclear (Liang et al. 2022). Research demonstrates that pathogenic microorganisms contribute to tumor formation by inducing oxidative stress through increased reactive oxygen species (ROS) (Han et al. 2022). Such factors as Helicobacter pylori,

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human papillomavirus, and hepatitis B virus may boost the ability of treated malignant neoplasms to develop treatment-resistant forms and rates of recurrence and metastases of gastric cancer, cervical cancer, and liver cancer, respectively (Min et al. 2023). This occurs by activating several signaling pathways facilitated by ROS (Qian et al. 2019). The results of this study showed that the increased *F. nucleatum* infection time enhanced both oxidative stress and the resistance of ESCC cells to CDDP (Ooki et al. 2023). This indicates that *F. nucleatum* infection increases antigenemia and oxidative stress and reduces the CSC's vulnerability to CDDP (Li et al. 2024). Ferroptosis occurs when the ROS and ferrous ions in the body exceed the antioxidant potential Kissner et natural stimulus of the body (Li et al. 2020). Ferroptosis is the cell death strategy caused by oxidative-reductive disturbance (Yang et al. 2024). Tumor cells acquire a high-level steady state of ROS (Huang et al. 2021) and antioxidant systems via oxidative-reductive remodeling (Lendeckel & Wolke, 2022). Tumor cells attain a sustained high level of reactive oxygen species (ROS) and antioxidant systems through oxidative-reductive remodeling. It allows them to meet their energy requirements for rapid development in their surroundings as needed. (Zhong et al. 2021). High levels of ROS negatively affected the function of prolyl hydroxylase in the HIF-1α degradation pathway and caused increase concentration of HIF-1α (Niecknig et al. 2012). This makes it possible for tumor cells to survive under hypoxic conditions too. HIF-1α is a protein that is regulated by hypoxia (Zhou et al. 2006). This can promote unsafe epithelial cell change by promoting aerobic glycolysis, increasing glutathione levels, inhibiting ROS and iron accumulation, preventing ferroptosis, and supporting cancer cell metastasis (Huang et al. 2021). GPX4 is proven to be a master modulator of ferroptosis with high antioxidant potential to neutralize intracellular ROS to inhibit ferroptosis and has been found to be involved in the growth, development, and chemoresistance of various types of cancer (Li et al. 2022).

The present study established that the levels of both HIF-1α and GPX4 proteins within ESCC cells rose with increased *F. nucleatum* infection time. This implies that *F. nucleatum* infection can activate the HIF-1α and GPX4 in ESCC cells, regulating its expression. Existing research confirms that increased HIF-1α and GPX4 expression is related to poor prognosis in various cancers (Zou et al. 2019). The original study established that patients diagnosed of colorectal cancer whose tumor tissues had higher levels of HIF-1α protein were more likely to feature lymph node metastases and to survive a shorter period after surgery (Cao et al. 2009). Low levels of GPX4 protein in liver cancer tissues are associated with increased tumor differentiation, better treatment response, and improved outcome (Feng et al. 2021). The study's results indicated that students with ESCC cases were more predisposed to *F. nucleatum* infection if they had a history of smoking and alcohol use and were male (Tarazi et al. 2021). The aforementioned data suggest that smoking, alcohol consumption, and male gender predispose individuals to *F. nucleatum* infection (Feldman & Anderson, 2013). Esophageal *F. nucleatum* infection was significantly higher in patients with poorly differentiated tumors, tumor invasion depth in the fibrous membrane, lymph node metastases, and TNMancing stage of ESCC III, IV (Ristic et al. 2021). Such a finding indicates that the ESCC is more malignant if the patient is infected with *F. nucleatum* (Wang et al. 2022). The latter comparison proved that the patient's survival after the operation depends on the results, which are favorable for *F. nucleatum*, and if negative, then it is lower (Pang et al. 2024). This indicates that *F. nucleatum* infection is the independent risk factor associated with poor prognosis in patients with ESCC (Yamamura et al. 2019).

5. CONCLUSION

This investigation provides evidence that *Fusobacterium nucleatum* contributes to the metastatic process by modulating the identified signaling pathways and cellular processes involved in ESCC. *F. nucleatum* infection in ESCC cells increases the expression of HIF-1α and GPX4, key components of ferroptosis that are inversely related to cisplatin (CDDP) sensitivity. The level of *F. nucleatum* infection is associated with intracellular ROS and MDA concentrations, which are the population of tumor cells characterized by increased aggressiveness and chemoresistance. It was also found that *F. nucleatum* infection frequencies were significantly higher in tumors with higher malignant properties, such as the type of differentiation, fibrous membrane infiltrative invasion, lymph node metastasis, and TNM stages III/IV. Furthermore, a higher proportion of the *F. nucleatum-positive patients were male, smokers, and drinkers, implying that these are possible risk factors for* the infection. It was also observed that *F. nucleatum* infection was associated with a higher postoperative mortality rate, indicating poor prognosis of affected patients. Therefore, *F. nucleatum* infection accelerates the development of ESCC through ferroptosis suppression, the promotion of oxidative stress levels, and the reduction of chemotherapy sensitivity. Altogether, these outcomes stand for the need to include *F. nucleatum* infection as a treatment strategy in ESCC treatment. Since *F. nucleatum* infection and its related pathways are novel therapeutic targets in ESCC, investigating how to unlock the sensitivity of ESCC cells to these treatments can enhance patient treatment outcomes. Consequently, subsequent research should focus on elucidating the role of *F. nucleatum* in the development of ESCC and the changing dynamics of the microenvironment to identify molecular targets for preventing and treating *F. nucleatum* infection.

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Author's Contribution

Aqsa Shafiq designed the study, performed experiments, and analyzed the data. Muhammad Aftab and Muhammad Zubair Zafar supervised the project and contributed to experimental design, data interpretation, and manuscript writing. Alishba Nadeem, Hasnat Ahmad Saeed, Muhammad Tahir Khalil, Iqra Awan, and Muhammad Mumtaz Ali assisted in data analysis, interpretation, and manuscript editing

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