ELUCIDATING IFI6 AND RSAD2 PROTEIN EXPRESSION IN ORAL SQUAMOUS CELL CARCINOMA USING IMMUNOHISTOCHEMISTRY AND APTAHISTOCHEMISTRY WITH NOVEL IN SILICO DNA-APTAMERS.

DANIAL QASIM BUTT

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by

DANIAL QASIM BUTT

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LIST OF ABBREVIATIONS

2D	Two-Dimensional
3D	Three-Dimensional
A	Adenine
ADCC	Antibody-dependent cellular cytotoxicity
AHC	Aptahistochemistry
AI	Artificial Intelligence
AJCC	American Joint Committee on Cancer
ASE	Allred score expression
ASL	Allred score levels
ATM	Ataxia-telangiectasia Mutated
ATR	ATM and Rad3 Related
BAK	BCL2 Antagonist/Killer 1
BAX	BCL2-Associated X Protein
BM	Basement membrane
BST2	Bone Marrow Stromal Antigen 2
C	Cytosine
cGAS	Cyclic GMP-AMP synthase
CHK1	Checkpoint kinase 1 homolog
CIITA	Class II Major Histocompatibility Complex Transactivator
COE	Conventional Oral Examination
Cryo-EM	Cryo-Electron Microscopy
CSA	Cancer specific antigen
CTL	Cytotoxic T Lymphocytes
CTL	Cytotoxic T-lymphocytes
CXCL10	C-X-C Motif Chemokine Ligand 10

CXCL10	C-X-C Motif Chemokine Ligand 10
CXCL9	C-X-C Motif Chemokine Ligand 9
CXCR3	CXC Chemokine Receptor 3
DC	Dendritic cell
DOI	Depth of invasion
EC	Escherichia coli
ECM	Extracellular matrix
EIF2AK2	Eukaryotic Initiation Factor 2-Alpha Kinase 2
ENE	Extranodal extension
ER	Endoplasmic reticulum
FADD	FAS-Associated Death Domain Protein
FAS	Fas Cell Surface Death Receptor
FGF-2,	Fibroblast growth factor-2
G	Guanine
GBP1	Guanylate-Binding Protein 1
GROMACS	GROningen MAchine for Chemical Simulations.
H	Hydrogen Atom
H-bond	Hydrogen bond
H&E	Hematoxylin and Eosin
HLA-DRA	Major Histocompatibility Complex, Class II, DR Alpha
HNSCC	Head and Neck Squamous Cell Carcinoma
HPV	Human Papilloma Virus
HS	Homosapiens
ICD-O-3	International Classification of Diseases of Oncology

ICs	Immune cells
IDO1	Indoleamine 2,3-Dioxygenase 1
IFI27	Interferon Alpha-Inducible Protein 27
IFI6	Interferon Alpha-Inducible Protein 6
IFIT1	Interferon-Induced Protein With Tetratricopeptide Repeats 1
IFITM1	Interferon-Induced Transmembrane Protein 1
IHC	Immunohistochemistry
IL-1β,	Interleukin-1 beta
IL-28RA	Interleukin-28 Receptor Alpha
IL-6	Interleukin-6
IRF	Interferon Regulatory Factor
IRF1	Interferon Regulatory Factor 1
IRF7	Interferon Regulatory Factor 7
ISG	Interferon-Stimulated Gene
ISG15	Interferon-Stimulated Gene 15
ISG20	Interferon-Stimulated Gene 20
LY6E	Lymphocyte Antigen 6 Complex, Locus E
M	Median cut-off value
MD	Molecular Dynamic
MD-OSCC	Moderately differentiated - Oral Squamous Cell Carcinoma
MDSCs	Myeloid-Derived Suppressor Cells
MHC	Major Histocompatibility Complex
MM	Mus Musculus
MMPs	Matrix metalloproteinases
MUC1	Mucin 1
MX1	Myxovirus Resistance Protein 1

NCCN	National Comprehensive Cancer Network
NKC	Natural killer cell
NKG2D	Natural-killer group 2, member D
NMR	Nuclear Magnetic Resonance
OAS1	2'-5'-Oligoadenylate Synthetase 1
OH	Hydroxyl Group
OSCC	Oral Squamous Cell Carcinoma
PD-OSCC	Poorly differentiated - Oral Squamous Cell Carcinoma
PDB	Protein Data Bank
PDGF	Platelet-Derived Growth Factor
PKR	Protein Kinase R
PKR	Protein Kinase R
PLIP	Protein Ligand Interaction Profiler
PMN-MDSCs	Polymorphonuclear Myeloid-Derived Suppressor Cells
Ps	Picosecond
RCSB	Research Collaboratory for Structural Bioinformatics
Rg	Radius of Gyration
RMSD	Root mean square deviation
RMSF	Root mean square fluctuation
ROS	Reactive Oxygen Species
RSAD2	Radical S-Adenosyl Methionine Domain Containing 2
ssDNA	Single-Stranded Deoxyribose nucleic acid
ssRNA	Single-Stranded Ribose nucleic acid

STAT	Signal Transducer and Activator of Transcription
T	Thymine
TAM	Tumor-Associated Macrophages
TAN	Tumor-Associated Neutrophils
TERT	Telomerase reverse transcriptase
TGF	Tumour growth factor
TGF-β1,	Tumour growth factor-Beta1
TGF-β2,	Tumour growth factor-Beta1
TME	Tumour microenvironment
TRAIL	TNF-Related Apoptosis-Inducing Ligand
Tregs	Regulatory T cells
TRIM22	Tripartite Motif Containing 22
Type II ISGs	Type II Interferon-Stimulated Genes
Type III ISGs	Type III Interferon-Stimulated Genes
U	Uracil
vdW	Van der Waal
WD-OSCC	Well differentiated - Oral Squamous Cell Carcinoma
ZAP	Zinc Finger Antiviral Protein
ZAP	Zinc Finger Antiviral Protein
ΔG	Gibbs free energy

LIST OF APPENDICES

MENJELASKAN EKSPRESI PROTEIN IFI6 DAN RSAD2 DALAM KARSINOMA SEISKUAMOSA ORAL MENGGUNAKAN IMUNOHISTOKIMIA DAN APTAHISTOKIMIA DENGAN APTAMER DNA IN SILIKO YANG NOVEL

ABSTRAK

Penyelidikan terkini menonjolkan gen rangsangan interferon (ISG) seperti IFI6 dan RSAD2 yang berpotensi sebagai biomarker protumorigenik, menyumbang kepada proliferasi dan kemandirian kanser. Walaupun peranan mereka dalam nyahpengawalaturan apoptosis telah didokumenkan dalam pelbagai kemaglinanan, penglibatan mereka dalam pengelakan imun, khususnya dalam karsinoma sel skuamosa oral (OSCC), masih belum diterokai sepenuhnya. Oleh itu, memahami pengaruh mereka dalam mikropersekitaran tumor adalah penting. Kemajuan dalam teknologi pengesanan biomarker menawarkan kelebihan yang ketara dalam meningkatkan spesifisiti, sensitiviti, dan kebolehulangan, di samping mengatasi cabaran kos dan variasi kelompok dalam diagnostik OSCC berasaskan antibodi. Kajian ini bertujuan untuk meneliti ekspresi protein IFI6 dan RSAD2 dalam sel kanser dan sel imun (neutrofil, sel plasma, dan limfosit) dalam mikropersekitaran tumor OSCC menggunakan teknik imunohistokimia (IHC) dan aptahistokimia (AHC), dengan menggunakan aptamer DNA perolehan secara in silico. Selain itu, kajian ini juga bertujuan untuk mereka bentuk, mencirikan, dan mengesahkan aptamer DNA ini bagi aplikasi dalam AHC. Ekspresi protein IFI6 dan RSAD2 dianalisis dalam sampel tisu OSCC (n=23) dan tisu sihat (n=7) menggunakan IHC dan AHC. Hubungan antara ekspresi protein, kehadiran sel kanser dan imun, serta

gred histologi tumour dinilai secara statistik. Aptamer DNA untuk IFI6 dan RSAD2 direka bentuk secara in silico, dan interaksi pengikatannya dicirikan menggunakan pemodelan dok molekul (tenaga pengikatan), PyMOL, dan Protein-Ligand Interaction Profiler (PLIP) untuk analisis ikatan hidrogen. Simulasi dinamik molekul di dalam GROMACS menilai kestabilan kompleks (RMSD) dan fleksibiliti aptamer (RMSF). Aptamer DNA berstem-gelung pin rambut (35–50 mer) berjaya dibangunkan untuk IFI6 dan RSAD2. Di antaranya, aptamer IFI6 (35- dan 50-mer) dan aptamer RSAD2 (35- dan 45-mer) menunjukkan afiniti pengikatan yang tinggi (-15.7 hingga -18.7 kcal/mol), ikatan hidrogen yang kukuh (<4 Å), kestabilan RMSD (<0.4 nm), dan kawasan gelung yang fleksibel (RMSF >0.4 nm). Analisis IHC dan AHC menunjukkan ekspresi IFI6 dan RSAD2 yang signifikan dalam tisu OSCC manakala tidak dikesan dalam sampel sihat ($p<0.05$). Tahap ekspresi menunjukkan peningkatan yang ketara dalam gred histologi tumor yang rendah pembezaan serta dalam sel imun yang berkaitan dengan tumour ($p<0.05$). Kajian ini mengesahkan ekspresi IFI6 dan RSAD2 dalam sel kanser dan sel imun OSCC serta hubungannya dengan gred tumour yang lebih tinggi. Penemuan ini menyokong potensi IFI6 dan RSAD2 sebagai biomarker prognostik dalam mikropersekitaran tumor. Aptamer DNA perolehan in silico menunjukkan ciri pengikatan yang kukuh dan berpotensi untuk digunakan dalam pengesanan OSCC berasaskan AHC, menawarkan alternatif diagnostik yang lebih kos efektif dan boleh dipercayai.

ELUCIDATING IFI6 AND RSAD2 PROTEIN EXPRESSION IN ORAL SQUAMOUS CELL CARCINOMA USING IMMUNOHISTOCHEMISTRY AND APTAHISTOCHEMISTRY WITH NOVEL IN SILICO DNA-APTAMERS

ABSTRACT

Emerging research highlights interferon-stimulated genes (ISGs) such as IFI6 and RSAD2 as potential protumorigenic biomarkers, contributing to cancer proliferation and survival. While their role in apoptotic dysregulation has been documented in various malignancies, their involvement in evading immune evasion, particularly in oral squamous cell carcinoma (OSCC), remains largely unexplored. Understanding their influence within the tumour microenvironment is, therefore, imperative. Advances in biomarker detection technologies offer substantial advantages in improving specificity, sensitivity, and reproducibility while mitigating cost and batch variation challenges in antibody-based OSCC diagnostics. This study aims to examine IFI6 and RSAD2 protein expression in cancer and immune cells (neutrophils, plasma cells, and lymphocytes) within the OSCC tumour microenvironment using immunohistochemistry (IHC) and aptahistochemistry (AHC), employing in silico-derived DNA aptamers. Additionally, it seeks to design, characterise, and validate these DNA aptamers for potential application in AHC. IFI6 and RSAD2 protein expression was analysed in OSCC (n=23) and healthy (n=7) tissue samples via IHC and AHC. The correlation between protein expression, cancer, immune cell presence, and histological tumor grades was statistically assessed. DNA aptamers for IFI6 and RSAD2 were designed in silico, and their binding interactions were characterised using molecular docking (binding energy), PyMOL, and Protein-

Ligand Interaction Profiler (PLIP) for hydrogen bonding analysis. Molecular dynamics simulations in GROMACS assessed complex stability (RMSD) and aptamer flexibility (RMSF). Stem-hairpin loop DNA aptamers (35–50 meters) were successfully developed for IFI6 and RSAD2. Among them, 35- and 50-mer IFI6 and 35- and 45-mer RSAD2 aptamers exhibited high binding affinity (-15.7 to -18.7 kcal/mol), strong hydrogen bonding (<4 Å), stable RMSD (<0.4 nm), and flexible loop regions (RMSF >0.4 nm). IHC and AHC analyses revealed significant IFI6 and RSAD2 expression in OSCC tissues while absent in healthy samples ($p<0.05$). Expression levels were markedly elevated in poorly differentiated tumour grades and tumour-associated immune cells ($p<0.05$). This study confirms IFI6 and RSAD2 expression in OSCC cancer and immune cells, correlating with higher tumour grades. These findings support their potential as prognostic biomarkers in the tumour microenvironment. The in silico-derived DNA aptamers demonstrate strong binding characteristics and hold promise for AHC-based OSCC detection, offering a cost-effective and reliable diagnostic alternative.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Cancer is a complex and heterogeneous disease characterised by the uncontrolled proliferation of abnormal cells, leading to local invasion and distant metastasis (Brown et al., 2023). It remains a leading cause of mortality worldwide, particularly in individuals under 70 years of age, due to its aggressive nature and high recurrence rates (Ferlay et al., 2021). Oral squamous cell carcinoma (OSCC), the predominant malignancy of the oral cavity, accounts for over 90% of oral cancers. It arises from the squamous epithelial cells of the oral mucosa and is known for its rapid progression and high metastatic potential (Wang et al., 2022). The development of OSCC is a multistep process triggered by carcinogenic exposures, including tobacco, alcohol, radiation, and oncogenic pathogens, leading to genetic and epigenetic alterations (Zhang et al., 2024). These alterations contribute to key hallmarks of cancer, such as evasion of apoptosis, uncontrolled proliferation, and immune evasion, ultimately worsening prognosis.

Recent advancements in cancer immunology highlight the tumour microenvironment (TME) as a pivotal determinant in cancer progression and response to therapy (Wang et al., 2017). Under normal conditions, the immune system recognises and eliminates malignant cells through innate and adaptive immune responses. Dendritic cells activate interferon-stimulated genes (ISGs) via type I interferons (IFN-I), which serve as the first line of defence against tumour progression (Li et al., 2021; Diamond and Kanneganti, 2022). However, cancer cells manipulate immunoediting processes to evade

immune surveillance and establish an immunosuppressive TME (Borroni and Grizzi, 2021). This adaptation enables tumour cells to escape immune-mediated destruction and sustain uncontrolled growth (Gubin and Vesely, 2022).

This study is grounded in the immunoediting framework, focusing on how OSCC exploits ISGs to establish immune-resistant TME. Interferon Alpha Inducible Protein 6 (IFI6) and Radical S-Adenosyl Methionine Domain Containing 2 (RSAD2) have been identified in various malignancies, including esophageal, pancreatic, lung, gastric, and breast cancers. These proteins play a crucial role in inhibiting apoptosis and evading immunosurveillance, mechanisms that contribute to cancer progression, and have been associated with poor prognosis and decreased survival rates (Liu et al., 2020; Choi et al., 2022; Huang et al., 2023; Liu et al., 2023). The involvement of these proteins suggests that they have protumorigenic characteristics (Butt et al., 2024).

Specifically, this study aligns with best practices in biomarker validation by investigating IFI6 and RSAD2 expression within the TME of OSCC tissues, encompassing both cancerous and immune cells. Utilising immunohistochemistry (IHC) and aptahistochemistry (AHC), this research aims to elucidate their expres, offering novel insights into OSCC pathogenesis and contributing to the development of innovative diagnostic strategies.

This thesis is structured into nine chapters. After the introduction, Chapter 2 presents a comprehensive literature review, outlining the study's conceptual framework on the research gap, research question, hypothesis, objectives, and justification. Chapter

3 details the materials and methodologies employed for immunohistochemistry (IHC) and aptahistochemistry (AHC). The subsequent chapters focus on the study's findings: Chapter 4 reports the IHC results and Chapter 5 presents a method for *in silico* DNA aptamer development, as it is a novel finding and is considered a result. Chapter 6 describes the *in silico* DNA aptamer development process, and its outcomes, and Chapter 7 presents the AHC findings. Chapter 8 provides a critical discussion of the results in the context of existing literature, and finally, Chapter 9 concludes the study, summarising key insights, their implications, and future recommendations.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This chapter presents an overview of OSCC, an in-depth review of the concept of cancer immunology, including the tumour microenvironment and its key players, interferon-stimulated genes (ISGs), and the regulation of apoptosis. A focused review of the IFI6 and RSAD2 proteins and their potential roles in tumour progression and immune evasion highlights the gap in the understanding. Following the conceptual framework on the research gap, the research question, hypothesis, objectives, and justification are presented.

2.2 Cancer

Cancer is a highly complex and heterogeneous disease characterised by the unregulated growth of abnormal cells, leading to local tissue invasion and the spread to distant sites (Brown et al., 2023). Owing to its aggressive nature and high recurrence rates, it remains a major cause of mortality worldwide, particularly among individuals under the age of 70. Various cancer types, including carcinoma, sarcoma, leukaemia, and lymphoma, originate from different tissues and exhibit distinct biological behaviours. Among them, OSCC is a highly aggressive malignancy of the head and neck, often associated with poor prognosis due to late-stage diagnosis and resistance to therapy (Tan et al., 2023). Understanding the molecular mechanisms underlying cancer progression,

including the role of immune modulation and tumour-associated biomarkers, is crucial for improving early detection, prognostic evaluation, and treatment strategies.

2.3 Brief overview of oral squamous cell carcinoma

2.3.1 Epidemiology

Oral cancer is the sixth most common cancer worldwide, with an annual incidence of 377713 cases per year in 2020, and OSCC represents 2-5% of head and neck cancers (GLOBOCON, 2020). Cancer of the tongue in women and oropharyngeal in men is also rising (Tota et al., 2017). The past 30 years saw an increasing number of young patients aged 40-45 years being diagnosed with OSCC (Steffen et al., 2022). It has been suggested that the etiology and pathogenesis of cancer in young patients are characteristically and clinically distinctive from older age groups; typically, because of tobacco and alcohol abuse (Hussein et al., 2017; Herck et al., 2021).

2.3.2 Anatomical Sites of OSCC

SCC of the head and neck is one of the most common heterogeneous groups of cancers in the world, ranking seventh in the world. The oral cavity is the most common anatomically affected area in the head and neck region (Johnson et al., 2020).

OSCC affects the mucosal lining of lips, the hard and soft palate, the floor of the mouth, salivary glands, and the gingivobuccal complex comprising the gingiva, retromolar trigone area, and buccal mucosa.

About 40% of OSCCs begin on the floor of the mouth or the lateral and ventral surfaces of the tongue (Bubliy et al., 2021). Cancer of the lip accounts for 25-30% of all cancers in the oral cavity while cancer of the gingivobuccal complex accounts for 10% per cent of all cancers in North America and Western Europe with 40% of its prevalence in Southeast Asia, China, and Africa (Montero and Patel, 2015).

2.3.3 Risk factors

OSSC has been associated with risk factors such as alcohol, smoking, human papillomavirus infection, sun exposure, betel quid, and areca nut (Ahmad et al., 2021). Smoking and alcohol consumption have been linked to 71% and 14% of deaths from OSCC, respectively (Ndayisabye et al., 2022). Other possible risk factors include chronic mechanical irritations like poorly fitting dentures and drinking hot beverages (Loomis et al., 2016; Gupta et al., 2021).

2.3.4 Signs, symptoms, and diagnosis

OSCC is initially painless and unnoticeable but becomes aggressive, painful, and prone to bleeding as it progresses. Clinically, it presents in various colour configurations, appearing red (erythroplakia), white (leukoplakia), or mixed (erythroleukoplakia) (Thavarool et al., 2019; Burgess, 2022). The lesion may be flat, plaque-like, lump-like, or ulcerative with raised exophytic margins. In advanced stages, submaxillary and cervical lymph nodes become palpable and eventually fixed, indicating possible metastasis. Prognosis and treatment rely on TNM grading and histological classification (Muthu et al., 2018).

Diagnosis involves a clinical assessment of tumour size, site, and spread to adjacent structures, with MRI and CT scans aiding in staging and treatment planning. Histopathological analysis assesses the degree of tissue differentiation, which is one of the determinants of prognostic value in OSCC (Cariati et al., 2022). The gold standard for diagnosis is histological assessment through tissue biopsies. Incisional biopsies are performed for large lesions, while excisional biopsies are used for smaller, exophytic lesions without neck metastasis (Abhyankar et al., 2020; Ellis et al., 2022). Multiple biopsy sites are preferred for non-homogeneous lesions, ensuring representative sampling. Early detection improves survival rates significantly, increasing cure rates from 50% to 80%, and reducing the need for aggressive treatment, improving the patient's quality of life (Haj-Hosseini et al., 2022).

2.3.5 Histopathological grading of OSCC

Cancer grading determines how much cancer cells differ from normal healthy cells. A common method involves staining tissue slides with H&E and examining them under a microscope. Cancer cells that closely resemble normal cells are classified as well-differentiated, while those that look less like normal cells are considered poorly differentiated. According to WHO classification, OSCC is graded as well-differentiated (G1), moderately differentiated (G2), or poorly differentiated (G3) (Appendix A) (Huang and O'Sullivan, 2017; Jie et al., 2018).

2.3.6 Treatment modalities

Surgery and radiotherapy are the primary treatments for OSCC patients and the treatment modalities rely on the site of a lesion, the stage of the disease, and the overall health of a patient (Abbas et al., 2018). Chemotherapy is a cancer treatment where the medicine is used as an adjunct to the primary treatment or as a primary treatment for metastatic cancer and blood cancer.

Surgery is the main treatment of OSCC followed by radiation and chemotherapy for Stages 1 to 2, and for stages 3, 4b, and 4c, radiotherapy and chemotherapy or a combination of both are recommended. Recently, a study has shown the promising results of neoadjuvant immunotherapy before surgery in OSCC improving the prognosis of cancer in patients (Olmos et al., 2021).

Photodynamic therapy is a new approach and is currently being studied for the management of OSCC (Ketabat et al., 2019). Screening, diagnosis, and treatment are important in determining the prognosis of OSCC patients. Early detection and treatment offer a better prognosis.

The development of diagnostic methods and treatments that rely on cancer-specific biomarkers, particularly chemotherapy, and immunotherapy, is attributed to research aimed at understanding cancer progression from its initiation to the late stages at the tissue, cellular, and molecular levels (Passaro et al., 2024). For example, in cancer immunology, the understanding is based on investigating biomarkers at the tissue level, to assess their

prognostic and predictive role in cancer patients to develop immune-checkpoint inhibitors therapy against cancer patients (Bai et al., 2020; Sankar et al., 2022).

2.4 Overview of research in cancer progression

There are three major areas in cancer research; one, studies focusing on the understanding of the disease such as epidemiology, risk factors, clinicopathology, and pathogenesis; two, studies on developing diagnostic methods and tools to diagnose the disease such as identifying biomarker study at the tissue and cellular level (Arip et al., 2022), and three, studies on developing and experimenting with treatment methods such as surgical resection, chemotherapy, radiotherapy, immunotherapy, and palliative (McKean et al., 2020). The initiation, progression, and metastasis of cancer is known as tumorigenesis, or oncogenesis or carcinogenesis (Maiti, 2015).

Six recognisable characteristics of cancers lead to tumorigenesis, progression, and metastasis in the host, each of which affects the immunological properties against tumour cells. First, by creating autocrine and paracrine growth factors with immunosuppressive properties, tumours can become self-sufficient in growth signals. Second, tumours lose their sensitivity to antigrowth signals that cause local immunosuppression, such as transforming growth factor (TGF). Third, cancers overexpress mitochondrial cell-death inhibitors, which are potential tumour antigens, and inhibit caspase activation, which is necessary for immunogenic cell death, to elude apoptosis. Fourth, excessive TERT (telomerase reverse transcriptase) expression and p53 mutation, two possible cancer antigens, are linked to limitless replication. Fifth, sustained angiogenesis entails the

creation by tumours of angiogenic substances that prevent dendritic-cell maturation and T-cell activation, such as vascular endothelial growth factor. Sixth, epigenetically reprogrammed cytokines in TME that aid cancer cells to evade immunity (Hanahan, 2022).

The current understanding of tumorigenesis suggests that the immune system is involved in the progression and development of cancer; hence, originating the science of cancer immunology.

Cancer immunology is the study of the interaction between cancer cells and immunity and contributed to the two important areas of research, which led to the understanding of cancer immunotherapy; immunosurveillance and immunoediting (Borroni and Grizzi, 2021).

2.5 Immunosurveillance

Immunosurveillance is a process whereby the immune cells identify and eliminate pathogen-infected or cancerous cells recognised as antigen-presenting cells by activating the innate and acquired immune cells (Kupper and Fuhlbrigge, 2004). Burnet in 1967 hypothesised that long-lived animals with multicellular systems might use the immune system to deal with the development of somatic mutation and possible neoplasia, and this idea gave rise to the term "immunological surveillance" (Burnet, 1967; Agraharkar et al., 2004). The significance of immunosurveillance is supported by the rise in the prevalence of malignant illnesses in immunocompromised individuals and laboratory animals (Agraharkar et al., 2004).

Two important processes occur within the tumour microenvironment (TME): immunosurveillance and immunoediting (Pandya et al., 2016). This microenvironment is occupied by macromolecules of extracellular matrix (ECM), basement membrane (BM), and immune cells that are surrounding tumours (Bożyk et al., 2022).

Several tumour-suppressing systems, many of which work in a cell-intrinsic way, prevent cancer growth. The DNA-damage response is triggered by the activation of oncogenes, and it regulates pre-malignant lesions by activating several different molecules, including DNA-damage sensors like ATM (Ataxia-telangiectasia Mutated) and ATR (ATM and Rad3 Related), checkpoint kinases like CHK1 (checkpoint kinase 1 homolog) and CHK2, and the tumour-suppressor protein p53. ATM, ATR, and CHK2 can also cause cancer cells to express ligands for NKG2D (natural-killer group 2, member D), which in turn triggers an immune surveillance response from NKG2D-expressing lymphocytes (Zingoni et al., 2018; Liu et al., 2019; Takeuchi et al., 2019).

The detection and elimination of cancer cells involve a large number of innate and adaptive immune effector cells in the TME. The immune cells respond by releasing cytokines that mediate apoptosis in cancerous cells and remove them from the host tissue environment, thus, inhibiting tumorigenesis.

The immune cells, particularly white blood cells (leukocytes), are the first line of defence and play a pivotal role in defending against cancer in TME. These cells are derived from bone marrow stem cells, these immune cells circulate through the blood and lymphatic systems. They are typically divided into two functional categories: innate

immune cells, which provide a first line of defence, and adaptive immune cells, responsible for targeted, long-term immunity (Marshall et al., 2018).

2.5.1 Innate immune cells

Innate immune cells in TME, such as macrophages, neutrophils, dendritic cells, and natural killer (NK) cells, play a crucial role in identifying and eliminating pathogenic invaders, including cancer-specific antigens (Li et al., 2024). These cells form a vital component of the innate immune system, serving as the body's first line of defence by recognising and responding rapidly to antigens, thereby preventing the spread of infection and triggering subsequent adaptive immune responses (Maiorino et al., 2022).

Neutrophils originating from bone marrow hematopoietic stem cells are a vital component of innate immunity. These granular, multilobed white blood cells act through phagocytosis, releasing antimicrobial enzymes and reactive oxygen species (Andrés et al., 2022). In the context of cancer, neutrophils are recruited to the tumour site by chemokines, such as CXCL8, to perform immunosurveillance. Though generally considered anti-tumorigenic, their role in the TME is nuanced, as they can also contribute to tumour progression under certain conditions (Cambier et al., 2023).

2.5.2 Adaptive immune cells

Lymphocytes, a crucial component of adaptive immunity, are a type of white blood cell characterised by their large, rounded nucleus and scant cytoplasm. They play a significant role in the immune system, with the two primary types being B cells and T cells. B cells are responsible for producing antibodies that neutralise pathogens, while T

cells mediate cellular immunity by eliminating diseased or malignant cells and activating other immune cells (Fanous et al., 2022).

Helper T cells (CD4+ T cells) are a specialised subset of T lymphocytes that develop in the thymus after originating from hematopoietic stem cells. These cells are distinguished by their round nucleus and minimal cytoplasm. Their primary role is to coordinate immune responses by releasing cytokines that activate B cells, cytotoxic T cells, and macrophages. Chemokines such as CCL3 and CCL5 direct helper T cells to sites of infection, where they assist in immune activation. In the context of cancer, helper T cells may enhance immunity against tumours; however, tumour cells can inhibit their function, hindering their ability to mount an effective immune defence (Zhang et al., 2020).

Cytotoxic T cells, also known as CD8+ T cells, originate from hematopoietic stem cells in the bone marrow and mature in the thymus. They are small lymphocytes with a large, round nucleus and sparse cytoplasm. Upon activation by CD4+ helper T cells and other immune cells, cytotoxic T cells contribute to adaptive immunity by targeting and destroying malignant or infected cells. Chemokines such as CXCL9 and CXCL10 attract cytotoxic T cells to sites of infection or tumours, where they directly attack pathogens or cancerous cells (Tokunaga et al., 2018).

Regulatory T cells (Tregs) represent a specialised subset of T lymphocytes which are derived from hematopoietic stem cells and mature in the thymus. These cells play a critical role in maintaining immune homeostasis by suppressing excessive immune

responses, thus preventing autoimmunity and modulating inflammation. Tregs are recruited to the inflammatory or tumour sites through chemokines like CCL22 and CCL17 (Sarkar et al., 2021). Their primary function is to regulate and dampen immune responses, ensuring that inflammation does not damage healthy tissues.

Plasma cells, which are differentiated B cells, are characterised by their oval shape, eccentric nucleus, and abundant cytoplasm. These cells specialize in the production and secretion of large quantities of antibodies. Plasma cells are derived from activated B cells during an immune response to a specific antigen. They play a critical role in humoral immunity by producing antibodies that neutralise cancer antigens and label them for destruction by other immune cells (Chi et al., 2024).

2.5.3 Immunosurveillance and elimination of cancer cells

In this phase, the immune cells from both innate and acquired immunity play a role in eliminating the mutated cells by releasing cytokines produced by dendritic cells and consolidating the immunosurveillance via apoptosis.

In TME, interferon has been reported to trigger the expression of two types of proteins. One is tumour suppressor proteins (p53 and retinoblastoma) which are present in both cancer and healthy cells that triggers apoptosis via the carcinogenic stimuli and instigate malignant transformation. Two, anti-tumour ISG proteins, such as pro-apoptotic (IRF family, TRAIL, and FAS), anti-metastatic (IRF7), and anti-angiogenic (CXCL9, CXCL10, and CXCL11) for initiating the apoptotic process (Cheon et al., 2014; Waight et al., 2014; Yang et al., 2017). These ISGs inhibit the development of cancer by inducing

apoptosis through BAX and BCL2 in the intrinsic apoptosis pathway (Ozaki and Nakagawara, 2011; Wang et al., 2023). Similarly, in breast cancers, ISG (ISG12a) activates caspase-mediated apoptosis and inhibits tumour progression (Avraham et al., 2013). In addition, ISGs activate signaling cascades by triggering CD8+ T cells to eliminate cancer cells from TME (Yeung et al., 2018; Duong et al., 2022).

However, immunosurveillance can be impaired in the later stages of tumorigenesis by epigenetic alterations. Due to this, cancer cells begin to control the immune cells to escape and metastasise (Noonepalle et al., 2021).

2.6 Immunoediting

Immunoediting is a concept in which tumour cells escape immunosurveillance and undergo immunogenic sculpting. There are three phases of immunoediting: elimination phase, equilibrium phase, and escape phase. The immunosurveillance dominates the elimination phase. The cancer cells evade immunosurveillance through immunoediting during the equilibrium and escape phases (Dunn and Schreiber., 2004; Borroni and Grizzi, 2021).

2.6.1 Equilibrium Phase

The innate and acquired immune cells in this phase could not control the genetically unstable new cancer cell variants as the latter continue to replicate and become resilient; this allows them to survive immune elimination. The tumour is clinically undetectable in this phase and enters into dormancy.

The immune system's ongoing challenges may serve as catalysts for the positive selection of cancer mutations that can change into a non-immunogenic tumour phenotype. As a result, the equilibrium phase is characterised by protracted intervals of clinical activity that cannot be seen. The prolonged latency period of many human malignancies serves as evidence that this is likely attributable to repeated elimination and creation of malignant cells over numerous stages of the elimination phase. This resistance leads to epigenetic silencing of ISGs because of resistance to IFN by cancer cells. Like for example, the loss of STAT2, a promoter of ISG transcription, was reported in breast cancer cells, the axis gets affected during the phase and hinders the production of proapoptotic ISGs (Figure 2.1) (Walter et al., 2020).

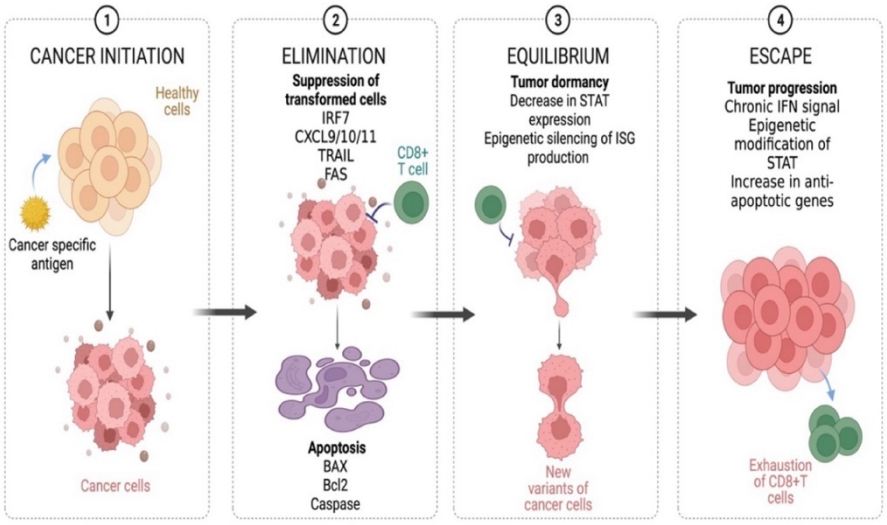

Figure 2.1: Immunoediting process (Borroni and Grizzi, 2021). The image is created with BioRender.com.

2.6.2 Escape Phase

The new cancer cell variants are undetectable by immune cells and have immunosuppressive properties. They replicate and escape the immune cell attack. In this phase, the cancer cells are clinically detectable (Figure 2.1).

In TME, prolonged exposure to interferons (IFNs) leads to the overexpression of STAT proteins (STAT2/3/5), potentially inducing protumorigenic ISGs (Cheon et al., 2022). This process is driven by the epigenetic reprogramming of cancer cells. The phosphorylated STAT in leukemic cancer cells causes the activation of anti-apoptotic genes in the cancer cell's nucleus. However, the histone modification protein P66 epigenetically changed phosphorylated STAT3 (Zeinalzadeh et al., 2021). In addition, CD8 T-cell exhaustion caused by STAT-caspase activation allows cancer cells to evade cytotoxicity (Sun et al., 2023).

The cancers cells in the escape phase recruit and reprogram the function of immune cells, as a part of the immunoediting process by secreting growth factors and cytokines (including IL-6, IL-1β, TGF-β1, TGF-β2, FGF-2, and PDGF), as well as enzymes such as matrix metalloproteinases (MMPs) in reshaping and remodeling the ECM and BM, by activating tumour-associated dendrites, tumour-associated macrophages, myeloid-derived suppressive cells, tumour-associated neutrophils, and pro-tumour Tregs disrupting the harmony in TME and leading to tumour progression by evading cytotoxicity of immune cells (McQuitty et al., 2020; Neophytou et al., 2021; Glabman et al., 2022).

By inhibiting the anti-tumour immune response, Tregs, which are essential for preserving immunological tolerance, can encourage the growth of tumours. Tregs lessen the immune system's capacity to identify and eliminate cancer cells in the tumour microenvironment by suppressing the activity of cytotoxic T cells, NK cells, and dendritic cells. Additionally, they release cytokines that suppress the immune system, such as TGF-β and IL-10, which reduce inflammation and encourage immune evasion (Li et al., 2020). As a result of their function in establishing an immunosuppressive niche, elevated Treg presence is frequently linked to a poor prognosis in cancer patients.

Polarised neutrophils are pathologically activated immature neutrophils (N2) with pro-tumour activity and are often referred to as a subset of myeloid-derived suppressor cells (MDSCs) (Veglia et al., 2021). These cells, particularly the granulocytic or polymorphonuclear MDSCs (PMN-MDSCs), are derived from immature neutrophils and can suppress the immune response, promoting tumour growth (Lu et al., 2024). They do this by inhibiting T cell function, producing reactive oxygen species (ROS), and fostering an immunosuppressive TME.

The cancer growth is significantly aided by pro-tumour plasma cells, which have polarised within the TME. These polarised plasma cells (P2) can take on a tumour-associated character that promotes immunosuppression and is frequently activated by signals from tumours. (Wei et al., 2019). P2 may release immunosuppressive cytokines like IL-10 and TGF-β, which prevent cytotoxic T-lymphocytes (CTLs) and other anti-tumour immune cells from launching an efficient anti-tumour immune response (Hu et al., 2024). By helping to create an immunosuppressive TME, this polarisation enables cancer

cells to avoid immune recognition and removal. Additionally, by promoting angiogenesis and aiding in the survival of cancer stem cells, polarised plasma cells may promote tumour growth and ultimately aid in tumour progression and metastasis.

Cancer cells in the equilibrium and escape phases survived immunosurveillance because genetic mutations and epigenetic modifications helped them to be unrecognised (Bhatia and Kumar, 2011; Arrieta et al., 2018).

Due to these triggering factors, epigenetic modifications occur causing phenotypic changes in gene regulation by altering chromosomal structure without changing the DNA sequence (Tomasi et al., 2006). At the time of cancer development, the epigenetic mechanism alters the phenotypic characteristics of cytokines such as interferons and interleukins present as anti-tumour proteins in immunosurveillance due to DNA methylation (Falvo et al., 2013; Das et al., 2021). In other words, the normal control of these cytokines is hijacked by cancer cells due to epigenetic modifiers in TME. In addition, these epigenetic modifications help the cancer cells to produce a new set of cytokines that could assist them in escaping immunosurveillance. Due to this, cancer cells begin controlling the immune cells to escape and metastasise (Noonepalle et al., 2021).

2.7 Interferon-stimulated gene (ISG)

Interferon-stimulated genes are first discovered in 1984 as transcriptional genes produced by interferon when researchers investigated the methods to trigger interferon production in fibroblast cells as a potential treatment for viral infections (Larner et al., 1984). The characterisation of numerous ISGs evolved gradually, and

their functional roles were discovered between 1985 and 1987 (Haas et al., 1987). ISGs are produced by the immune cells, including macrophages, dendritic cells, T-cells, neutrophils, plasma cells, natural killer cells, and other connective tissues, including vascular endothelial cells and fibroblasts, and regulated by specific interferons. ISGs are part of innate immunity and have a role in inducing the death of virally infected cells (Schoggins and Rice, 2011). The ISG transcription is induced by interferons, which are proteins in the family of cytokines produced by dendritic cells to activate host defence (Boukhaled et al., 2021). There are three known families of IFNs: Type-I (IFN-α and IFN-β), Type-II (IFN-γ), and Type-III (IFN-λ) (Kopitar-Jerala, 2017).

ISGs are a set of complex genes that defend the host against cancer-specific antigens (CSAs) and viral pathogens (Schneider et al., 2014). They are induced by IFN binding on the IFN activating receptor (IFNAR1) on newly mature naïve cell (T-cell) by phosphorylating Janus kinases (JAK) and tyrosine kinase (TYK), and signal transducer and activator of transcription proteins (STAT) gets activated and enter into the nucleus forming a complex with interferon regulatory factor-9 (IRF9) to form IFN-stimulated gene factor-3 (ISGF3). The ISGF3 binds with DNA, subsequently, leading to the transcription of hundreds of ISGs (Figure 2.2) (Chyuan et al., 2019). As a part of acquired immunity, interferon releases hundreds of ISGs that defend against pathogenic infections and cancer cells (Table 2.1).

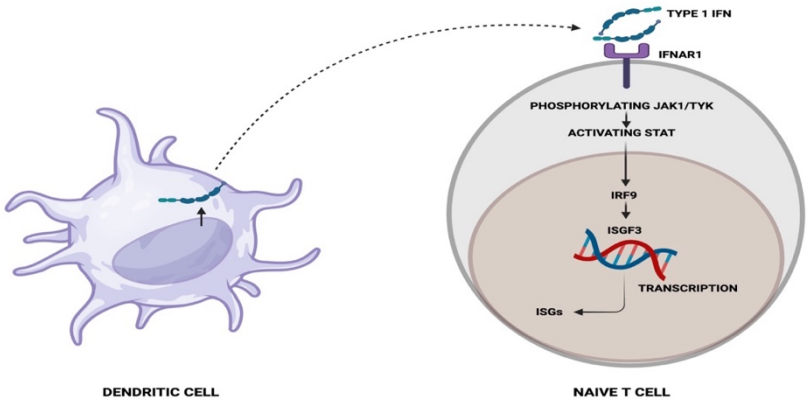

Figure 2.2: ISG production from type 1 IFN. The image was created with BioRender.com.

Table 2.1: Types of ISGs likely linked to immunosurveillance.

Type I ISGs	Origin	Function	Reference
MX1	Epithelial cells, macrophages	Inhibits viral replication by targeting viral nucleocapsid	(Jung et al., 2019)
OAS1	Macrophages, dendritic cells	Activates RNase L, leading to degradation of viral RNA	(Drappier and Michiels, 2015)
PKR (EIF2AK2)	Most nucleated cells	Phosphorylates eIF2α, inhibiting both viral and host protein synthesis	(Cesaro and Michiels, 2021)
IFITM1	Epithelial cells, lymphocytes	Restricts viral entry by altering membrane integrity	(Gómez-Herranz et al., 2023)
RSAD2	Dendritic cells, macrophages	Inhibits viral replication by disrupting lipid rafts and viral budding	(Lim et al., 2023)
IFI6	Endothelial cells, epithelial cells	Protects mitochondrial integrity, prevents apoptosis induced by viral infections	(Qi et al., 2015)
ISG15	Most nucleated cells	Acts as a ubiquitin-like protein, modifying viral and host proteins (ISGylation)	(Perng and Lenschow, 2018)
IFIT1	Dendritic cells, macrophages	Binds and sequesters viral RNA lacking 2′-O-methylation, inhibiting translation	(Hyde and Diamond, 2015)
GBP1	Macrophages, dendritic cells	Promotes clearance of intracellular pathogens via autophagy	(Fisch et al., 2020)
TRIM22	Monocytes, lymphocytes	Inhibits viral replication by targeting viral proteins for degradation	(Pietro et al., 2013; Zu et al., 2022)
BST2	Epithelial cells, T cells	Restrains virion release from infected cells	(Wan et al., 2019)

Table 2.1 Continued

Type 1 ISGs	Origin	Function	Reference
CXCL10	Macrophages, fibroblasts, endothelial cells	Recruits immune cells (e.g., T cells, NK cells) to sites of infection	(Elemam et al., 2022; Trifilo et al., 2004)
IRF7	Dendritic cells, macrophages	Amplifies type I IFN signaling by inducing more IFN-α/β production	(Sin et al., 2020)
IFI27	Epithelial cells, dendritic cells	Promotes apoptosis and contributes to the antiviral response, involved in regulating cell death in infected cells	(Ullah et al., 2021)
LY6E	Epithelial cells, macrophages	Restricts viral fusion and entry, especially for coronaviruses and flaviviruses	(Xu et al., 2014; Xuesen Zhao et al., 2020)
ISG20	Epithelial cells, macrophages	Degrades viral RNA, inhibiting replication	
Type II ISGs	**Origin**	**Function**	**References**
IRF1	Macrophages, dendritic cells, T cells	Enhances antigen presentation by inducing MHC class I and II molecules	(Lu et al., 2019)
CIITA	Dendritic cells, B cells	Master regulator of MHC class II expression, enhances antigen presentation	(Nakamura, 2014)
CXCL9	Monocytes, endothelial cells	Chemokine that recruits activated T cells to sites of infection or inflammation	(Duque and Descoteaux, 2014)
CXCL10	Macrophages, fibroblasts	Recruits immune cells (T cells, NK cells) to sites of infection or tumours	(Elemam et al., 2022b)
IDO1	Dendritic cells, macrophages	Catabolizes tryptophan, limiting pathogen growth and regulating immune responses	(Mellor et al., 2017)
HLA-DRA	Dendritic cells, B cells, macrophages	Component of MHC class II, crucial for antigen presentation to CD4+ T cells	(Reinisch et al., 2003)
Type III ISGs	**Origin**	**Function**	**References**
IL-28RA	Epithelial cells, dendritic cells	Component of the IFN-λ receptor, enhances IFN-λ signaling to amplify antiviral responses	(Syedbasha and Egli, 2017)
ZAP (PARP13)	Epithelial cells, macrophages	Binds to viral RNA and mediates degradation to inhibit viral replication	(Busa et al., 2024)
MUC1	Epithelial cells (lung, gut)	Protects mucosal surfaces by enhancing barrier function and immune signaling	(Iverson et al., 2022)

Table 2.1 Continued

MX1: Myxovirus Resistance Protein 1, OAS1: 2'-5'-Oligoadenylate Synthetase 1, PKR: Protein Kinase R, EIF2AK2: Eukaryotic Initiation Factor 2-Alpha Kinase 2, IFITM1: Interferon-Induced Transmembrane Protein 1, RSAD2: Radical S-Adenosyl Methionine Domain Containing 2, IFI6: Interferon Alpha-Inducible Protein 6, ISG15: Interferon-Stimulated Gene 15, IFIT1: Interferon-Induced Protein With Tetratricopeptide Repeats 1, GBP1: Guanylate-Binding Protein 1, TRIM22: Tripartite Motif Containing 22, BST2: Bone Marrow Stromal Antigen 2, CXCL10: C-X-C Motif Chemokine Ligand 10, IRF7: Interferon Regulatory Factor 7, IFI27: Interferon Alpha-Inducible Protein 27, LY6E: Lymphocyte Antigen 6 Complex, Locus E, ISG20: Interferon-Stimulated Gene 20, IRF1: Interferon Regulatory Factor 1, CIITA: Class II Major Histocompatibility Complex Transactivator, CXCL9: C-X-C Motif Chemokine Ligand 9, IDO1: Indoleamine 2,3-Dioxygenase 1, HLA-DRA: Major Histocompatibility Complex, Class II, DR Alpha, IL-28RA: Interleukin-28 Receptor Alpha, ZAP: Zinc Finger Antiviral Protein, MUC1: Mucin 1.

Recent reports suggest that some ISGs function/work against defence mechanisms and exhibit anti-defensive characteristics that promote tumour progression. Studies on the TME of breast and colorectal cancer, which comprises cancerous and non-cancerous cells, including fibroblasts, immune cells, blood vessel-forming cells, and proteins, have shown that the upregulation of ISGs is associated with the inhibition of apoptosis and depletion of CD8 T-cells, which are crucial for eliminating cancer cells (Butt et al., 2024). The reports suggest that the ISGs' behaviour contradicts their anti-defensive role mentioned earlier, nevertheless, whether they are of different variants, i.e., defensive or protumorigenic, is unclear. In the case of the latter, the change in the role of ISGs from defensive to anti-defensive is likely the result of transmutation following immunoediting.

2.7.1 Apoptosis by ISGs

Apoptosis is a programmed cell death regulated by the immune system to maintain normal tissue homeostasis and body defence mechanisms in the event of cellular injury or pathogenic exposure. Cell self-destruction via apoptosis involves the caspase cascade pathway and leads to proteolytic degradation/condensation of the nucleus and cytoplasm, which results in cell death (Fuchs and Steller, 2015).

In normal healthy cells, the apoptosis process is characterised by the collapse of cells featuring DNA fragmentations, chromatin condensation, and cell shrinkage, membrane blebbing (Shi, 2002). These apoptotic cells are engulfed by macrophages eliminating the cells from the system (Schrijvers et al., 2005; Elmore, 2007). The process of apoptosis depends on the activation of caspase proteases to induce extensive cleavage of hundreds of cellular substrates such as lamin, cytoskeletal proteins, and caspases themselves and causes rapid cell death (Castillo Ferrer et al., 2021). The apoptosis process defends against cancer with the help of immunosurveillance. ISG aids the process in both virally infected and cancer cells via three caspase-mediated pathways: the extrinsic, intrinsic, and endoplasmic reticulum stress pathways (Butt et al., 2024).

The extrinsic pathway is initiated when pathogens stimulate IFN-1 production, triggering the transcription of ISGs such as ISG15 and IFI27. These ISGs enhance the recruitment and activation of FADD (Figure 2.3). FADD is an adaptor molecule that transmits apoptotic signals from death receptors on the cell surface, such as the TRAIL receptors to initiate the caspase cascade (Ganesan et al., 2019; Xie et al., 2022).