MECHANISM OF NEURAL PRECURSOR CELL EXPRESSED DEVELOPMENTALLY DOWN-REGULATED 4-LIKE (NEDD4L) IN MODULATING FERROPTOSIS VIA UBIQUITINATION OF SOLUTE CARRIER FAMILY 7 MEMBER 11 (SLC7A11) IN OESOPHAGEAL SQUAMOUS CELL CARCINOMA

CHEN ZHEN

UNIVERSITI SAINS MALAYSIA

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by

CHEN ZHEN

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LIST OF ABBREVIATIONS

ACSL4	Acyl-CoA synthetase long-chain family member 4
ANOVA	Analysis of variance
AP	Ammonium persulfate
ATCC	American Type Culture Collection
BALB/c	Bagg Albino nude mice
CCCP	Carbonyl cyanide-m-chloro phenylhydrazone
CCK8	CCK-8 cell counting
CHX	Cycloheximide
CO_2	Carbon dioxide
CRT	Chemo-radiotherapy
DAPI	FluoroshieldTM containing 4’,6’-diamidino-2-phenylindole
DCFH-DA	2, 7-dichlorodihydrofluorescein diacetate
DECR1	2,4-Dienoyl-CoA Reductase 1
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ddH_2O	Double-distilled water
EGFR	Epidermal growth factor receptor
FAGs	Ferroptosis-associated genes
FBS	Fetal bovine serum
Fe^{3+}	Ferric ions
FC	Fold change
FITC	Fluorescin isothiocyanate
FL	Full length

FTH1	Ferritin heavy chain 1
GEO	Gene Expression Omnibus
GEPIA	Gene Expression Profiling Interactive Analysis
GPX4	Glutathione peroxidase 4
GSH	Glutathione
GTEx	Genotype-Tissue Expression
GWAS	Genome-wide association study
HECT	Homologous to the E6AP carboxyl terminus
HMOX1	Heme Oxygenase 1
IF	Immunofluorescence
IP	Immuno-precipitation
IREB2	Iron-responsive element binding protein 2
JC-1	5,5V ,6,6V -tetrachloro-1,1V ,3,3V-tetraethylbenzimidazolylcarbocyanine iodide
KEAP1	Kelch like ECH associated protein 1
LPCAT3	Lysophosphatidylcholine acyltransferase 3
MDA	Malondialdehyde
MG132	Carbobenzoxy-Leu-Leu-Leucinal
MMP	Mitochondrial membrane potential
mRNA	Messenger RNA
NEDD4L	Neural precursor cell expressed, developmentally down-regulated 4-like
OAC	Oesophageal adenocarcinoma
OD	Optical density
OS	Overall survival
OSCC	Oesophageal squamous cell carcinoma
PBS	Phosphate buffer saline
PDT	Photodynamic therapy

PE	Phycoerythrin
PFS	Progression-free survival
PMSF	Phenylmethylsulfonyl fluoride
PI	Propidium iodide
PTMs	Post-translational modifications
PUFAs	Polyunsaturated fatty acids
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative real-time polymerase chain reaction
RT	Radiotherapy
RNA	Ribonucleic acid
RNA-seq	RNA Sequencing
ROS	Reactive oxygen species
RPMI 1640	Roswell Park Memorial Institute 1640
SAGE	Serial analysis of gene expression
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SDT	Sonodynamic therapy
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SLC7A11	Solute carrier family 7 member 11
STR	Short tandem repeat
TCGA	The Cancer Genome Atlas
TEM	Transmission electron microscopy
TEMED	Tetramethylethylenediamine
UALCAN	University of Alabama at Birmingham Cancer
Ub	Ubiquitin
VDAC	Voltage-Dependent Anion Channel

LIST OF SYMBOLS

α	Alpha
β	Beta
℃	Degree Celsius
~	Approximately
*	Asterisk
–	Dash
=	Equals
>	Greater than
-	Hyphen
<	Less than
/	Or
±	Plus-minus
×	Times
%	Percentage
KDa	Kilodalton
µL	Microliter
µM	Micromolar
µg	Microgram
mL	Milliliter
nM	Nanomolar
rpm	Revolutions per minute
Δ	Delta
mA	Milliampere

MEKANISMA *NEURAL PRECURSOR CELL EXPRESSED DEVELOPMENTALLY DOWN-REGULATED 4-LIKE* (NEDD4L) DALAM MODULASI FEROPTOSIS MELALUI PENGUBIKUITINAN *SOLUTE CARRIER FAMILY 7 MEMBER 11* (SLC7A11) DALAM SEL KARSINOMA SKUAMUS ESOFAGUS

ABSTRAK

Pengesanan sel karsinoma skuamus esofagus (OSCC) pada peringkat awal menimbulkan cabaran besar disebabkan presentasinya yang halus. Ketiadaan terapi sasaran yang diluluskan dan prognosis yang lemah turut menambah kesukaran dalam merawat OSCC. Feroptosis, dengan keupayaannya untuk menghalang percambahan sel kanser, menunjukkan potensi dalam terapi kanser, dan gabungannya dengan kemoterapi mungkin dapat meningkatkan keberkesanan rawatan. Bukti baharu menunjukkan bahawa NEDD4L ialah ligase ubiquitin E3 yang memainkan peranan penting dalam penindasan tumor dengan mengawal atur laluan feroptosis melalui ubiquitinasi. Walau bagaimanapun, hubungan dan mekanisma pengawalaturan khusus mereka dalam OSCC masih tidak diketahui. Oleh itu, kajian ini direka untuk menjelaskan peranan NEDD4L dalam OSCC, serta mekanisma pengawalaturan khusus antara NEDD4L dan feroptosis dalam OSCC. RNA penggangu kecil digunakan terlebih dahulu untuk mengkaji kesan penyenyapan NEDD4L pada OSCC. Hasil kajian menunjukkan bahawa penyenyapan NEDD4L mempercepatkan migrasi dan invasi sel EC9706, serta menggalakkan percambahan sel EC9706 dan Eca109. Model mencit xenograf telah dibangunkan melalui suntikan subkutaneus sel EC9706 yang dijangkiti dengan rambut pendek NEDD4L. Selepas 30 hari, berat dan isipadu tumor mencit diukur dan hasilnya mengesahkan bahawa penyenyapan NEDD4L

mencetuskan pertumbuhan tumor dalam OSCC. Tambahan pula, DCFH-DA yang sangat sensitif digunakan untuk mengesan spesies oksigen reaktif (*Reactive Oxygen Species atau ROS*). Data menunjukkan bahawa penyenyapan NEDD4L menghalang pengumpulan ROS dalam sel EC9706 dan Eca109. Di bawah rawatan erastin, kesan NEDD4L pada penunjuk feroptosis yang lain termasuk kematian sel, perubahan morfologi mitokondria, dan potensi membran mitokondria (*mitochondrial membrane potential atau MMP*) dikesan masing-masing melalui pewarnaan Annexin V/PI, mikroskopi elektron transmisi dan mikroskopi pendarfluor. Didapati bahawa penyenyapan NEDD4L menunjukkan peranan penghalangan dalam feroptosis. Selain itu, hubungan yang berpotensi antara NEDD4L dan SLC7A11 telah ditemui melalui penjujukan berkeluaran tinggi, yang disokong oleh asai penyelamatan, imunopendarfluor (*immunofluorescence atau IF*) dan pengimunopemendakan bersama (*co-immunoprecipitation atau Co-IP*). Asai sikloheksimida (*Cycloheximide atau CHX*) dan asai MG132 dilakukan untuk menganalisis kesan penyenyapan NEDD4L pada kestabilan protein SLC7A11. Terbukti bahawa penyenyapan NEDD4L memanjangkan separuh hayat SLC7A11 dan pengawalaturan sedemikian bergantung pada proteasom. Ekspresi berlebihan NEDD4L meningkatkan ubiquitinasi berkaitan K48, sambil mengurangkan ubiquitinasi berkaitan K63 pada SLC7A11 dalam sel HEK293T. Keputusan ini ditunjukkan melalui asai IP berasaskan *ubiquitin*. Tambahan pula, mutan NEDD4L^{C942A}, yang kekurangan aktiviti pemangkin, tidak dapat mempengaruhi ekspresi, separuh hayat, serta ubiquitinasi SCL7A11, menunjukkan kesan NEDD4L dalam mengawal atur SLC7A11 bergantung pada aktiviti ligase E3. Kesimpulannya, NEDD4L menyumbang kepada feroptosis dan perencatan pertumbuhan sel dengan memudahkan ubiquitinasi SLC7A11 dalam OSCC. Kajian *in vitro* dan *in vivo* lanjut boleh dijalankan pada ubat pengganggu NEDD4L, peningkatan NEDD4L, yang mengakibatkan penindasan SLC7A11 dan pengaktifan laluan

feroptosis, mungkin boleh menjadi sasaran terapeutik yang berpotensi untuk pesakit OSCC.

MECHANISM OF NEURAL PRECURSOR CELL EXPRESSED DEVELOPMENTALLY DOWN-REGULATED 4-LIKE (NEDD4L) IN MODULATING FERROPTOSIS VIA UBIQUITINATION OF SOLUTE CARRIER FAMILY 7 MEMBER 11 (SLC7A11) IN OESOPHAGEAL SQUAMOUS CELL CARCINOMA

ABSTRACT

Detecting oesophageal squamous cell carcinoma (OSCC) in early stages poses significant challenges due to its subtle presentation. The absence of approved targeted therapies and poor prognosis further compound the difficulties in treating OSCC. Ferroptosis, with its capacity to inhibit the proliferation of cancer cells, highlights potential in cancer therapy, and its combination with chemotherapy may improve treatment effectiveness. Emerging evidence showed that NEDD4L is an E3 ubiquitin ligase that plays a curial role in tumour suppression by regulating ferroptosis pathway through ubiquitination. However, their relationship and specific regulatory mechanism in OSCC remains unknown. Therefore, this study was designed to elucidate the role of NEDD4L in OSCC, as well as the specific regulatory mechanism between NEDD4L and ferroptosis in OSCC. The small interfering RNA was used first to investigate the impact of silencing NEDD4L on OSCC. The results showed that silencing NEDD4L accelerated the migration and invasion of EC9706 cells, as well as promoted the proliferation of EC9706 and Eca109 cells. Xenograft mouse models were established through the subcutaneous injection of EC9706 cells infected with short hairpin NEDD4L. After 30 days, the tumour weight and volume of the mice were measured and the results confirmed that silencing NEDD4L triggered tumour growth in OSCC. Moreover, highly sensitive DCFH-DA was used to detect reactive oxygen species

(ROS). The data showed that the silencing NEDD4L prevented the accumulation of ROS in EC9706 and Eca109 cells. Under the treatment of erastin, the effects of NEDD4L on other indicators of ferroptosis including cell death, mitochondrial morphological changes, and mitochondrial membrane potential (MMP) were detected by Annexin V/PI staining, transmission electron microscopy and fluorescence microscopy, respectively. It was found that silencing NEDD4L exhibited an inhibitory role in ferroptosis. Moreover, a potential connection between NEDD4L and SLC7A11 was uncovered through high-throughput sequencing, which was further supported by rescue, immunofluorescence (IF) and co-immunoprecipitation (Co-IP) assays. Cycloheximide (CHX) assay and MG132 assay were performed to analyse the effect of silencing NEDD4L on SLC7A11 protein stability. It was proved that silencing NEDD4L prolonged the half-life of SLC7A11 and such regulation was proteasome-dependent. NEDD4L overexpression increased K48-linked ubiquitination, while decreased K63-linked ubiquitination of SLC7A11 in HEK293T cells. These results were demonstrated by ubiquitin-based IP assays. Furthermore, NEDD4L^{C942A} mutant, which lacks catalytic activity, was unable to affect the expression, half-life, as well as ubiquitination of SCL7A11, indicating the effect of NEDD4L on modulating SLC7A11 depended on its E3 ligase activity. To conclude, NEDD4L contributed to ferroptosis and cell growth inhibition by facilitating SLC7A11 ubiquitination in OSCC. Further *in vitro* and *in vivo* studies can be conducted on NEDD4L interfering drugs, the upregulation of NEDD4L, which resulted in the suppression of SLC7A11 and activation of the ferroptosis pathway, may serve as a potential therapeutic target for OSCC patients.

CHAPTER 1

INTRODUCTION

1.1 Research background

Oesophageal cancer ranks as the seventh most prevalent cancer in the world with oesophageal squamous cell carcinoma (OSCC) accounting for over 90% of cases (Sung *et al*., 2021; Abnet *et al*., 2018). Although many optical technologies are available for diagnosing OSCC, endoscopic with biopsy remains the standard. However, its invasiveness causes patient discomfort, poor compliance and challenges in detecting early-stage OSCC (Codipilly, 2022; Leng *et al*., 2020). To date, surgical resection combined with adjuvant therapy such as chemotherapy and radiotherapy is the main treatment method for advanced OSCC. The emerging therapy such as immunotherapy hold the potential to enhance patient outcomes. Nevertheless, treatment responses vary among different patients and the costs are high. Meanwhile, the lack of follow-up data makes it challenging to assess long-term effectiveness and safety (Codipilly, 2022; Leng *et al*., 2020). To sum up, despite notable advancements in the diagnosis and therapy of OSCC, the overall burden of OSCC has continued to rise over time, and the five-year survival rate remains less than 15%, largely attributed to its high propensity for invasion and metastasis (Shi *et al*., 2021). Hence, it is imperative to explore more effective and targeted treatment methods for OSCC.

Ferroptosis was first defined by Brent R. Stockwell in 2012 as an iron-dependent type of cell death that is driven by lipid reactive oxygen species (ROS) (Dixon *et al.,* 2012; Dixon *et al*., 2015; Doll *et al*., 2019). Over the past two decades, ferroptosis has been extensively studied in a variety of malignancies, and it has been shown to inhibit cancer progression through post-translational modifications (PTMs) (Chen *et al*., 2023; Ji *et al*., 2022; Jun *et al*., 2023; Zhang *et al*., 2022), especially

ubiquitination (Wei *et al*., 2022). Ubiquitination is a vital PTMs that chemically attaches the 76-amino acid ubiquitin protein (contains seven lysine residues K6, K11, K27, K29, K33, K48 and K63) to a target protein in a covalent manner. It mainly involves three types of enzymes: ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3). E1 initiates activation and transfers ubiquitin to E2, whereas E3 is responsible for recruiting substrate proteins to facilitate the transfer of the ubiquitin component (Jeong *et al*., 2023; Morgan & Crawford, 2021). Among them, E3 ubiquitin ligases are considered the most influential because of their capacity to selectively attach and enlist specific target substrates for the process of ubiquitination (Jeong *et al*., 2023; Morgan & Crawford, 2021). Given the ability of ubiquitination involves in all aspect of cellular functioning, it is not surprising that its deregulation connects with multiple diseases, especially cancer. However, there is still a lack of evidence for the relationship between E3 ubiquitin ligases and ferroptosis in OSCC.

Solute carrier family 7 member 11 (*SLC7A11*), is a core gene of ferroptosis that encodes a protein involved in the transport of amino acids across cell membranes (Koppula *et al*., 2021; Lin e*t al*., 2020). It is responsible for the transport of cystine, a crucial amino acid, into cells. By promoting the production of glutathione, *SLC7A11* contributes to maintain cellular redox balance and protects against ferroptosis (Chen *et al*., 2021). Previous studies have found that the degradation of SLC7A11 can be promoted through the ubiquitination pathway, thereby activating ferroptosis. For instance, the suppressor of cytokine signalling 2 (SOCS2) was identified as a biomarker that predicted the sensitivity of hepatocellular carcinoma (HCC) to radiation therapy by enhancing the ubiquitin-mediated degradation of SLC7A11 and facilitating ferroptosis (Chen *et al*., 2023). Recent evidence showed that the 3-

hydroxy-3-methylglutaryl reductase degradation (HRD1) suppressed the development of tumours and facilitated ferroptosis by promoting the degradation of SLC7A11 in ovarian cancer (Wang *et al*., 2023). However, no investigation has been conducted to elucidate the relationship between SLC7A11 and ubiquitination in OSCC. The search for the potential ubiquitin ligases is still ongoing and the underlying mechanisms involved in the ubiquitination and SLC7A11 in human cancers remains largely unknown.

NEDD4L (neural precursor cell expressed, developmentally downregulated 4-like), is a member of the NEDD4 family of E3 ubiquitin ligases, which plays vital role in the regulation of protein degradation and cellular processes (Wang *et al*., 2020; Xie *et al*., 2021). NEDD4L protein contains C2 domain, 4 WW domains, and homologous to the E6AP carboxyl terminus (HECT) domain (Manning & Kumar, 2018; Wang *et al*., 2020). These domains enable the protein to bind to specific target proteins and transfer ubiquitin molecules onto them, leading to their degradation through the proteasome pathway. Previous study have indicated that NEDD4L inhibited cell proliferation, migration and invasion in bladder cancer and prostate cancer (PC) (Feng *et al*., 2023; Wu *et al*., 2023). Interestingly, recent study further confirmed that NEDD4L functioned as a positive regulator in glutathione peroxidase 4 (GPX4) ubiquitination, thereby promoted ferroptosis in non-small cell lung cancer (NSCLC) cells (Cheng *et al*., 2023). Nevertheless, to date, there has been no report on its ubiquitination regulation of ferroptosis in OSCC. Notably, the Ubi Browser database predicts that NEDD4L is the E3 ubiquitin ligase most closely associated with SLC7A11. Therefore, the present study aimed to determine the specific mechanism of NEDD4L regulated the SLC7A11/ferroptosis pathway in OSCC. Furthermore, the effect of silencing NEDD4L on OSCC progression was investigated by establishing

xenograft mouse models, thereby establishing a foundation and reference for targeted therapy in OSCC.

1.2 Problem statement

Detecting OSCC in its early stages poses a significant challenge due to its subtle presentation. Additionally, the absence of approved targeted therapies and poor prognosis further compound the difficulties in treating OSCC (Reichenbach *et al*., 2019; Sheikh *et al*., 2023). Despite the increasing evidence indicating a connection between genetic factors and OSCC, the regulation of tumours involves numerous signalling pathways and is susceptible to various PTMs. Consequently, diagnosing and treating for OSCC continue to pose major challenges.

To date, accumulating evidence indicated ferroptosis acted as a tumour suppressor mechanism and influenced the cell cycle, proliferation and progression of human cancers. In addition to blood-related cancers (Birsen *et al*., 2022; Jin & Tong, 2021), ferroptosis also acts as a tumour inhibitor in multiple solid tumours, including lung cancer (Tang *et al*., 2021), breast cancer (Yang *et al*., 2021), pancreatic cancer (Ye *et al*.,2021), colorectal cancer (CRC) (Yang *et al*., 2020), liver cancer (Zhu *et al*., 2021), GC (Wang *et al*., 2021) and melanoma (Yao *et al*., 2021). It is worth noting that ferroptosis also plays a vital role in the treatments for multiple cancers, such as chemotherapy and immunotherapy (Chen *et al*., 2023). If ferroptosis indeed plays a similar role in OSCC, investigating its mechanisms and function may provide promising avenues for advancing the diagnosis and treatment of OSCC.

Despite the established role of ferroptosis as a tumour suppressor *via* the ubiquitination pathway in numerous cancer types, there is a research deficiency when it comes to its influence on OSCC. Until now, several studies have scrutinised data

from The Cancer Genome Atlas (TCGA) and the Gene Expression Omnibus (GEO) database, identifying a correlation between the anomalous expression of ferroptosis-related genes (like *EBLN3P, APOA1-AS* and *SCP2*) and prognosis in OSCC (Lu *et al*., 2021; Zhu *et al*., 2022). However, these findings have yet to be subjected to further validation.

Emerging evidence showed that NEDD4L is an E3 ubiquitin ligase that plays a curial role in tumour suppression by regulating ferroptosis pathway through ubiquitination (Cheng *et al*., 2023; Nie *et al*., 2022). For example, lactic acid could enhance GPX4 stability and reduce the sensitivity of NSCLC cells to ferroptosis by suppressing NEDD4L expression (Cheng *et al*., 2023). In addition, NEDD4L has been found to express at low levels in glioma clinical samples and was associated with poor prognosis. Further in *vitro* and *in vivo* analysis showed that overexpression NEDD4L could reduce cell viability and trigger ROS accumulation, leading to ferroptosis and tumour growth inhibition (Nie *et al*., 2022). Nevertheless, there is lack of evidence when it comes to NEDD4L and ferroptosis in OSCC and the specific regulatory mechanism remains largely unknown. Therefore, this study aimed to evaluate the role of NEDD4L in ferroptosis and OSCC. Additionally, *in vivo* study was performed in supporting the effect of silencing NEDD4L in OSCC. A detailed study was conducted to clarify the special mechanism of NEDD4L regulate ferroptosis through ubiquitination of SLC7A11 in OSCC. The results of this study will provide a new perspective on the role and mechanism of NEDD4L regulated SLC7A11 in OSCC. The upregulation of NEDD4L, which resulted in the suppression of SLC7A11 and activation of the ferroptosis pathway, may serve as a potential therapeutic target for OSCC patients.

1.3 Hypothesis

It is hypothesised that NEDD4L could interact with SLC7A11 and modulate its stability *via* ubiquitin-proteasome pathway, as well as regulating ferroptosis in OSCC.

1.4 Objectives of the study

1.4.1 General objective

The main objective of this study was to elucidate the role of NEDD4L in OSCC and ferroptosis, as well as the specific mechanism of NEDD4L regulating SLC7A11.

1.4.2 Specific objectives

1. To determine the impact of silencing NEDD4L on cell migration, invasion and proliferation in EC9706 and Eca109 cell lines.
2. To verify the effect of silencing NEDD4L on *in vivo* tumourigenic of OSCC within BALB/c nude mice.
3. To analyse the effect of silencing NEDD4L on ferroptosis in EC9706 and Eca109 cell lines.
4. To investigate the interaction between NEDD4L and SLC7A11 in EC9706 and Eca109 cell lines.
5. To elucidate the specific regulatory mechanism of NEDD4L on SLC7A11 in EC9706 and HEK293T cell lines.

CHAPTER 2

LITERATURE REVIEW

2.1 Epidemiology of OSCC

2.1.1 Statistics and risk factors

Cancer is a complex group of diseases characterised by the uncontrolled growth and spread of abnormal cells. These abnormal cells can form tumours, invade nearby tissues, and spread to other parts of the body through the bloodstream or lymphatic system, a process known as metastasis (Brown *et al*., 2023). The development of cancer is typically a multi-step process involving various genetic and environmental factors. Mutations or changes in the DNA of cells can disrupt the normal regulatory mechanisms that control cell growth, division and death. As a result, affected cells may continue to divide uncontrollably, eventually forming tumours (Huang & Zhou, 2021). Cancers can occur in almost any part of the body and have many different forms, depending on the type of cells involved and where they are located. The intricate complexity of cancer, combined with challenges in early detection, treatment resistance, and its impact on overall health, contributes to its high incidence and status as the leading cause of mortality worldwide (Pulumati *et al*., 2023; Sung *et al*., 2021). It is reported that in 2020, there were 19.3 million new cancer cases and about 10 million people died from cancer (Sung *et al*., 2021). Female breast cancer ranks as the most frequently diagnosed cancer, accounting for 11.7% of total cases, closely followed by lung cancer at 11.4%, CRC at 10.0%, PC at 7.3%, stomach cancer at 5.6%, liver cancer at 4.7% and oesophageal cancer at 3.1%. Lung cancer stands as the foremost cause of cancer-related deaths, comprising 18.0% of the total cancer cases, followed by CRC at 9.4%, liver cancer at 8.3%, stomach cancer at 7.7%, female breast cancer at 6.9% and oesophageal cancer at 5.5% (Sung *et al*., 2021).

Of note, certain types of cancer exhibit significant regional differences, especially oesophageal cancer. Specifically, oesophageal cancer ranks seventh and sixth in global morbidity (604,000 cases) and mortality (544,000 cases) in 2020, respectively (Figure 2.1) (Sung *et al*., 2021). Traditionally, oesophageal cancer has two most prevalent histologic subtypes, OSCC and oesophageal adenocarcinoma (OAC). Among them, OSCC accounts for more than 90% of all cases. However, there is marked disparity in the incidence of OSCC and OAC, which are characterised by distinct underlying causes. For example, in high-income countries such as the United States and Australia, OAC comprises approximately two-thirds of oesophageal cancer cases. Key risk factors for OAC include excess body weight, gastroesophageal reflux disease and Barrett's oesophagus (Lv *et al*., 2019). In developing countries, particularly in China where OSCC constitutes the predominant form of oesophageal cancer, alcohol consumption, tobacco, economic growth, enhanced dietary conditions, the use of hot beverages and the consumption of pickled vegetables are believed to be the primary factors contributing to this trend (Ishiguro *et al*., 2009; Zhao *et al*., 2023). Interestingly, the occurrence of OSCC may also be influenced by other factors, including the presence of underlying gastrointestinal diseases, human papillomavirus infection (Petrelli *et al*.,2021) and inadequate oral hygiene (Dar *et al*., 2013).

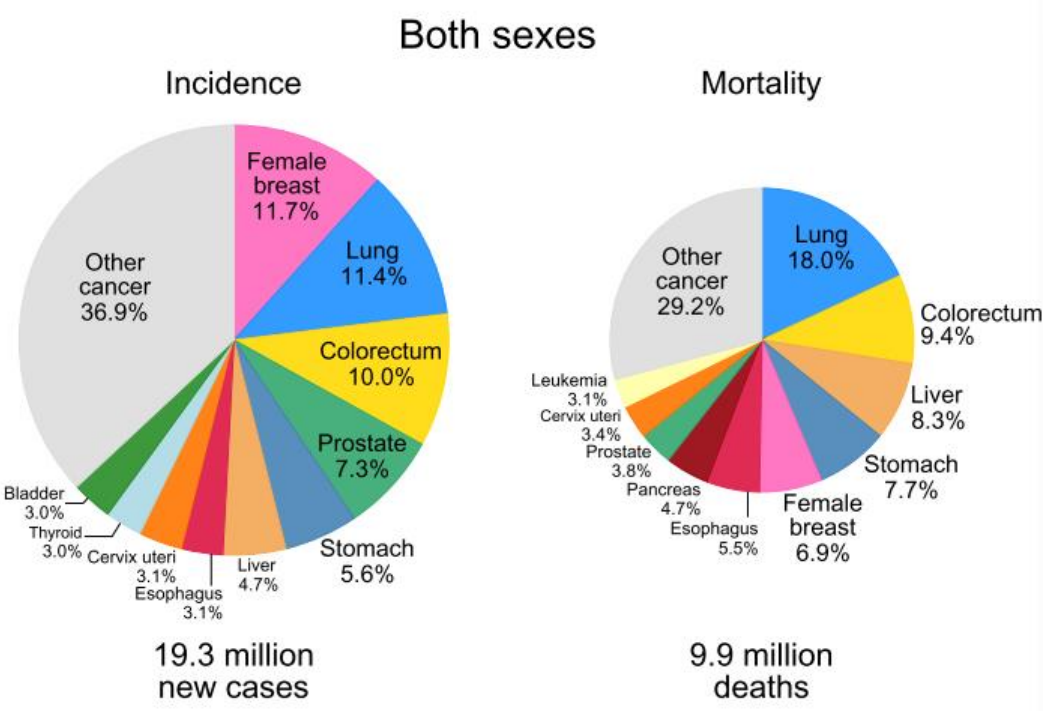

Figure 2.1: Distribution of cases and deaths for the top 10 most common cancers in 2020 for both sexes. Adapted from (Sung *et al*., 2020).

2.1.2 Genetic factors

Numerous studies have shown that family history increased the risk of OSCC, implying that genetic factors may play an essential role in the development of OSCC (Chen *et al*., 2015; Yang *et al*., 2021). Subsequent studies further identified the relationship between genetic and OSCC by using high-throughput genotyping technology. The notable study from our team uncovered that phospholipase c epsilon 1 gene (*PLCE1*) as a risk factor for OSCC *via* genome-wide association study (GWAS) (Wang *et al*., 2010). Further analysis observed that the 2q33 mutation located in the *CASP8*/*ALS2CR12*/*TRAK2* gene region is strongly associated with the risk for incidence of OSCC (Abnet *et al*., 2012). In addition, by including a larger scale of OSCC samples for GWAS analysis, human leukocyte antigen (HLA) class II gene, *ATP1B2*/*TP53* and *TMEM173* were further confirmed as risk factors for OSCC (Wu *et al*., 2014). Based on this, GWAS conducted by another research group showed that

PDE4D, *RUNX1*, *ALDH2* and *ADH1B* were susceptibility genes for OSCC (Wu *et al.*, 2011) (summarised in Table 2.1) .

Table 2.1: The genes identified as susceptibility factors in OSCC.

Gene	Loci	rsID	Number of patients	Reference
PLCE1	10q23.33	rs2274223	2,810 (1,077 cases and 1,733 controls)	(Wang *et al*., 2010)
CASP8/*ALS2CR12*/*TRAK2*	2q33	rs13016963	6,361 (2,961 cases and 3,400 controls)	(Abnet *et al*., 2012)
PDE4D	5q11	rs10052657	4,075 (2,031 cases and 2,044 controls)	(Wu *et al*., 2011)
RUNX1	21q22	rs2014300	4,075 (2,031 cases and 2,044 controls)	(Wu *et al*., 2011)
ALDH2	12q24	rs671	4,075 (2,031 cases and 2,044 controls)	(Wu *et al*., 2011)
HLA class II gene	6p21.32	rs35597309	11,124 (5,337 OSCC cases and 5,787 controls)	(Wu *et al*., 2014)
ATP1B2/*TP53*	17p13.1	rs1642764	11,124 (5,337 OSCC cases and 5,787 controls)	(Wu *et al*., 2014)
TMEM173	5q31.2	rs7447927	11,124 (5,337 OSCC cases and 5,787 controls)	(Wu *et al*., 2014)
ADH1B	4q23	rs1042026	4,075 (2,031 cases and 2,044 controls)	(Wu *et al*., 2012)

In addition to GWAS analysis, some *in vivo* and *in vitro* studies have also proven the connection between genetic factors and OSCC. For example, Song *et al* (2014) identified *p53*, family with sequence similarity 135, member B (*FAM135B*), *RB1*, *NFE2L2*, *PIK3CA*, *CDKN2A*, *NOTCH1*, and *ADAM29* genes are mutated, and histone regulator genes are highly altered in OSCC. Of note, subsequent *in vitro* assays

confirmed FAM135B is overexpressed in multiple OSCC cells. FAM135B depletion highly inhibited the proliferation, migration and invasion abilities of OSCC cells, suggesting FAM135B had a strong connection with OSCC. Interestingly, real-time PCR (RT-PCR) assays and histopathological analysis conducted by Fardi Golyan & Forghanifard (2021) proposed that the simultaneous decrease in inositol polyphosphate 5-phosphatase (INPP5A), accompanying the increased expression of matrix metalloproteinase 2 (MMP2)/epidermal growth factor receptor (EGFR)/TWIST1 were associated with various adverse prognostic indicators (tumour stage, invasion and metastasis) in OSCC through targeting epithelial-mesenchymal transition (EMT) signalling pathway. Similarly, glycoprotein nonmetastatic melanoma protein B (GPNMB) is a recently uncovered factor implicated in promoting tumours, potentially facilitating the progression of tumour cells through the activation of the phosphoinositide 3-kinase (PI3K)/AKT pathway *via* EGFR. The latest evidence showed that GPNMB was associated with EGFR and p-PI3K, and they were increasingly correlated with OSCC progression (Wang *et al*., 2022). Consistent with these findings, evidence from *in vivo* and *in vitro* confirmed that the metastasis and invasion ability of OSCC can be improved by ring finger protein-128 (RNF128) *via* targeting EGFR/MMP2/mitogen-activated protein kinase (MAPK) signalling pathway (Jing *et al*., 2019). Interestingly, recent research found that silencing calmodulin1 (CALM1) increased the susceptibility of OSCC cells to afatinib (an EGFR inhibitor) followed by obviously tumour suppression (Liu *et al*., 2021).

Despite emerging evidence suggesting a correlation between predisposing genetic conditions and the occurrence of OSCC (Li *et al*., 2021; Qu *et al*., 2017; Waters & Reznik, 2022), the specific mechanism is still requires further elucidation and how they can be translated into clinical practice is worthy of exploration.

2.2 Diagnosis of OSCC

A vast range of optical technologies is accessible for OSCC diagnosis, endoscopic screening method is the main and recognised method including white light endoscopy (WLE), chromoendoscopy, narrow band imaging (NBI), transnasal endoscopy, endocytoscopy and microendoscopy (Table 2.2). At present, the combination of endoscopic monitoring and histopathological assessment is the most widely recommend diagnosis method for OSCC. Although conventional WLE is effective in detecting invasive oesophageal cancer, it has limited sensitivity in identifying oesophageal squamous dysplasia, which is the precursor to OSCC and a key focus of screening. Squamous dysplasia often exhibits similarities to normal squamous mucosa, posing challenges for accurate detection using WLE (Hur & Yachimski, 2019). On the contrary, chromoendoscopy is considered the prevailing standard for highlighting areas of abnormality, greatly enhancing the ability to detect oesophageal squamous dysplasia. Chromoendoscopy employs iodine, which selectively binds to glycogen in the squamous epithelium. As immature and rapidly dividing cells, such as those encountered in oesophageal squamous dysplasia or esophagitis, have lower glycogen levels, they appear unstained compared to normal epithelium. This enables targeted biopsies to be performed on the non-stained areas, distinguishing dysplastic and inflammatory oesophageal mucosa from the normal epithelium (Mwachiro *et al.*, 2016). Chromoendoscopy is a cost-effective and relatively straightforward procedure that can be easily performed and interpreted. In contrast, one expensive method named NBI could utilize 415 nm and 540 nm that correspond to the absorption peaks of haemoglobin. It enables a more distinct visualisation of capillary networks and superficial mucosal patterns (Gono, 2015). In this process, oesophageal squamous dysplasia or early OSCC is distinguished by

brownish appearance accompanied by abnormal vascular patterns of intracapillary papillary loops. Similarly, endocytoscopy was found to exhibit a sensitivity of 100% and a specificity of 80% in the detection of OSCC without biopsy (Kumagai *et al*., 2012). Notably, microendoscopy is widely recognised by developing countries for its affordability and high sensitivity. It is reported that the feasibility of this approach for detecting OSCC has been established, with sensitivity ranging from 84% to 93% and specificity ranging from 92% to 97% (Leoncini *et al*., 2014; Shin *et al*., 2015). More than that, transnasal endoscopy is a well-tolerated diagnostic method for clinic patients. Despite its limited implementation, transnasal endoscopy has attracted increased attention due to its significant advantages, which include the elimination of sedation requirements, the eradication of sedation-related risks and cost reduction.

Table 2.2: Endoscopic screening methods in the diagnosis of OSCC.

Endoscopic Screening Methods	**Advantages**	**Disadvantages**
White light endoscopy	Easily accessible in developed regions	Low sensitivity and specificity for precursor lesions
Chromoendoscopy	High sensitivity of precursor lesions; high visibility; Affordable	Low specificity for precursor lesions; need for dye; allergic reactions
Narrow band imaging	High sensitivity of precursor lesions; high visibility; high accuracy; no need for dye	Special training required; expensive
Transnasal endoscopy	Narrow pipe diameter, high pass rate; affordable	Not standard for screening and not widely used; not yet evaluated for precursor lesions

Table 2.2: Continued.

Endoscopic Screening Methods	Advantages	Disadvantages
Endocytoscopy	Safe; accurate	Small field of view; not yet evaluated for precursor lesions; special training required; expensive
Microendoscopy	High sensitivity and specificity for the detection of dysplasia and early-stage cancer; affordable	Small field of view; special training required

These endoscopic screening methods have their own benefits and shortages. One main limitation of using endoscopic screening methods in the diagnosis of OSCC is the potential challenge in detecting early-stage lesions, especially in regions that are difficult to access or visualise comprehensively. Limited visibility and the possibility of overlooking subtle abnormalities can hinder the efficacy of endoscopic screening in identifying precancerous or early-stage OSCC. Hence, there is a need to explore effective and efficient diagnostic methods. Emerging evidence suggested several advanced technologies that have shown potential as alternative options include wide area transepithelial sampling with 3-dimensional tissue analysis, breath testing utilising an electronic nose device and cancer cell biomarkers (Chaber-Ciopinska *et al*., 2016; Liu *et al*., 2020). Among them, biomarkers have been actively studied these years (summarised in Table 2.3). Specifically, several proteins have been proved to serve as biomarkers in OSCC. For instance, L1 cell adhesion molecule (L1CAM) expression was found to be elevated in OSCC tissues compared to normal epithelium, and this high level of L1CAM in OSCC tissues was associated with poorer patient survival (Xu *et al*., 2018). Similarly, the levels of serum insulin-like growth factor binding protein-1 (IGFBP-1) were markedly increased in OSCC. More importantly,

serum IGFBP-1 exhibited excellent diagnostic accuracy for the early-stage and advanced-stage group of OSCC (Xu *et al*., 2020). In addition, a lot of circRNAs also have been actively studied in recent years. For example, circGSK3β overexpression showed a positive association with advanced clinical stage and adverse outcomes in individuals suffering from OSCC. The expression of circGSK3β in plasma served as a biomarker for detecting both OSCC and early stages of OSCC (Hu *et al*., 2019). Similarly, higher levels of circSLC7A5 in plasma are correlated with an increased TNM stage, which in turn is associated with a tendency towards shorter overall survival (OS) compared to individuals with lower levels of circSLC7A5 in OSCC (Wang *et al*., 2020). On the other hand, some miRNAs also proved to involve in OSCC. For instance, OSCC patients exhibited a notable rise in plasma miR-19a levels when compared to control group. For early stages of OSCC, miR-19a displayed a sensitivity rate of 68.09% (Bai *et al*., 2017). Additionally, the sensitivity and specificity of lncRNAs POU3F3 in diagnosing OSCC were 72.8% and 89.4%, respectively (Tong *et al*., 2015). Other lncRNAs including lncRNAs NR_003353, NR_036133, NR_039819, ENST00000442416.1 and ENST00000416100.1 were shown to be downregulated in patients with OSCC after surgery (Jiao *et al.*, 2020).

Although existing studies have suggested that these biomarkers can be used for the diagnosis of OSCC, there is no clinical evidence of their effectiveness. Therefore, it is imperative to discover more efficient biomarkers and incorporate them into clinical practice, especially those suitable that can be used in the early diagnosis of OSCC.

Table 2.3: Potential diagnostic biomarkers for OSCC.

Biomarkers	Methods	References
L1CAM	Enzyme-linked immunosorbent assay	(Xu *et al*., 2018)
Serum IGFBP-1	Enzyme-linked immunosorbent assay	(Xu *et al*., 2020)
CircGSK3β	Microarrays; droplet digital PCR	(Hu *et al*., 2019)
Circ-SLC7A5	Quantitative reverse transcription polymerase reaction	(Wang *et al*., 2020)
miR-19a	Quantitative reverse transcription polymerase reaction	(Bai *et al*., 2017)
POU3F3	Quantitative reverse transcription polymerase reaction	(Tong *et al*., 2015)
lncRNAs NR_003353, NR_036133, NR_039819, ENST00000442416.1, and ENST00000416100.1	Quantitative reverse transcription polymerase reaction	(Jiao *et al*., 2020)

2.3 Staging of OSCC

After establishing the diagnosis, it is essential to carry out staging in order to determine the appropriate course of treatment. Staging is conducted by employing the TNM classification system, which has been outlined by American Joint Committee on Cancer (AJCC). The TNM staging system incorporates three key aspects: the extent of tumour invasion (T-staging), the involvement of lymph nodes (N-staging) and the presence of metastatic spread (M-staging). Moreover, T4 lesions are categorized as either resectable (T4a) or unresectable (T4b), and the classification of N-staging is determined by the involvement of lymph nodes. Notably, Tis lesions have been redefined as high-grade dysplasia, and M-staging is divided into two categories: no

distant metastases (M0) or presence of distant metastases (M1) (Figure 2.2) (Rice *et al*., 2010). The standard staging procedure should encompass various diagnostic tests such as endoscopic ultrasound (EUS), CT scans of the chest, abdomen, pelvis or neck, as well as FDG-PET/CT. These modalities have the capability to provide distinct and complementary information for the determination of cancer stage.

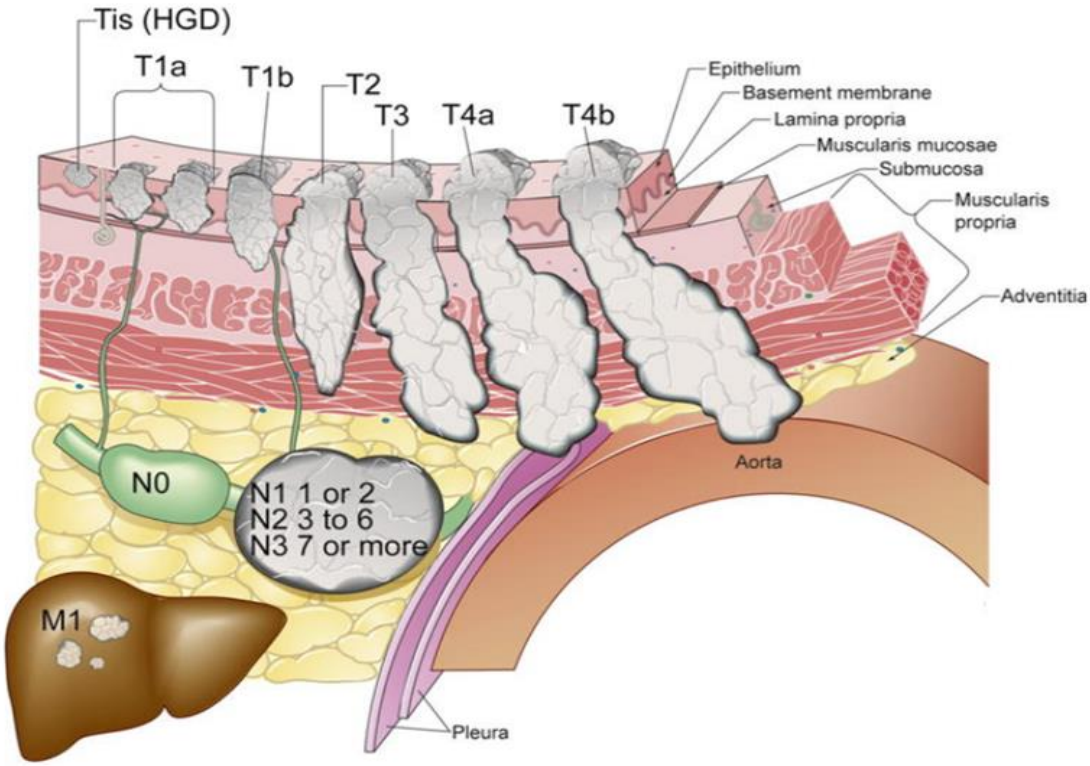

Figure 2.2: TNM staging diagram of OSCC. Adapted from (Rice *et al.*, 2017).

2.4 Therapy for OSCC

At present, the therapy for OSCC mainly include surgery, chemotherapy, immunotherapy, radiotherapy and molecular-targeting therapy.

2.4.1 Surgery

Surgery is extensively employed to achieve local and regional control, playing a crucial role in the OSCC therapy. Patients diagnosed with early-stage OSCC and without lymph node metastasis may undergo endoscopic mucosal resection or

endoscopic submucosal dissection (Waters & Reznik, 2022). Esophagectomy may be contemplated for patients with multifocal disease or those who decline endoscopic treatment. One of the esophagectomy called transhiatal esophagectomy involves an abdominal approach along with a cervical incision and cervical esophagogastrostomy. It is often used for cervicothoracic oesophageal cancer or elderly patients who cannot tolerate transthoracic surgery (Waters & Reznik, 2022). Distal oesophageal tumours are suitable for Mckeown esophagectomy, which procedure begins with a right thoracic approach, followed by incisions in the abdomen and left neck, ultimately concluding with a cervical esophagogastrostomy. On the other hand, it can be also achieved by Ivor Lewis esophagectomy starts with an abdominal approach, then proceeds with a right chest approach, culminating in an intrathoracic esophagogastric anastomosis (Waters & Reznik, 2022). Notably, another method named minimally invasive esophagectomy (MIE) is gaining popularity because of its less invasive approach, reduced risk of perioperative pneumonia and intensive care unit stay (Groth *et al*., 2018). More importantly, all the surgical methods mentioned above can be achieved through MIE.

2.4.2 Chemotherapy

Chemotherapy, serving as the primary treatment method for OSCC, providing multiple benefits such as tumour growth inhibition and prevention of distant metastasis. Cisplatin (DDP), 5-fluorouracil (5-FU) and doxorubicin (Dox) are the chemotherapy drugs most commonly used for the treatment of OSCC (He *et al*., 2021; Hirano, 2019). DDP-based chemotherapy is typically the primary choice for treating OSCC, and patients who have undergone surgery and developed recurrence or metastasis usually show an initial response to DDP treatment. Certain genes have been confirmed to be associated with the effectiveness of DDP in OSCC cell lines. For example, by inducing

the differentiation of fibroblasts into cancer-associated fibroblasts (CAFs), lncRNA *POU3F3* enhanced cisplatin resistance in OSCC (Tong *et al*., 2020). Similarly, the alleviation of DDP resistance can be achieved by circRNA *DOPEY2*, which promoted the ubiquitination and degradation of cytoplasmic polyadenylation element binding protein in OSCC cells (Liu *et al*., 2021). 5-FU is used as the first-line treatment for OSCC. Nevertheless, single 5-FU therapy is no longer suitable for OSCC patients due to the increasing dosage, toxicity and drug resistance (He *et al*., 2021). Hence, the combination DDP and 5-FU has been widely implemented as a treatment plan for OSCC (Wang *et al*., 2019; Zhang *et al*., 2017). In addition, Dox is a widely prescribed antitumor medication known for its ability to generate ROS. In xenograft models, Dox can be effectively administered to the tumour site and exhibited a notable inhibitory impact on tumour growth (Zhang *et al*., 2017). Interestingly, it is reported that the combined treatment of DDP, 5-FU, and Dox has the potential to significantly improve the prognosis by suppressing specific progression events while maintaining a higher rate of oesophagus preservation in stage II/III of OSCC (Yamashita *et al*., 2017).

2.4.3 Immunotherapy

Over the past decades, immunotherapy has gained significant attention as a promising therapeutic strategy for diverse forms of cancer (Ribas & Wolchok, 2018). By utilizing the patient's immune system, immunotherapy employs the inhibition of the immune checkpoint pathway to combat cancer cells (Joyce & Fearon, 2015). Specifically, the inhibition of immune checkpoints such as Programmed Death 1 (PD-1)/Programmed Death Ligand 1 (PD-L1) has demonstrated notable responses and clinical benefits in OSCC (Gao *et al*., 2021; Mahmoudian *et al*., 2021). For example, nivolumab, a humanized IgG4 monoclonal antibody, exhibits high affinity for the PD-1 receptor. A phase II clinical trial evaluated the effect of nivolumab in 65 patients

with advanced OSCC and found that the one-year and two-year OS rates of patients after nivolumab treatment were 45% and 17%, respectively (Kudo *et al*., 2017). Similarly, pembrolizumab, a fully humanized IgG4-k monoclonal antibody, is a highly selective and potent agent that targets the PD-1receptor (Wolchok, 2015). The multicohort phase IB study, known as the KEYNOTE-028 trial, enrolled 18 patients with OSCC and 5 patients with OAC who had experienced chemotherapy failure and tested positive for PD-L1. Under pembrolizumab treatment, the six-month and twelve-month OS rates were 60% and 40%, respectively (Doi *et al*., 2018). Although immunotherapy shows potential as a second-line treatment for OSCC, further direct evidence and comparisons are necessary to evaluate its effectiveness.

2.4.4 Radiotherapy

To date, radiotherapy (RT) has been the preferred choices for OSCC patients who refuse, not suitable for surgery or advanced stage of OSCC. On the one hand, RT can improve anti-tumour response by activating the new tumour antigens and promoting the dendritic cell maturation (Ahmed & Tait, 2020; Sadeghzadeh *et al*., 2020). On the other hand, RT could stimulate the formation of an anti-immunogenic microenvironment through the recruitment of M2 tumour-associated macrophages, and myeloid-derived suppressor cells, thereby triggering the invasion and metastasis of tumours (Jarosz-Biej *et al*., 2019; Shevtsov *et al*., 2019). Clinically, RT can significantly improve OS and disease-free survival (DFS) of OSCC patients (Wang *et al*., 2016). However, radioresistance caused by abnormal cell death and DNA damage greatly reduces the effect of RT. In view of this, many studies are beginning to explore the combination of RT and other therapies. For instance, a survey based on 5185 patients with OSCC showed that compared to RT group, patients who received chemo-radiotherapy (CRT) had better survival rates (p<0.001) (Li *et al*., 2021). Similar

conclusion was supported by a retrospective cohort study, they found the median progression-free survival (PFS) of CRT group was longer than RT group (15.3 months *vs* 10.6 months), and the OS in the CRT group was better than RT alone (24.6 months *vs* 19.4 months) for elder OSCC patients (Zhao *et al*., 2017). In addition, many studies have found that targeted immune checkpoint inhibitors can effectively reverse RT resistance. By activating T cells, PD-1 inhibitors can block G2/M phase and promote DNA damage, thereby improving immune response and radiosensitivity (An *et al*., 2022). Therefore, RT combines with other therapy may be a potential and effective option in the future treatment of OSCC.

2.4.5 Molecular targeted therapy

Molecular targeted therapy is a form of cancer treatment that focuses on specific molecules or molecular pathways involved in the growth and progression of cancer cells. It identifies specific molecules or proteins that play crucial roles in cancer development, such as growth factor receptors, enzymes or signalling proteins. Currently, clinical trials of molecular targeted therapy for OSCC mainly based on targeting EGFR and PI3K (Table 2.4).

The most representative is the development and application of inhibitors against EGFR, which is high expression (30%-70%) in all OSCC and connected with poor response to therapy (Codipilly & Wang, 2022; Kuwano *et al*., 2005). Cetuximab, known for its strong affinity for EGFR, has been shown to improve efficacy when used in combination with cisplatin–5-FU (CF) (Lorenzen *et al.*, 2009). Previously, a clinical phase 2 trial examined the effect of gefitinib in oesophageal cancer and found that the disease control rate of gefitinib is positively correlated with EGFR expression (Janmaat *et al*., 2006). Further clinical study found that combined gefitinib with radiation has a better median OS than radiation alone (14.0 months *vs* 9.3 months)

(Cooper *et al*., 1999; Mahmoudian *et al*., 2021). Nimotuzumab is a humanised recombinant monoclonal IgG1 antibody by targeting EGFR and exerts anti-tumour effect. A reported by Ling *et al* (2012) confirmed that nimotuzumab combined with CF has good efficacy and tolerable side effects in the treatment of advanced OSCC. This finding was further supported by a clinical phase 2 trial (Lu *et al*., 2016). When nimotuzumab was combined with DDP and paclitaxel to treat patients with advanced and metastatic OSCC, the disease control rate was 92.9% and the median OS was 20.2 months (Lu *et al*., 2016). Another inhibitor of EGFR called erlotinib was used to treat inoperable OSCC. It was found that erlotinib, paclitaxel and combined concurrent RT were more effective in OSCC patients with EGFR expression (Zhao *et al*., 2016). Recent evidence uncovered that erlotinib together with elective nodal irradiation and CRT resulted in increased long-term survival rates among patients with locally advanced OSCC (Xie *et al*., 2020). In addition, given that the PI3K is frequently elevated in OSCC, researchers have initiated the validation effectiveness of inhibitors targeting PI3K in the treatment of advanced OSCC. For example, BKM120 is a potent and highly specific oral PI3K inhibitor that has been shown to exert anticancer effects in a variety of cancers (Maira *et al*., 2012). BKM120 treatment alone showed favourable efficacy (median OS and PFS were 2 months and 9 months, respectively) and manageable toxicity (Kojima *et al*., 2022). Despite the remarkable success of these attempts, there are still numerous molecules awaiting discovery and confirmation.

Table 2.4: Evaluation of representative clinical trials of molecular targeted therapy for OSCC.

Agents	Targets	Response rate	Median PFS (months)	Median OS (months)	Clinical phase	Reference
Cetuximab	EGFR	19%	5.9	9.5	3	(Lorenzen *et al*., 2009)
Gefitinib	EGFR	90%	7	14.0	2	(Mahmoudian *et al*., 2021)
Nimotuzu-Mab	EGFR	51.8%	No reported	20.2	2	(Lu *et al*., 2016)
Erlotinib	EGFR	85.6%	14.3	22.9	2	(Zhao *et al*., 2016)
BKM 120	PI3K	2.4%	2.3	9.0	2	(Kojima *et al*., 2022)

2.5 NEDD4L

2.5.1 Molecular structure and function of NEDD4L

NEDD4L serves as an E3 ubiquitin ligase and stands out as the most closely related homolog to NEDD4, which is considered the prototype within its family (Yang & Kumar, 2010). NEDD4L protein contains C2 domain, 4 WW domains, and HECT domain (Figure 2.3) (Manning *et al*., 2020). The main functions of the C2 domain include binding Ca^{2+}, targeting membranes and facilitating interactions between proteins. The WW domains play a crucial role in recognising and binding specific substrates targeted by NEDD4L. The HECT domain is the catalytic core of NEDD4L and responsible for transferring ubiquitin molecules onto target proteins. This domain forms a thioester intermediate with ubiquitin before transferring it to the target protein substrate, thereby facilitating the ubiquitination process (Figure 2.3) (Manning & Kumar, 2018; Mari *et al*., 2014; Todaro *et al*., 2017; Wang *et al*., 2020).

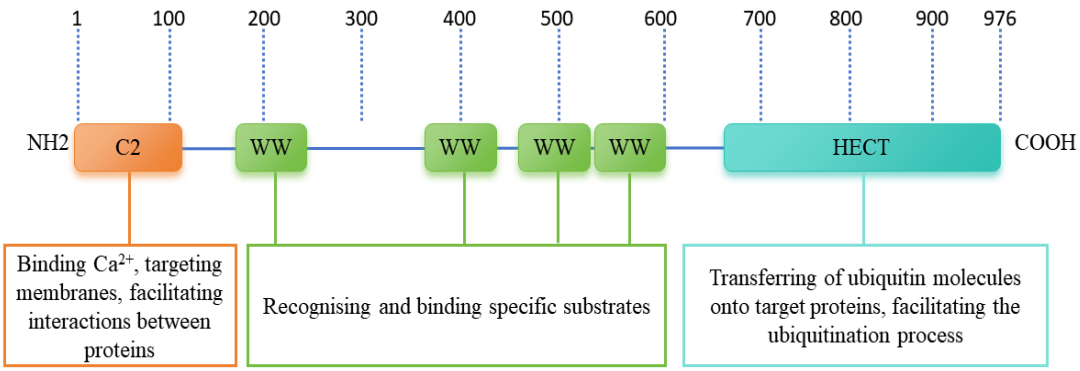

Figure 2.3: The structure of NEDD4L.

The most well-known function of NEDD4L is its regulation of the epithelial sodium channel (ENaC), particularly in the context of kidney function and blood pressure regulation. NEDD4L regulates ENaC by promoting its ubiquitination and subsequent degradation, thereby controlling the number of active ENaC channels at the cell membrane (Kamynina *et al.*, 2001; Zhang *et al.*, 2023). Additionally, increasing evidence suggested that NEDD4L involves in a range of biological processes, including cardiovascular diseases, DNA repair, antiviral immunity, inflammatory responses, especially tumour progression (Gao *et al.*, 2021; Li *et al.*, 2022; Mathieu *et al.*, 2021; Wang *et al.*, 2022; Xia *et al.*, 2023).

2.5.2 Role of NEDD4L in cancer

2.5.2(a) Role of NEDD4L in cell proliferation

According to the existing research, NEDD4L has two sides in regulating cell proliferation. On the one hand, NEDD4L has been shown to inhibit cell proliferation in multiple human cancers. For instance, the absence of NEDD4L enhanced the expression of PIK3CA, subsequently activating the PI3K-AKT signalling pathway and stimulating cell proliferation in glioma (Wang *et al.*, 2016). Interestingly, further study uncovered that NEDD4L suppressed the proliferation of lung adenocarcinoma cells both *in vivo* and *in vitro* by promoting the degradation of ubiquitin-conjugating