MECHANISM OF NEURAL PRECURSOR CELL EXPRESSED DEVELOPMENTALLY DOWN-REGULATED 4-LIKE (NEDD4L) AS A TUMOUR SUPPRESSOR BY DEGRADING YES-ASSOCIATED PROTEIN (YAP) IN OESOPHAGEAL SQUAMOUS CELL CARCINOMA THROUGH HIPPO PATHWAY

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by

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LIST OF SYMBOLS

%	Percent
°C	Degree Celsius
µL	Microliter
µM	Micromolar
µg	Microgram
mA	Milliampere
mL	Millilitre
rpm	Revolutions Per Minute
V	Volt
h	Hour

LIST OF ABBREVIATIONS

5-FU	5-fluorouracil
AI	Artificial intelligence
CHX	Cycloheximide
CSC	Cancer stem cell
CSCO	Chinese Society of Clinical Oncology
DDP	Cisplatin
EMR	Endoscopic mucosal resection
EMT	Epithelial-mesenchymal transition
ESD	Endoscopic submucosal dissection
FBS	Fetal bovine serum
GLOBOCAN	Global Cancer Incidence, Mortality and Prevalence
GWAS	Genome-wide association study
HCC	Hepatocellular carcinoma
HPV	Human papilloma virus
miRNA	MicroRNA
NEDD4L	Neural precursor cell expressed, developmentally downregulated 4-like
NSCLC	Non-small cell lung cancer
OSCC	Oesophageal squamous cell carcinoma
PTX	Paclitaxel
PLCE1	Phospholipase C epsilon 1
PTM	Post-translational modification
PROTAC	Proteolysis Targeting Chimeras
RT	Radiotherapy
shRNA	Short hairpin RNA

siRNA	Small interfering RNA
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
PTX	Paclitaxel

LIST OF APPENDICES

MEKANISMA NEURAL PRECURSOR CELL EXPRESSED DEVELOPMENTALLY DOWN-REGULATED 4-LIKE (NEDD4L) SEBAGAI PERENCAT TUMOR MELALUI DEGRADASI YES-ASSOCIATED PROTEIN (YAP) DALAM SEL KARSINOMA SKUAMUS ESOFAGUS MELALUI LALUAN HIPPO

ABSTRAK

Kanser esofagus merupakan salah satu daripada sepuluh kanser dengan kadar insiden dan kematian tertinggi di dunia. Sel karsinoma skuamus esofagus (OSCC) adalah subtaip kanser esofagus yang paling lazim di China, meliputi lebih 90% daripada semua kes kanser esofagus. Pada masa ini, rawatan utama OSCC adalah pembedahan, disokong oleh radioterapi, kemoterapi dan imunoterapi. Walau bagaimanapun, kadar kemandirian lima tahun bagi pesakit yang didiagnosis dengan OSCC kekal rendah, iaitu kira-kira 20%. Oleh itu, terdapat keperluan mendesak untuk mengenal pasti sasaran dan pendekatan terapeutik baharu bagi rawatan OSCC. Kajian terdahulu menunjukkan bahawa NEDD4L boleh memainkan peranan dwifungsi dalam tumor dan menyumbang secara signifikan kepada perkembangan kanser. Namun, peranan khusus NEDD4L dalam OSCC masih belum jelas. Objektif kajian ini adalah untuk menyiasat peranan dan mekanisme berkaitan NEDD4L dalam OSCC. Tahap ekspresi mRNA NEDD4L dalam OSCC dinilai menggunakan pangkalan data TCGA dan Firebrowse, yang mengesahkan bahawa NEDD4L diekspresikan lebih tinggi dalam tisu para-kanser berbanding dengan tisu kanser. Selanjutnya, didapati bahawa keupayaan proliferasi sel-sel OSCC meningkat selepas penyahekspresian NEDD4L secara *in vivo* dan *in vitro*, manakala keupayaan migrasi dan invasi OSCC juga bertambah dalam sel titisan OSCC. Penjujukan RNA dilakukan untuk meneroka

mekanisme berpotensi NEDD4L dalam OSCC. Analisis pengayaan KEGG mendedahkan beberapa laluan isyarat berubah selepas penyahekspresian NEDD4L, termasuk laluan Hippo. Menariknya, pangkalan data ramalan ligase ubiquitin bernama Ubibrowser meramalkan bahawa NEDD4L adalah ligase ubiquitin E3 yang mengawal selia protein teras YAP dalam laluan isyarat Hippo. Untuk mengesahkan sama ada YAP adalah protein sasaran NEDD4L dalam OSCC, eksperimen lanjut dijalankan dan membuktikan bahawa NEDD4L mengawal selia secara negatif tahap protein YAP dan tahap mRNA gen sasaran YAP termasuk CTGF dan CYR61 dalam OSCC. Ujian ko-imunopresipitasi menunjukkan bahawa NEDD4L dan YAP boleh berinteraksi antara satu sama lain dalam OSCC, dan domain WW serta domain HECT pada NEDD4L adalah penting untuk interaksi mereka, manakala domain WW YAP juga diperlukan. Selain itu, ujian kestabilan protein termasuk ujian MG132 dan ujian sikloheksimida menunjukkan bahawa NEDD4L mengawal kestabilan protein YAP melalui laluan proteasom, dan ujian ubiquitinasi menunjukkan NEDD4L meningkatkan poliubiquitinasi berpautan K48 YAP pada tapak K497. Akhirnya, ujian penyelamatan dijalankan dalam sel EC9706 untuk mengesahkan bahawa NEDD4L mengawal perkembangan OSCC bergantung kepada YAP. Penemuan ini mendedahkan mekanisme baharu di mana NEDD4L menyekat perkembangan OSCC dan mencadangkan potensi strategi terapeutik yang mensasarkan paksi NEDD4L-YAP.

MECHANISM OF NEURAL PRECURSOR CELL EXPRESSED DEVELOPMENTALLY DOWN-REGULATED 4-LIKE (NEDD4L) AS A TUMOUR SUPPRESSOR BY DEGRADING YES-ASSOCIATED PROTEIN (YAP) IN OESOPHAGEAL SQUAMOUS CELL CARCINOMA THROUGH HIPPO PATHWAY

ABSTRACT

Oesophageal cancer is one of the top ten cancers with the highest incidence and mortality rates in the world. Oesophageal squamous cell carcinoma (OSCC) is the most important prevalent subtype of oesophageal cancer in China, accounting for more than 90% of all oesophageal cancer cases. At present, the treatment of OSCC is mainly surgery, supplemented by radiotherapy, chemotherapy and immunotherapy. However, the 5-year survival rate for those diagnosed with OSCC remains low, at approximately 20%. Hence, there is a critical need to identify novel therapeutic targets and approaches for the treatment of OSCC. Previous studies have shown that NEDD4L can play a dual role in tumours and significantly contribute to cancer progression. However, the function-specific role of NEDD4L in OSCC remains unclear. The objective of this study was to investigate the role and related-mechanism of NEDD4L in OSCC. Firstly, the mRNA expression level of NEDD4L in OSCC was evaluated using the TCGA database and Firebrowse database, which confirmed that NEDD4L was highly expressed in para-cancerous tissues compared with cancer tissues. Furthermore, it was determined that the proliferation ability of OSCC was increased after NEDD4L depletion both *in vivo* and *in vitro*, while the migration ability and invasion ability of OSCC were also improved in OSCC cell lines. After that, RNA-sequencing was performed to explore the potential mechanism of NEDD4L in OSCC.

KEGG enrichment analysis revealed several signalling pathways were changed after NEDD4L knockdown, including the Hippo pathway. Interestingly, a ubiquitin ligase prediction database named Ubibrowser predicted that NEDD4L is an E3 ubiquitin ligase that closely regulates the core protein YAP of the Hippo signalling pathway. To verify whether YAP is the target protein of NEDD4L in OSCC, further experiments were carried out and proved that NEDD4L can negatively regulate the level of YAP protein and the mRNA level of the YAP target gene including genes CTGF and CYR61 in OSCC. Co-immunoprecipitation assay demonstrated that NEDD4L and YAP could interact with each other in OSCC, and the WW domain and HECT domain of NEDD4L were essential for their interaction, while the WW domain of YAP was also indispensable. Furthermore, protein stability assays including MG132 assay and cycloheximide assay determined that NEDD4L regulates YAP protein stability *via* the proteasome pathway, and the ubiquitination assays showed NEDD4L could increase K48-linked polyubiquitination of YAP at the K497 site. Finally, phenotype-related assays were conducted in EC9706 cells to verify that NEDD4L regulates OSCC progression dependent on YAP. These findings reveal a novel mechanism by which NEDD4L suppresses OSCC progression and suggest potential therapeutic strategies targeting the NEDD4L-YAP axis.

CHAPTER 1

INTRODUCTION

1.1 Background

According to the global cancer statistics report, oesophageal cancer ranks seventh in cancer incidence and sixth in mortality rate, respectively (Sung *et al.*, 2021). There are mainly two types of oesophageal cancer: oesophageal squamous cell carcinoma (OSCC) and oesophageal adenocarcinoma (OAC) (Grille *et al.*, 2021). Unlike in Western countries where adenocarcinoma is common, Eastern countries have a higher susceptibility to getting OSCC, especially in China (Liu *et al.*, 2023a). The 2024 cancer report from the China Cancer Center revealed that China has an annual incidence of over 4 million new cases and a mortality rate of over 2.5 million deaths (Han *et al.*, 2024). In Malaysia, the incidence rate of oesophageal cancer is rather low, ranking 17th among male cancers and 19th among female cancers (Siti-Azrin *et al.*, 2016). OAC was prevalent among Malays, whereas OSCC was more frequent within Indians (Lim *et al.*, 2022). It was found that OSCC is associated with various variables, including genetic inheritance, human papilloma virus (HPV) infection, riboflavin deficiency, smoking, alcohol usage, and so on. Genetic factors may hold a significant position among them (Codipilly and Wang, 2022; Oyouni, 2023).

OSCC is currently confronted with two significant challenges. Firstly, the capacity to detect OSCC at an early stage is inadequate, and a significant number of patients are already in advanced stages upon diagnosis (Codipilly *et al.*, 2018). Secondly, the treatment methods for patients with advanced are limited and lack effective treatment options (Puhr *et al.*, 2023). In terms of OSCC diagnosis, it was evidenced that endoscopy plus biopsy is the gold standard for diagnosing OSCC (Wen

et al., 2022). Nevertheless, because of the low adherence and significant financial burden associated with endoscopy and the absence of apparent symptoms in individuals with early-stage OSCC, only a small number of patients choose yearly endoscopy (Codipilly *et al.*, 2018). As a result, the current pressing issue is to search for a more convenient and cost-effective technology or a more efficient molecular marker for the diagnosis of OSCC. Furthermore, according to previous reports, the 5-year survival rate of patients with OSCC is still not optimistic, only around 20% (Wen *et al.*, 2022). In the treatment of oesophageal cancer, in addition to traditional surgical treatment and radiotherapy, immunotherapy and molecular targeted therapy are increasingly attracting the interest of experts (Waters and Reznik, 2022). However, the treatment of advanced OSCC still lacks of effective strategies and targets so far. Therefore, it is imperative to discover novel targets for the treatment of OSCC.

Given that ubiquitination is the most important modification mode for regulating YAP expression, investigating the process of ubiquitination modification of YAP in OSCC is crucial to show the correlation of ubiquitination with the study. Ubiquitination is simply the process of connecting ubiquitin molecules to target proteins, which requires the consumption of adenosine triphosphate (Sun *et al.*, 2020). However, ubiquitination is a crucial process in the body that is strongly linked to several functions such as inflammatory response, immune regulation, protein degradation, signal transduction, protein localisation, and more (Liang *et al.*, 2022; Heo *et al.*, 2023; Huang *et al.*, 2023a). In the ubiquitination process, adenosine triphosphate supplies energy for the activation of ubiquitin molecules by the ubiquitin-activating enzyme (E1), E1 then transfers the activated ubiquitin molecules to the ubiquitin-binding enzyme (E2). Finally, ubiquitin ligase (E3) attaches the ubiquitin bound to E2 to the target proteins (Lacoursiere *et al.*, 2022). According to available

data, the amount of E1 and E2 is minimal, however, the number of E3 can reach more than 600 (Nakamura, 2018). Furthermore, E3 ligases are responsible for selecting the specific substrate protein to which the ubiquitin molecule will bind. Based on the above reasons, the function of E3 ubiquitin ligase is extremely important and extensive in organisms. In recent years, reports have linked E3 ligases to the advancement of tumours. For instance, TRIM50, an E3 ligase, has been documented to suppress cancer progression *via* degrading the PGK1 protein in gastric cancer (Gu *et al.*, 2024). A separate investigation demonstrated the involvement of TRIM50 in the ubiquitination process of JUP at the K57 location. Consequently, the transport of JUP from the cytoplasm to the nucleus was prevented, suppressing the MYC signalling pathway. Consequently, the malignancy of gastric cancer cells was suppressed (Hu *et al.*, 2023). Interestingly, two different studies on TRIM26 have demonstrated its ability to selectively target certain proteins, hence either inhibiting or promoting cancer development in clear cell renal cell carcinoma (Zheng *et al.*, 2024) and non-small cell lung cancer (NSCLC) (Sun *et al.*, 2023c). Based on the previously mentioned reports, it can be inferred the following outcomes: E3 ligase performs an essential part in the initiation and progression of tumours. Furthermore, the role of the same E3 ligase in various cancers could differ. Moreover, it is worth noting that the identical E3 ligase exhibits many substrates within the same tumour. In this case, it becomes particularly important to comprehensively explore the function of E3 ubiquitin ligase in different tumours.

Neural precursor cell expressed, developmentally downregulated 4-like (NEDD4L) belongs to the HECT family of E3 ubiquitin ligases, and it has a crucial function in controlling protein degradation and cellular activities (Blackburn *et al.*, 2024; Shi *et al.*, 2024b). According to reports, the effect of NEDD4L in tumours may

either prevent or promote cancer, potentially influenced by the specific substrate proteins that NEDD4L targets. For example, it was found that NEDD4L can degrading PHD Finger Protein 8, thus inhibiting cancer cell proliferation in prostate cancer (Feng *et al.*, 2023). Besides, NEDD4L has also been reported to block the occurrence of skin tumours by suppressing the IL-6/GP130 pathway, indicating its suppressive role in cancer (Liu *et al.*, 2024a). Nevertheless, recent investigations have verified that NEDD4L can function as a positive regulatory element for tumours. For instance, an investigation on gallbladder cancer revealed that NEDD4L can raise MMP1 and MMP13 expression levels, resulting in the heightened invasion of tumour cells (Takeuchi *et al.*, 2011). Furthermore, the impact of NEDD4L on the regulation of ferroptosis is diverse. It was found that NEDD4L could function as a suppressor of ferroptosis *via* lactotransferrin (LTF) protein degradation, thus promoting proliferation in pancreatic cancer and ovarian cancer (Wang *et al.*, 2020d). Before the start of this investigation, there were few studies regarding the function and mechanism of NEDD4L in OSCC. This work identified NEDD4L as a tumour suppressor and found that YAP severed as a targeted protein of NEDD4L in OSCC.

YAP is the core protein of the Hippo signalling pathway and also is a transcriptional co-activator, which can synergistically activate target gene transcription with transcription factors (Ko *et al.*, 2022). YAP has been demonstrated to serve crucial roles in the body, including regulating organ size, immunological modulation, carcinogenesis, cancer progression, renewing stem cells, and more (Ma *et al.*, 2019; Driskill and Pan, 2023; Piccolo *et al.*, 2023; Gao *et al.*, 2024). Besides its involvement as an oncogene in tumours, there is a growing amount of research indicating that YAP can also exhibit anti-cancer effects, highlighting its multifaceted significance. Furthermore, the *in vivo* expression level of YAP is mostly controlled by

post-translational modifications. Hence, investigating the post-translational changes of YAP is essential for understanding the regulatory mechanisms of YAP.

This study revealed that NEDD4L functions as a ubiquitin ligase that controls the expression of YAP in OSCC. NEDD4L facilitated the process of ubiquitination and subsequent degradation of YAP at the K497 site. This study can provide important evidence for the use of NEDD4L and YAP as molecular markers and drug targets for OSCC.

1.2 Rationale of the study

Early-stage OSCC presents significant clinical challenges due to its non-specific symptomatology and limited therapeutic options, resulting in poor patient survival rates. Unlike breast cancer, which has well-established molecular subtypes, OSCC's complex aetiology and pathogenesis have hindered the development of comprehensive molecular classification systems. This lack of molecular stratification impedes the implementation of precise, targeted treatments for OSCC. Consequently, both diagnosis and treatment of OSCC remain formidable challenges in clinical oncology. Therefore, there is an urgent need to identify novel therapeutic targets and robust biomarkers for OSCC to improve patient outcomes and enable more personalised treatment approaches.

The occurrence and development of OSCC involve multiple signalling pathways, and targeting these crucial pathways could be a promising therapy approach. Previous sequencing based on the clinical sample has demonstrated the Hippo signalling pathway is often dysfunctional in OSCC, suggesting it may be a key signalling pathway for OSCC (Gao *et al.*, 2014). In addition, YAP is a key component of the Hippo signalling pathway and plays a crucial role in carrying out its functions.

It was reported that YAP has different functions in tumours. For example, YAP acts as an oncogene in colorectal cancer (Ni *et al.*, 2019), hepatocellular carcinoma (Zhu *et al.*, 2020), breast cancer (Luo *et al.*, 2023), and lung cancer (Guo *et al.*, 2017), while YAP plays a tumour suppressor role in lung squamous cell carcinoma (Huang *et al.*, 2017), prostate cancer (Li *et al.*, 2023), and ER-positive breast cancer (Li *et al.*, 2022d). Our recent study showed that YAP overexpression can result in immune evasion of cancer cells in OSCC. Additionally, it was found that elevated levels of YAP were linked with poorer overall survival rates of OSCC patients. Furthermore, the application of verteporfin, a YAP inhibitor, resulted in a reduction in the occurrence of OSCC in mouse models (Zhou *et al.*, 2024). Given the importance of YAP in OSCC, targeting YAP seems to be a good choice. However, due to the disorder of YAP structure, it is very difficult to develop small molecule inhibitors that directly target YAP. Therefore, targeting YAP upstream may be a new approach. Furthermore, given that ubiquitination is the most important modification mode for regulating YAP protein expression, investigating the process of ubiquitination modification of YAP in OSCC is crucial. In addition, a new protein degradation technology, Proteolysis Targeting Chimeras (PROTAC), has been proposed in recent years. PROTAC can ligate E3 ubiquitin ligase with the target protein, thereby achieving the degradation of the specific protein (Alabi *et al.*, 2021). Therefore, investigating E3 ubiquitin ligases that target YAP in OSCC could establish a foundation for developing therapy options using PROTAC in the future.

In this study, based on database analysis, NEDD4L is predicted to be an E3 ligase that potentially regulates YAP. RNA-sequence analysis confirms that NEDD4L can indeed control Hippo/YAP in OSCC. This strongly suggests a potential link between NEDD4L and YAP. The objective of this project was to elucidate the function

of NEDD4L in OSCC and uncover the molecular mechanism *via* which NEDD4L regulates YAP. This research seeks to generate novel insights for the treatment of OSCC.

1.3 Hypothesis

NEDD4L enhances the degradation of YAP protein through the ubiquitin-proteasome pathway, thus suppressing cancer progression in OSCC.

1.4 Objectives of the study

1.4.1 General objective

To elucidate the function of NEDD4L in OSCC and the specific mechanism by which NEDD4L regulates YAP.

1.4.2 Specific objectives

1. To investigate the effects of NEDD4L suppression on migration, invasion, and proliferation in EC9706 and KYSE150 OSCC cell lines
2. To evaluate the impact of NEDD4L knockdown *via* short hairpin RNA on OSCC tumourigenicity *in vivo* using a xenograft mouse model
3. To characterise the molecular interactions between NEDD4L and YAP in EC9706 and KYSE150 OSCC cells
4. To elucidate the mechanistic pathway by which NEDD4L regulates YAP protein levels and activity in HEK293T cells, focusing on ubiquitination and proteasomal degradation processes

5. To determine whether NEDD4L regulates OSCC progression through YAP by comparing the effects of individual and combined silencing of NEDD4L and YAP on EC9706 cell behaviour and tumour growth

CHAPTER 2

LITERATURE REVIEW

2.1 Epidemiology of OSCC

2.1.1 Overview

Cancer is a condition characterised by uncontrolled cell proliferation in the body. Under typical physiological circumstances, cell division occurs exclusively when cells reach a state of ageing or damage, resulting in the production of new cells to replace the old ones. However, when the normal physiological condition is disrupted, it can lead to uncontrolled cell growth and the subsequent development of tumours (Zhang *et al.*, 2024c).

The development and progression of cancer is a highly intricate and challenging process to comprehend within a limited timeframe. Extensive research has demonstrated that genetic variables are significant contributors to the formation and progression of cancers. These factors mostly involve proto-oncogenes and tumour suppressor genes (Porta-Pardo *et al.*, 2020; Zhang *et al.*, 2020b). Typically, the proto-oncogenes found in the genome are not actively expressed and have significant physiological roles. However, when specific circumstances arise, such as viral infection, exposure to chemical carcinogens, or radiation exposure, proto-oncogenes have the potential to acquire abnormally activated and converted into oncogenes. This transformation can then trigger the process of cell carcinogenesis. Tumour suppressor genes exert a negative control on cell proliferation and growth (Zhou *et al.*, 2020). However, their function can be impaired and suppressed, causing the development of tumours. It is worth noting that the gene mutation of the tumour suppressor gene will also lead to its transformation into an oncogene. The most typical example is p53,

which is a tumour suppressor gene, but it can promote tumour growth as an oncogene due to its gene mutation (Liebl and Hofmann, 2021).

Due to the unclear pathogenesis, genetic inheritance, environmental factors, lack of early detection methods, and treatment resistance, the incidence rate and mortality of cancers are gradually increasing all over the world. Reports indicated that in 2020, breast cancer and lung cancer exhibited the highest rates of occurrence and death compared to other types of tumours (Figure 2.1 and Figure 2.2). Oesophageal cancer ranked seventh in terms of incidence among all types of cancer and was the sixth greatest cause of mortality (Sung *et al.*, 2021).

For oesophageal cancer, men account for about 70% of cases, and the gap in mortality and incidence rates between the sexes is two to three times. East Asia has the greatest incidence rate of men and women because of the significant pressure placed on China (Sung *et al.*, 2021). Interestingly, oesophageal cancer shows significant regional distribution differences. The regions with the highest incidence rate of oesophageal cancer are eastern Africa to southern Africa, and central and northern China to northern Iran (Thrift, 2016). Worldwide, OSCC is the most prevailing oesophageal cancer subtype, which accounts for about 87% of all cases of oesophageal cancer (Liu *et al.*, 2023a). Although OSCC is the predominant subtype of oesophageal cancer worldwide, with the highest occurrence rates in Eastern Asia and Eastern Africa, OAC is the most prevalent subtype of oesophageal cancer in western countries (Arnold *et al.*, 2020).

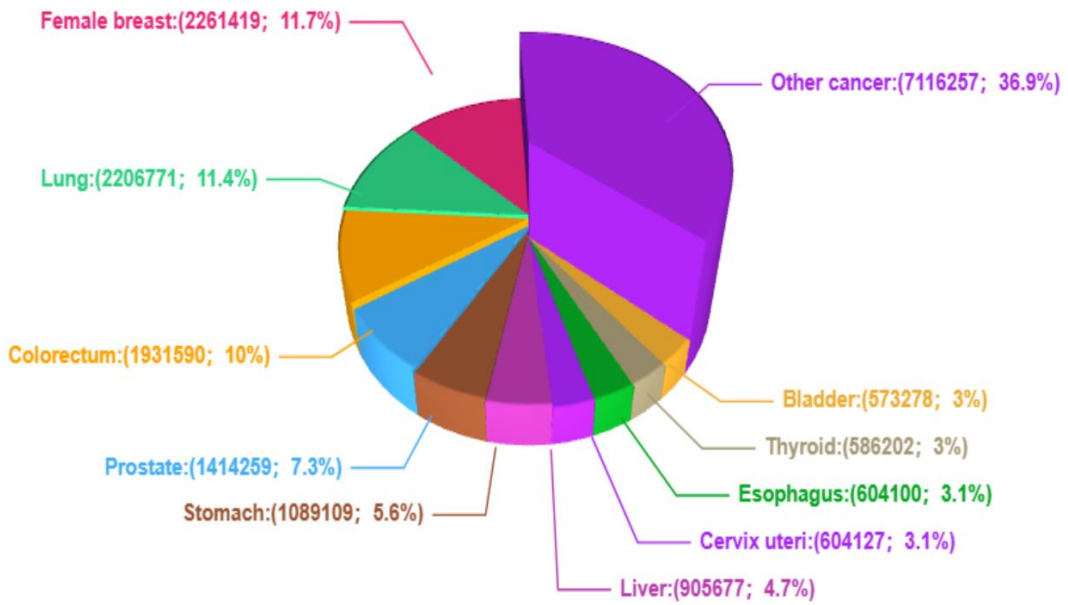

Figure 2.1 The new cases of the top 10 cancers in 2020. (Modified from GLOBOCAN 2020)

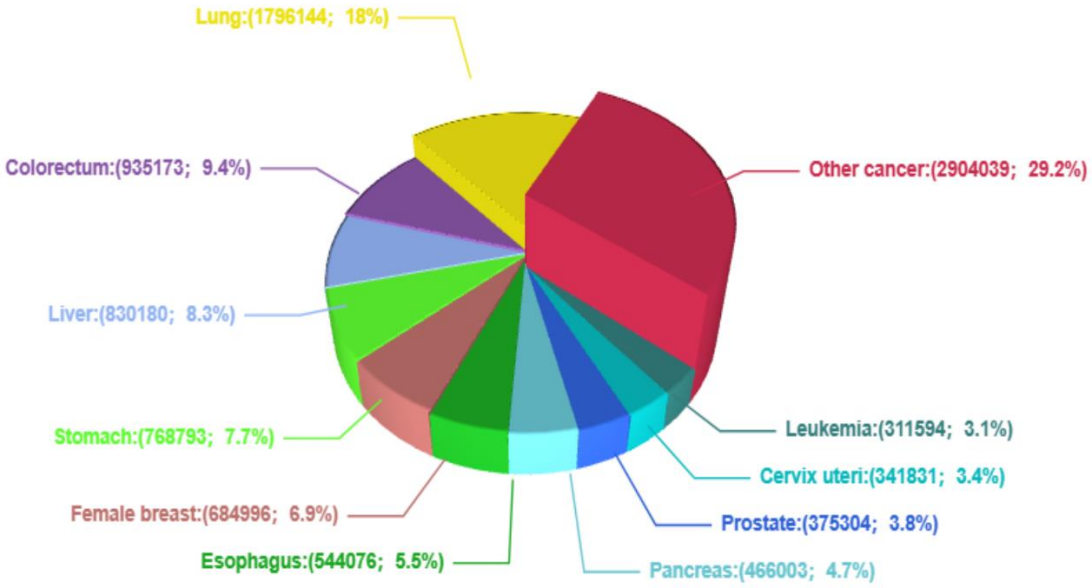

Figure 2.2 The deaths of the top 10 cancers in 2020. (Modified from GLOBOCAN 2020)

2.1.2 Risk factors of oesophageal squamous cell carcinoma

OSCC is defined as a tumour in the squamous epithelium that lines the normal oesophagus, which always occurs in the middle of the oesophagus. An increasing

number of studies showed several risk factors contribute to OSCC, such as genes, tobacco use, alcohol, HPV, Helicobacter pylori (HP), etc.

2.1.2(a) Genetic factor

An extensive number of studies report that genetic factor is a significant risk factor for the OSCC. Herein, we will summarise the genes that are associated with OSCC in a large trial (Table 2.1).

A genome-wide association study (GWAS) conducted in 2009 involving Japanese individuals revealed the presence of two functional single nucleotide polymorphisms (SNPs) in the *ADH1B* and *ALDH2* genes. These genes encode metabolic enzymes responsible for processing alcohol and carcinogens, and their presence is associated with an elevated risk of developing OSCC (Cui *et al.*, 2009). Furthermore, Wang et al. identified Phospholipase C epsilon 1 (*PLCE1*) and *C20or54* as susceptibility genes of OSCC by genotyping more than one thousand OSCC patients and control *via* using the GWAS (Wang *et al.*, 2010a). Abnet's research also confirmed that PLCE1 functions as a susceptibility gene at the same time (Abnet *et al.*, 2010). A further study showed PLCE1 could increase proliferation, migration, and invasion ability *via* upregulating Snail in OSCC cell lines, suggesting PLCE1 likely has a significant impact on OSCC (Zhai *et al.*, 2017). C20or54, also called human riboflavin transporter 2 (RFT2), is responsible for the riboflavin transport and intestinal absorption of riboflavin (Yamamoto *et al.*, 2009). Of note, riboflavin deficiency has been considered one of the reasons for the high incidence of OSCC in Linzhou City (China) in the long term. In 2011, Wu et al. discovered that *ALDH2*, *RUNX1*, and *PDE4D* are susceptibility genes by analyzing 2,031 cases and 2,044 controls of OSCC (Wu *et al.*, 2011). A study from UMAR's group demonstrated *CASP8* as a new

susceptibility gene of OSCC through 259 cases and 259 controls (Umar *et al.*, 2011). Furthermore, *CASP8/ALS2CR12/TRAK2* were substantially related to the risk of OSCC by Abnet et al (Abnet *et al.*, 2012). In addition, it was reported that *ADH* genes, *JUP/HAP1*, *SMG6*, *TMEM188/HEATR3/PAPD5*, *XBP1/CHEK2*, *ST6GAL1*, *PTPN2*, *IGFBP2*, and *SLC10A2* were associated with OSCC risk (Wu *et al.*, 2012). Based on a joint analysis of GWAS, the human leukocyte antigen (HLA) class II gene, *ATP1B2*, and *TMEM173* were verified as risk factors of OSCC in a large number of samples (Wu *et al.*, 2014). Furthermore, three independent groups discovered that *KLF5*, *PADI4*, and *BTLA* are confirmed as risk factors for OSCC, respectively (Chang *et al.*, 2015; Wang *et al.*, 2017a; Cao *et al.*, 2020). Recently, a new study conducted in 2,031 cases and 2,044 controls showed *MYO1B*, and *CHEK2* were associated with OSCC. Interestingly, this study uncovered a new susceptibility locus of *PLCE1* (rs7099485), which confirmed that *PLCE1* is a susceptibility gene of OSCC (Chen *et al.*, 2023). Although emerging research has shown several genes are closely associated with the occurrence of OSCC, its underlying mechanism is still not clear and needs furthermore exploration in the future.

Table 2.1 The genetic factors of OSCC

Genes	rsID	Loci	Number of cases	Reference
ADH1B	rs1229984	4q21-q23	188 cases and 938 controls	Cui et al., 2009
ALDH2	rs671	12q24	188 cases and 938 controls	Cui et al., 2009
PLCE1	rs2274223	10q23	1,077 cases and 1,733 controls (Wang et al.) 2,115 cases and 3,302 controls (Abnet et al.)	Wang et al., 2010 Abnet et al., 2010
C20or54	rs13042395	20p13	1,077 cases and 1,733 controls	Wang et al., 2010
RUNX1	rs2014300	21q22	2,031 cases and 2,044 controls	Wu et al., 2011
PDE4D	rs10052657	5q11	2,031 cases and 2,044 controls	Wu et al., 2011
UNC5CL	rs10484761	6p21	2,031 cases and 2,044 controls	Wu et al., 2011
CASP8	rs3769818	2q33	259 cases and 259 controls	UMAR et al., 2011
CASP8/ALS2CR12/TRAK2	rs13016963	2q33	2,961 cases and 3,400 controls	Abnet et al., 2012
ADH genes	rs1042026	4q23	2,031 cases and 2,044 controls	Wu et al., 2012
TMEM188/HEATR3/PAPD5	rs4785204, rs7206735	16q12.1	2,031 cases and 2,044 controls	Wu et al., 2012
JUP/HAP1	rs6503659	17q21	2,031 cases and 2,044 controls	Wu et al., 2012
XBP1/CHEK2	rs2239612	22q12	2,031 cases and 2,044 controls	Wu et al., 2012
ST6GAL1	rs2239612	3q27	2,031 cases and 2,044 controls	Wu et al., 2012

SMG6	rs17761864	17p13	2,031 cases and 2,044 controls	Wu et al., 2012
PTPN2	rs2847281	18p11	2,031 cases and 2,044 controls	Wu et al., 2012
IGFBP2	rs9288520	2q22	2,031 cases and 2,044 controls	Wu et al., 2012
SLC10A2	rs17450420	13q33	2,031 cases and 2,044 controls	Wu et al., 2012
HLA class II gene	rs35597309	6p21.32	5,337 cases and 5,787 controls	Wu et al., 2014
ATP1B2	rs1642764	17p13.1	5,337 cases and 5,787 controls	Wu et al., 2014
TMEM173	rs7447927	5q31.2	5,337 cases and 5,787 controls	Wu et al., 2014
KLF5	rs1924966, rs115797771	13q22.1	2,031 cases and 2,044 controls	Chang et al., 2015
PADI4	rs2240337	1p36	629 cases and 686 controls	Wang et al., 2017
BTLA	rs3112270, rs2171513	3q13	721 cases and 1208 controls	Cao et al., 2020
FAM120A	rs12379660	chromosome 9	1,686 cases and 3,217 controls	Chen et al., 2023
MYO1B	rs142741123	chromosome 2	1,686 cases and 3,217 controls	Chen et al., 2023
PLCE1	rs7099485	chromosome 10	1,686 cases and 3,217 controls	Chen et al., 2023
CHEK2	rs1033667	chromosome 22	1,686 cases and 3,217 controls	Chen et al., 2023

2.1.2(b) Tobacco use and alcohol

In addition to the genetic factor, tobacco use and alcohol were considered traditional risk factors of OSCC, and they had been verified by several studies. A study involving more than 3000 participants revealed smoking and excessively drinking of alcohol every day were important risk factors for oesophageal precancerous lesions. Former and current tobacco use is related to a 2- to 4-fold greater risk of OSCC when compared to nonsmokers (Wang *et al.*, 2017b). In addition, Alcohol use increases the risk of OSCC by 2- to 9-fold compared with never-drinkers (Codipilly and Wang, 2022). A study using multicentre case-control studies discovered smoking could increase ESCC risks (Simba *et al.*, 2023).

2.1.2(c) Other factors

In addition to the above factors, emerging evidence has shown that other factors are also involved in the risk of OSCC, such as HPV, and HP (Conway *et al.*, 2023). For example, a retrospective study was conducted, involving 225 patients diagnosed with OSCC and 224 controls, to investigate the correlation between HPV infection and OSCC. The result indicated that HPV infection does not present an independent risk for developing OSCC in those who do not smoke or consume alcohol. However, it does elevate the possibility of OSCC occurrence in smokers (Qi *et al.*, 2013). Two studies examining HPV in general indicated a higher incidence of OSCC by 1.56 and 6.4 times, respectively (Guo *et al.*, 2012; Yang *et al.*, 2014). In addition, the potential association between HP and OSCC has been investigated. On the one hand, Li et al. uncovered that the HP infection rate was very high overall in three OSCC high-risk regions of China, and proved the HP infection rate among OSCC patients was more than double that of the normal group (Li *et al.*, 2014). On the other

hand, a multivariate analysis verified that the presence of HP infection in Huaian City is linked to a risk of acquiring OSCC that is more than three times higher (Wang *et al.*, 2006). Interestingly, the incidence of OSCC may also be affected by the consumption of salted meat, pickled vegetables, and tea, as well as dental cleanliness, consumption of fresh produce, and lifestyle choices (Gopakumar *et al.*, 2019; Zhao *et al.*, 2019; Lin *et al.*, 2020b).

2.2 Diagnosis and screening methods of OSCC

Although many technologies including computed tomography (CT) scans, barium contrast studies, and molecular markers contribute to a diagnosis of OSCC, the primary tool for OSCC diagnosis is endoscopy with biopsies (DiSiena *et al.*, 2021). OSCC can often be diagnosed with white light endoscopy (WLE), Lugol's Chromoendoscopy, narrow band imaging (NBI), and endoscopic ultrasonography (EUS) (Codipilly *et al.*, 2018). Furthermore, the utilisation of molecular marker, Papanicolaou stain, especially DNA methylation detection of tumour suppressors holds promising potential for oesophageal cancer screening.

WLE is the main method for diagnosing oesophageal cancer. In clinical practice, after screening ordinary patients and discovering suspicious lesions, further examination can be conducted to clarify the nature of the lesions. Advanced oesophageal cancer is easy to identify, but early oesophageal cancer has no specific manifestations under ordinary white light endoscopy, making it difficult to distinguish its nature (Su *et al.*, 2013).

Lugol's Chromoendoscopy is the most effective method in OSCC screening at present. Under the ordinary white light microscope, OSCC can show the morphological changes of the oesophagus after bulge, depression, redness or inflation.

In the early stage of lesions, these changes are relatively mild and easy to ignore, especially for small flat lesions. When screening for OSCC, spraying 1.2%~2.5% iodine on oesophageal mucosa can greatly improve the detection rate of early OSCC and precancerous lesions (Hashimoto *et al.*, 2005). The interaction between iodine molecules and glycogen in cells is the mechanism of iodine staining. Normal squamous epithelial cells contain enough glycogen to interact with iodine molecules, and the mucosa after reaction is brown (Mansour and Anandasabapathy, 2018). When epithelial cells are defective or abnormal, glycogen content decreases, which affects its interaction with iodine molecules, and mucosa is not stained (Cotton and Choksi, 2020).

The NBI technique employs specific filters to narrow the wavelength of white light emitted by hernia lamps. This limits the penetration of red, green, and blue light to the surface of the mucosa, resulting in enhanced clarity and sharp contrast of the reconstructed capillaries compared to the surrounding normal mucosa (Di Maio *et al.*, 2020). Consequently, this technique improves the ability to detect early superficial cancer. However, due to the filtering out of some light, the intensity of NBI's light source is weakened and the image is darker, which limits the observation of a wider mucosal area and has certain defects. NBI utilises spectral changes to achieve endoscopic "light staining" without the need for auxiliary drugs, and there are no contraindications or discomfort during the examination process. Moreover, it was reported that adding Narrow Band Imaging Magnifying Endoscopy to conventional endoscopy can improve the diagnostic accuracy of OSCC (Katada *et al.*, 2019).

EUS is also used for the auxiliary diagnosis of early OSCC. Conventional endoscopy is limited to visualizing lesions on the surface of the mucosa, and cannot detect lesions within the mucosal layer and submucosa. When the lesions penetrate the

innermost layer of the oesophageal wall, it can lead to misdiagnosis and delay in diagnosing and treating patients. However, EUS can partially address these issues. EUS is a synergistic integration of endoscopic and ultrasonic methodologies. It relies on the use of endoscopy. The ultrasonic probe is positioned at the top end of the endoscope. The ultrasonic probe is employed to precisely identify and determine the specific position of the lesion. Subsequently, a high-resolution ultrasonic scan is conducted to acquire the histology ultrasonic image features of the tube wall at various levels, as well as the ultrasonic pictures of the neighbouring organs (Iglesias-Garcia *et al.*, 2022). EUS allows for the direct visual observation of the distinct layers of the oesophagus wall with the naked eye.

In addition, an emerging non-invasive technique for imaging the gastrointestinal tract is video capsule endoscopy. There has been discussion of utilising this as an OSCC screening technique. However, in a high-risk group of patients with head and neck cancer, a preliminary investigation comparing the use of video capsule endoscopy to Lugol's chromoendoscopy discovered that the technology had poor sensitivity and specificity in identifying neoplastic lesions in the oesophagus (Heresbach *et al.*, 2010).

Although the above methods can be used in the early stage of oesophageal cancer, the cost of the examination is still high, and the accuracy of the examination outcomes relies greatly on the operator's level of expertise. Therefore, it is crucial to find new low-cost, simple and feasible methods. In recent years, biomarkers of cancer cells have received more and more attention from experts. For example, it was reported that serum DSG2 was significantly higher in OSCC than in controls, and DSG2 may be a diagnostic biomarker for OSCC (Liu *et al.*, 2022b). Based on an analysis of 45 pairs of OSCC tissues and normal tissues from Iranian patients, it was found that

Maelstrom could be a diagnostic marker for OSCC (Abbaszadegan *et al.*, 2021). Moreover, a separate investigation examined OSCC data from GEO and TCGA databases and merged it with ELISA experiments. The findings revealed that the concentration of anti-CXCL8 autoantibody in OSCC patients was significantly elevated compared to the control group, suggesting that Anti-CXCL8 autoantibody has the potential to serve as a biomarker for diagnosing OSCC (Chen *et al.*, 2022a). In addition, a similar study demonstrated that anti-POSTN and anti-TIMP1 autoantibodies might be used as diagnostic indicators of OSCC (Xie *et al.*, 2022). Another investigation analysed samples from over 100 cases of OSCC at various stages of advancement. It was discovered that the methylation of the p16 gene increased as OSCC progressed, indicating that p16 methylation can function as an indicator for the diagnosis of OSCC (Fan *et al.*, 2022). Of note, there is increasing research on the methylation of tumour suppressor genes as a biomarker for early diagnosis of tumours, and many tumour methylation detection kits have passed clinical trials. According to incomplete statistics, from 2021 to 2023, more than 20 methylation detection kits for different tumours had been approved by the China Food and Drug Administration in China, demonstrating good specificity and sensitivity for tumour diagnosis in clinical trials. However, it is interesting that there is currently no approved methylation detection kit for oesophageal cancer in China, which is a direction that needs further efforts in the future. More importantly, the tumour methylation detection kit can be used for large-scale screening in areas with high incidence of oral squamous cell carcinoma, because it is simple and easy to operate, and only needs blood.

In addition to endoscopy, many studies have been conducted on non-endoscopic oesophageal sample devices as low-cost OSCC screening methods. A brush, a deflated balloon, or a sponge enclosed in a gelatin capsule and connected to a

cannula or thread that is tethered outside the mouth are swallowed by the awake patient. Once it gets to the stomach, the sponge swells and the balloon becomes inflated or the gelatin capsule breaks. Then, the cell collection device is dragged up the oesophagus and out, collecting the oesophageal superficial squamous cells. Thereby, slides containing the recovered material are coated with Papanicolaou stain for cytologic analysis (Codipilly *et al.*, 2018). These methods are appealing because they can be used widely in environments with low resources and without the need for endoscopy or anaesthesia (Patel *et al.*, 2017). However, their sensitivity and specificity are not as ideal as expected (Pan *et al.*, 2008).

Apart from the above techniques, artificial intelligence (AI) has attracted research in the auxiliary diagnosis of cancer in recent years. AI can help doctors improve the accuracy of early cancer diagnosis of oesophageal cancer. For example, using traditional WLI endoscopy as a basis, Cai et al. developed a novel system for early OSCC identification by convolution neural network. The results demonstrated that this system's diagnostic accuracy is 97.8%, which is higher than senior endoscopists (88.8%) and junior endoscopists (77.2%) (Cai *et al.*, 2019). Similarly, a group designed an AI model to identify the early OSCC in 2022. To train and evaluate the AI model, 13,083 WLI pictures from 1,239 patients were utilised, and a result of AI-assisted WLI shows good accuracy for the diagnosis of OSCC (Liu *et al.*, 2022a). In addition, another team combined AI and WLI for the diagnosis of OSCC, and this model was found to perform exceptionally well in identifying early OSCC, with a sensitivity of 0.979 and a specificity of 0.886 (Tang *et al.*, 2021). Endoscopists' diagnosis accuracy improved considerably after using this model's prediction outputs. Given the outstanding performance of AI in assisting with the diagnosis of

oesophageal cancer, it is likely that AI will have a significant role in the early detection of this type of cancer in the future.

2.3 The staging and therapy of OSCC

2.3.1 Staging of OSCC

Staging of OSCC is the most important indicator of disease prognosis. Formal standards that follow the American Joint Committee on Cancer guidelines are commonly utilised for staging based on tumour invasion (T), lymph node involvement (N), and metastatic spread (M) (Figure 2.3) (Rice *et al.*, 2017). Oesophageal cancer in T1 and T2 stages without lymph node metastasis and distant metastasis was usually considered as early oesophageal cancer (Iriarte *et al.*, 2021). In addition, the staging of oesophageal cancer is an important indicator for selecting the treatment of oesophageal cancer. For instance, for individuals diagnosed with high-grade dysplasia (Tis), mucosal (T1a), and submucosal carcinoma (T1b), endoscopic therapy, which includes endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD), was an effective and preferred treatment method (Borggreve *et al.*, 2018).

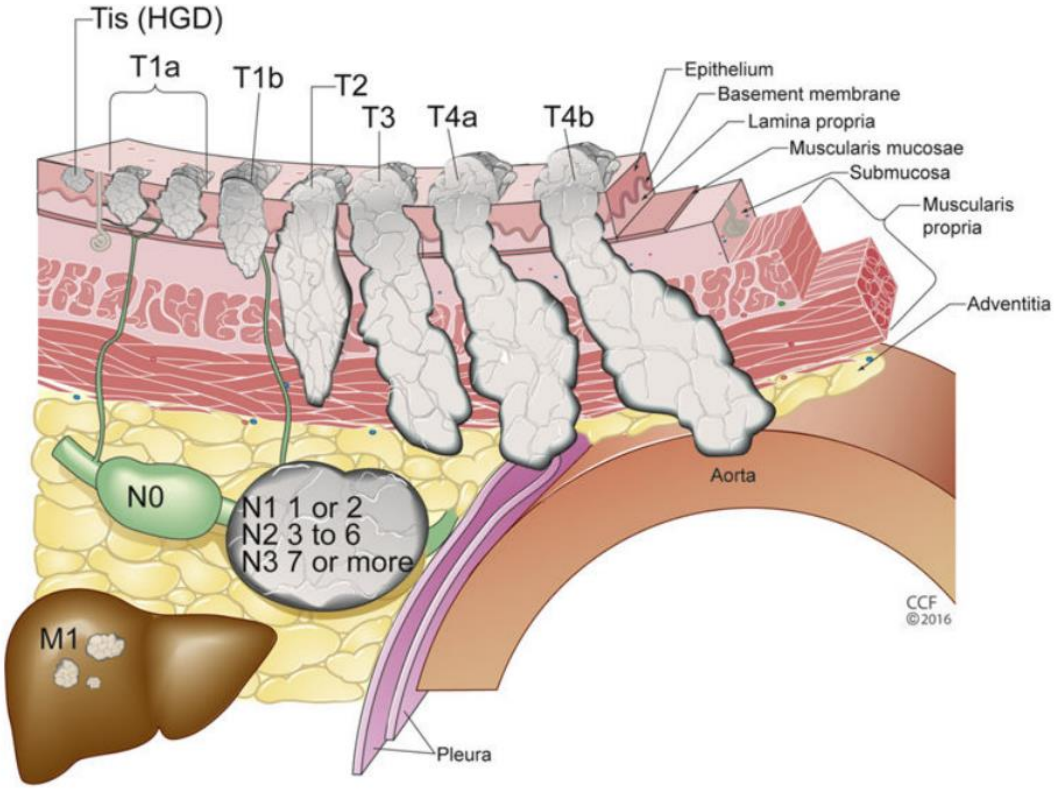

Figure 2.3 TNM categories of oesophageal cancer. Source: (Rice *et al.*, 2017).