MOLECULAR AND CELLULAR ANALYSIS OF HEPG2 HEPATOCELLULAR CARCINOMA UPON DEUBIQUITIN ENZYMES OTUB1 AND OTUB2 KNOCKDOWN

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by

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LIST OF SYMBOLS

$\Delta\Delta C_t$	Comparative Threshold Cycle
$2^{-\Delta\Delta Ct}$	Fold change
°C	Degree Celsius
µg	Microgram
µL	Microliter
µM	Micromolar
bp	Base pair
CO_2	Carbon dioxide
C_T	Threshold Cycle
CT	Computed tomography
kb	Kilo-base pair
mL	Milliliter
ng	Nanogram
nm	Nanometer

LIST OF ABBREVIATIONS

AFP	Alpha-fetoprotein
BIRC3	Baculoviral IAP repeat-containing protein3
cDNA	Complementary DNA
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CXCL2	Chemokine (C-X-C motif) ligand 2
CXCL3	Chemokine (C-X-C motif) ligand 3
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
FBS	Foetal bovine serum
FOS	Protein c-Fos
GC-content	Guanine-cytosine content
GFP	Green fluorescent protein
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HepG2	Human liver cancer cell line
IL6R	Interleukin-6 receptor
IL-17	Interleukin 17
GO	Gene Ontology
JAMMs	JAB1/MPN/MOV34 metalloproteases
JUN	Transcription factor Jun
KEGG	Kyoto Encyclopedia of Genes and Genomes
MAPK	Mitogen-activated protein kinase
MINDY	Motif interacting with ubiquitin-containing novel DUB family
MJDs	Machado-Joseph disease proteases
MRI	Magnetic resonance imaging
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OTUB1	Otubain-1
OTUB2	Otubain-2
OTUs	Ovarian tumor proteases
padj	Adjusted *P*-value
PAM	Protospacer Adjacent Motif

PBS	Phosphate Buffered Saline
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
Poly-A	Polyadenylation
qRT-PCR	Quantitative Real-time Polymerase Chain Reaction
RELB	Transcription factor RelB
RT	Real-time
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
Rpm	Revolutions per minute
sgRNA	Single guide RNA
TAE	Tris-acetate-EDTA
TACE	Transcatheter arterial chemoembolization
TARE	Transarterial Radioembolization
TNF	Tumour necrosis factor
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3
TNFRSF9	Tumor necrosis factor receptor superfamily member 9
UCHs	Ubiquitin carboxy-terminal hydrolases
USPs	Ubiquitin-specific proteases
$HepG2^{WT}$	Wild-type human liver cancer line
$HepG2^{OTUB1\text{-}KD}$	Liver cancer cell lines with OTUB1 knockdown
$HepG2^{OTUB2\text{-}KD}$	Liver cancer cell lines with OTUB2 knockdown

ANALISIS MOLEKULAR DAN SELULAR BAGI HEPG2 HEPATOSELULAR KARSINOMA DENGAN PENYAHFUNGSIAN ENZIM DEUBIKUITINASI OTUB1 AND OTUB2

ABSTRAK

Hepatoma atau karsinoma hati (HCC) ialah salah satu kanser yang paling lazim dan membawa maut di seluruh dunia. Enzim deubiquitin, Otubain-1 (OTUB1) dan Otubain-2 (OTUB2) telah dikaitkan dengan pertumbuhan kanser, menjadikan enzim ini sasaran terapeutik yang berpotensi. Kajian ini menyelidik kesan penyahfungsian ekspresi OTUB1 dan OTUB2 terhadap daya hidup sel, migrasi, dan laluan ekspresi gen dalam sel HepG2, dengan tujuan untuk menerokai peranan mereka dalam kanser hati. Turutan RNA berpandu yang menyasarkan OTUB1 dan OTUB2 direka dan diklonkan ke dalam plasmid pSpgRNA. Konstruk-konstruk ini dipindahkan ke sel $HepG2^{KRAB}$ menggunakan Lipofectamine^{TM} 3000. PCR kuantitatif (qPCR) digunakan untuk mengesahkan keberkesanan penurunan ekspresi. Ujian penyembuhan luka dilakukan untuk menilai tingkah laku migrasi sel manakala daya hidup sel dinilai menggunakan ujian MTS setelah penurunan ekspresi. RNA-seq dilakukan untuk meneroka perubahan dalam ekspresi gen, diikuti oleh analisis pengayaan fungsi menggunakan Analisis Gen Terekspresi Berbeza (DEGs), Gene Ontology (GO), dan analisis laluan KEGG. Penyahfungsian ekspresi OTUB1 dan OTUB2 disahkan melalui qPCR, yang menunjukkan pengurangan ketara dalam tahap mRNA mereka, dengan kedua-dua ekspresi gen menurun sebanyak 2 kali ganda (masing-masing $p<0.0001$ dan $p<0.05$). Ujian penyembuhan luka menunjukkan bahawa penyahfungsian ekspresi kedua-dua gen ini menyebabkan kadar penutupan luka yang ketara perlahan berbanding dengan kawalan pada semua tempoh masa (24 jam, 48 jam

dan 72 jam). Ujian MTS menunjukkan penurunan daya hidup sel selepas penyahfungsian gen OTUB1 dan OTUB2 dalam sel HepG2. Penyahfungsian OTUB1 menyebabkan penurunan daya hidup sel yang ketara pada 72 jam (daya hidup 40%, $p < 0.01$), manakala penyahfungsian OTUB2 menghasilkan daya hidup sebanyak 50% ($p < 0.0001$). Sel kawalan ($HepG2^{WT}$ dan $HepG2^{KRAB}$) mengekalkan daya hidup yang stabil, mengesahkan bahawa pengurangan pertumbuhan sel yang diperhatikan adalah disebabkan oleh penurunan ekspresi OTUB1/OTUB2. Analisis RNA-seq mendedahkan perubahan laluan yang ketara. Dalam sel dengan penyahfungsian ekspresi OTUB1, analisis KEGG menunjukkan peningkatan laluan isyarat IL-17, laluan isyarat NF-kB, laluan isyarat TNF dan interaksi Cytokine. Penyahfungsian ekspresi OTUB2 juga mempengaruhi interaksi Cytokine dan laluan keradangan. Analisis GO menonjolkan proses yang terlibat dalam regulasi imun, migrasi sel dan apoptosis. Penyahfungsian ekspresi OTUB1 dan OTUB2 menjejaskan fungsi sel utama dalam sel HepG2 dengan signifikan, terutamanya mengurangkan daya hidup sel dan membataskan migrasi. Kajian ini menunjukkan bahawa OTUB1 dan OTUB2 penting untuk mengekalkan daya hidup dan migrasi sel HepG2 melalui penglibatan mereka dalam laluan berkaitan keradangan dan kanser. Menyasarkan OTUB1 dan OTUB2 berpotensi sebagai strategi terapeutik baru untuk melawan HCC dengan mengganggu mekanisme yang mengaktifkan tumor ini.

MOLECULAR AND CELLULAR ANALYSIS OF HEPG2 HEPATOCELLULAR CARCINOMA UPON DEUBIQUITIN ENZYMES OTUB1 AND OTUB2 KNOCKDOWN

ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most prevalent and deadly cancers worldwide. The deubiquitinating enzymes Otubain-1 (OTUB1) and Otubain-2 (OTUB2) have been linked to cancer progression, making them potential therapeutic targets. This study investigates the effects of OTUB1 and OTUB2 knockdown on cell viability, migration, and gene expression pathways in HepG2 cells, aiming to explore their roles in liver cancer. Guide RNA sequences targeting OTUB1 and OTUB2 were designed and cloned into the pSpgRNA plasmid. These constructs were transfected into HepG2^{KRAB} cells using Lipofectamine^{TM} 3000. Quantitative PCR (qPCR) was employed to confirm the knockdown efficiency. A wound healing assay was performed to assess migratory behavior, while cell viability was evaluated using an MTS assay upon knockdown. RNA sequencing (RNA-seq) was conducted to explore changes in gene expression, followed by functional enrichment analysis using Differentially Expressed Gene Analysis (DEGs), Gene Ontology (GO), and KEGG pathway analysis. The knockdown of OTUB1 and OTUB2 was confirmed by qPCR, showing significant reductions in their mRNA expression, with both gene expression reduced by 2-fold ($p<0.0001$ and $p<0.05$, respectively). The wound healing assay revealed that both knockdowns led to significantly delayed wound closure compared to controls at all timepoints (24h, 48h, and 72h). MTS assay revealed reductions in cell viability following the knockdown of OTUB1 and OTUB2 in HepG2 cells. OTUB1 suppression led to a pronounced decline at 72h (40% viability, $p < 0.01$), while

OTUB2 knockdown resulted in 50% viability ($p < 0.0001$). In contrast, control cells ($HepG2^{WT}$ and $HepG2^{KRAB}$) maintained stable viability, confirming that the observed growth inhibition was specific to OTUB1/OTUB2 downregulation. RNA-seq analysis revealed significant pathway alterations. In OTUB1 knockdown cells, KEGG enrichment showed upregulation of the IL-17 signaling pathway, NF-kB signaling pathway, TNF signaling pathway, and cytokine-cytokine receptor interaction. OTUB2 knockdown similarly affected cytokine-cytokine receptor interaction and inflammatory pathways. GO analysis highlighted processes involved in immune regulation, cell migration, and apoptosis. The knockdown of OTUB1 and OTUB2 significantly affects key cellular functions in HepG2 cells, notably reducing cell viability and impairing migration. This study demonstrates that OTUB1 and OTUB2 are essential for maintaining HepG2 cell viability and migration, likely through their involvement in inflammatory and cancer-related pathways. Targeting OTUB1 and OTUB2 may provide a novel therapeutic strategy for combating HCC by disrupting these tumor-promoting mechanisms.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Hepatocellular carcinoma (HCC) is the most frequent liver cancer in humans and one of the main causes of cancer death globally, according to the Global Cancer Observatory. According to GLOBOCAN 2020, primary liver cancer is the sixth most prevalent cancer to be diagnosed, accounting for over 906,000 new cases and 830,000 cancer deaths worldwide. Rates of incidence and mortality in most provinces were documented to be higher among men compared to among women, approximately 2 to 3 times higher (Sung et al., 2021). It has been established that the incidence of liver diseases is typically higher in developing countries (Starley et al., 2010). In the United States, liver cancer is the fifth most prevalent and the only one of the top five deadliest cancers to experience an annual percentage surge in occurrence (Siegel et al., 2019).

HCC could be detected by a few early biomarkers such as serum α-fetoprotein (AFP), Glypican-3 (GPC3), and tumor-associated antigens (TAAs) (Nakatsura et al., 2003; Wun & Dickinson, 2003; Xing et al., 2021). Unfortunately, most people were unable to be diagnosed and treated at an early stage (Couri & Pillai, 2019). Usually, HCC patients in early and intermediate stages were suggested to undergo hepatic resection and liver transplantation since they were the most effective treatments (Chen et al., 2020). However, surgical therapy must consider factors such as the patient's physical state and tumour stage, and it could be inappropriate for some patients with certain issues. Systemic therapy and some adjuvant therapies of clinical surgery, such as transarterial chemoembolization (TACE), transarterial radioembolization (TARE), external beam radiation therapy, and oncolytic virus are currently being researched for the treatment of HCC, but the results of these therapies were not optimal (Chen et al.,

2020; Lawal et al., 2021). Thus, systemic drug therapy has emerged as a crucial component of today's liver cancer treatments (Foerster & Galle, 2021). Numerous targeted medications, including Lenvatinib, Regorafenib (Stivarga), and Nexavar (sorafenib), are now approved for the clinical treatment of HCC patients (Zhai & Sun, 2013; Tai et al., 2014; El-Khoueiry et al., 2017). These medications undeniably offered alternative options for HCC patients. Unfortunately, despite significant advances in HCC treatment that have been established and approved, these drugs have only extended survival by a few months. Additionally, patients with advanced liver cancer were also at risk for drug resistance and tumor invasion as well as metastasis (Zhao et al., 2020).

Ubiquitination is a post-translational modification of a protein that modulates a sequence of biological activities and physiological responses by targeting the degradation of substrate protein, regulating its activity and role, changing its subcellular location, as well as influencing DNA repair. Deubiquitinases (DUBs) are a superfamily of deubiquitinating enzymes that catalyze deubiquitination, a process that reverses ubiquitination. DUBs are extensively distributed in cells and could hydrolyze the link between substrates and ubiquitin chains, which negatively regulates protein breakdown, subsequently affecting cell development, differentiation, migration, and other processes (Mevissen & Komander, 2017). Deubiquitination was reported to facilitate the growth of cancer, according to past studies (Mcclurg & Robson, 2015). Thus, targeting DUBs is a viable approach for the development of anticancer drugs because it has been demonstrated that DUBs are intimately associated with the occurrence and progression of malignancies (Zhou et al., 2018).

Recent research has shown that OTUB1 was highly expressed in the tumour tissues of patients with breast, lung, prostate, colon, and ovarian cancer. According to these findings, OTUB1 overexpression promoted tumour metastasis and was linked to a poor prognosis and quality of life. Meanwhile, OTUB2 was discovered to be elevated in liver cancer, and patients with high OTUB2 expression had a lower overall survival, according to a study conducted by Gu et al. (2020). OTUB2 knockdown significantly reduced liver cancer cell proliferation at the cellular level by reducing nuclear factor-kappa B (NF-κB) signalling (Gu et al., 2020).

1.2 Problem Statement

The global burden of cancer continues to rise, with hepatocellular carcinoma (HCC) remaining one of the leading causes of cancer-related mortality. Despite advancements in therapeutic options, treatment outcomes for HCC are still limited by poor prognosis, high recurrence rates, and growing resistance to conventional therapies. For instance, while transarterial chemoembolization (TACE) has shown modest improvements in survival for intermediate-stage HCC patients, and sorafenib remains the standard of care for advanced stages, clinical benefit is often short-lived and limited to a subset of patients, with resistance typically developing within six months (El-Serag et al., 2008).

Furthermore, long-term use of current chemotherapeutic agents is associated with significant adverse effects, including hypertension, haemorrhage, and immunosuppression (Zhu et al., 2017), underscoring the urgent need for novel, targeted approaches in HCC management. Recent studies have highlighted the role of deubiquitinating enzymes (DUBs) in regulating tumor progression and immune evasion pathways. In particular, OTUB1 has been identified as a key player in

stabilizing immune checkpoint proteins such as PD-L1, as well as modulating tumor suppressor proteins, including p53. By contrast, OTUB2, although structurally related, exhibits distinct regulatory roles, influencing FOXM1 stabilization and NF-κB signaling. Despite growing evidence implicating OTUB1 and OTUB2 in tumorigenesis, their specific functions in HCC progression remain incompletely understood.

To bridge this gap, this study investigates the effects of OTUB1 and OTUB2 knockdown in HepG2 liver cancer cells using CRISPR/Cas9 genome editing technology. Subsequent analyses, including wound healing and MTS assays, were employed to assess changes in cell migration and proliferation. In parallel, RNA sequencing and bioinformatics tools were used to identify key genes and cancer-related pathways disrupted by the loss of OTUB1 and OTUB2. By dissecting the distinct roles of these DUBs, this study aims to elucidate their contributions to HCC pathogenesis, offering new insights into potential molecular targets for more effective and personalized cancer therapies.

1.3 Research Objective

The ultimate aim of this study is to investigate the molecular and cellular effects of knocking down the deubiquitin enzymes OTUB1 and OTUB2 in hepatocellular carcinoma (HepG2) cell lines, with a focus on understanding their roles in gene expression, cell proliferation, migration, and survival. Additionally, this study aims to utilize Next-Generation Sequencing (NGS) – RNA sequencing to predict cancer-associated pathways affected by the knockdown, exploring the potential implications for therapeutic targeting in liver cancer.

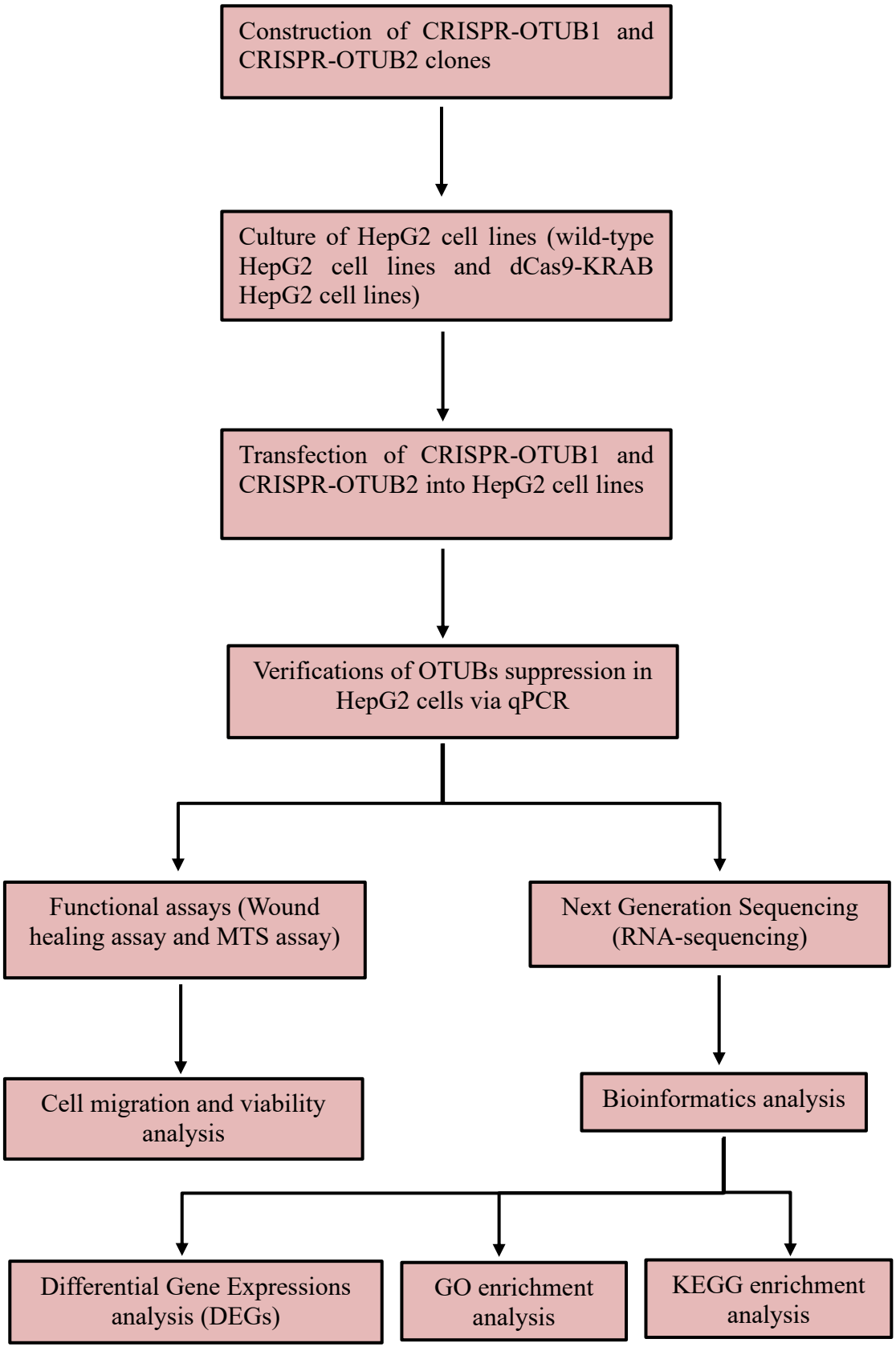
1.3.1 Specific objectives

1. To knockdown OTUB1 and OTUB2 expression using CRISPR/dCas9 technology (CRISPR-OTUB1 and CRISPR-OTUB2) in HepG2 cells.
2. To analyze the effects of OTUB1 and OTUB2 knockdown on cell proliferation and cell metastasis.
3. To elucidate the gene expression and cancer-associated pathways affected by OTUB1 and OTUB2 knockdown using RNA-seq.

1.4 Hypothesis

The expression of OTUB1 and OTUB2 in HepG2 cells will be significantly reduced by a CRISPR/dCas9-mediated gene knockdown strategy in HepG2 cell lines. A significant differential expression of other genes upon OTUB1 and OTUB2 knockdown from RNA sequencing is expected to be discovered. Using bioinformatic analysis, it is postulated that OTUB1- and OTUB2-related cancer-associated pathways can be revealed.

1.5 Flowchart of study



CHAPTER 2

LITERATURE REVIEW

2.1 Liver Cancer

Cancer is ranked as a leading cause of death and a significant barrier to increasing life expectancy in every country worldwide (Bray et al., 2021). According to reports from the World Health Organization (WHO) in 2019 (World Health Organization, 2020), cancer was the first or second leading cause of death before the age of 70 in 112 out of 183 countries, and it ranked third or fourth in another 23 countries (Bray et al., 2021). The growing prominence of cancer as a leading cause of death was partly due to the notable declines in stroke and coronary heart disease mortality rates, compared to cancer, in many countries. Overall, the global burden of cancer incidence and mortality is rapidly increasing (Sung et al., 2021).

Primary liver cancer was the sixth most frequently diagnosed cancer globally in 2020, ranking as the third leading cause of cancer-related deaths. Approximately 906,000 new cases and 830,000 deaths were reported in that year. The rates of both incidence and mortality were 2 to 3 times higher in men compared to women in most regions **(Figure 2.1)**. For men, liver cancer was the fifth most common cancer and the second leading cause of cancer deaths worldwide. Incidence rates in men were 2.4 times higher in more developed regions, but the highest rates overall were found in less developed countries. It was the most common cancer in 11 diverse countries, including Mongolia (with rates far higher than other nations), Southeast Asia (e.g., Thailand, Cambodia, Vietnam), and parts of Northern and Western Africa (e.g., Egypt, Niger). Liver cancer is the top cause of cancer deaths in countries like Mongolia, Thailand, Cambodia, Egypt, and Guatemala for both men and women and in an additional 18 countries for men only (Sung et al., 2021). Liver cancer mainly included hepatocellular

carcinoma (HCC), which accounted for 75%-85% of cases, and intrahepatic cholangiocarcinoma (10%-15%), with some rarer types. The primary risk factors for HCC included chronic hepatitis B (HBV) or hepatitis C (HCV) infections, aflatoxin exposure, heavy alcohol consumption, excess body weight, type 2 diabetes, and smoking (Thomas et al., 2018). These risk factors varied by region, and HBV infection and aflatoxin exposure were key factors in HCC in areas such as China, South Korea, and sub-Saharan Africa. In contrast, in countries like Japan, Italy, and Egypt, HCV infection was the primary cause. In Mongolia, the high liver cancer burden was due to HBV, HCV, co-infections with hepatitis delta viruses, and alcohol use (Chimed et al., 2017).

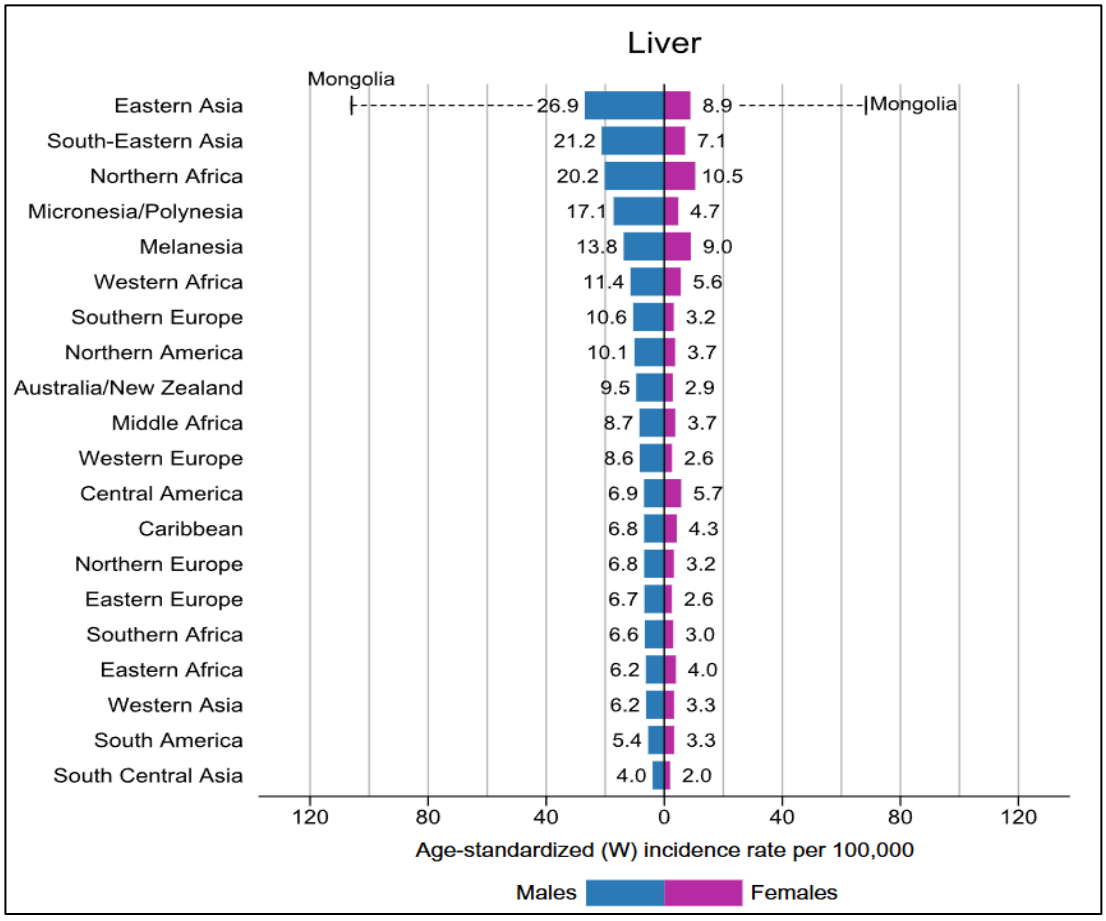


Figure 2.1 Region-Specific Incidence Age-Standardized Rates by Sex for Liver Cancer in 2020. Rates are shown in descending order of the world (W) age-standardized rate among men, and the highest national rates among men and women are superimposed. Source: GLOBOCAN, 2020.

2.1.1 Diagnosis for Liver Cancer

2.1.1(a) Imaging

Patients who showed abnormal results during surveillance, such as a liver nodule detected through abdominal ultrasound or elevated serum α-fetoprotein levels (greater than 20 ng/mL), were considered high-risk and required prompt diagnostic follow-up. Typically, a 3-month period was adequate for re-evaluation of most lesions. For lesions that were 1 cm or larger, a quadruple-phase CT or dynamic contrast-enhanced MRI was recommended (Marrero et al., 2018; Simon et al., 2020). On CT or MRI, HCC lesions typically appeared brighter than the surrounding liver tissue during the arterial phase and less bright during the venous and delayed phases due to the tumor's differing blood supply compared to the rest of the liver (Shinmura et al., 2005). This phenomenon, known as "arterial enhancement and delayed washout," was highly sensitive (89%) and specific (96%) for HCC, serving as a radiological indicator that could confirm the diagnosis without needing histological verification (van der Pol et al., 2019). However, MRI using hepatobiliary contrast agents had shown lower specificity than extracellular agents, and its utility in non-invasive HCC diagnosis remained debated (Paisant et al., 2020). Increasingly, biopsy was recommended to molecularly characterize HCC (Marrero et al., 2018). Notably, imaging criteria for diagnosing HCC were only applicable to at-risk individuals, including those with cirrhosis or chronic HBV infection. Advanced imaging techniques, such as magnetic resonance imaging (MRI) and computed tomography (CT), are widely used for the early detection and diagnosis of hepatocellular carcinoma (HCC). Multisequence MRI and three-dimensional multifrequency magnetic resonance elastography have shown potential in enhancing preoperative assessments and predicting PD-1/PD-L1 expression in HCC

(Kucukkaya et al., 2023; Liu et al., 2023). Additionally, contrast-enhanced CT has been utilized to develop preoperative prediction models for macrotrabecular-massive HCC (He et al., 2023). Furthermore, deep machine learning models based on baseline MRI have demonstrated accuracy in predicting tumor recurrence in patients with early-stage HCC (Kucukkaya et al., 2023).

Recent research has also investigated the use of novel biomarkers, including microRNAs and long non-coding RNAs, for more accurate and earlier detection of HCC (Gabbia & De Martin, 2023). Advances in targeted medicine and bioengineering have played a significant role in improving personalized treatments for hepatocellular carcinoma. Through the use of biomarkers, genetic testing, nanotechnology, and drug delivery systems, clinicians could customize treatment plans for individual patients, leading to better outcomes. However, there are still challenges in the field, such as the need for more precise diagnostic tools and the development of new targeted therapies for HCC (Sun et al., 2023).

2.1.1(b) Histopathology

Although imaging revealed typical features for most HCCs, around 10% of tumors and up to 30% of tumors measuring 1-2 cm presented atypically, lacking the hallmark imaging characteristics of HCC. The International Consensus Group for Hepatocellular Neoplasia had identified major histological features of HCC, which included stromal invasion, increased cell density, intratumoral portal tracts, unpaired arteries, pseudo-glandular patterns, and diffused fatty changes (Kojiro et al., 2009). If HCC was suspected but imaging was inconclusive, a biopsy or repeat contrast-enhanced study should be conducted (Marrero et al., 2018). The sensitivity of the biopsy was about 70%, which decreased for smaller tumors.

Histopathological analysis of liver biopsy specimens remained the gold standard for diagnosing HCC. However, biopsies come with risks, including complications, and might not be suitable for all patients. They were practical in estimating fibrosis levels and diagnosing conditions like inflammation, steatosis, and necrosis. However, the procedure carries risks, including severe pain and complications, with mortality occurring in about 0.1% of cases and prolonged hospital stays for observation in around 5% of patients (Addissouky et al., 2019). As a result, there was growing interest in non-invasive methods, such as advanced imaging techniques and liquid biopsy, for HCC diagnosis (Gabbia & De Martin, 2023; Liu et al., 2023).

Despite these challenges, histopathological analysis remained crucial for characterizing HCC and identifying important prognostic markers, such as tumor grade and vascular invasion (Jose et al., 2022). One case highlighted an uncommon type of HCC, cirrhotomimetic hepatocellular carcinoma, which can mimic liver cirrhosis. This case underscores the need for clinicians to consider such tumors in differential diagnoses, especially in patients with chronic liver disease. It emphasized the importance of thorough imaging and pathological evaluation for accurate diagnosis and treatment. The findings from this case contribute to a better understanding and management of cirrhotomimetic HCC, potentially improving patient outcomes through early detection and proper treatment (Aby et al., 2023; Addissouky et al., 2024).

2.1.2 Current Treatment for Liver Cancer

2.1.2(a) Surgical Interventions

Surgical treatments, which encompassed both liver resection and transplantation, had traditionally served as the primary curative therapies for hepatocellular carcinoma (HCC), achieving the highest success rates with a 5-year survival rate of approximately 70-80% (Galle et al., 2018; Marrero et al., 2018). Determination between resection and transplantation depended on various factors, including the patient's liver function, the severity of portal hypertension, overall performance status, and tumor specifics such as size, number, and vascular involvement. Additionally, local regulations regarding the availability and distribution of organs must be factored into the decision-making process. Western guidelines emphasize the importance of selecting ideal candidates to achieve optimal outcomes for surgical resection while directing non-ideal candidates toward alternative treatments (Galle et al., 2018; Marrero et al., 2018). However, recent studies challenged this approach, suggesting that resection in less-than-ideal candidates might yield comparable or superior results to locoregional therapies (Llovet et al., 2021).

Hepatic resection was the preferred treatment for hepatocellular carcinoma (HCC) in patients without cirrhosis and a low risk of postoperative liver failure (Shrager et al., 2012; Galle et al., 2018; Marrero et al., 2018). However, resection in non-cirrhotic patients with non-alcoholic fatty liver disease (NAFLD) could lead to significant complications (Pawlik & Soubrane, 2015; Piscaglia et al., 2016). For cirrhotic patients, guidelines recommended resection only for those with a single tumor, good liver function, no significant portal hypertension, and a favorable performance status, resulting in a 5-year survival of about 70% and low perioperativertality (Zhou et al.,

2001). Despite guidelines, many resections were performed on patients who do not meet these criteria, with some studies indicating that having one risk factor did not greatly affect survival (Roayaie et al., 2015). However, patients with both portal hypertension and elevated bilirubin had worse outcomes (Ishizawa et al., 2008; Berardi et al., 2020). Additionally, the recurrence of HCC after resection was a major concern, with rates reaching 70% in 5 years (Roayaie et al., 2015). Recurrences were classified as early or late, with surgical techniques showing variable success in reducing recurrence rates (Shi et al., 2007; Hidaka et al., 2020). Current approaches for managing HCC recurrence, including various therapies, have outcomes comparable to primary HCC (Tabrizian et al., 2015). Lastly, pre-emptive liver transplantation was being considered for high-risk patients (Ferrer-F Abrega et al., 2016).

Patients with cirrhosis and limited tumor burden, defined by the Milan Criteria (a single tumor ≤5 cm or 2-3 tumors ≤3 cm without vascular invasion), were candidates for liver transplantation (Incenzo Azzaferro et al., 1996). This procedure showed excellent outcomes, with 5-year and 10-year survival rates of 70% and 50%, respectively, and a recurrence rate of 10-15% at 5 years (Tabrizian et al., 2022). Liver transplantation generally offers better long-term results than resection, which has a 70% recurrence rate and a 10-year survival of 7-15% (Franssen et al., 2014). However, organ shortages led to long waiting times, causing patients to drop out due to tumor progression, making resection outcomes comparable if dropout rates exceeded 20% (Cucchetti et al., 2020). Neoadjuvant therapies like TACE or ablation aimed to reduce tumor burden while patients waited for transplantation (Yao et al., 2015; Kulik et al., 2018). A multicenter analysis found a 10-year survival of 52% for patients down-staged to Milan criteria (Tabrizian et al., 2022). Responses to these therapies might assist in candidate selection for transplantation and predicted post-transplant outcomes

(Cucchetti et al., 2020), but further validation was needed before they could be integrated into guidelines. To address the organ shortage, the use of marginal donors (e.g., older donors or those with diabetes) and living donors has been proposed (Kulik et al., 2012). While living donors yielded similar survival rates as deceased donors, some studies indicated higher recurrence rates due to the rapid transplantation process, preventing the identification of aggressive HCC types (Miltiadous et al., 2015). The application of marginal organs was associated with an increased risk of recurrence (Yao et al., 2015; Kulik et al., 2018). The 10-year recurrence rate after transplantation was 10-15% for tumors within Milan criteria and around 20% for those down-staged to these criteria (Tabrizian et al., 2022). Currently, no adjuvant treatments have been shown to prevent recurrence post-transplant, with one RCT revealing no significant differences in overall survival or recurrence-free survival between sirolimus-based and standard immunosuppression (Geissler et al., 2016). In summary, adherence to Milan criteria leads to low recurrence rates, but further improvements through molecular therapies have yet to be established (Llovet et al., 2021).

2.1.2(b) Chemotherapy for Liver Cancer

Systemic therapy, which included tyrosine kinase inhibitors (TKIs) and immune checkpoint inhibitors (ICIs), was commonly used for advanced HCC or as adjuvant therapy following surgery or ablation. TKIs like sorafenib and lenvatinib were recommended as first-line treatments for advanced HCC. For patients whose disease has progressed after TKI treatment, ICIs such as nivolumab and pembrolizumab were approved options. Recently, new combination therapies, such as TKIs with ICIs or TKIs combined with anti-angiogenic agents, have shown the potential to improve patient outcomes (Pan et al., 2023).

Sorafenib, a widely recommended oral multi-kinase inhibitor, served as the primary treatment for advanced stages of liver cancer (HCC) globally (Kane et al., 2009; Jelic & Sotiropoulos, 2010). It hindered tumor angiogenesis, cell division, and proliferation by targeting various proteins, including Raf-1, PDGFR-β, cKIT, FLT-3, VEGF receptors, and RET (Cainap et al., 2015; Song et al., 2019). The FDA approved sorafenib in 2007, though it only marginally increased patient survival by 3 to 5 months (Llovet et al., 2008; Cheng et al., 2009; Bruix et al., 2012), accompanied by adverse effects such as increased serum lipase, amylase, hypertension, and other side effects. Combination treatments, such as transarterial chemoembolization plus sorafenib, showed better results than sorafenib alone (Zhu et al., 2017). Ongoing clinical trials explored combining sorafenib with other drugs for improved outcomes and fewer side effects (Choi et al., 2013).

To address sorafenib's limited efficacy, ongoing trials explored combinations with other drugs like vorinostat and doxorubicin, aiming for synergistic effects and reduced resistance development. Preliminary findings suggested improved progression-free and overall survival (Zhang et al., 2008; Richly et al., 2009). Evaluation of tivozanib, a drug that suppresses VEGF receptors, has been limited in liver cancer, with some positive effects similar to sorafenib. A current trial examined tivozanib's impact on advanced HCC, while past trials were inconclusive (Jamil et al., 2015), another citation. The drug 5-FU, known for inhibiting cancer growth, faced challenges of chemoresistance in liver cancers (Luo et al., 2017). Despite inhibiting cell progression and up-regulating p53, resistance mechanisms involving long non-coding RNAs and liver cancer stem cells were prominent (Huo et al., 2017). Combining 5-FU with cisplatin, especially through hepatic arterial infusion, showed increased survival rates

for HCC patients (Nouso et al., 2013). Ongoing clinical trials explored the efficacy of cisplatin and 5-FU in HCC patients with sorafenib resistance (Anwanwan et al., 2020).

2.1.2(c) Immunotherapy for Liver Cancer

Immunotherapy has emerged as a promising approach for treating cancers by modifying patients' immune systems. The techniques included recognizing specific antigens on cancer cells, blocking immune checkpoints to enhance immune activity, using cancer vaccines, and employing non-specific cancer immunotherapies. This field, acknowledged as the "Advanced Therapy of the Year" in 2013, allowed for combination with existing liver cancer drugs, showing potential synergistic effects in clinical trials involving monoclonal antibodies such as ramucirumab and bevacizumab (NCT00410956, NCT01180959, NCT01246986) (Couzin-Frankel, 2013).

Immune checkpoint inhibitors, focusing on proteins like PD-1 and PD-L1, target mechanisms suppressing immune responses against cancer cells. High PD-L1 expression was associated with poor prognosis in HCC patients, leading to increased recurrence and vascular invasion. Combining anti-PD-1, anti-CXCR4, and sorafenib demonstrated improved outcomes, emphasizing the role of immunosuppression (Ho et al., 2019). Targeting PD-L1/PD-1 blockade might mitigate hepatitis B and C infections, reducing HCC risk factors. While autoimmune diseases were possible, checkpoint inhibitors like nivolumab, FDA-approved for various cancers, show promise. Clinical trials combined nivolumab with ipilimumab, an FDA-approved drug for melanoma and colorectal cancer (Mataki et al., 2007; Hodi et al., 2018; Iñarrairaegui et al., 2018; Overman et al., 2018). Monoclonal antibodies (mAbs) offered a targeted approach against cancer antigens. Antibodies against the immune checkpoint regulator PD-1, exemplified by nivolumab and ipilimumab, stimulated T-cell activity, boosting the

immune system against cancer cells. These mAbs were under clinical evaluation, comparing their efficacy with sorafenib (NCT01658878) (Hirano et al., 2005; Keir et al., 2008).

Attacking cancer stem-like cells, resistant to traditional treatments, was crucial for reducing multidrug resistance, metastasis, and recurrence in HCC. Annexin A3-transfected dendritic cells induce T-cell activation, improving the effectiveness of current treatments. Oncolytic immunotherapeutics like Pexa-Vec, in combination with common therapies, showed promise in improving HCC patient survival in clinical trials (NTC03071094, NTC02562755) (Heo et al., 2013; Breitbach et al., 2015; Pan et al., 2015). **Figure 2.2** shows the schematic infographic of interactions between HCC and immune cells.

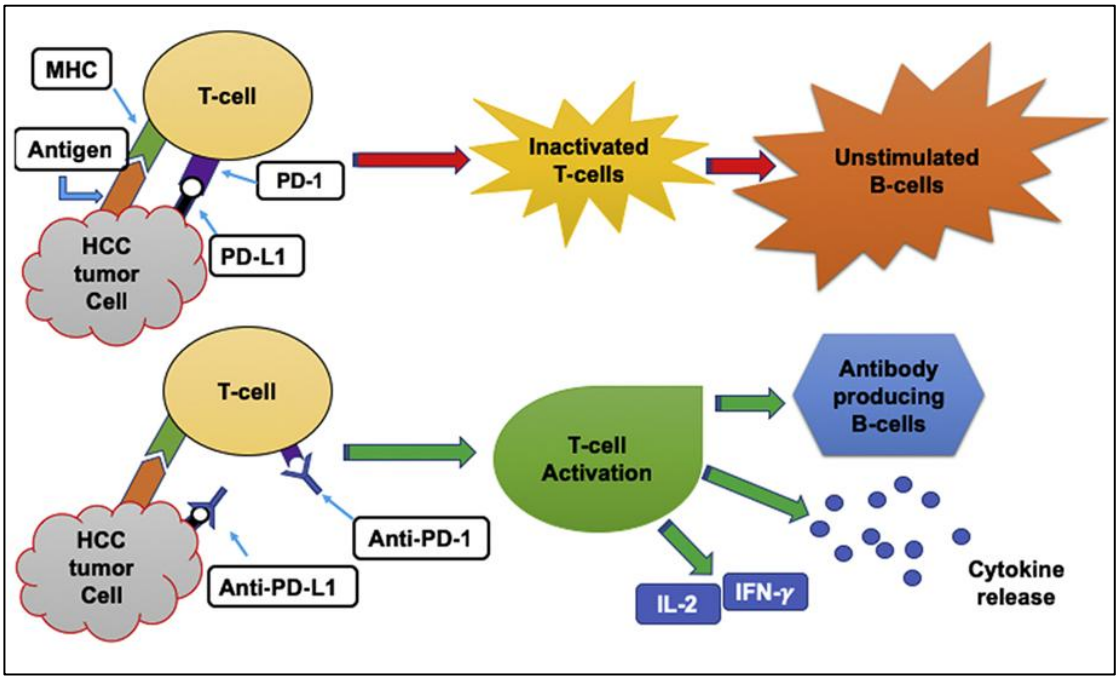


Figure 2.2 A schematic showing how HCC cells activate T-cells, while inactivating others and leaving B-cells unstimulated, which is essential for class switching. Activated T-cells trigger the immune response through IFN-gamma, IL-2, cytokine release, and stimulate B-cells to undergo class switching. Image adapted from Anwanwan et al., 2020.

2.1.2(d) Nanotechnology-Based Therapy for Liver Cancer

Nanotechnology advancements have significantly improved the efficacy of liver cancer treatments. Various nanocarrier-based drug delivery systems have been developed, allowing for reduced drug amounts, minimized systemic toxicity, prolonged drug release, and enhanced targeting of liver cancer cells. Luminescent, organic dye-doped nanoparticles demonstrated selective binding to HepG2 liver cancer cells *in vitro*, showcasing the potential for improved outcomes in treating liver cancers (He et al., 2004).

A unique mesoporous silica structure loaded with docetaxel exhibited remarkable effectiveness against HepG2 cells and in mice. This method required only 7% of the docetaxel amount compared to the free form, showing low systemic toxicity and a 15% improvement in tumor inhibition for mice transplanted with hepatocarcinomas (Li et al., 2010). Drug-eluting microspheres reduced the required doxorubicin amount for hepatic tumors, resulting in significantly lower plasma concentrations upon intra-arterial administration, with increased tumor necrosis and minimal side effects (Hong et al., 2006).

Nanoparticles have enhanced combination therapies for chemosensitizing drug-resistant cancer cells. The delivery of doxorubicin and the chemosensitizer curcumin as lipid nanoparticles showed sustained release and potential synergy, indicated by higher cytotoxicity and decreased IC50 for HepG2 cells. In diethylnitrosamine-induced liver cancers, the doxorubicin/curcumin approach exhibited synergistic tumor growth inhibition compared to free doxorubicin/curcumin (Zhao et al., 2014).

Clinical trials were underway for innovative nanoparticle formulations. NBTXR3, a hafnium oxide crystal formulation activated by stereotactic body radiation therapy (SBRT), showed promising results for HCC patients with liver metastases.

Another trial involving lipid-based nanoparticles, MTL CEBPA, targeted the CEBPA gene, demonstrating positive effects on HepG2 cells and cirrhotic/HCC rats with multifocal liver tumors (Reebye et al., 2014; Chajon et al., 2018). These advancements offered potential improvements in liver cancer treatment without compromising liver function.

2.2 Overview Functions of Deubiquitinating Enzymes (DUBs)

Ubiquitylation stood out as one of the most widespread post-translational modifications, exerting control over the functions of numerous cellular proteins. This process played a critical role in a myriad of physiological activities, encompassing cell survival, proliferation, differentiation, as well as innate and adaptive immunity (Popovic et al., 2014). This process was executed sequentially by three distinct enzymes — ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2, and ubiquitin ligase E3 — the relatively small 76-amino-acid protein ubiquitin (Ub) was ultimately appended to specific substrates, guiding them towards distinct outcomes (Hershko & Ciechanover, 1998). The catalytic reactions involved a series of steps: Ub was initially activated by an E1 in an ATP-dependent fashion, subsequently transferred to the active cysteine residue of an E2 through a trans-(thio) esterification reaction, and ultimately linked to a substrate via an isopeptide bond by an E3, thereby imparting specificity in substrate recognition (Gallo et al., 2017; Zhang & Jiang, 2021).

The ubiquitination process is dynamic and reversible through deubiquitination, a mechanism catalyzed by a group of proteins known as ubiquitin hydrolases or deubiquitinating enzymes, commonly referred to as DUBs (Karunarathna et al., 2016). Mechanistically, DUBs can directly interact with substrates or recognize Ub chain structures to remove Ub moieties from either the ends or within the chains. By

counteracting the function of E3 ligases, DUBs manage protein balance and activities, thereby influencing a range of physiological and pathological processes, including development, metabolism, immune response, and tumorigenesis **(Figure 2.3)** (Bufalieri et al., 2020). This action could lead to various outcomes, with the primary consequence of safeguarding proteins from degradation. For instance, if ubiquitination were non-proteolytic, DUBs could halt a signaling event initiated by ubiquitin conjugation. In essence, DUBs represented the flip side of the coin in protein ubiquitination, contributing significantly to the dynamic interplay between ubiquitin addition and removal across the entire proteome (Hewings et al., 2017).

DUBs, as the primary characteristic, functioned as proteases, constituting approximately 20% of the ~500 protease-encoding genes in the genome and representing the largest protease family in humans. DUBs fall into two enzymatic groups: cysteine-based and metalloprotease DUBs. The cysteine-based DUBs encompassed six families, each defined by distinct enzymatic domains within the group – ubiquitin-specific proteases (USPs), ovarian tumor proteases (OTUs), ubiquitin carboxy-terminal hydrolases (UCHs), Machado–Joseph disease proteases (MJDs), the recently identified motif interacting with ubiquitin-containing novel DUB family (MINDY) (Abdul Rehman et al., 2016), and ZUP1 (Haahr et al., 2018; Hermanns et al., 2018; Hewings et al., 2018; Kwasna et al., 2018). On the other hand, metalloprotease DUBs included only the zinc-dependent JAB1/MPN/MOV34 metalloproteases (JAMMs) family. Fundamentally, the primary role of DUBs is to counteract ubiquitination, contributing to the equilibrium between substrate conjugation and deconjugation to regulate cellular pathways. Essentially, their function was akin to that of phosphatases in kinase signaling pathways (Bonacci & Emanuele, 2020).

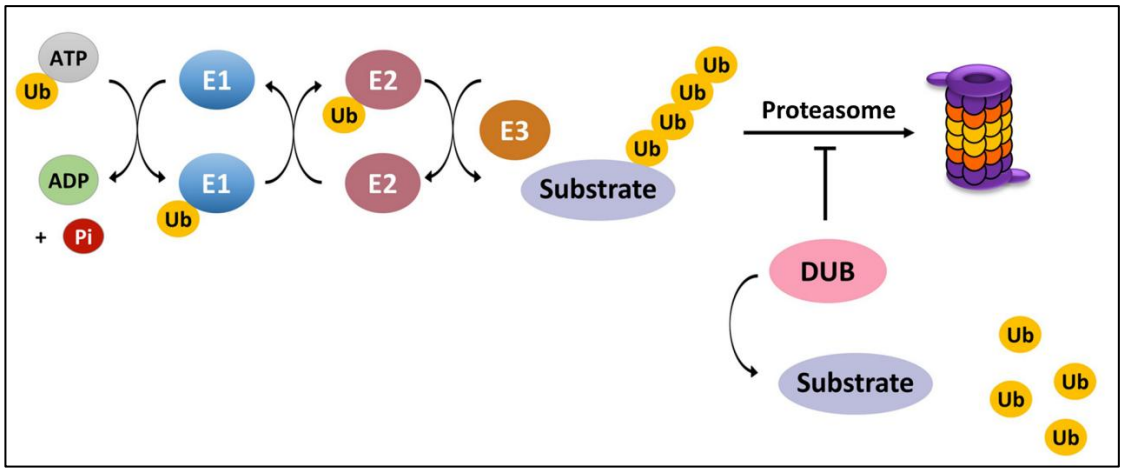


Figure 2.3 The role of DUBs in the ubiquitin-proteosome system. Image adapted from Bonacci & Emanuele, 2020.

2.2.1 Ovarian Tumor Proteases (OTUs)

In contrast to other DUBs that cleaved all types of polyubiquitin chains, the OTU deubiquitinase subfamily exhibited an inclination for specific linkage types. There are 16 (Keusekotten et al., 2013) to 18 recognized (Mevissen et al., 2013; Sun et al., 2020) active OTU family DUBs in humans, which are classified into four subfamilies – the Otubains subfamily (OTUB1 and OTUB2), which contains OTU domain-containing ubiquitin aldehyde-binding proteins, the Ovarian tumor domain-containing proteins (OTUDs) subfamily (OTUD1, OTUD2/YOD1, OTUD3, OTUD4, OTUD5/DUBA, OTUD6A, OTUD6B, ALG13), the A20-like subfamily (A20, Cezanne/OTUD7B, Cezanne2/OTUD7A, TRABID/ZRANB1, VCPIP), and the OTU DUB with linear linkage specificity (OTULIN) as shown in **Figure 2.4**.

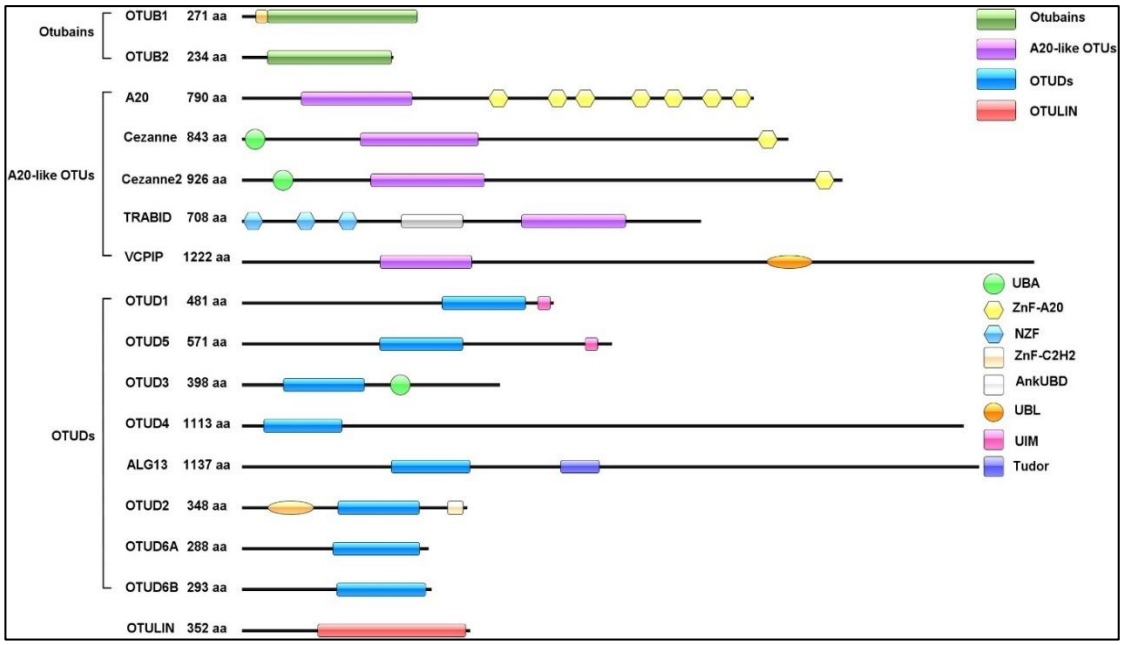


Figure 2.4 The OTUs' family members' schematic domain structure. The structure was generated by the Illustrator for Biological Sequences (IBS) (http://ibs.biocuckoo.org/). Image adapted from Zhu et al., 2021.

2.2.2 Otubains – Ovarian Tumor Deubiquitinylases Otubain-1 (OTUB1) and Otubain-2 (OTUB2)

OTUB1 and OTUB2 are categorized within the cysteine protease class, sharing a sequence identity of 48% and a similarity of 70%. Notably, OTUB1 featured a 37-residue-long N-terminal region that was absent in OTUB2 (Edelmann et al., 2009). Despite significant sequence conservation and structural similarity, discernible substrate specificity was evident (Edelmann et al., 2009). OTUB1 exhibited specific affinity for K48-linked ubiquitin chains that could lead to proteasomal degradation. In contrast, OTUB2 has a broader range, showing a preference for K63-linked ubiquitin chains that could lead to the turning off or on of various signalling pathways (Altun et al., 2015). Furthermore, OTUB1 interacted with a subset of E2 ubiquitin-conjugating enzymes, inhibiting ubiquitin transfer independently of its catalytic activity. The N-terminal ubiquitin-binding domain unique to OTUB1, absent in OTUB2, governed the

recognition of ubiquitin by E2 enzymes (Wiener et al., 2012). The binding of free ubiquitin to the distant site on OTUB1 induced conformational changes in its OTU domain, forming a ubiquitin-binding helix in the structure spanning residues 25 – 44 in the OTUB1 N-terminus. This conformational shift enhanced the binding of the conjugated donor ubiquitin in the E2-Ub complex to OTUB1, effectively obstructing ubiquitin transfer from E2s to the substrate (Juang et al., 2012; Wiener et al., 2012). Moreover, the same set of E2s reinforced the affinity of OTUB1 for K48-linked polyubiquitin substrates by supporting the N-terminal helix of OTUB1 when the E2s lacked ubiquitin charge (Wiener et al., 2013). **Figure 2.5** shows the specific affinity for K48-linked ubiquitin chains for OTUB1 and K63-linked ubiquitin chains for OTUB2.

OTUB1 and OTUB2 exhibited notable distinctions in the P'-side region, particularly in the area where the binding of a leaving lysyl group from a target substrate or a ubiquityl group from a chained substrate was anticipated. Differences in surface charge properties were evident in this region between the two proteins, and the α2 helix of OTUB2 displayed a kink, a characteristic not found in the structure of OTUB1. Additionally, the structural flexibility of the catalytic cleft in OTUB1 was constrained by the proximity of Pro87 to Cys91, a situation not mirrored in OTUB2, where Gly47 occupied the corresponding position. The larger side chain in OTUB1 might contribute to its preference for cleaving isopeptide bonds over ubiquitin C-terminal fusions, a feature not observed in OTUB2 (Nanao et al., 2004). **Figure 2.6** shows Otubains' protein-interacting domains and modification residues.

In terms of domain composition, OTUB2 differed relatively from OTUB1. OTUB2 exclusively featured a carboxyl-terminal OTU catalytic active domain, whereas OTUB1 included an additional amino-terminal ubiquitin-binding domain. This additional domain in OTUB1 facilitated interactions with E2 enzymes, allowing it to

regulate ubiquitination events within cells (Nakada et al., 2010; Du et al., 2020). The absence of extra domains in OTUB2 might restrict its potential for common post-translational modifications like hydroxylation and phosphorylation, which were prevalent in other deubiquitination enzymes (Zhu et al., 2021). Currently, the sole confirmed modification of OTUB2 is SUMOylation at the Lys233 site. This modification has been observed to interact with Yes-associated protein/transcriptional co-activator with PDZ-binding motif (YAP/TAZ), resulting in heightened OTUB2 activity and the transfer of ubiquitin (Zhang et al., 2019).

OTUB1 could control the target protein expression and cancel its role via inhibition of ubiquitination (Wiener et al., 2012; Sivakumar et al., 2020). Previous research had displayed that plenty of essential cellular processes were regulated by OTUB1, including DNA repair, cell signalling transduction, cell proliferation, and apoptosis (Nakada et al., 2010; Liu et al., 2019). In the field of cancer, OTUB1 also demonstrated a progressively significant and unique role (Zhu et al., 2021). OTUB2 has been revealed to be related to numerous human diseases (Kudo et al., 2010; Li et al., 2010; Beck et al., 2013), inclusive of cancer, significantly stimulates metastasis through transcriptional regulators YAP and TAZ deubiquitination (Zhang et al., 2019). OTUB2 was found to have activity against K11 and K48 chains, although it prudently cleaved K63-linked polyubiquitin chains (Mevissen et al., 2013). Zhu et al. (2021) summarized that both OTUB1 and OTUB2 portrayed essential functions in cellular processes and human diseases. It was also discussed that otubains could function dependently and independently based on their enzyme activity via interaction with collective substrates and corresponding proteins (Zhu et al., 2021).