

Determination of Intracellular Iron in Oral Squamous Cell Carcinoma Using Perl's Prussian Blue Iron Stain

Abstract

Oral squamous cell carcinoma (OSCC) has a 50% 5-year survival rate which depends on several factors including micronutrients such as iron. Further, iron not only has profound effects on the growth and development of OSCC but also can influence the progression and prognosis of OSCC an important role in the progression of OSCC. In the study, FFPE (formalin-fixed paraffin-embedded) OSCC tissue segment samples were examined for the presence of intracellular iron. From the archives, previously diagnosed FFPE were retrieved, and 3-micron tissue sections were made. The tissue section is then subsequently stained with Perl's Prussian blue stain. A trained pathologist evaluated and interpreted the stained tissue section. Histopathological analysis showed scarce iron deposits within OSCC cells after staining with Perl's Prussian blue stain which showed the blue-colored deposits with the tissue cells. There is the presence of small traces of intracellular iron in OSCC which supports the view that iron might play a role in OSCC growth and proliferation. Determining intracellular iron using Perl Prussian blue stain is a convenient and inexpensive method to determine the outcome of the treatment of the cases with OSCC.

Keywords: Iron, Oral squamous cell carcinoma, Perl's Prussian blue, Ferroptosis, Prognosis

Introduction

Oral squamous cell carcinoma (OSCC) is a major disease responsible for millions of deaths all over the world.^[1] OSCC is diagnosed in the progressive stage because of the delay in seeking treatment.^[2] Further, several factors such as iron not only have profound effects on the growth and development of OSCC but also can influence the progression and prognosis of OSCC.^[2, 3] Furthermore, iron plays an important role in the metabolic function of cancer cells.^[4]

During tumor growth, tumor tissue increases the uptake of iron in OSCC. Cancer cells reprogram their iron metabolism to increase net iron influx.^[5-8] Upregulating proteins for iron uptake such as transferrin receptor (Tf) accomplishes this.^[9] The Tf/TfR system represents one of the major routes for iron acquisition, both in normal and malignant cells.^[10] Intracellular iron accumulation can trigger the activation of tumor necrosis factor- α and nuclear factor- κ B which provide a favorable environment for carcinogenic compounds production.^[11] Further, iron accumulation also initiates oxidative stress by promoting

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reactive oxygen species (ROS) generation.^[5] ROS activates the cancer cell survival signaling cascade which in turn activates the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), matrix metalloproteinase (MMPs), and vascular endothelial growth factor (VEGF).^[12] Thereby accelerating the growth of tumors requiring more iron which may lead to iron deficiency anemia.

Conversely, since tumor cells are strongly dependent on iron for their growth/proliferation, they are more sensitive to iron depletion than normal cells leading to iron-dependent cell death called ferroptosis.^[13] Ferroptosis is a type of cell death usually accompanied by a large amount of iron accumulation and lipid peroxidation during the cell death process.^[13] Moreover, in the past, several studies reported the development of iron deficiency anemia in OSCC^[14] and reported higher levels of serum ferritin^[3, 15] which is contradictory to serum ferritin levels present in iron deficiency anemia. This clearly shows a discordant view. Further, little is known about the intracellular presence of iron. It is not clear

**Chandan Panigrahi¹,
Monal Yuwanati^{2*},
Senthil Murugan M³,
Priyadharshini R¹**

¹Department of Pathology, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences (SIMATS), Saveetha University, Chennai – 600077, India.

²Department of Oral Pathology and Microbiology, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical sciences (SIMATS), Saveetha University, Chennai-600077, India.

³Department of Oral Oncology & Reconstructive Microsurgery, Dentistry, Saveetha Dental College and Hospitals, Chennai-600077, India.

Address for correspondence:

Monal Yuwanati,
Department of Oral Pathology,
Saveetha Dental College and
Hospitals,
Saveetha Institute of Medical and
Technical sciences (SIMATS),
Saveetha University,
Chennai- 600077, India.
E-mail:

monalyuwanati.sdc@saveetha.com

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whether cancer cells have higher intracellular iron accumulation or whether cells are selective in the timing of intracellular uptake of iron from serum.

There is limited evidence on the intracellular presence of iron in oral cancer cells. Tanvir *et al.* investigated the iron expression in different grades of OSCC.^[16] This study observed a decrease in intracellular iron levels with an increase in the degree of differentiation. One possible explanation for this is that during the keratinization process in well-differentiated OSCC, cancer cells may enhance iron intake for epithelial cell maturation.^[17, 18] This needs further exploration to understand the role of iron metabolism in OSCC. Further, clinicians use iron supplements to treat iron deficiency and researchers are working on drugs that target the ferroptosis pathway to treat OSCC.^[13] However, it is crucial to know whether there is an iron buildup within the cells; otherwise, the aforementioned treatment may unintentionally cause toxicity or accelerate OSCC.

Therefore, the primary aim of the study was to investigate the intracellular iron in OSCC tissue with a secondary aim to check the feasibility of Pearl's Prussian blue stainability to detect ferroptosis in cancer cells.

Materials and Methods

The present observational study was conducted on archival Formalin-Fixed Paraffin-embedded (FFPE) OSCC tissue.

Tissue sample

Ten histopathologically confirmed well-Differentiated OSCC along with clinical and medical history, tumor location; clinical manifestation, histopathology stage, etc. were taken from Dental College.

Iron staining and assessment

The FFPE tissue block was cut into a 4-micron thin tissue section and transferred to a glass slide. Tissue sections are deparaffinized using Xylene. Sections are immersed in equivocal amounts of potassium ferrocyanide (1%) and hydrochloric acid (1%). Followed by 3 washings in distilled water and counterstained with 1% neutral red for 5 minutes. Tissues were again dehydrated at 90 % and 100 % and passed through 2 changes of xylene before mounting and coverslipping. A pathologist assessed the presence of iron and assigned a score of a present (+) or absence (-).

Results and Discussion

Detailed characteristics of OSCC cases are given in **Tables 1 and 2**. The mean age of patients with OSCC was 47.6 years. All cases were of Well-Differentiated grade. Eight and two males and females, respectively. The site was buccal mucosa (n=6), gingiva (n=2), and tongue (n=2). All were in TNM stage II. The mean Hb % was 11.28 Hb %. Out of 10 OSCC, cases only three were positive for intracellular iron (**Table 2 and Figure 1**).

Table 1. Clinicopathological Characteristics of included OSCC samples

Clinicopathological Parameter	N (%)
Mean Age	47.55 years.
Gender (F)	8 (2)
Site	
Buccal Mucosa	6
Gingiva	2
Tongue	2
TNM stage	
II	10
Mean Hb%	11.8%

Table 2. Descriptive features of OSCC samples and iron expression in OSCC cells

OSCC Id	Gender	Age	Histopathology Grade	TNM	Site	Hb%	Iron staining
1	Female	61	WDSSC	II	Tongue	7.80	Negative
2	Male	46	WDSSC	II	gingiva	11.60	Positive
3	Male	36	WDSSC	II	Buccal mucosa	8.60	Negative
4	Male	47	WDSSC	II	Buccal mucosa	12.30	Negative
5	Male	53	WDSSC	II	Buccal Mucosa	11.80	Negative
6	Male	45	WDSSC	II	gingiva	11.20	Negative
7	Male	54	WDSSC	II	Buccal Mucosa	13.40	Positive
8	Female	47	WDSCC	II	Tongue	11.90	Negative
9	Male	52	WDSCC	II	Buccal Mucosa	11.50	Negative
10	Male	35	WDSCC	II	Buccal Mucosa	12.70	Positive

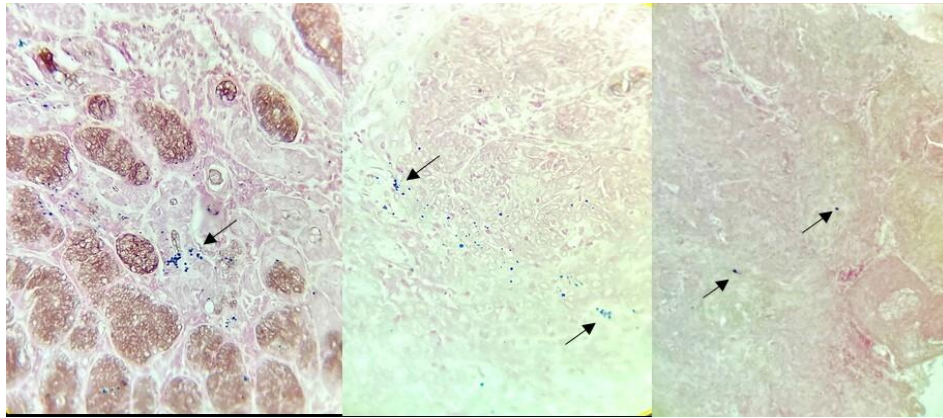


Figure 1. Perl's Prussian blue-stained tissue sections showing the blue-stained iron within the OSCC cells.

The present study observed the presence of small traces of intracellular iron in OSCC which support the view that iron might play a role in OSCC growth and proliferation. Iron is an essential mineral required in several biochemical interactions including the proliferation of epithelial cells, and metabolism. Iron utilization occurs within the mitochondria, cytoplasm, and nucleus in cells. Several cellular functions depend upon iron including the cell signaling involved in cell division and maturation. Identification of intracellular iron deposits in OSCC compared to normal mucosa widened the scope of iron in OSCC progression. However, intracellular iron presence was very less in OSCC. This may be because of the selection of OSCCs with good differentiation, and the majority of the cells may have achieved complete maturity and keratinization.

Ferroptosis involves the rapid removal of intracellular iron and triggering the apoptosis process. It is characterized by intracellular iron ion accumulation and reactive oxygen species (ROS)-induced lipid peroxidation.^[19] The presence of small amounts of iron may indicate that OSCC cells might use different pathways to overcome ferroptosis by preventing the excessive accumulation of iron which might be responsible for increased serum ferritin. In OSCC, especially in T3, T4 stage, and recurrence, serum ferritin levels increase as compared to normal tissue and oral potentially malignant disease.^[15] Further, serum ferritin has a correlation with lymph node metastasis in OSCC.^[20] Ferritin is the major regulator of intracellular iron and is stored in the form of Fe₂ within the serum. Decrease levels indicate the presence of iron deficiency anemia. Unfortunately, several studies reported the presence of iron deficiency anemia in OSCC which contradicts high serum ferritin levels. This can explain the basis of selective uptake for iron by OSCC tissue to favor the growth and progress without affecting the serum iron levels.

Identification of intracellular iron in OSCC tissue can help in predicting prognosis to a certain extent. However, it needs expensive equipment and materials such as fluorescent dye to identify the ferroptosis process within cells. Perl's Prussian blue histochemical stain may be used as a cost-effective alternative. However, a future study with large sample size is warranted before recommending its use as routine practice and investigation.

The present study had certain limitations. First, we did not have an appropriate source to confirm the ferroptosis process within OSCC cells, as it requires an expensive confocal fluorescent microscope. Since it was a retrospective study, we could not collect and correlate serum ferritin levels with perls Prussian blue staining

Conclusion

There is the presence of intracellular iron in OSCC although in small quantities. Determining intracellular iron using Perl Prussian blue stain is a convenient and inexpensive method to determine the outcome of the treatment of the cases with OSCC as well as an alternative to expensive fluorescent or confocal microscopy. Further, it can be used as a potential prognostic marker for OSCC. However, future studies with a large cohort are essential.

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Conflict of interest

None.

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None.

Ethics statement

The study was approved by Institutional ethics committee (Number: IHEC/SDC/UG-2001/22/GPATH/613).

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