Adiponectin Receptors and LDLR Axis in Papillary thyroid carcinoma: Therapeutic Implications of AdipoRon

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Dissertation presented by Changda Li *to apply for the doctorate degree from the* Autonomous University of Barcelona

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2025

ABBREVIATIONS

ACC	Acetyl CoA carboxylase
AGR	Albumin to globulin ratio
AKT	Protein Kinase B
AMPK	AMP activated protein kinase
AO	Acridine orange
APC	Annual Percent Change
APC (2)	Adenomatous Polyposis Coli
APN	Adiponectin
ASR	Age standardized incidence rate
ATC	Anaplastic thyroid carcinoma
AVOs	Acidic vesicular organelles
BP	Biological Process
BPA	Bisphenol A
BRAF	V-Raf murine sarcoma viral oncogene homolog B1
Bregs	Regulatory B cells
BSA	Bovine serum albumin
C cell	Parafollicular
CAP1	Cyclic AMP activated protein 1
Cd	Chronic cadmium
CDH1	Cadherin 1
CM	Conditioned media
DAPI	4′,6-diamidino-2-phenylindole
DAPK	Death Associated Protein Kinase
DEHP	Di (2 ethylhexyl) phthalate

DHGTC	Differentiated high grade thyroid carcinoma
DII	Dietary Inflammatory Index
DMSO	Dimethyl sulfoxide
Dvl	Dishevelled
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbent assay
EMT	Epithelial mesenchymal transition
ER	Endoplasmic reticulum
ERK	Extracellular Signal Regulated Kinase
ERs	Estrogen receptors
FAK1	Focal adhesion kinase 1
FAO	Fatty acid β-oxidation
FASN	Fatty acid synthase
FBS	Fetal bovine serum
FDNs	Follicular cell derived neoplasms
FIB	Fibrinogen
FNAB	Fine needle aspiration biopsy
FNMTC	Familial non-medullary thyroid cancer
fT3	Free Triiodothyronine
fT4	Free Thyroxine
FTA	Follicular thyroid adenoma
FTC	Follicular thyroid carcinoma
G-CSF	Granulocyte colony-stimulating factor
GDC	Genomic Data Commons

GEO	Gene Expression Omnibus
GLR	Glucose to lymphocyte ratio
GSEA	Gene Set Enrichment Analysis
GTEx	Genotype Tissue Expression
Gy	Gray
H&E	Hematoxylin and Eosin
HDL	High density lipoprotein
HDLc	High density lipoprotein cholesterol
HIF-1α	Hypoxia inducible factor-1α
HMW	High molecular weight
HSCSP	Santa Creu i Sant Pau Hospital
HT	Hashimoto's thyroiditis
IGF-1	Insulin like Growth Factor 1
IL-1ra	IL-1 receptor antagonist
IL-6	Interleukin-6
ILs	Interleukins
KBr	Potassium bromide
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein cholesterol
LDLR	LDL receptor
LMR	Lymphocyte to monocyte ratio
LMW	Low molecular weight
LXRs	Liver X receptors
MAPK	Mitogen Activated Protein Kinase
MEK	Mitogen Activated Protein Kinase Kinase

MMPs	Matrix metalloproteinases
MMW	Middle molecular weight
MUFA	Monounsaturated fatty acids
N0	Lymph node negative
N1	Lymph node positive
NF-κB	Nuclear Factor kappa light chain enhancer of activated B cells
NIS	Sodium/iodide symporter
NLR	Neutrophil to lymphocyte ratio
O/N	Overnight
OC	Oncocytic carcinoma of the thyroid
OCPs	Organochlorine pesticides
ox-LDL	Oxidized Low density lipoprotein
P/S	Penicillin/Streptomycin
p38 MAPK	P38 mitogen activated protein kinase
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PCBs	Polychlorinated biphenyls
PDTC	Poorly differentiated thyroid carcinoma
PE	Phosphatidylethanolamine
PHB	Prohibitin
PI3K	Phosphoinositide 3-Kinase
PLR	Platelet to lymphocyte ratio
PLT	Platelet count
PMSF	Phenylmethylsulphonyl fluoride
PNI	Prognostic nutritional index

POX	Proline oxidase
PPARs	Peroxisome proliferator activated receptors
PPARγ	Peroxisome proliferator activated receptor gamma
Prok1	Endocrine gland derived vascular endothelial growth factor
pSS	Primary Sjögren's Syndrome
PTC	Papillary thyroid carcinoma
PTC3	Papillary Thyroid Carcinoma type 3 fusion
PTEN	Phosphatase and Tensin Homolog
RA	Rheumatoid Arthritis
RAF	Rapidly Accelerated Fibrosarcoma
RARβ2	Retinoic Acid Receptor Beta 2
RAS	Rat Sarcoma virus
RASSF1A	Ras Association Domain Family Member 1 isoform A
RET	Rearranged during Transfection
ROS	Reactive oxygen species
SAT	Subacute thyroiditis
SCD	Stearoyl-CoA desaturase
SII	Systemic immune inflammation index
SIR	Standardized Incidence Ratio
SLC5A8	Solute Carrier Family 5 Member 8
SLE	Systemic Lupus Erythematosus
SMOC2	SPARC related modular calcium binding protein 2
SNPs	Single nucleotide polymorphisms
SREBPs	Sterol regulatory element-binding proteins
SSc	Systemic Sclerosis

T3	Triiodothyronine
TBST	Tris Buffered Saline with Tween 20
TCGA-THCA	The Cancer Genome Atlas-Thyroid Carcinoma
TGF-α	Transforming growth factor alpha
TGFβ1	Transforming growth factor β1
TIMP3	Tissue Inhibitor of Metalloproteinases 3
TKIs	Tyrosine kinase inhibitors
TME	Tumor microenvironment
TNF-α	Tumor necrosis factor α
TPM	Transcripts Per Million
TPO	Thyroid peroxidase
Tregs	Regulatory T cells
TSH	Thyroid Stimulating Hormone
TSP1	Thrombospondin-1
TZDs	Thiazolidinediones
uPA	Urokinase type plasminogen activator
UPS	Ubiquitin proteasome system
VEGF	Vascular Endothelial Growth Factor
VEGFA	Vascular endothelial growth factor A
VLDL	Very low-density lipoprotein
WB	Western blot
^{131}I	Iodine 131

SUMMARY

Thyroid cancer (TC) is among the fastest-growing solid malignancies worldwide, with papillary thyroid carcinoma (PTC) accounting for approximately 90% of all cases. Although the prognosis for most PTC patients is favorable, a subset still faces risks of aggressive progression and recurrence, highlighting the need for deeper understanding of its molecular mechanisms and novel therapeutic strategies. In recent years, obesity-associated chronic inflammation has been recognized as a major driver of PTC development. Adipose tissue, functioning as an endocrine organ, secretes various adipokines that play critical roles in tumor immunity, metabolic reprogramming, and microenvironment modulation. Among them, adiponectin (APN) is a key anti-inflammatory adipokine with regulatory functions in metabolism, autophagy, and cellular proliferation. Through its receptors, AdipoR1 and AdipoR2, APN activates signaling pathways such as AMPK and PPAR-α, contributing to energy homeostasis, autophagy induction, and modulation of the PI3K/Akt/mTOR axis. However, the poor stability of the APN protein limits its clinical application, prompting growing interest in its small-molecule receptor agonist, AdipoRon. With high oral bioavailability and stability, AdipoRon has shown potential in inducing apoptosis and autophagy across multiple tumor models. Recent studies also suggest an interaction between APN signaling and low-density lipoprotein (LDL) metabolism. Particularly in BRAF-mutated PTC, LDL promotes tumor progression via the MAPK/ERK pathway, while APN may counteract these effects by enhancing LDLR degradation. Thus, investigating the regulatory role of AdipoRon in PTC, especially in relation to lipid metabolism and LDL signaling, holds significant scientific and therapeutic value. **This study aims** to comprehensively investigate the expression patterns, biological functions, and molecular mechanisms of adiponectin (APN) and its receptors (AdipoR1 and AdipoR2) in thyroid cancer, particularly PTC. Special emphasis is placed on the interplay between APN signaling and LDL metabolism, and the antitumor potential of AdipoRon in promoting apoptosis and/or autophagy.

A combination of bioinformatics and cell-based functional experiments was employed to explore the role of APN signaling in PTC. Public databases including TCGA, GTEx, and GEO were utilized to analyze the differential expression of APN receptor-related genes (*ADIPOR1, ADIPOR2*) across normal thyroid tissue, benign tumors, and PTC, along with correlations with clinicopathological parameters and prognosis. Two thyroid cancer cell lines with distinct mutational backgrounds (BRAF-mutated BCPAP and RET/PTC1-rearranged TPC-1) were treated with AdipoRon to evaluate its effects on cell proliferation and migration. Western blotting was used to assess the expression of key proteins involved in apoptosis, autophagy, and relevant signaling pathways (AKT, mTOR, ERK). Furthermore, combined treatment with LDL and AdipoRon was applied to examine the modulation of LDL-induced tumor-promoting effects and explore the potential synergistic mechanisms under dysregulated lipid metabolism conditions.

Our findings reveal a significant role of APN signaling in the pathogenesis and progression of PTC. APN and its receptors (ADIPOR1/2) are differentially expressed in PTC compared to benign thyroid tissues, with functional divergence: ADIPOR1 is mainly involved in cell death and immune regulation, whereas ADIPOR2 is closely linked to metabolic remodeling. In PTC patients, perithyroidal adipose tissue exhibited elevated APN secretion along with altered inflammatory cytokine profiles, suggesting its contribution to tumor transformation via the local inflammatory microenvironment. AdipoRon effectively inhibited proliferation and migration of PTC cells, induced apoptosis and autophagy, and showed mutation-dependent activity. Under high LDL conditions, AdipoRon suppressed LDLR expression and interfered with AKT/mTOR/ERK signaling, reversing LDL-induced

proliferative effects. Collectively, these findings highlight the critical role of the APN–LDL axis in metabolic regulation of PTC and support the therapeutic potential of AdipoRon as a targeted intervention in metabolically dysregulated thyroid cancer.

I. INTRODUTION

I. 1. Overview of Thyroid Cancer

I. 1.1 Epidemiology of Thyroid Cancer

Over the past two decades, the thyroid cancer incidence has risen dramatically worldwide; In 2022, approximately 821,214 new cases of thyroid cancer were reported worldwide, corresponding to an age standardized incidence rate (ASR) of 9.1 per 100,000, it ranks as the seventh most prevalent cancer worldwide. The global mortality ranked 24th, with 47,507 deaths and an ASR of 0.44. Incidence was significantly higher among females than males (20.2% vs. 7.5%) （Figure 1）. Europe accounted for 9.6% of new cases and 12.4% of deaths worldwide (Figure 2)(1). Thyroid cancer incidence rates increase sharply after age 40, peaking in the 65–74 age group (Figure 3). Despite of some authors suggest that this increment could be attributed to an overdiagnosis effect, this type is projected to rise to the fourth position among all cancers by 2030(2).

Figure 1. Thyroid cancer incidence rate by sex in 2022. From GLOBOCAN 2020, Global Cancer Observatory, IARC. https://gco.iarc.fr/today

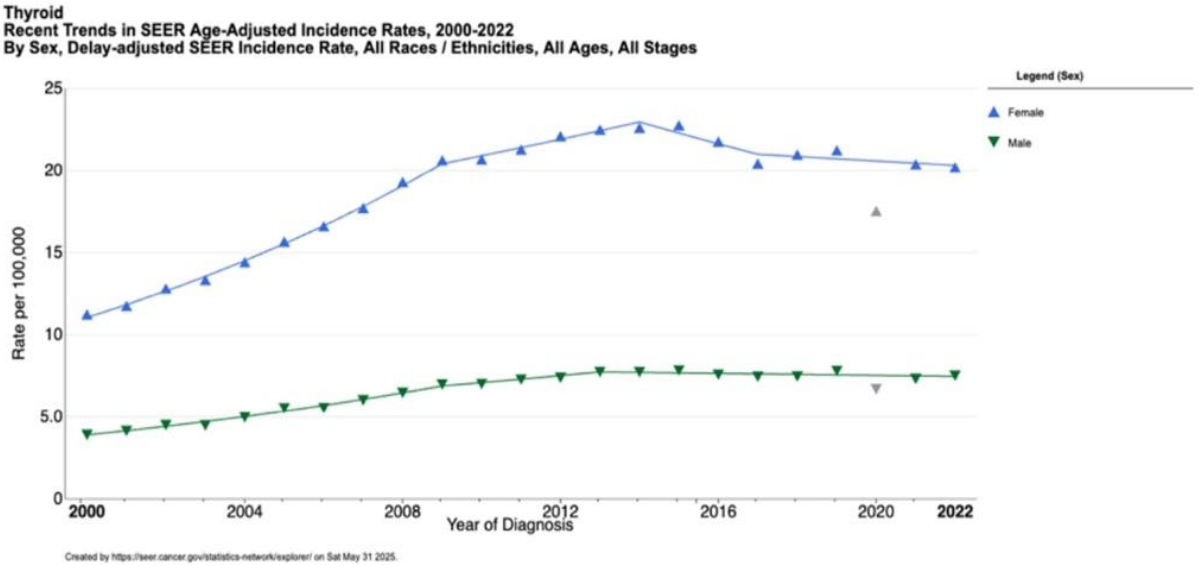


Figure 2. Thyroid cancer incidence and mortality rate in 2022. From GLOBOCAN 2020, Global Cancer Observatory, IARC. https://gco.iarc.fr/today

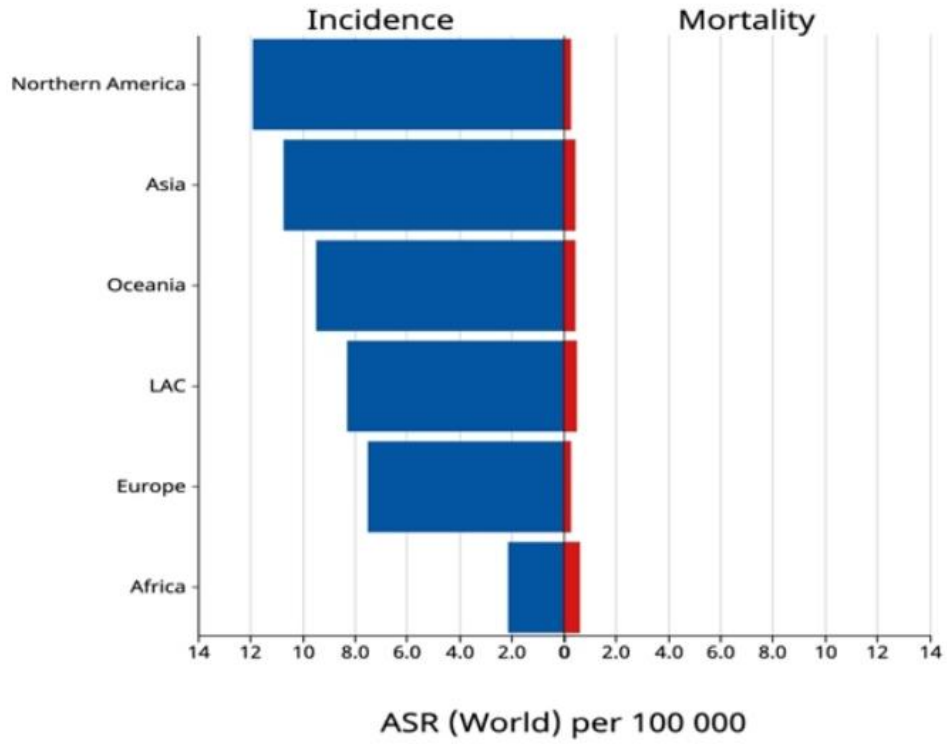
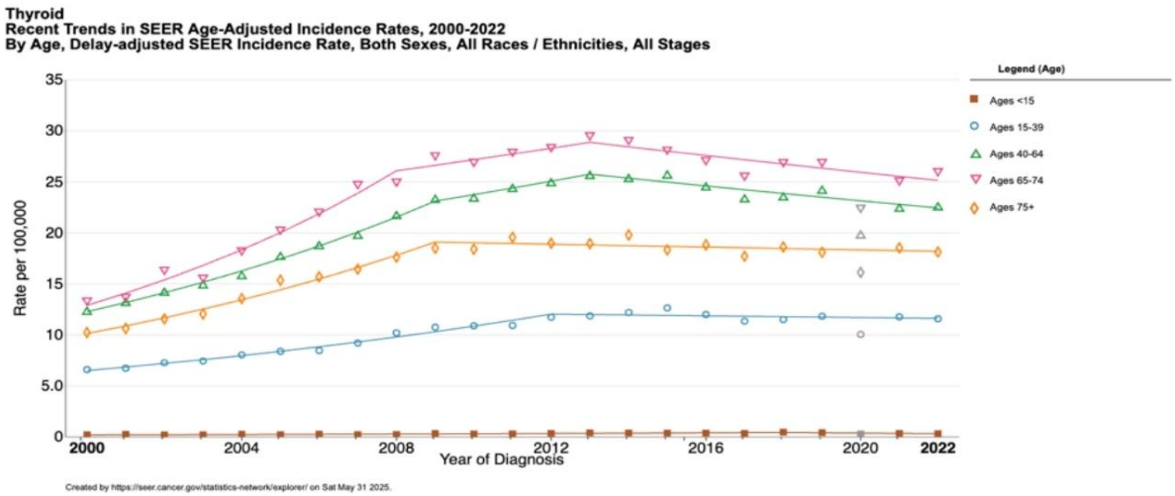


Figure 3. Thyroid cancer incidence rate by age in 2022. From GLOBOCAN 2020, Global Cancer Observatory, IARC. https://gco.iarc.fr/today

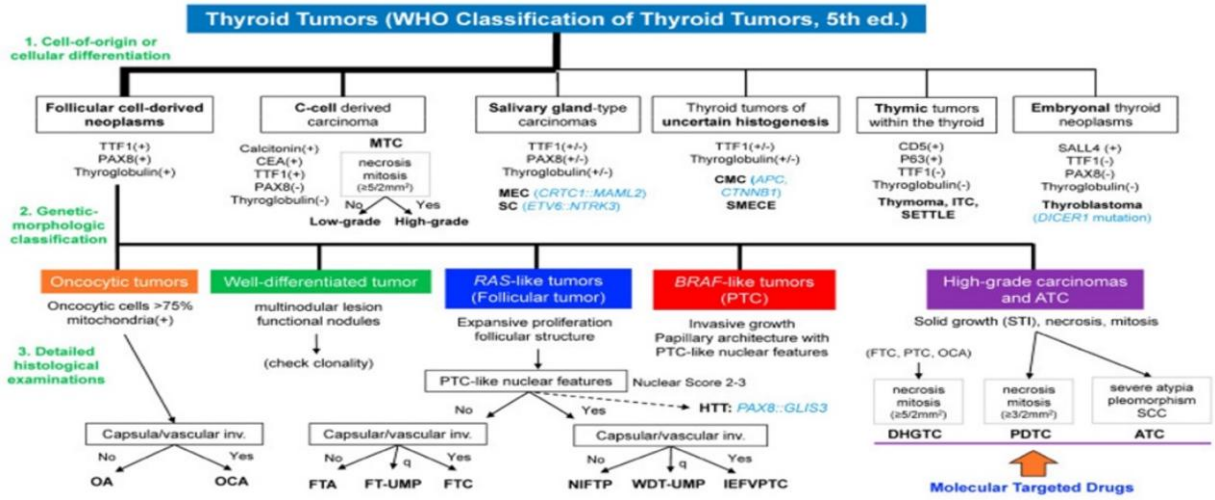


I. **1.2 Classification of Thyroid Cancer**

According to the 2022 WHO Classification of Thyroid Neoplasms, thyroid tumors are categorized based on their cell of origin into three primary groups: follicular cell derived neoplasms (FDNs), parafollicular (C cell) derived tumors, and miscellaneous tumors. FDNs are further classified by biological behavior into: Benign tumors, Low risk neoplasms and Malignant neoplasms, including: Follicular thyroid carcinoma (FTC), Invasive encapsulated follicular variant of papillary thyroid carcinoma, Papillary thyroid carcinoma (PTC), Oncocytic carcinoma of the thyroid (OC), Anaplastic thyroid carcinoma (ATC) and High grade follicular derived carcinomas: Differentiated high grade thyroid carcinoma (DHGTC) which is newly defined, Poorly differentiated thyroid carcinoma (PDTC). Notably, DHGTC is a newly proposed entity characterized as an aggressive PTC/FTC/OC tumor exhibiting ≥5 mitoses per 2 mm² and/or tumor necrosis, but lacking anaplastic features (Figure4)(3,4).

Figure 4. Algorithm for diagnosis of thyroid tumors (WHO 5th edition)

From *Chiba, T. Molecular Pathology of Thyroid Tumors: Essential Points to Comprehend Regarding the Latest WHO Classification. Biomedicines 2024, 12, 712.* https://doi.org/10.3390/biomedicines12040712



I. **1.3 Clinical Characteristics of PTC**

PTC accounts for approximately 90% of all thyroid cancers. Its incidence varies according to tumor size, with tumors larger than 2 cm exhibiting distinct clinical behavior. Since 2014, the overall incidence of PTC particularly localized tumors and microcarcinomas ≤1.0 cm has declined, accompanied by a significant decrease in the rate of distant metastasis (APC = –17.86). However, the incidence of tumors >2.0 cm has remained relatively stable. Despite the reduction in early stage diagnoses, mortality related to PTC based on incidence has continued to rise from 2000 to 2018 (APC = 1.35), indicating a decline in overdiagnosis of indolent tumors but limited advances in the detection or treatment of advanced stage disease(5). These findings suggest that the incidence of advanced PTC has not been significantly affected by recent improvements in diagnostic or therapeutic technologies.

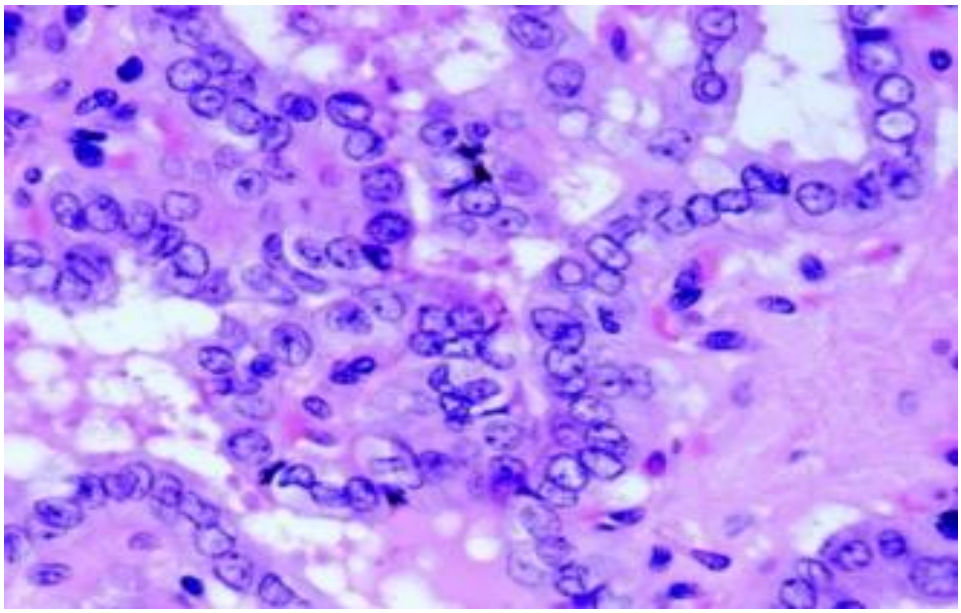
PTC is a well differentiated thyroid carcinoma that, along with FTC and OC, forms the group of DTC. DTCs are generally localized and associated with excellent prognoses, with 5 year survival rates exceeding 98%(6). In contrast, PDTC, a more aggressive subtype, has a markedly lower 5 year survival rate of about 76%(7). Due to the indolent and localized nature of DTC, larger tumors often

display low risk features such as univocality, confinement within the thyroid gland, and absence of lymph node metastasis. In these cases, the extent of thyroidectomy may not significantly influence oncologic outcomes, and lobectomy may suffice. However, for patients with DTC tumors measuring between 1 and 4 cm, total thyroidectomy is associated with a reduced risk of recurrence(8).

I. **1.4 Histopathology and Variants of PTC**

The diagnosis of PTC primarily relies on the distinctive nuclear features of thyroid tumor cells rather than on architectural patterns alone. Although various structural variants exist and underscore the importance of architectural changes, diagnostic emphasis remains firmly on nuclear morphology. The characteristic nuclear features include (Figure5)(9,10): Enlarged, elongated nuclei with crowding and overlapping; Irregular nuclear contours; Clear or vesicular chromatin with chromatin margination, resulting in the distinctive “Orphan Annie eye” nuclei; Multiple small nucleoli situated beneath the nuclear membrane; Nuclear grooves resulting from irregular nuclear contours visible in two dimensional sections; Intranuclear pseudo inclusions formed by cytoplasmic invaginations into deep nuclear grooves. These nuclear characteristics enlarged and overlapping nuclei, chromatin clearing, irregular nuclear contours, grooves, and intranuclear cytoplasmic pseudo inclusions are hallmark features of PTC and are essential for its histopathological diagnosis (10).

Figure 5. Characteristic Nuclear Features of PTC. Adapted from *WHO Classification of Tumours Editorial Board: Endocrine and Neuroendocrine tumours*, vol. 8. 5th edn. (International Agency for Research on Cancer, Lyon, France, 2022) https://tumourclassification.iarc.who.int.



I. **1.5 Risk factors for thyroid cancer**

The risk of developing thyroid cancer is multifactorial. However, some factors may increase this risk, and the most important ones are described below.

I. **1.5.1 Internal Factors**

Family History

Having a first degree relative, especially a sibling, with thyroid cancer increases the risk of sporadic PTC(11). Female relatives have a 2% cumulative risk, nearly triple that of the general population;

male relatives show a 1% risk with a Standardized Incidence Ratio (SIR) of 2.5. In families with ≥2 early onset PTC cases (<60 years), the risk rises to 10% in females and 24% in males. Twin studies show a 23 fold increased risk(12). Familial non medullary thyroid cancer (FNMTC), defined by ≥3 affected family members, often presents younger, with multifocality, early onset, and higher recurrence and aggressiveness(13–15). Compared to sporadic cases, familial PTC is typically more invasive and recurrent(16–21), though some studies report no significant difference(21–26). Even micropapillary forms can be aggressive in familial cases. Familial cases account for ~5% of all thyroid cancers, mainly PTC, and may involve chromosomal alterations(27)

Hormonal factors

The levels of thyroid stimulating hormone (TSH) is associated with a higher risk in euthyroid patients with nodules (28). Rising thyroglobulin levels may serve as an indicator of increased risk, while TSH shows inconsistent associations (29). Some studies link higher TSH with cancer diagnosis, but results vary. Cancer patients often show lower triiodothyronine (T3) and higher free thyroxine (fT4); an fT4/fT3 ratio >3.3 increases risk 3.6 fold (30,31). Higher free T3 (fT3) is linked to lower risk, while fT4 >2.2 ng/dL significantly increases it(32) .

Age and gender

Age

The majority of thyroid cancer cases occur in adults between 25 and 65 years old, with a peak incidence in people aged 40 to 50. It is less frequent in very young children and older adults(33). Age is recognized as a crucial prognostic indicator in DTC(34).
Evidence suggests that survival rates decline with increasing age in patients with PTC, and DTC exhibits more aggressive clinical behavior in elderly individuals(35,36).
Furthermore, in ATC, as a key prognostic factor, age has a significant influence on patient outcomes, with individuals under 50 years of age exhibiting the most favorable prognoses (37)

Gender
Thyroid cancer, especially PTC, is more frequent in females. Its incidence rises after puberty and peaks again during the perimenopausal period, declining after menopause, likely due to estrogen level changes(38). In women under 45, late menarche (≥14 years), long menstrual cycles (>30 days), recent pregnancy (within 5 years), and parity (2–3 births) increase PTC risk, while long term breastfeeding (>12 months) is also linked to higher risk(39–41) Oral contraceptive use appears protective, particularly in younger women(42,43) Family history also increases susceptibility(44).
These sex based differences suggest that thyroid cancer may be influenced by endogenous estrogen levels, especially among obese or overweight women(45) . In adipose tissue, increased aromatase expression promotes the synthesis and conversion of endogenous sex steroids (46), leading to elevated estrogen levels that contribute to thyroid cancer development (47). Estrogen primarily acts through nuclear estrogen receptors (ERs), with ER-α and ER-β having opposing effects on thyroid cancer cell proliferation (48). Differential expression of these receptors allows estrogen to regulate cell proliferation via multiple signaling pathways, including PI3K/AKT, MEK/ERK, vascular endothelial growth factor (VEGF), and NF-κB (49). Estrogen can also stimulate cell proliferation through non-ER pathways (50), and influence tumor progression by modifying the tumor

microenvironment. For example, it may affect oxidative stress responses, autophagy, and VEGF secretion, thereby enhancing angiogenesis in thyroid cancer(51) .

Racial Disparities

There has been a striking 150.2% surge in thyroid cancer incidence since 1973 in Whites and 73.2% in Blacks with consistently lower rates in Black populations, partly due to healthcare disparities, though intrinsic factors likely contribute(52). Compared to Whites, A higher incidence of extrathyroidal extension has been observed in Black and Hispanic patient populations, while Asian patients show similar rates(53) . Treatment patterns also differ: Black patients are more likely to receive radioactive iodine, while Hispanic patients are less likely(54). African Americans face lower survival, possibly due to more aggressive tumor types and larger tumors at diagnosis(55) . Asian/Pacific Islanders have higher rates of brain metastases(56) , and Hispanics are often diagnosed younger, with greater lymph node involvement and more advanced disease than Whites(57) .

I. **1.5.2 External Factors**

Ionizing Radiation

Exposure to iodine 131 (^{131}I) during childhood significantly increases the risk of TC due to the gland's pronounced ability to absorb ^{131}I (58). After the Chernobyl accident, pediatric population (aged 0–19) in contaminated areas experienced a sharp upward trend in the occurrence of TC, a risk that has persisted for decades(59–63). Contaminated milk was the main source of radiation, with doses up to 42 Gy, though many studies rely on retrospective questionnaires, which may cause bias (64–66). It is estimated that by 2065, Chernobyl radiation may cause around 16,000 thyroid cancer cases(67). Molecular studies show frequent RET/PTC3 gene rearrangements in both radiation induced and sporadic PTC (64–66). Even low dose radiation exposure, such as from the Hiroshima event, increases cancer risk(68) . Living near nuclear plants shows no statistically significant increased risk(69) , yet ^{131}I therapy remains a standard treatment for thyroid cancer (70). Ionizing radiation causes DNA damage, with children's thyroids being particularly sensitive(71–74). Medical imaging like CT scans also raises the risk of micropapillary carcinoma(75). Mobile phone use alone shows no clear link to thyroid cancer, but certain DNA repair gene variants may increase risk(76,77). Radiofrequency radiation induces oxidative stress and DNA damage, contributing to carcinogenesis(78). Minimizing radiation exposure in children and avoiding unnecessary imaging is critical to reduce thyroid cancer risk.(79)

Smoking

Current smoking has been associated with a reduced risk of thyroid cancer(80). Negative correlations have been reported between smoking and the risk of thyroid nodules as well as PTC(81) . Nonetheless, some studies have found little evidence supporting significant associations between hormonal factors, smoking, or alcohol consumption and thyroid cancer risk (82).

Endocrine Disruptors

Endocrine disruptors are harmful environmental agents linked to thyroid cancer, though their effects on thyroid hormone levels remain unclear(83) . Dioxins and dioxin like compounds, such as polychlorinated biphenyls (PCBs), disrupt thyroid function and are associated with increased thyroid cancer risk(84,85). They interfere with thyroid hormone transport, cause cytotoxic damage, and induce metabolic enzymes(86,87). PCBs promote cancer cell growth by affecting genes like PIK3R1

and PI3K/Akt pathways, key in thyroid cancer processes(88). Phthalates, common plasticizers, enter the body via ingestion, inhalation, or skin contact and disrupt thyroid function(89,90). Exposure to Di(2 ethylhexyl) phthalate (DEHP), a phthalate, is linked to higher differentiated thyroid cancer risk and PTC incidence(91,92). Bisphenol A (BPA) acts as a weak antagonist to thyroid hormone receptors, inhibiting their activity(93,94). BPA exposure correlates with elevated TSH levels, which may increase thyroid cancer risk through hormone disruption and TSH overstimulation(95). According to the fetal origin hypothesis, fetuses and infants exhibit increased susceptibility to BPA's harmful effects(96). Chronic cadmium (Cd) exposure is linked to thyroid issues like multinodular goiter, disrupted thyroglobulin secretion, including hyperplastic changes in parafollicular cells(97). Studies show Cd disrupts thyroid function even at low levels and may induce thyroid cancer and autoimmune thyroid diseases(98–104). Its toxicity mainly involves oxidative stress triggering apoptosis via DNA damage and Bax activation(101). A South Korean study found high Cd in thyroid tissue correlates with advanced cancer stages, possibly linked to dietary habits (104).

Manganese, mainly ingested through diet, is essential for metabolism but excessive exposure during pregnancy can disrupt thyroid hormone balance, affecting offspring neurodevelopment (105–107). However, links between manganese and thyroid cancer remain unclear (108). Lead's carcinogenic role is debated; in PTC patients, serum lead negatively correlates with TSH and positively with T3, suggesting complex effects on thyroid pathology(109). Vanadium, especially toxic as V2O5, may influence thyroid inflammation by inducing Th1 chemokines, but its direct link to thyroid cancer lacks conclusive evidence (110–113). Selenium, an essential micronutrient, is vital for thyroid function, yet excess intake may negatively impact thyroid hormone metabolism(113,114).

Epidemiological studies show that occupational exposure to insecticides and fungicides is connected to greater incidence of thyroid cancer, with a marked effect on papillary thyroid microcarcinoma (tumors ≤1 cm) (115). Exposure to biocides in the workplace also elevates thyroid cancer risk, though a direct link remains unconfirmed between pesticide use and thyroid cancer in women(115). While PCBs and organochlorine pesticides (OCPs) have not been directly linked to PTC (116), overall pesticide exposure is considered a potential risk factor for thyroid cancer (117). Pesticides may disrupt thyroid function by inhibiting iodine uptake, increasing hormone clearance, interfering with thyroid enzyme activities, altering hormone cellular uptake, and modifying gene expression(118), potentially promoting tumor development. Common pesticides like quizalofop-p-ethyl, 2,4-D, chlorpyrifos, glyphosate, and imazethapyr have been evaluated but generally show no significant carcinogenicity related to thyroid cancer(119,120). Certain occupations, including healthcare workers, technicians, medical professionals, nurses, construction workers, cleaners, pest control staff, and customer service employees, exhibit higher thyroid cancer rates, particularly papillary microcarcinomas and larger tumors (121).

Volcanic Eruptions and Pollution

Several studies report the increment in the incidence of TC in in volcanic regions such as; Iceland and the Philippines report thyroid cancer incidences exceeding those in similar non volcanic areas(82,122,123) or Near Mount Etna, Sicily, volcanic plume exposure may raise thyroid cancer rates(124) . Most of causes it is because in these volcanic areas show elevated trace elements in water, air, and urine, including cadmium, mercury, uranium, and vanadium, which correlate strongly and may contribute to thyroid carcinogenesis(125) . These populations also face increased DNA damage risk, especially in children's (126).

Others elements

Nitrates

Nitrate pollution disrupts thyroid function by inhibiting iodide uptake, potentially raising TSH levels (127). While animal studies link elevated TSH to thyroid disease and cancer(128), a cohort study found no strong association between high nitrate in water and thyroid dysfunction in postmenopausal women(129). However, epidemiological data suggest long term nitrate nitrogen exposure (>5 mg/L) in drinking water may increase thyroid cancer risk by 2.6 fold (130).

I. **1.5.3 Other Factors**

Diet

Dietary factors influence thyroid cancer risk beyond just iodine intake(131). For instance, an increased risk of thyroid cancer has been correlated with overconsumption of particular fruits and vegetables(132), although no specific dietary pattern has yet been conclusively linked to thyroid cancer(133). Diet also plays a direct role in modulating inflammation; diets rich in fats and processed sugars contribute to obesity, which can promote tumor development(134). Given the complex interactions among dietary components, the Dietary Inflammatory Index (DII) was designed to more accurately quantify the link between diet and inflammation(135). Higher DII scores indicate a more pro inflammatory diet and have been associated with an elevated cancer risk(136). Latest research supports an association between pro-inflammatory diets and an higher likelihood of DTC, particularly in overweight individuals(137).

Overweight/obesity

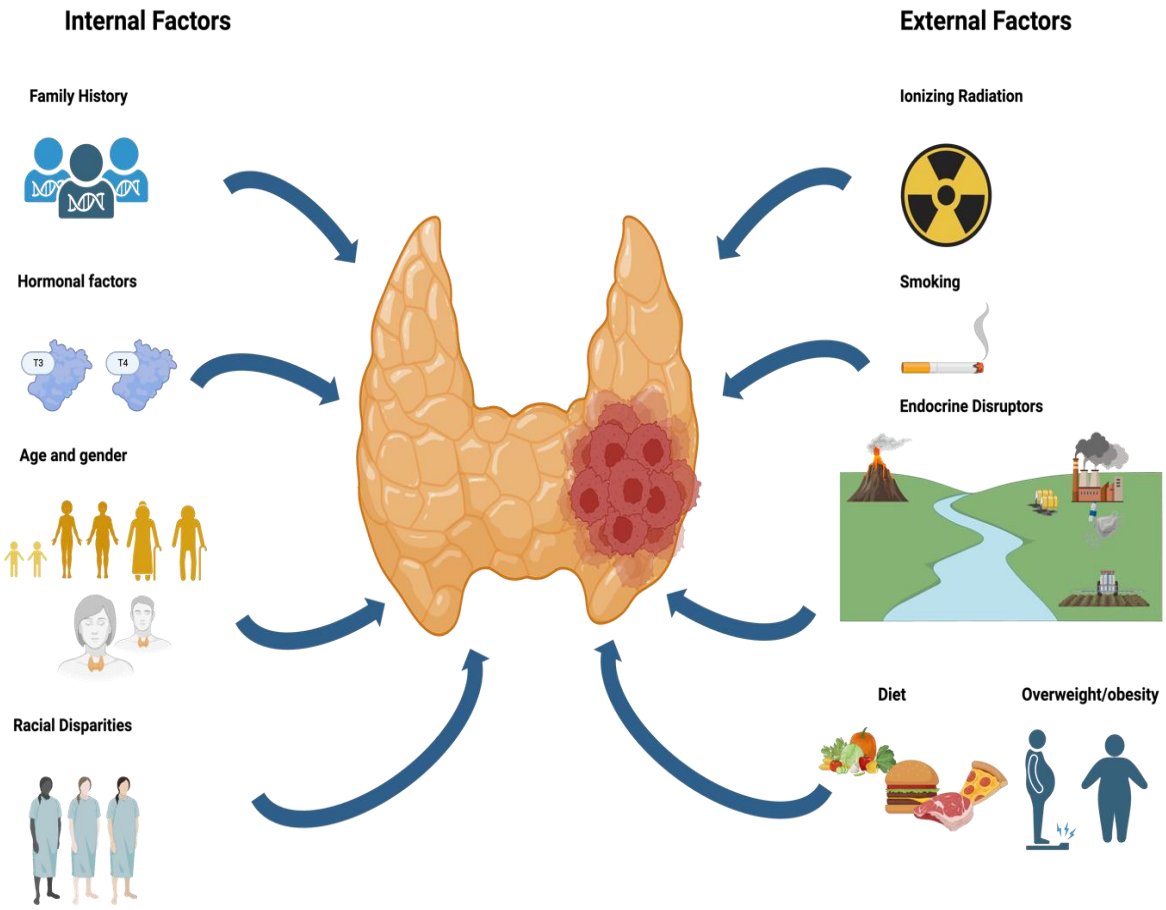
As obesity has reached epidemic levels worldwide, the incidence of both obesity or/ overweight and thyroid cancer, especially with the histological pattern PTC, has increased simultaneously, highlighting their potential connection.

Despite the biological mechanisms between Overweight and obesity should be explored in deep. Meta-analysis performed with 22 prospective studies in relation between thyroid cancer incidences in association with different anthropometric factors; including BMI, suggested that the excess adiposity throughout adulthood could be promoted an elevated incidence across most common forms of TC, including the rare ATC(138). This link may involve hormonal imbalances such as insulin resistance, disrupted hormone synthesis, and altered signaling pathways(139). Furthermore, excess adipose tissue creates chronic local and systemic inflammation that fosters tumor growth (140). Both obesity and benign thyroid conditions elevate thyroid cancer risk (141), with overweight and obesity strongly associated with PTC(142).

Obesity and overweight cause’s insulin resistance and hypoadiponectinemia, raising insulin and Insulin like Growth Factor 1(IGF-1) levels, which may increase thyroid cancer risk. Insulin resistance is central to this association, interacting with IGF-1, adipokines, and TSH to promote tumor development(143). Diabetes also raises DTC risk(144), and insulin resistance correlates with its prevalence (145). Metabolic syndrome and insulin resistance further contribute to thyroid cancer risk(146). A Mendelian randomization study linked type 2 diabetes to thyroid cancer risk, however, found no evidence of a connection between obesity and thyroid cancer(147).

Among the risk factors for TC, a growing body of evidence suggests that inflammation plays a significant role in the occurrence and progression of it. Additionally, diet and obesity are linked with low grade chronic inflammation, which affects thyroid cancer through inflammatory mediators, various cytokines, and adipokines. Therefore, the mechanisms by which chronic inflammation influences TC warrant further attention in future research.

Figure 6. Schematic model of Risk factors for thyroid cancer. (BioRender.com)



I. 1.6 Molecular basis in thyroid cancer

I. 1.6.1 Common genetic alterations in thyroid cancer

BRAF Mutation

V-Raf murine sarcoma viral oncogene homolog B1 (BRAF) is located on human chromosome 7(148). The major hotspot mutation in BRAF occurs in exon 15, specifically BRAF's T1799A mutation leads to the V600E amino acid alteration, known as the classic V600E mutation(149).
BRAF encodes a serine/threonine protein kinase characterized by significant single nucleotide polymorphisms (SNPs), and is frequently activated through somatic point mutations. It is considered a key oncogenic driver in various human cancers(150). The $BRAF^{V600E}$ mutation is the most prevalent genetic change reported in adult sporadic PTC, occurring almost exclusively in this subtype(151–153). Importantly, this mutation arises independently of RET/PTC rearrangements or RAS mutations(154).

RAS mutation

The RAS gene family, including *HRAS*, *KRAS*, and *NRAS*, encodes the small GTPase p21, which is essential for transmitting extracellular signals to the MAPK and PI3K/AKT signaling pathways(155). In thyroid cancer, point mutations can occur in all three RAS genes, with *NRAS* being the most frequently mutated(156). RAS mutations observed in approximately 10–20% of PTCs and 25% of poorly differentiated thyroid carcinomas (PDTCs)(157). These mutations are particularly enriched in the follicular variant of PTC (FVPTC) (158,159). Additionally, RAS mutations have been detected in about 40% of follicular adenomas (FAs) and 68% of sporadic medullary thyroid carcinomas (MTCs) that lack RET mutations(160,161).

Current evidence suggests that RAS mutations represent an independent oncogenic pathway, which is mutually exclusive from other driver alterations such as $BRAF^{V600E}$ mutations and PAX8-PPARγ rearrangements(162). As an early and pivotal molecular event, RAS mutations are implicated across various stages of thyroid tumor development, ranging from benign FA and FVPTC to FTC, PDTC, and even ATC, supporting a continuum model of tumor progression(163,164). In advanced thyroid cancers, RAS mutations frequently co-occur with alterations in genes such as TP53, β-catenin, PTEN, and PIK3CA(165–167), suggesting a cooperative role in promoting malignant progression and dedifferentiation. However, the natural course and clinical outcome of RAS positive thyroid tumors remain unclear, and their precise biological behavior warrants further investigation(168).

RET/PTC1 rearrangement

RET/PTC rearrangement is a specific chromosomal translocation event involving genes located on chromosome 1q21–q22. These genes belong to the receptor tyrosine kinase family and mediate extracellular signal transduction pathways that regulate cell growth(169–171). RET/PTC rearrangements are frequently observed in PTC, occurring in approximately 25% of adult and up to 50% of pediatric PTC cases. To date, 16 distinct RET fusion types have been identified, with

RET/PTC1 and RET/PTC3 being the most prevalent subtypes(172).

Among them, RET and its fusion partner gene CCDC6 (termed H4) are typically co-expressed in a colocalized pattern(173). The oncogenic mechanism mainly involves the fusion of the RET tyrosine kinase domain with heterologous partner proteins such as CCDC6 or NCOA4, leading to constitutive kinase activation. In this context, the chimeric RET fusion gene is transcriptionally activated under the control of the partner gene's promoter, circumventing the need for ligand dependent activation characteristic of wild type RET(174,175).

This constitutive activation continuously stimulates classical oncogenic pathways such as the RAS/MAPK and PI3K/AKT signaling cascade(176). As a result, RET/PTC rearrangements induce misexpression of RET in thyroid follicular cells, conferring several malignant phenotypes, including growth factor independent proliferation, resistance to apoptotic stimuli, and enhanced migration and invasiveness(177).

Importantly, RET/PTC activation is considered A hallmark early alteration during PTC pathogenesis, while subsequent mutations in BRAF, RAS, or the TERT promoter may act synergistically to drive tumor progression and aggressiveness(178,179). Clinically, RET/PTC rearrangements are more frequently observed in younger patients, and are associated with classic papillary histological features, locally aggressive behavior, along with an increased frequency of lymph node metastasis(172,180), underscoring their distinct biological and clinical significance in PTC.

I. **1.6.2 Altered signaling pathways in thyroid cancer**

MAPK Signaling Pathway

BRAF as a key element, is integral to the classical growth factor signaling cascade, specifically the RAS/MAPK pathway(181,182). The RAF protein family shares a similar structural organization, with a catalytic domain located at the C-terminus and regulatory elements distributed in the N-terminal region. In its inactive state, RAF usually exists as a monomer in the cytoplasm. Its activation is a complex process involving membrane localization, homo- or heterodimerization with other family members, and multi-site phosphorylation and dephosphorylation(183).

The BRAF ^{V600E} mutation has been shown to constitutively activate the MAPK pathway, enhancing ERK signaling to drive aberrant cancer cell proliferation and transformation(184). Furthermore, this mutation can potentiate its oncogenic effects through several downstream MAPK branches, promoting the upregulation of various cancer related proteins. For example: Promotes lymph node metastasis via upregulation of CXC chemokines(185);Induces vascular endothelial growth factor A (VEGFA) expression, enhancing tumor invasiveness(186);Stimulates matrix metalloproteinases (MMPs), increasing extracellular matrix degradation and invasion capability(187);Activates the urokinase type plasminogen activator (uPA) and its receptor uPAR, leading to FAK/PI3K/Akt pathway activation, which inhibits senescence and enhances cancer cell proliferation and migration(188);Increases secretion of transforming growth factor β1 (TGFβ1), indirectly suppressing sodium/iodide symporter (NIS) expression, thereby promoting thyroid cancer progression and dedifferentiation(189,190); Induces endocrine gland derived vascular endothelial growth factor (Prok1) expression(191);Upregulates hypoxia inducible factor 1α (HIF-1α) even in normoxic conditions, activating metabolism and angiogenesis related pathways(192);Modulates proteins

such as prohibitin (PHB)(193), vimentin(194), and thrombospondin 1 (TSP1)(195), collectively enhancing cancer cell metastasis and survival.

In summary, the $BRAF^{V600E}$ mutation drives tumor cell proliferation, growth, migration, invasion, and angiogenesis primarily through sustained activation of the MAPK pathway and its downstream oncogenic effectors. It represents one of the most critical driving events in the pathogenesis of several solid tumors, particularly thyroid cancer.

NF-κB Signaling Pathway

In thyroid cancer, the $BRAF^{V600E}$ mutation can activate the NF-κB signaling pathway (196,197). As a classical pro-inflammatory pathway, NF-κB has been confirmed to play a crucial role in the development and progression of various tumors. It can promote the transition from chronic inflammation to cancer or provide a tumor promoting inflammatory microenvironment (198). Studies have shown that NF-κB is significantly activated in thyroid tumors, especially in ATC, and in vitro experiments further demonstrate that its activation is closely associated with tumor cell proliferation(199,200).

Notably, studies have found that $BRAF^{V600E}$ can directly couple with the NF-κB signaling pathway, and this mechanism is independent of the traditional MEK-ERK signaling cascade (201). Moreover, in vitro experiments with MEK inhibitors have shown that their antitumor efficacy is significantly enhanced when combined with NF-κB pathway inhibition, suggesting that NF-κB activation may contribute to the increased aggressiveness of $BRAF^{V600E}$ driven thyroid cancer (202).

PI3K/Akt Signaling Pathway

The PI3K/Akt signaling pathway plays a pivotal role in the initiation and progression of thyroid cancer. In PTC, mutations in PI3K pathway related genes, such as PIK3CA, have been reported at relatively high frequencies (203). In ATC and FTC, genetic alterations involving both PI3K/Akt and MAPK pathways are more prevalent (204). Among them, Akt activation has been observed in most ATC samples harboring PIK3CA mutations(205) , indicating a critical role for this pathway in malignant progression.

Moreover, research has highlighted that alterations in PI3K/Akt pathway related genes are mutually exclusive between FTC and follicular thyroid adenoma (FTA) (206), suggesting that this pathway may be involved in distinguishing between benign and malignant tumors. PIK3CA and AKT1 mutations are relatively common in advanced thyroid cancers, particularly in metastatic or recurrent cases (207). Interestingly, AKT1 mutations had not been previously reported in thyroid cancer, highlighting a potentially novel oncogenic mechanism.

BRAF mutations play a crucial role in the development of ATC and its lymph node metastasis, whereas PIK3CA mutations tend to appear in the later stages of ATC(208) . It has been shown that mutations in PI3K/Akt pathway related genes can coexist with BRAF mutations and may act synergistically to promote the progression from PTC to ATC (209). Moreover, aberrant activation of the PI3K/Akt pathway may contribute to resistance mechanisms against BRAF/MEK targeted therapies, further supporting the oncogenic collaboration between these two pathways (210)

From a molecular perspective across different thyroid cancer subtypes, the MAPK and PI3K/Akt pathways also show a division of labor in differentiated thyroid cancers. MAPK pathway mutations are more likely to drive tumor development toward PTC, while PI3K/Akt pathway mutations are associated with the formation of FTA and FTC. With the accumulation of genetic mutations, both

pathways may be activated simultaneously, driving the evolution of tumors into poorly differentiated thyroid carcinoma (PDTC) and even ATC (211). Furthermore, additional oncogenic mutations, such as in TP53, CTNNB1, and ALK, may accelerate this malignant transformation process.

Wnt/β-catenin Signaling Pathway

The Wnt/β-catenin signaling pathway is crucial for various cancers, and its dysregulation is closely related to the process of cell proliferation, tumor progression, and prognosis. When this pathway is inactivated or key components are absent, it may contribute to tumor initiation and development(212–214). For example, elevated cytoplasmic expression of Wnt5A has been closely linked to melanoma progression and poor clinical outcomes(215), whereas increased nuclear β-catenin expression is typically associated with enhanced cell proliferation, reduced tumor size, and improved survival outcomes(216).

In ATC, three independent studies have reported nuclear localization of β-catenin in approximately 40–60% of cases, indicating the significant role of this pathway in ATC(217–219). These studies further identified mutations in β-catenin and the Axin1 gene. Two of the studies found that all β-catenin mutations were located at conserved serine/threonine residues that are normally phosphorylated by CK1 and GSK3β, resulting in increased β-catenin stability as well as its sustained accumulation within the cytoplasmic and nuclear compartments. Nuclear accumulation of β-catenin is generally associated with high tumor cell proliferation and loss of differentiation, indicating poor prognosis(217,218).

Although β-catenin is widely recognized for promoting cell proliferation, subsequent studies have noted that its expression is absent in a subset of ATC samples(220). These findings suggest that ATC is not a uniform entity but rather comprises multiple subtypes, each characterized by distinct and sometimes mutually exclusive driver mutations. For instance, some subtypes may harbor mutations primarily in the Wnt/β-catenin pathway, whereas others may feature TP53 mutations(221) or promotion of the PI3K/Akt pathway(222). These three types of alterations constitute some of the most common oncogenic events in ATC. Therefore, clarifying whether mutual exclusivity exists among key driver gene mutations is essential for more precise classification of ATC.

In contrast to β-catenin, mutations in Axin1 occur more frequently, with over half of ATC samples reported to carry mutations in the functional domains of Axin1(218). These mutations may affect its binding domains with APC, β-catenin, and Dvl, as well as its regulatory region for G proteins, thereby impairing its function as a negative regulator of β-catenin. Such discrepancies across studies may reflect genetic differences among patient populations.

Activation of the Wnt/β-catenin pathway in thyroid cancer mainly occurs via two mechanisms: disruption of cell adhesion structures and activation of upstream oncogenic signals. E-cadherin, a calcium dependent adhesion molecule, maintains cell polarity and adhesion by linking β-catenin to the cytoskeleton(223). Downregulation or functional inhibition of E-cadherin is believed to promote the release and cytoplasmic accumulation of β-catenin, thereby inducing epithelial–mesenchymal transition (EMT). In various thyroid cancers, including papillary, follicular, and anaplastic types, E-cadherin downregulation is common and may result from promoter methylation or oncogene activation, ultimately weakening cell–cell adhesion and enhancing migration and invasion capabilities(224–226). Therefore, activation of the Wnt/β-catenin pathway may stem from loss of adhesion function or be directly induced by oncogenic signals stabilizing and activating β-catenin—two mechanisms that may act synergistically in tumorigenesis and progression.

I. **1.6.3 Epigenetics**

RASSF1A represents one of the tumor suppressor genes most often epigenetically suppressed in sporadic human tumors(227,228). As a critical component of several key oncogenic signaling pathways, including Ras/PI3K/AKT, Ras/RAF/MEK/ERK, and Hippo loss of RASSF1A function is believed to play a pivotal role in the initiation and progression of various solid tumors(229,230). In FTC, RASSF1A promoter hypermethylation is negatively correlated with BRAF mutations(231,232). Moreover, higher levels of RASSF1A promoter methylation have been observed in multifocal lesions, thyroid capsule invasion, and regional lymph node involvement, suggesting its involvement in tumor progression(233,234). Furthermore, this methylation pattern has been strongly correlated with advanced stages of thyroid cancer(235), potentially influencing the progression of PTC and its transition to a more invasive phenotype via the PI3K/AKT pathway(236).

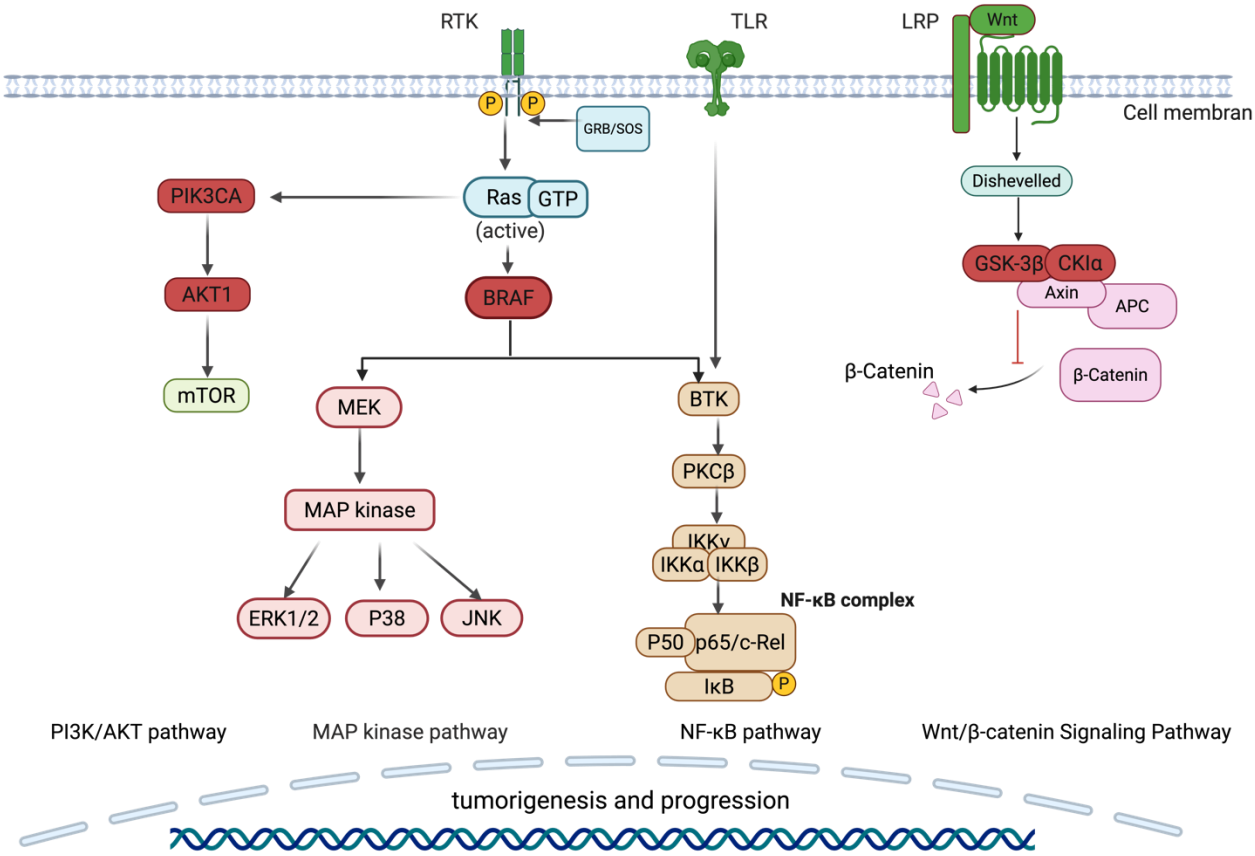
Solute Carrier Family 5 Member 8 (SLC5A8), a sodium/monocarboxylate cotransporter, is also considered a tumor suppressor. In PTC, it is frequently silenced through promoter methylation, which is inversely correlated with BRAF mutations(237). While high methylation of the RARB2 promoter has not been observed in PTC or FTC, it is significantly elevated in ATC, implying that RARB2 promoter methylation may be associated with tumor aggressiveness(238). Furthermore, its methylation level is positively correlated with $BRAF^{V600E}$ mutations(239).

SPARC related modular calcium binding protein 2 (SMOC2) is a secreted matricellular protein that enhances angiogenic factor activity(240), promotes cell cycle progression(241), and facilitates cell adhesion and migration(242). Despite generally lower SMOC2 expression in PTC compared to NT tissue, even in cases harboring BRAF mutations cellular experiments have shown the opposite trend: SMOC2 expression slightly increases in normal thyroid cell lines (Nthy-ori 3-1) following transfection with mutant BRAF. Additionally, significantly reduced SMOC2 expression occurs in lymphocytic thyroiditis and follicular neoplasms, including adenomas and carcinomas(243).

Tissue Inhibitor of Metalloproteinases 3 (TIMP3) and Death Associated Protein Kinase (DAPK) are often silenced through abnormal promoter methylation in PTC, and their methylation status is closely associated with BRAF mutations(244). Similarly, promoter methylation of PTEN is elevated in follicular tumors but nearly absent in normal thyroid tissues. This methylation pattern may be linked to alterations in genes involved in the PI3K/AKT signaling pathway(245).
In both thyroid tissues and peripheral blood of PTC patients, PTEN expression is strongly correlated with lymph node metastasis(246).

A meta-analysis revealed that high methylation levels of the promoter regions of RASSF1A, PTEN, DAPK, CDH1, and RARβ2 are significantly associated with an increased risk of thyroid cancer(247). Collectively, these findings suggest that BRAF mutations not only drive tumorigenesis through genetic alterations but may also promote thyroid cancer development and progression by orchestrating genome wide hyper and hypomethylation events that regulate oncogene and tumor suppressor gene expression(248).

Figure 7. Schematic model of signaling pathways in thyroid cancer. (BioRender.com)



I. **2. Chronic Inflammation and Thyroid Cancer**

I. **2.1 Overview of the Inflammatory Process and cancer**

Inflammation is a complex defensive response initiated by the body to restore tissue homeostasis when faced with infections, tissue damage, stress, or dysfunction (249). However, in the 19th century, Virchow was the first to suggest a connection between inflammation and cancer, based on his observation of inflammatory cells in tumor biopsies(250). Over time, cancer related chronic inflammation has come to be recognized as a key factor, with clear evidence linking chronic inflammatory processes to malignant progression in most types of cancer. The biological mechanisms underlying this relationship involve changes in the tumor microenvironment (TME) such as: the DNA damage, angiogenesis and tissue Remodeling and suppression of anti-tumor immunity(251).

Additionally, chemokines (e.g., IL-8/CXCL8) form concentration gradients to guide the recruitment and directed migration of leukocytes, mainly neutrophils and monocytes, by promoting their rolling, adhesion, and transmigration through the vessel wall to the site of inflammation(252), thereby facilitating pathogen clearance and initiating tissue repair and also will be involved in the spread the tumoral cells (253).

In the acute inflammation, resolution occurs after the elimination of the causative agents through a series of active “pro-resolving” mechanisms, including the replacement of pro-inflammatory lipid mediators with pro-resolving lipid mediators. Neutrophil recruitment and activation are inhibited, and they undergo programmed cell death (apoptosis), after which they are recognized and engulfed by macrophages to prevent the release of intracellular inflammatory mediators, reducing secondary tissue damage and promoting inflammation resolution and tissue repair. Moreover, pro-inflammatory signaling pathways such as NF-κB are negatively regulated by anti-inflammatory cytokines like IL-10 and transforming growth factor-β (TGF-β), which reduce the expression of pro-inflammatory mediators (254). In addition to soluble mediators, regulatory immune cells such as regulatory T cells (Tregs) and regulatory B cells (Bregs) produce immunosuppressive factors to maintain immune homeostasis. Endothelial cells restore barrier function to limit leukocyte extravasation, and extracellular matrix remodeling offers a scaffold for cell proliferation and tissue repair(249). However, when acute inflammation persists due to continual stimulation by pathogens, foreign bodies, toxins, or autoantigens, and cannot be fully resolved(255), dysregulation of immune homeostasis may lead to autoimmune diseases. A deficiency in pro-resolving mediators impairs macrophage mediated clearance of apoptotic neutrophils, causing the inflammatory process to "stall." Macrophages, depending on their M1 or M2 polarization state, can secrete large amounts of pro-inflammatory and pro-fibrotic factors, resulting in persistent inflammation that can last for weeks, months, or longer. This chronic state leads to repeated cycles of tissue damage and repair, activation of fibroblasts, promotion of collagen deposition, and ultimately fibrosis and irreversible tissue remodeling(256).

I. 2.2 Chronic Inflammation and Risk Factors for Thyroid Cancer

I. 2.2.1 Autoimmune Thyroid Diseases and Thyroid Cancer

Hashimoto's thyroiditis (HT) , a widespread autoimmune disease, is a key factor in the development of hypothyroidism. Its epidemiological characteristics are similar to those of thyroid tumors, both showing a marked sex difference, with significantly higher incidence in females than in males(257,258). However, male patients with TC tend to exhibit more aggressive behavior at the time of diagnosis(259). After puberty, the incidence of thyroid nodules increases significantly in children(260), and the presence of nodules in this population indicates a higher risk of PTC(261). Studies have found that children with coexisting HT and PTC are more likely to experience extrathyroidal extension, capsular invasion, and lymph node metastasis(262). HT is a T-cell mediated autoimmune disease in which aberrant T-cell attacks lead to lymphocytic and plasmacytic infiltration of thyroid tissue(263). Consequently, increasing attention has been paid in recent years to the potential link between HT and PTC, with the hope of gaining new insights into the relationship between chronic inflammation and tumorigenesis.

However, current studies on the association between HT and PTC remain inconclusive. On one hand, several studies have reported an increased risk of PTC in patients with HT(264–266), with those having nodular HT being more likely to develop PTC compared to those with non-nodular HT(267). However, other studies have found no notable correlation between HT and PTC(268,269). However, HT is only one type of “chronic thyroiditis.” Other forms of chronic inflammation or systemic inflammatory conditions may also be linked with thyroid tumorigenesis and its advancement, though the evidence is less well established than for HT(270).

I. 2.2.2 Other Chronic Inflammatory Conditions and Thyroid Cancer Risk

Subacute thyroiditis (SAT), typically triggered by viral infections in genetically predisposed individuals, primarily affects middle aged women(271,272). Though rare, SAT can coexist with PTC, and its ultrasound features may resemble malignancy, potentially masking PTC(273,274). Fine needle aspiration biopsy (FNAB) is advised for nodules with malignant potential in SAT cases(275). Beyond HT, other autoimmune diseases, such as idiopathic inflammatory myopathies, RA, SSc, pSS, and SLE, may also raise thyroid cancer risk through systemic inflammation, especially in younger women(276–279).

I. 2.3 Clinical Relationship Between Inflammation and Thyroid Cancer

I. 2.3.1 Clinical Significance of Inflammation Related Markers and Immune Cells in Thyroid Cancer

In recent years, the relationship between inflammation and tumor development has received considerable attention. Inflammatory mediators in the blood, such as neutrophils, lymphocytes, monocytes, and platelets, have become important indicators for evaluating prognosis and metastatic

risk in TC patients due to their accessibility and low cost. Inflammatory scores like neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), lymphocyte-to-monocyte ratio (LMR), and systemic immune inflammation index (SII) have demonstrated significant prognostic value in various cancers(280–282). NLR reflects the dynamic balance between systemic inflammatory response and immune defense, with higher NLR often associated with poor response to chemotherapy and immunotherapy, as well as unfavorable prognosis(283–286). PLR has also shown prognostic potential in renal cancer, lung cancer, and colorectal cancer, with platelets releasing chemokines and growth factors that may promote tumor progression(287–290). LMR reflects host immune homeostasis, and lower LMR is generally linked to worse outcomes, especially in anaplastic thyroid carcinoma and PTC(291–293). SII tends shown to be upregulated in patients diagnosed with DTC and may correlate with tumor multifocality, though its relationship with invasive tumor features remains unclear(294). Additionally, indicators such as lymphocyte and neutrophil counts(295), platelet count (PLT)(296), fibrinogen (FIB)(297), glucose-to-lymphocyte ratio (GLR)(298), prognostic nutritional index (PNI)(299), and albumin-to-globulin ratio (AGR) have also been studied for prognostic evaluation(280).

Despite efforts to establish clinical diagnostic models based on various inflammatory indicators for predicting thyroid cancer, current studies face several limitations. These include a limited range of evaluated markers, inconsistent results across studies, and a lack of large scale, multicenter research to reach consensus. Moreover, further investigation is needed to determine the optimal thresholds, key marker molecules, and the precise relationship between these inflammatory indicators and thyroid cancer prognosis and metastatic risk(300–302).

I. **2.3.2 Molecular Link between Inflammation and Thyroid Cancer**

I. **2.3.2.1 Expression of Inflammatory Cytokines and Their Association with Tumor Staging, Invasiveness, and Prognosis**

Inflammatory cytokines/factors serve a key function in orchestrating immune regulation and inflammation. These include cytokines, chemokines, and growth factors, which can exert either pro-inflammatory or anti-inflammatory effects. In the context of chronic inflammation, these factors also contribute to tumor initiation, progression, metastasis, and immune evasion, thereby exhibiting significant tumor promoting properties(303).
In thyroid cancer, cytokines, particularly interleukins (ILs), are considered critical in tumorigenesis and disease progression. These molecules not only directly promote tumor cell proliferation and invasion but also facilitate tumor advancement and immune escape by inducing angiogenesis and suppressing anti-tumor immune responses. Studies have shown significantly altered serum levels of IL-1, IL-6, IL-8, and IL-27 in patients with thyroid cancer(304–308), suggesting their potential as biomarkers for assessing malignancy and prognosis (305).

I. **2.3.2.2 Molecular Mechanisms Linking Inflammation and Thyroid Cancer**

Numerous previous studies have enriched the understanding of the interplay between inflammation and tumors. Dysregulation of inflammatory molecules and pathways is closely associated with tumorigenesis. Under the influence of pro-inflammatory cytokines such as interleukin 6 (IL-6), tumor

necrosis factor α (TNF-α), and epidermal growth factor (EGF), signaling pathways including nuclear factor-κB (NF-κB), AKT/mTOR, and MAPK are activated, or autophagy is regulated to affect cellular metabolic reprogramming, thereby promoting thyroid cancer cell survival, proliferation, invasion, and metastasis.

Oxidative Stress

Cells generate reactive oxygen species (ROS) using oxygen, which are categorized into two types: free radicals, such as superoxide anion (O2-) and hydroxyl radical (OH-), and non-radical molecules like hydrogen peroxide (H2O2). These ROS are essential for many biological functions, including intracellular signal transduction, control of apoptosis, transcriptional activity, immune defense, and endocrine function(309). Normally, the redox regulatory system in the human body controls and balances ROS homeostasis to prevent oxidative stress(310). Oxidative stress can affect multiple signaling pathways related to cell proliferation(311), and ROS can influence tumorigenesis and progression through mechanisms such as DNA damage, regulation of apoptosis, and autophagy(312).

In thyroid cancer, redox imbalance has been observed in the blood(313), increased oxidative stress(314), and an imbalance of oxidative/antioxidative systems in tissues(315). Cellular experiments have also found reduced expression of reductases, which is associated with elevated free radical levels in tumor tissues(316,317). Taken together, the evidence points to an association between oxidative stress and thyroid malignancy.

Oxidative DNA Damage

Oxidative stress plays a central role in inducing DNA damage that facilitates tumorigenesis(318). Direct interactions between ROS and DNA may result in oxidative base damage in DNA(319). In mouse models, elevated oxidative DNA damage in the thyroid promotes somatic mutations within the thyroid(320). Compared to normal tissues, higher oxidative DNA damage has been found in FTC and PTC, potentially related to malignant progression of thyroid cancer(321). Dysregulation of related signaling pathways has been identified in malignant thyroid tumors(322,323). Studies suggest oxidative DNA damage may lead to BRAF gene mutations(324) and RET/PTC rearrangements(325). Analysis of TCGA data shows that thyroid peroxidase (TPO) related genes are differentially expressed among thyroid tumors of various differentiation statuses, with lower expression in tumors harboring the $BRAF^{V600E}$ mutation, suggesting a link to PTC tumor differentiation(326).

Interleukins

Abnormal proliferation of tumor cells is a key step in tumor initiation and progression. Studies have shown that interleukins play an important role in regulating the proliferation of thyroid cancer cells.

The IL-1 family includes the pro-inflammatory cytokines IL-1α and IL-1β, as well as the inhibitory cytokine IL-1 receptor antagonist (IL-1ra). IL-1α and IL-1β exert their functions by binding to type I IL-1 receptor (IL-1R), thereby activating downstream signaling pathways and inducing immune and inflammatory responses(327). IL-1 exerts a bidirectional regulatory effect on thyroid cancer cell proliferation, with variations observed among different cell lines(328–332). $CD4^+$ T cell subsets can

release IL-22, which binds to its receptor and activates multiple signaling pathways, including JAK/STAT3 and MAPK, as well as transcription factor regulation, thereby promoting tumor progression(333,334).

In addition, several interleukins can induce epithelial mesenchymal transition (EMT) in thyroid cancer cells, enhancing their invasiveness.As a pro-inflammatory chemokine, IL-8 binds to its receptor and induces EMT through the IL-8–Akt–Slug pathway, thereby promoting tumor invasion and progression(335). IL-11, through its receptor, can promote the migration, invasion, and EMT process of ATC cells via the PI3K pathway, possibly driving their high metastatic potential(336). In PTC, high expression of IL-13 receptors upregulates EMT-related molecules such as N-cadherin, Vimentin, and Snail, thereby enhancing cell migration and invasion. Knockdown of IL13Rα2 significantly reduces the invasiveness of PTC cells, suggesting that IL-13 may participate in thyroid cancer progression by regulating EMT, although the detailed mechanism requires further investigation(337,338).
IL-17 may be closely linked with the invasiveness of thyroid cancer through its pro-angiogenic effects(339). It can also inhibit tumor immune escape by suppressing the PD-L1 pathway(340). IL-10, on the other hand, promotes tumor immune escape through its immunosuppressive effects, thereby affecting thyroid cancer aggressiveness(341). IL-4 exerts a weak stimulatory effect on thyroid cancer cell proliferation. Its tumor promoting effect is linked with the upregulation of the Bcl-2 and the slight downregulation of the Bax, resulting in inhibition of apoptosis(342), and conferring resistance to thyroid cancer cells against the toxic effects of chemotherapy drugs(343).

Chemokines

Chemokines are a type of cytokine that guide cell movement and are key players in cancer development and spread. By binding to specific receptors, they help control tumor cell migration, invasion, and metastasis(344). They also affect how immune cells behave within the tumor environment. Studies have linked chemokines to lymph node metastasis in several cancers(345). Inflammatory cytokines like IL-6 and TNF-α can increase chemokine expression(346,347), which may trigger EMT, making tumor cells more invasive(348). Chemokines also influence immune cell infiltration and responses to immunotherapy, making them important targets in cancer diagnosis and treatment(349).

Within the CXC family, CXCR1 and CXCR2 share ligands like CXCL1, CXCL6, and CXCL8. These receptors are found in immune cells and thyroid cancer tissues, suggesting a role in tumor behavior. One study found that CXCR1 is overexpressed in PTC and linked to lymph node metastasis(350). However, other research showed no clear relationship between CXCR1/2 levels and clinical features(351). CXCL5, a CXCR2 ligand, can promote PTC cell migration and EMT, supporting a role for CXCR2 in tumor progression(352).

CXCR3 binds to interferon inducible ligands (CXCL9, CXCL10, and CXCL11) and was first studied in autoimmune thyroiditis(353–355). More recently, CXCR3 has been shown to be overexpressed in TC, especially PTC, and may aid metastasis by regulating immune responses and cell migration(356). Immunohistochemistry reveals higher CXCR3 levels in PTC tissues and tumor infiltrating lymphocytes, indicating a role in immune regulation. CXCR3 is also elevated in some thyroid lymphomas and linked to poor outcomes(357).

CXCR4, one of the earliest cancer related CXC receptors, promotes tumor growth by preventing cell death and supporting migration(358–360). High CXCR4 expression in TC is linked to lymph node metastasis, advanced stage, and poor prognosis, making it a promising therapeutic target(361).

CXCR7 binds to CXCL12 and CXCL11, supporting tumor cell adhesion, migration, and survival(362). In PTC, CXCR7 is associated with lymph node metastasis(363). It can also drive tumor progression through effects on the cell cycle and VEGF related angiogenesis(364,365).

The CXCL12/CXCR4/CXCR7 axis plays a major role in TC progression(366). Both CXCR4 and CXCR7 are highly expressed in thyroid tumors and promote invasiveness more than proliferation(367). In medullary thyroid carcinoma, this axis may regulate EMT(368). In FTC, high CXCR4/CXCR7 levels are found in distant metastases and are linked to worse survival. CXCL12 promotes FTC cell growth and EMT, while CXCR4 inhibitors reduce invasion(369).

CCR3, a CC chemokine receptor, binds to ligands like CCL5, CCL11, and CCL24. It is upregulated in PTC, showing increased levels in tumors relative to normal tissue (370), but it is not clearly associated with lymph node metastasis, suggesting a role in tumor growth rather than spread(371).

CCR6, the receptor for CCL20, is involved in both immune regulation and cancer cell movement. Its role in thyroid cancer is still being explored.

Epidermal growth factor (EGF)

Epidermal growth factor (EGF) exerts its function by binding to the epidermal growth factor receptor (EGFR). EGFR is a transmembrane glycoprotein with intrinsic tyrosine kinase activity that participates in signal transduction pathways regulating various cellular processes, such as growth, differentiation, and apoptosis. Several growth factor ligands have the ability bind to and activate EGFR, including EGF and transforming growth factor-alpha (TGF-α)(372).

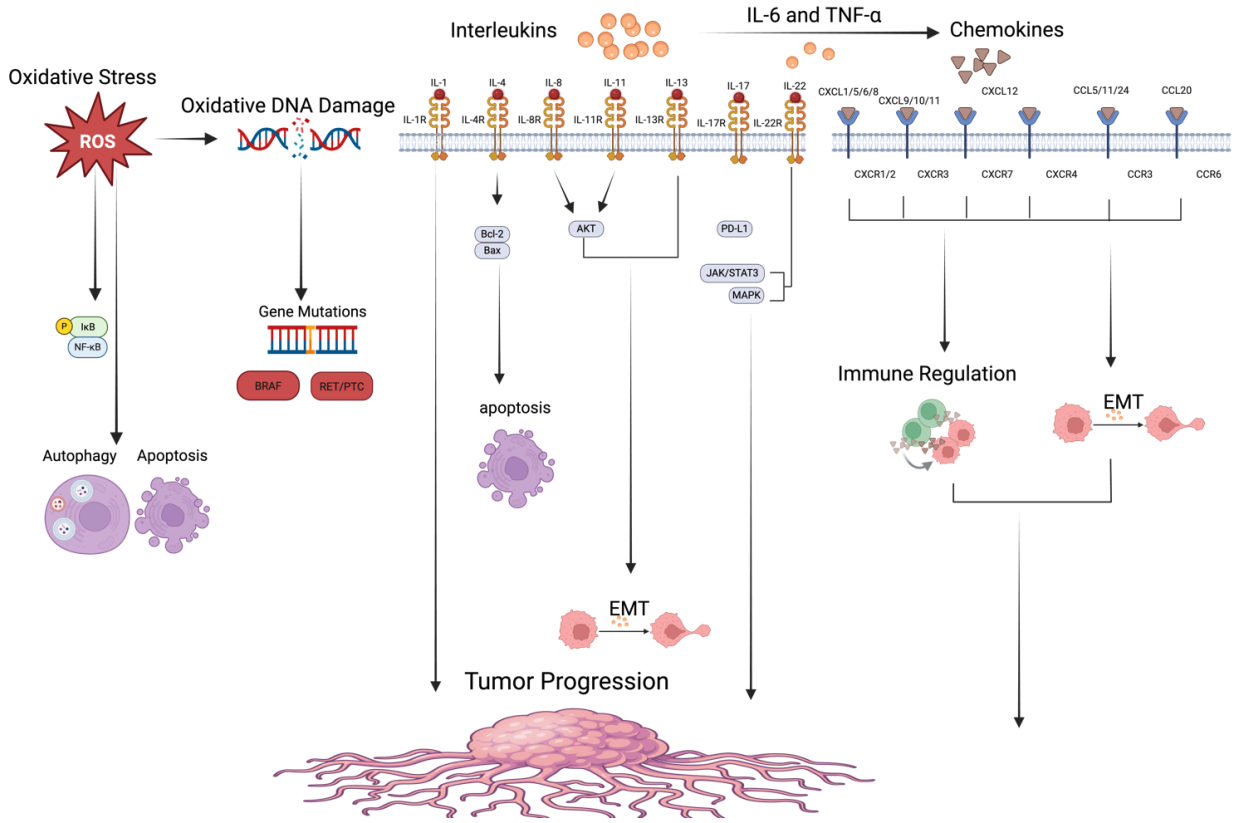
Upon activation, EGFR triggers phosphorylation and downstream signaling through multiple cascades, including the PI3K/AKT/mTOR pathway, RAS/MAPK, STAT, and NF-κB pathways(373), promoting cell proliferation and survival(374). In thyroid tumors, EGFR is frequently overexpressed(375), and its expression is associated with the proliferation and metastasis of thyroid cancer(376,377), aggressive tumor behavior, and TNM staging(378–380). In cases of ATC, studies have reported a correlation between EGFR expression and poor clinical outcomes(381). EGFR expression is also related to tumor dedifferentiation(382); however, its role as a prognostic marker for recurrence in PTC remains controversial(383,384). Although anti-EGF or anti-TGFα antibodies show limited effects on thyroid cancer cell proliferation(385), EGFR tyrosine kinase inhibitors (TKIs) can induce apoptosis in ATC cell lines(386).

TNF-α

TNF-α is another key pro-inflammatory cytokine, mainly secreted by adipose tissue and inflammatory cells. Although TNF-α can induce apoptosis, under chronic inflammatory conditions, it may instead promote tumorigenesis by upregulating granulocyte colony stimulating factor (G-CSF), interleukins,

prostaglandins, and other vasoactive mediators(387,388), enhancing the expression of VEGF(389), as well as adhesion molecules such as ICAM-1, E-selectin, and VCAM-1, thereby facilitating tumor metastasis(390). Elevated TNF-α expression has been observed in patients with DTC, and its levels decrease following ^{131}I treatment, indicating its potential as a diagnostic biomarker(391). However, some studies report no association between TNF-α and thyroid cancer risk(392), whereas meta-analyses suggest a strong link between the two(393). This discrepancy may be due to the dual role of TNF-α, which might contribute to tumor initiation but decline as the tumor progresses, coinciding with increased TGF-β expression that promotes EMT and enhances tumor cell migration(394), while diminishing the immune system's anti-tumor capacity. Therefore, further clinical and mechanistic studies are warranted to clarify these roles(395).

Figure 8. Schematic model of AdipoRon regulating inflammatory downstream effects. (BioRender.com)



Adipokines

Adipose tissue is recognized both for its capacity to store energy and its essential endocrine functions like the metabolism regulation, and endocrine signaling. In recent years, studies have shown that adipose tissue secretes a variety of bioactive molecules collectively known as adipokines, including leptin, APN, resistin, TNF-α, and IL-6, among others. These adipokines play key roles in numerous physiological processes, such as inflammatory responses, insulin sensitivity, and cell apoptosis(396).

Under normal conditions, adipose tissue maintains homeostatic functions. However, in obesity, adipocytes increase in size and number, immune cell infiltration intensifies, and the local microenvironment becomes disrupted. These changes result in abnormal adipokine secretion, leading to chronic low-grade inflammation. More importantly, adipose tissue dysfunction has been closely linked to the development of various diseases, particularly cancer. Aberrantly secreted adipokines can influence the tumor microenvironment through autocrine, paracrine, and endocrine mechanisms, thereby promoting tumor cell proliferation, migration, angiogenesis, and immune evasion(397).

These alterations in adipose tissue provide a possible mechanistic link between metabolism and cancer development and offer potential targets for cancer prevention and therapy. Therefore, in depth investigation of the endocrine functions of adipose tissue and its association with tumorigenesis is of great significance in understanding the mechanisms underlying obesity related cancers.

Resistin

Resistin is a small adipokine encoded by the RETN gene(398). In humans, resistin is primarily secreted by peripheral mononuclear cells(399), and its expression is closely associated with immune cell infiltration in adipose tissue(400). Increasing evidence suggests that resistin may act as a critical link between obesity and tumorigenesis by mediating chronic low grade inflammation, thereby promoting tumor initiation and progression(401).

At the molecular level, resistin regulates tumor related biological behaviors through multiple signaling pathways. It can activate TLR4, PI3K, and NF-κB pathways, inducing the expression of pro-inflammatory cytokines such as TNF-α and IL-6, thus amplifying inflammatory responses(402,403). These pro-inflammatory cytokines further activate the JAK/STAT and MAPK (including ERK) signaling cascades, promoting cancer cell proliferation, differentiation, and metastasis(404). Activation of the ERK pathway upregulates SOCS3, a negative regulator of the JAK2/STAT3 signaling, causing G1 phase cell cycle arrest and enhancing tumor cell drug resistance(405).

Furthermore, resistin can increase the expression of the chemokine SDF-1 (CXCL12) via MAPK and NF-κB pathways(406). SDF-1 interacts with its receptors CXCR4 and CXCR7 to facilitate tumor cell migration and angiogenesis(407). Resistin also activates the PI3K/AKT pathway, leading to phosphorylation and inactivation of pro-apoptotic proteins, thereby promoting cell survival(408). Regarding adhesion molecules, resistin upregulates ICAM-1 and VCAM-1 expression through NF-κB signaling, enhancing tumor cell adhesion and metastatic potential(409,410).

In addition to TLR4, studies have identified cyclic AMP activated protein 1 (CAP1) as receptor for resistin. CAP1 activation is closely linked to cell migration and invasion, and its high expression in various tumors correlates with increased metastatic risk and poor prognosis(411–413).
However, the specific roles and mechanisms of resistin in thyroid cancer remain to be further elucidated.

Leptin

Leptin is a circulating hormone mainly secreted by adipose tissue, playing a key role in regulating energy balance and body weight. By interacting with its hypothalamic receptors, leptin regulates energy storage and promoting satiety(414). Obese individuals often exhibit leptin resistance along with elevated leptin levels(415). Additionally, leptin activates intracellular signaling pathways such as AKT/PI3K and ERK/MAPK, which regulate the cell cycle, cell proliferation, migration and invasion, stimulate angiogenesis, and suppress anti-inflammatory cytokines(416).

Overexpression of leptin and its receptor (OB-R) has been observed in various cancers (417–420), including thyroid cancer, where elevated serum leptin levels have been reported(421) and linked to tumor staging(422), indicating a higher risk of occurrence(423). In PTC, leptin and OB-R expression levels are higher and associated with tumor invasiveness(424). Cell experiments show that leptin stimulates proliferation and inhibits apoptosis through activation of the PI3K/AKT signaling pathway(425), enhancing migratory phenotypes and promoting metastasis(426).

I. **3. Overview of Adiponectin**

Adiponectin (APN) is a multifunctional secretory protein primarily produced by differentiated mature adipocytes(427). In addition, other cell types such as cardiomyocytes, skeletal muscle cells, and lymphocytes also express and secrete APN(428–431). In humans, APN is encoded by the *ADIPOQ* gene located on chromosome 3q27. Structurally, APN contains an N-terminal signal peptide region, a variable region, a collagenous domain, and a C-terminal globular domain(382). The C-terminal globular domain is the main functional region of APN and can be cleaved to produce an independently active form called globular APN.
APN is synthesized as a monomer in adipocytes and then assembles into multimers. In the bloodstream, it exists mainly in three isoforms: low molecular weight (LMW) trimers, middle molecular weight (MMW) hexamers (two trimers), and (HMW) multimers composed of multiple trimers with a molecular weight exceeding 250 kDa. The HMW form is considered to have the strongest biological activity(432). Different isoforms exert diverse biological effects in different tissues by activating specific signaling pathways for example, the trimeric APN activates AMP activated protein kinase (AMPK) in skeletal muscle(433), whereas HMW APN can induce activation of the NF-κB pathway(434). Studies suggest that the HMW isoform mainly mediates pro-inflammatory effects, while the trimeric form is more associated with anti-inflammatory actions(435).

Moreover, globular APN is a cleaved form generated by proteolysis of the C-terminal domain, present at lower concentrations in plasma(436). This form is produced by neutrophil elastase

cleavage, with a molecular weight around 18–25 kDa, and also shows significant metabolic regulatory functions in various tissues(437).

I. **3.1 Regulation of APN Secretion**

The expression of APN is controlled by a variety of transcription factors, with the promoter region containing several active binding sites, including PPAR response elements, C/EBP binding sites, FOXO regulatory elements, and E-box sequences(438,439). Peroxisome proliferator activated receptor gamma (PPARγ), highly expressed in adipose tissue, acts as a positive regulator of APN expression. Its ligands, especially thiazolidinediones (TZDs), significantly promote APN expression and secretion(440–442).

Conversely, inflammatory factors such as reactive ROS, TNF-α, and IL-6 negatively regulate APN expression by suppressing promoter activity(443,444). In obesity, APN expression is commonly downregulated, and its serum levels are negatively correlated with insulin sensitivity. Insulin resistance promotes the release of inflammatory and vascular factors, further inhibiting APN gene expression(445–447).

The function of APN depends on its multimeric forms, post-translational modifications are critical for its activity regulation. A conserved cysteine residue at the N-terminus plays a key role in forming intermolecular disulfide bonds necessary for multimerization(448). Studies have shown that in diabetic patients, succinylation of this cysteine blocks disulfide bond formation, inhibiting multimerization and reducing plasma APN levels(449). Additionally, hydroxylation and glycosylation targeting multiple conserved lysines in the collagen region also affect multimer formation(450–452).

Certain endoplasmic reticulum (ER) molecules regulate APN multimerization and secretion. ERp44 forms disulfide bonds with APN cysteines, retaining multimers in early secretory compartments, while Ero1-Lα releases ERp44 bound APN, facilitating secretion of HMW forms(453,454). DsbA-L acts as a molecular chaperone that promotes APN multimerization in adipocytes and alleviates ER stress induced secretion impairment via an autophagy dependent mechanism, thereby enhancing HMW APN release(455). Mutations in the *ADIPOQ* gene can also disrupt APN multimerization, leading to secretion defects(456,457).

I. **3.2 APN Receptors and Mechanisms**

APN exerts its biological effects through membrane receptors, with different oligomeric forms showing tissue specific targeting. Current research identifies three main receptors: AdipoR1, AdipoR2, and T-cadherin(458,459). AdipoR1 is predominantly expressed in skeletal muscle, while AdipoR2 is mainly found in the liver. Structurally and functionally, AdipoR1 preferentially binds globular APN, whereas AdipoR2 primarily recognizes full length APN(460).

Both receptors possess seven transmembrane domains with intracellular C-termini and extracellular N-termini, containing zinc-binding catalytic sites on the cytoplasmic side of the plasma membrane(461). The globular domain of APN binds to the extracellular portion of these receptors, triggering downstream signaling pathways in target tissues such as skeletal muscle, liver, heart,

kidney, and pancreas(462). Key downstream molecules activated include AMPK, p38 mitogen activated protein kinase (p38 MAPK), and PPARα, which play crucial roles in glucose and fatty acid metabolism(461).

The adaptor protein APPL1 directly binds the intracellular regions of AdipoR1 and AdipoR2, regulating and initiating these signaling cascades(463). Although T-cadherin lacks an intracellular domain, it can bind MMW and HMW APN forms on the cell surface, clustering these highly active isoforms to enhance AdipoR1/2 binding and synergistically promote APN signaling(464).

Degradation mechanisms of APN remain incompletely understood. Besides exocytosis, APN multimers may be retained and degraded within the ER. ER stress induces autophagy activation, and APN itself promotes autophagy via AMPK dependent signaling. Additionally, suppression of MEK/ERK1/2 signaling in adipocytes leads to a significant reduction in intracellular and secreted APN, suggesting this pathway is involved in APN degradation regulation(465–467).

Figure 9. Schematic model of Overview of APN downstream effects. (BioRender.com)

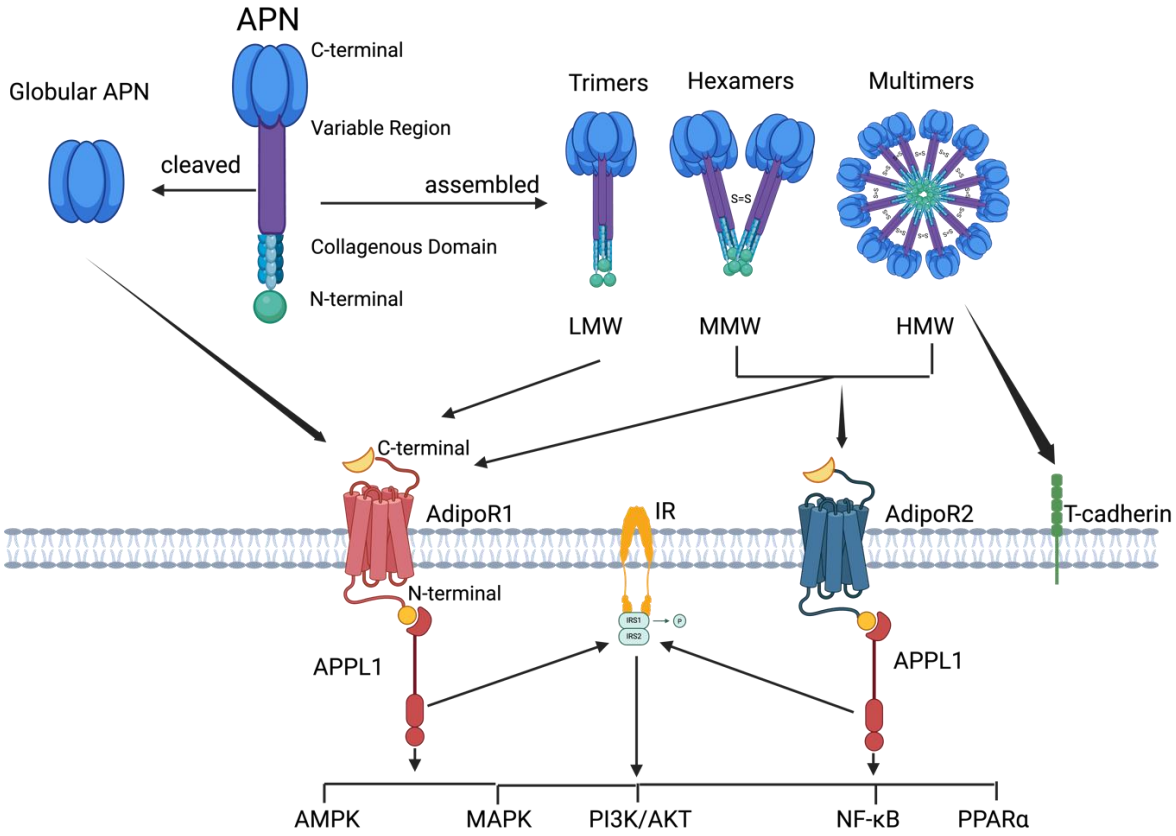


Figure 9. present schematic models summarizing the molecular and cellular pathways involved in APN signaling and their roles in cancer.

I. **3.3 Biological Functions and Therapeutic Potential**

APN exhibits multiple biological effects, including anti-inflammatory, anti-diabetic, insulin sensitizing, cardioprotective, anti-atherosclerotic, and immune regulatory functions(468–474). Therefore, developing agonists that activate APN receptors and their downstream signaling is considered a promising strategy for treating cardiovascular and metabolic diseases(475).
Studies show that insulin resistance and type 2 diabetes patients commonly have decreased APN levels(476). Supplementation with recombinant APN improves insulin resistance(477,478). In animal models, elevating APN levels alleviates insulin resistance induced by high fat, high sugar diets(479) and reduces hypertension induced by high salt diets(480). These findings highlight the significant potential and application prospects of APN in preventing and treating cardiometabolic disorders.

I. **4. APN and Thyroid Cancer**

APN is an adipocyte derived hormone with well-established roles in glucose regulation, lipid metabolism, anti-inflammatory response, and anti-tumor activity. Increasing evidence suggests that APN may influence the development and progression of various cancers, including TC

I. **4.1 Clinical Correlation between Serum APN Levels and Thyroid Cancer**

Despite there are few studies to analyzed APN and TC development. One studies found relationship between in APN levels in the serum of patients with various types of thyroid cancer were significantly reduced in both sexes(481) and a multicenter prospective study further indicated a negative correlation between serum APN levels and thyroid cancer risk in women(482). In both studies, the authors suggesting that this association may reflect the long term effects of low APN exposure(481,482). However, APN levels alone were not significantly correlated with tumor malignancy, size, or clinical stage(483,484).

Further research revealed that reduced APN levels were associated with the HOMA index, a marker of insulin resistance and metabolic syndrome. Interestingly, when APN was analyzed in combination with IGF-1 and its binding protein IGFBP-3, an independent correlation with tumor size emerged(485). Additionally, no correlation between APN levels and medullary thyroid carcinoma has been identified to date(486); whereas in differentiated thyroid cancers such as papillary and follicular types, serum APN levels were lower in patients compared to those with benign thyroid diseases or healthy controls(487).

Moreover; Meta-analyses have shown that obesity related indicators are significantly associated with the risk of developing papillary, follicular, and anaplastic thyroid carcinomas(488), suggesting that adipokines may play a potential role in thyroid cancer pathogenesis(489).

Despite the preliminary evidence suggesting a role of APN in thyroid cancer development, current studies face certain limitations, current studies on the clinical relevance of serum APN in thyroid cancer require further validation through large scale, systematic investigations, existing findings

suggest that APN, as an inflammation related factor derived from adipose tissue, may play a key role in the initiation and progression of thyroid cancer and thus warrants deeper exploration(490).

I. **4.2 APN Receptors (AdipoR1 and AdipoR2) in Thyroid Tissue (TC)**

The expression levels of AdipoR1 and AdipoR2 have been shown to exhibit tumor type specific associations with clinical parameters. In prostate cancer, these receptors are found to be downregulated (491). In contrast, both receptors have been detected in gastric(492,493) and lung cancers(494), where they are expressed in malignant tissues but are either absent or expressed at significantly lower levels in normal tissues. Notably, in gastric cancer, AdipoR1 appears to be more closely associated with patient prognosis than AdipoR2(493). In endometrial adenocarcinoma, decreased AdipoR1 expression has been linked to tumor invasion and lymph node metastasis(495), whereas in lung cancer, AdipoR2 is primarily expressed in non-small cell lung cancer, with its high expression correlating with advanced clinical staging. Similar findings have been reported in esophageal adenocarcinoma(496) and colorectal cancer(497), where AdipoR2 expression may be associated with lymphatic metastasis.

To date, studies investigating the expression of APN receptors in TC are limited. Existing evidence suggests that APN receptors are expressed in human thyroid tissues and are downregulated in thyroid tumor tissues compared to normal controls(498). Interestingly, a study by *Cheng et al*. further demonstrated that AdipoR1 and AdipoR2 are overexpressed in PTC tissues relative to adjacent non tumorous tissues(499). Moreover, the differential expression of these receptors in thyroid tumors has been linked with tumor invasiveness and clinical staging, indicating their potential as favorable prognostic biomarkers in TC.

These inconsistencies may reflect the tissue specific expression patterns of AdipoR1 and AdipoR2, as well as their involvement in distinct signaling pathways that mediate tumorigenesis differently across cancer types. Nevertheless, insufficient comprehensive studies examining the correlation between APN receptors and clinical features in thyroid cancer, particularly in the context of progression from differentiated to poorly differentiated subtypes. This transformation is often driven by the accumulation of genetic alterations, including BRAF ^{V600E}, RAS, or RET/PTC rearrangements, in combination with TERT promoter or TP53 mutations. Therefore, investigating the link between serum APN levels, APN receptor expression, and the clinical status of thyroid cancers with varying degrees of differentiation holds significant value. Such research may offer valuable insights for the development of novel targeted therapies for TC.

I. **4.3 Anti-tumor Mechanisms of APN**

APN exerts a variety of biological functions, including metabolic regulation, anti-inflammation, and cytoprotection, by binding to its receptors and activating several signaling pathways(500,501). The N-terminal intracellular region of APN receptors can interact with the adaptor protein APPL1, mediating downstream signal transduction. Among these pathways, AMPK and PPAR-α are two key effectors of APN signaling. The AMPK pathway is activated under conditions of energy deficiency to promote ATP production and inhibit energy consumption, thus maintaining cellular energy

homeostasis(502,503). APN primarily activates AMPK through AdipoR1, while stimulation of PPAR-α is mainly mediated via AdipoR2(504).

Studies have shown that trimeric forms of APN significantly activate the AMPK pathway via AdipoR1(505,506), while hexameric or HMW forms predominantly act on AdipoR2, activating PPAR-α. However, inhibition of either receptor alone cannot fully block downstream signaling, suggesting potential functional compensation between AdipoR1 and AdipoR2(507).

Activation of AMPK can upregulate tumor suppressor genes such as p53 and p21, leading to cell cycle arrest and apoptosis(508). Additionally, APN can inhibit Akt activity through the LKB1-AMPK axis, thereby blocking protein synthesis and cell growth in tumor cells, promoting autophagy, and counteracting the proliferative effects of the PI3K/Akt/mTOR pathway(509–512). Some studies suggest the existence of a negative feedback regulation within this pathway: APN may promote TSC2 phosphorylation by inhibiting Akt, which indirectly offsets AMPK-mediated mTOR suppression(513).

In terms of anti-inflammatory and anti-tumor effects, APN related pathways can also activate tumor suppressive factors such as protein phosphatase 2A (PP2A)(514,515) and S1P, thereby inhibiting pro-inflammatory and pro-tumor signaling pathways like JNK/STAT3(516) and NF-κB, reducing the expression of inflammatory factors in the tumor microenvironment, and suppressing tumor migration and angiogenesis (517). For instance, JNK is a key component of the MAPK cascade, playing an important role in regulating cell proliferation, apoptosis, and metabolic reprogramming (e.g., the Warburg effect) (518,519). APN can enhance JNK signaling and induce caspase-3 mediated apoptosis. The STAT3 pathway promotes tumor progression by supporting cell proliferation, inhibiting apoptosis, and suppressing anti-tumor immunity(516,520); however, APN or its receptor agonists can inhibit STAT3 signaling, antagonizing the tumor promoting effects of leptin(521).

Notably, APN may exhibit tissue specific dual roles in different tumors. For example, in hepatocellular carcinoma, APN has been shown to promote phosphorylation of BCKDK at Ser31, which further activates the ERK1/2 pathway and enhances tumor cell proliferation and migration(522).

Additionally, in some estrogen dependent tumors, APN can interfere with hormone signaling driven cancer cell proliferation by regulating the expression of ERα and its downstream targets(523,524). In breast cancer, APN suppresses the Wnt/β-catenin signaling pathway, thereby affecting the transcription of proliferation associated genes like cyclin D1(525). Meanwhile, the ubiquitin proteasome system (UPS), which regulates protein degradation, cell cycle, and apoptosis, is also involved: APN downregulates the expression of the deubiquitinating enzyme USP2 in tumor cells, promoting the degradation of cyclin D1 and exerting anti-tumor effects(526). Moreover, APN induces cell cycle arrest and apoptosis in cancer cells by upregulating pro-apoptotic protein Bax and downregulating anti-apoptotic protein Bcl-2(527).

This adipokina can also exert antitumor effects through indirect mechanisms by modulating various bioactive molecules, including other adipokines (such as leptin and resistin), inflammatory cytokines (such as TNF-α and IL-6), extracellular matrix components, pro-angiogenic factors (such as vascular endothelial growth factor, VEGF), and metabolic regulators such as insulin and IGF-I, thereby

contributing to insulin sensitization, immune modulation, and inhibition of tumor angiogenesis(528,529).

Notably, serum ANP levels have been found to correlate with IGF-I and its binding protein IGFBP-3 in thyroid cancer tumor volume, suggesting a potential link between APN and thyroid cancer via the IGF signaling axis. Dysregulation of the IGF pathway has been implicated in various malignancies, including breast cancer (530), pancreatic cancer (531), and bladder cancer(532), and is generally associated with poor prognosis in thyroid cancer(533,534). As a mitogenic factor, IGF-I is functionally regulated by IGFBP-3. Upregulation of IGF-I or downregulation of IGFBP-3 may promote tumor cell proliferation(535).

Currently, studies on the biological effects of ANP in TC cells remain limited. Mitsiades et al. reported that recombinant APN had no significant effect on the proliferation and apoptosis of TC cells(536). However, subsequent studies by Nigro et al. demonstrated that APN alone could inhibit thyroid cancer cell proliferation and migration(537). Additionally, other research has shown that APN can activate the AMPK phosphorylation pathway in K1 and BCPAP cell lines(538). More recent studies revealed that recombinant human APN can suppress proliferation and migration of PTC cells, potentially via the autophagy pathway mediated by ADIPOR2(539).

In summary, APN regulates a complex antitumor signaling network by activating multiple pathways, including AMPK, PI3K/Akt/mTOR, and STAT3/NF-κB. It plays an important role in the development of endocrine related tumors and represents a promising therapeutic target worthy of further investigation.

I. **4.4 APN and analogs (AdipoRon) as a therapeutic approach in Thyroid Cancer**

APN regulates multiple physiological processes through its receptors AdipoR1 and AdipoR2, including energy metabolism, inflammatory responses, and apoptosis. Given APN’s multifaceted anti-tumor effects, there has been active research in developing small molecule agonists that can mimic its biological functions. AdipoRon, an orally active small molecule APN receptor agonist, can simultaneously activate both AdipoR1 and AdipoR2, thereby inducing downstream AMPK signaling pathways and exerting biological effects similar to those of APN. Its molecular design ensures good stability and bioavailability, making it an ideal candidate drug for APN function replacement and treatment of related metabolic diseases(540). In the field of metabolic diseases, AdipoRon has demonstrated significant anti-diabetic and anti-obesity effects, improving insulin resistance and chronic inflammatory states(541,542). In recent years, increasing studies have focused on the anti-tumor potential of AdipoRon. Multiple preclinical models have shown that AdipoRon can regulate tumor cell proliferation, apoptosis, and autophagy by activating AMPK and inhibiting the PI3K/Akt/mTOR pathway. Additionally, it modulates the tumor microenvironment by suppressing the expression of pro-inflammatory factors and angiogenic factors, thereby inhibiting tumor growth and metastasis. Relevant research has made positive progress in various tumor models including breast cancer(543), ovarian cancer(544), and pancreatic cancer(545), demonstrating its broad anti-tumor applicability.

The role of AdipoRon in TC has yet to be clearly defined. Existing studies have confirmed that APN receptors AdipoR1 and AdipoR2 are expressed on the surface of TC cells, and AdipoRon exerts anti-tumor effects through activation of these receptors(546,547). Cellular experiments indicate that AdipoRon significantly inhibits TC cell, colony formation, migration, and invasion, while promoting tumor cell differentiation, suggesting its potential therapeutic value. Mechanistically, AdipoRon mainly acts through AdipoR2, promoting the expression and phosphorylation activation of the key autophagy regulator ULK1. Activation of ULK1 enhances cellular autophagy levels, thereby inhibiting tumor growth. It is noteworthy that ULK1 regulation involves multiple phosphorylation sites; studies have also found that the Ser638 site of ULK1 is phosphorylated after AdipoRon treatment, indicating a complex regulation of its activity. Moreover, the expression changes of autophagy related marker proteins such as LC3B at different time points reflect the dynamic nature of the autophagy process and the complex interplay of multiple signaling pathways. Overall, AdipoRon effectively restricts the malignant behavior of thyroid cancer cells by activating autophagy pathways(548), providing important theoretical support for the clinical application of APN receptor agonists in thyroid cancer.

Although autophagy is considered a key mechanism underlying AdipoRon’s anti-tumor effects, current studies suggest its roles extend beyond this. In other tumor models, AdipoRon can induce tumor cell growth arrest by delaying or blocking the G0/G1 phase of the cell cycle, but whether it directly mediates cell death through cytotoxicity remains unclear(544,545). Notably, several tumor cell experiments have observed increased expression of apoptosis related proteins following AdipoRon treatment; however, the precise apoptotic mechanisms remain controversial. For example, in pancreatic cancer, although an increase in Annexin V positive cells suggests apoptosis occurrence, caspase dependent apoptosis is not the primary mode of cell death(544,545). Besides apoptosis, AdipoRon has also been shown to induce other forms of cell death, such as RIPK1/ERK dependent necroptosis. Regarding the AMPK signaling pathway, AdipoRon activates AMPK and its downstream target acetyl-CoA carboxylase (ACC), but does not exert its effects through the mTOR pathway(544). Furthermore, in other tumor models, AdipoRon regulates multiple AMPK dependent and independent signaling pathways, for example inhibiting STAT3 signaling while activating the ERK1/2 pathway(545), indicating a complex and diverse mechanism of anti-cancer action. The specific mechanisms of AdipoRon in thyroid cancer remain to be further elucidated.

Considering the close association between overweigh/obesity, metabolic abnormalities, and thyroid cancer development, AdipoRon, as a small molecule APN receptor agonist, shows dual potential in regulating tumor metabolism and promoting cellular autophagy, offering new avenues for its application in thyroid cancer therapy. Moreover, AdipoRon holds promise as an innovative therapeutic strategy targeting obesity related tumors.

Figure 10. Schematic model of AdipoRon anti-tumor mechanisms. (BioRender.com)

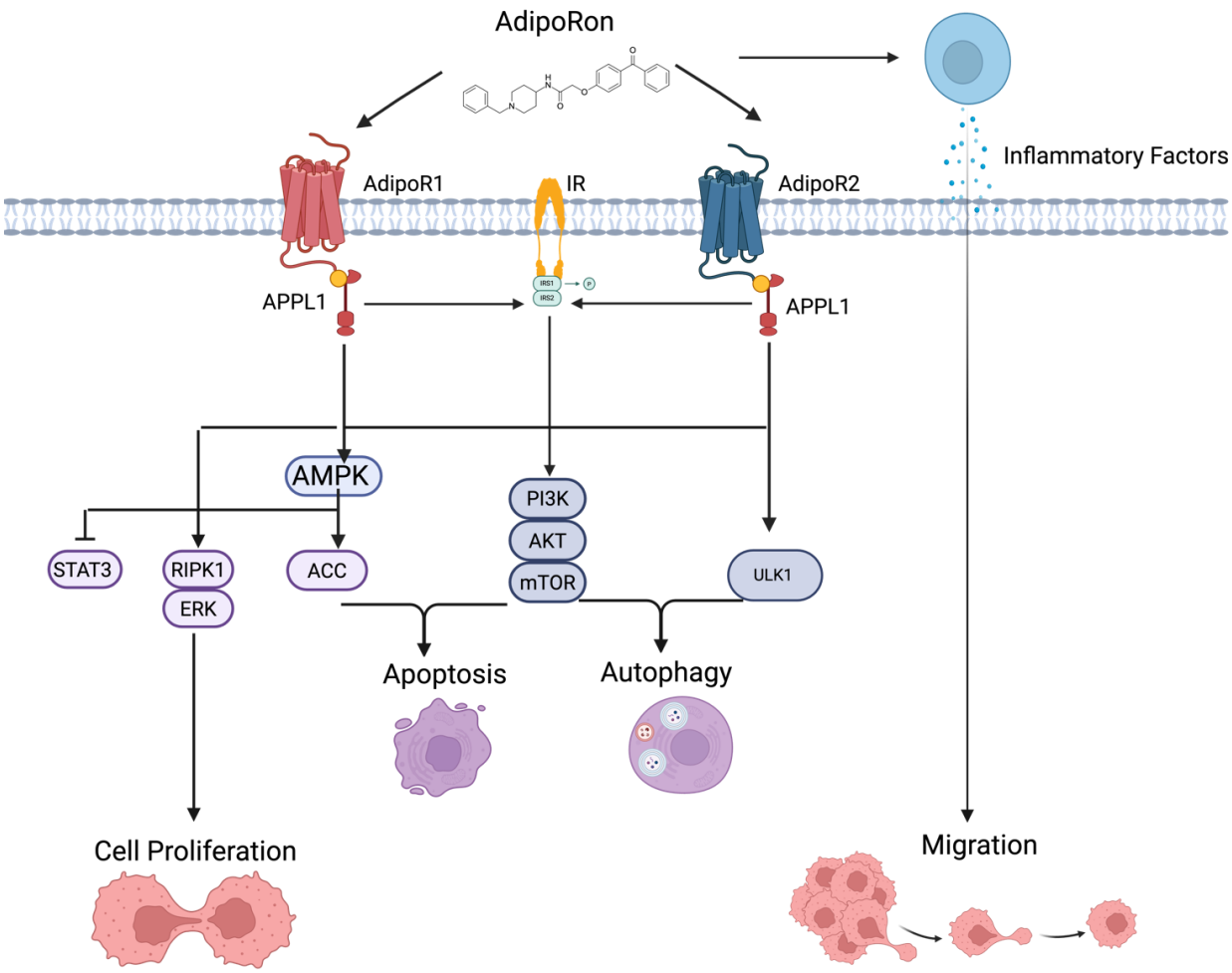


Figure 10. Schematic illustration of the proposed anti-tumor mechanisms of AdipoRon and its receptors. AdipoRon primarily acts through activation of the AMPK signaling pathway, promoting autophagy and apoptosis, while suppressing tumor cell proliferation and migration. In addition, it modulates the tumor microenvironment by downregulating pro-inflammatory and pro-angiogenic factors, and may exert its effects through both AMPK-dependent and AMPK-independent pathways.

I. 5. Low Density Lipoprotein Metabolism in Thyroid Cancer: From Lipid Regulation to Tumor Progression

I. 5.1 Lipid Metabolism in Cancer

Since Warburg first discovered that tumor cells tend to convert glucose into lactate under aerobic conditions, a phenomenon known as aerobic glycolysis, research into cancer metabolism has continued to deepen(549). As a critical component of metabolic reprogramming in tumors, lipid metabolism plays a pivotal role in TC. Thyroid cancer cells regulate lipid synthesis, degradation, and fatty acid oxidation to meet the energy demands and membrane biosynthesis required for rapid proliferation. Lipid metabolism is also closely intertwined with glucose and amino acid metabolism, collectively maintaining metabolic homeostasis in tumor cells(550). Moreover, dysregulation of lipid metabolizing enzymes and their metabolites not only supports tumor growth but may also influence the tumor microenvironment and activate oncogenic signaling pathways. Therefore, elucidating the aberrant mechanisms of lipid metabolism in thyroid cancer is of great significance for identifying novel metabolic therapeutic targets.

Lipids, alongside carbohydrates, serve as the primary energy source for most tissues and play crucial roles in energy storage, membrane construction, and signal transduction. Based on their chemical structures, lipids are categorized into triglycerides, steroids (such as cholesterol), phospholipids, and glycolipids, each with multifunctional roles in energy storage, signal regulation, and maintaining cellular structure(551–553). Epidemiological studies have shown that metabolic syndrome and dyslipidemia are associated with an increased risk of thyroid cancer(554,555). Furthermore, various lipoprotein types have been linked to thyroid cancer risk; for instance, reduced high density lipoprotein (HDL) levels are closely correlated with an elevated risk of thyroid cancer(556,557). A Mendelian randomization study reported associations between DTC risk and total cholesterol, HDL, apolipoprotein B, and the apolipoprotein B to apolipoprotein A1 ratio(558). Low density lipoprotein (LDL) levels are not only associated with tumor aggressiveness, but in vitro studies have confirmed that LDL can promote the proliferation and migration of thyroid cancer cells(559). Metabolically, compared to adjacent normal tissues, TC tissues exhibit significantly elevated levels of monounsaturated fatty acids (MUFA), whereas serum MUFA levels are relatively decreased(560,561). Additionally, receptors related to fatty acids are generally upregulated in TC tissues, suggesting enhanced lipid uptake capacity, which may be modulated by specific mutation types(562–565).

After being taken up by tumor cells, fatty acids are converted into acyl-CoA forms by acyl-CoA synthetases located on the outer mitochondrial membrane, and subsequently enter the mitochondria for oxidative metabolism. This process is commonly referred to as fatty acid β-oxidation (FAO). During FAO, peroxisome proliferator activated receptors (PPARs) serve as critical transcriptional regulators. As members of the nuclear receptor superfamily, PPARs act as lipid sensors and transcriptionally activate the expression of enzymes involved in FAO(566). In addition, the activity of FAO is tightly regulated by AMPK, which maintains cellular metabolic homeostasis by inhibiting biosynthetic pathways and the activation of degradative pathways involved in energy production(567). Upon activation, AMPK undergoes autophosphorylation and inactivates its

downstream target ACC, thereby promoting FAO(568). These mechanisms suggest that enhanced fatty acid metabolism may contribute to the progression of thyroid cancer(569).

Under conditions of energy deficiency, hepatic mitochondria can synthesize ketone bodies through FAO, including acetoacetate (AcAc), acetone, and β-hydroxybutyrate, which serve as alternative energy sources for extrahepatic tissues and organs(570,571). Metabolomic analyses have shown significantly elevated serum levels of β-hydroxybutyrate in patients with PTC, whereas acetone levels are notably decreased in FTC, indicating differential patterns of ketone body metabolism among thyroid cancer subtypes(572–574).

Moreover, tumor cells exhibit markedly enhanced lipogenesis, which not only provides energy and membrane biosynthesis precursors, but also participates in the regulation of multiple signaling pathways, thereby disrupting lipid metabolic homeostasis(575,576). Various key enzymes involved in lipogenesis have been found to be significantly upregulated in multiple types of cancer. Sterol regulatory element binding proteins (SREBPs), the master transcription factors for the synthesis of cholesterol, fatty acids, and triglycerides, play a central role in this process(577). In DTC, elevated SREBP1 expression is closely associated with tumor size and metastasis(578).

The activation of SREBP1c depends on the AKT/mTOR signaling pathway and can upregulate the expression of fatty acid synthase (FASN), thereby further enhancing lipogenesis(579). FASN has been confirmed to be highly expressed in various types of thyroid cancers, including PTC and ATC(580,581). ACC is another key enzyme involved in lipogenesis; it not only promotes fatty acid synthesis but also inhibits FAO(582). In PTC harboring the $BRAF^{V600E}$ mutation, ACC2 expression is lower than in wild type tumors, suggesting metabolic reprogramming through enhanced FAO(583). Activation of the AMPK pathway in PTC leads to increased pACC, further regulating lipid metabolic status(584). Additionally, stearoyl-CoA desaturase (SCD), particularly SCD1, is upregulated in both ATC and PTC, potentially regulated by DTX4(585,586).

Notably, phospholipid metabolism also plays an important role in thyroid cancer. Although related studies are limited, existing evidence shows abnormal distribution of phospholipid metabolism in thyroid cancer. Lipidomic analyses have revealed significant upregulation of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and sphingolipids in both PTC and FTC, suggesting their potential as metabolic biomarkers to distinguish benign and malignant thyroid tumors(587–590). Systematic investigation of lipid metabolism related enzymes in the future may uncover metabolic characteristics and potential therapeutic targets in thyroid cancer.

Phospholipid degradation is a crucial step in lipid metabolism and plays a key role in tumor progression. Phospholipases, the core enzymes catalyzing phospholipid hydrolysis, have carcinogenic potential in various cancers(591). Among them, PLCD3 is considered an oncogene that promotes tumor progression by modulating the Hippo signaling pathway, with potential diagnostic and therapeutic value(592). Moreover, the circ_0003747/miR-338-3p axis can regulate PLCD3 expression, and downregulation of this gene helps inhibit tumor growth, other enzymes involved in phospholipid degradation, such as sphingomyelinases, also warrant further investigation regarding their roles in tumors(593).

In recent years, lipid metabolism has garnered increasing attention in anticancer research, mainly focusing on inhibiting lipid synthesis and inducing ferroptosis via lipid peroxidation. Additionally, certain lipid molecules themselves exhibit potential in suppressing tumor progression. For example, ceramide can ameliorate drug resistance in FTC, while inhibition of glucosylceramide synthase enhances antitumor efficacy(594). The lipogenesis associated enzyme pyruvate carboxylase can activate the AKT/mTOR pathway, upregulating SREBP1c and FASN expression, thereby enhancing the anticancer response of PTC cells(595). Furthermore, sphingosine-1-phosphate has been found to inhibit migration of ATC cells, and activation of estrogen receptors also contributes to the suppression of PTC progression(596).

AdipoRon, a novel APN receptor agonist, exerts antitumor effects by inhibiting glucose and amino acid metabolism and inducing autophagy in thyroid cancer cells(597). Notably, autophagy is not only a key mechanism in maintaining cellular homeostasis but also participates in lipid metabolic regulation. Studies have shown that in breast cancer, AdipoRon regulates LDL receptor (LDLR) levels through autophagy induction, thereby modulating cholesterol homeostasis(598).

Figure 11. Schematic model of Lipid metabolism and their potential mediators in cancer. (BioRender.com)

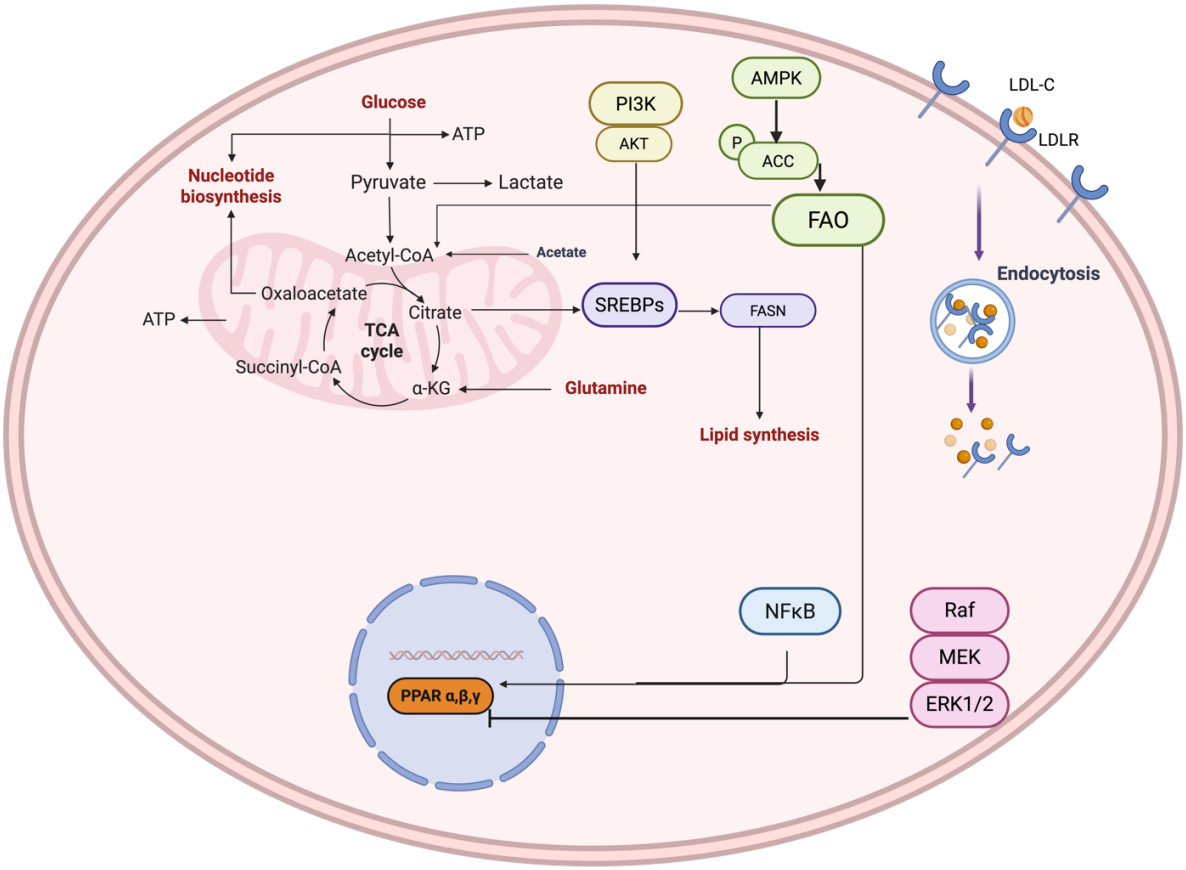


Figure 11. Tumor cells exhibit enhanced FAO and de novo lipogenesis, regulated by key pathways including AMPK, AKT/mTOR, and SREBPs. These metabolic alterations support tumor growth, survival, and progression, and may serve as potential therapeutic targets.

I. **5.2 LDL Metabolism in Cancer**

As previously mentioned, dysregulated lipid metabolism is closely associated with the risk of tumorigenesis and the invasive metastasis of various types of cancers. Lipid metabolism involves a wide array of enzymes and metabolic products, and it is intricately interconnected with glucose and amino acid metabolism. In vitro studies have provided multiple lines of evidence supporting the role of cholesterol metabolism in the development and progression of thyroid cancer, and its mechanisms of action have also been extensively explored in various tumor models.

In normal cells, cholesterol homeostasis is maintained through a combination of synthesis, transport, uptake, and metabolism(599). Cancer cells exhibit significant metabolic reprogramming, and cholesterol being a critical structural component of the cell membrane plays a role in maintaining membrane integrity and fluidity. During tumor cell proliferation, large amounts of cholesterol are required to support membrane biosynthesis and function. Moreover, certain cholesterol derived metabolites can modulate the tumor microenvironment by suppressing immune responses and promoting tumor progression(600,601).

When intracellular cholesterol levels drop, the activity of HMG-CoA reductase is upregulated, and SREBPs, located in the endoplasmic reticulum, are transported to the Golgi apparatus for processing and activation, thereby promoting cholesterol uptake. LDL is the primary circulatory carrier of cholesterol, responsible for transporting cholesterol synthesized in the liver to peripheral tissues. Upon increased expression of LDLR, its extracellular domain binds circulating LDL, which is then internalized into cells via endocytosis. The complex is subsequently delivered to lysosomes, where cholesteryl esters are hydrolyzed by lipases to release free cholesterol for cellular utilization. Accumulated intracellular cholesterol inhibits SREBP activation, reducing further cholesterol uptake in a negative feedback loop(602–604).

LDL and its oxidized form (ox-LDL) play particularly critical roles in regulating cholesterol homeostasis. LDL consists of a lipid core wrapped by apolipoprotein ApoB-100, and approximately 70% of circulating LDL cholesterol (LDL-C) is internalized and degraded through LDLR mediated endocytosis(605,606). LDLR activity is regulated by SREBP2, which upregulates the expression of key genes such as LDLR, HMGCR, and SQLE under cholesterol deprived conditions(607–610). Additionally, PCSK9 promotes the degradation of LDLR, thereby inhibiting LDL-C clearance(611). When intracellular cholesterol is in excess, it can be exported to the plasma via ABCA1 and ABCG1, a process regulated by liver X receptors (LXRs)(612). LXRs can also enhance E3 ubiquitin ligase activity to promote LDLR degradation, thus reducing LDL uptake(613,614).

The unsaturated fatty acids in LDL are susceptible to oxidation by reactive ROS and reactive nitrogen species (RNS), ox-LDL, which can further induce oxidative stress(615,616). The oxidatively modified ApoB-100 can no longer be recognized by LDLR and is instead taken up via scavenger receptors such as LOX-1, SR-A, and CD36, contributing to inflammatory responses and tumor progression(617,618).

Aberrant changes in blood cholesterol levels are among the key features of many types of tumors. Cancer cells often accumulate cholesterol by upregulating its synthesis or enhancing its uptake capacity(619,620). Multiple studies have demonstrated that cholesterol content is generally elevated in various tumor tissues, which may be associated with the overexpression of LDLR. This facilitates the uptake of LDL and is often accompanied by dysregulation of the negative feedback control of LDL-C levels(621–623). LDL not only serves as the primary carrier of cholesterol transport, but also plays a critical role in tumor initiation and progression.

An increasing number of studies have revealed that LDL can promote tumor cell proliferation, migration, and survival by activating several oncogenic signaling pathways, including PI3K/Akt, ERK, and STAT3(624–626). Among them, the PI3K/Akt pathway further activates mTOR signaling, which induces SREBP mediated cholesterol synthesis and uptake, thus providing metabolic support for cancer cells(627). In addition, LDL can enhance EMT, endowing tumor cells with stemness and invasiveness, and increasing their resistance to chemotherapy and immunotherapy(628,629).

LDL also plays a significant role in tumor immune evasion. Studies have shown that LDL can suppress the anti-tumor activity of γδT cells, thereby increasing the risk of tumor metastasis(630). Clinical data indicate that elevated LDL levels are associated with poor prognosis in chronic lymphocytic leukemia and with poor response to PD-1/PD-L1 immune checkpoint therapy(631,632). Moreover, LDL can activate endothelial cells, promoting tumor angiogenesis and lymphatic metastasis(633), and enhance the stem like phenotype of tumor cells(634).

ox-LDL, as a key mediator of lipid metabolism disorders and chronic inflammation, is also believed to play an important role in tumorigenesis. On one hand, ox-LDL can induce oxidative stress and promote the accumulation of DNA damage, thereby driving tumor initiation; on the other hand, activation of hypoxia-inducible factors and inflammatory cytokines by ox-LDL can reshape the tumor microenvironment and promote metastasis(635–637). A growing body of evidence supports the pro-tumorigenic roles of ox-LDL in promoting cancer cell proliferation, invasion, and metastasis.

The tumor promoting effects of ox-LDL are mainly mediated by its receptors, scavenger receptors CD36 and LOX-1. Both receptors are highly expressed in various metabolism related diseases, indicating their key roles under metabolic dysregulation(638). In tumors, the synergistic interaction between ox-LDL and LOX-1 can induce the production of reactive oxygen species (ROS), leading to oxidative DNA damage. Through activation of the NF-κB signaling pathway, this process upregulates the expression of VEGF, MMP-2, and MMP-9, thereby enhancing tumor cell proliferation, invasion, and angiogenesis(639,640).

CD36, another receptor for ox-LDL, is not only involved in lipid uptake but also plays roles in immune recognition, inflammatory responses, cell adhesion, and apoptosis(641). Studies have shown that ox-LDL can be internalized via CD36, leading to the accumulation of oxysterols and the induction of cardiotrophin 1 (CT-1) expression. This, in turn, enhances inflammation, cell proliferation, and angiogenesis, promoting the growth of glioblastoma xenografts(642). Furthermore, the binding of ox-LDL to CD36 can activate focal adhesion kinase 1 (FAK1) and the small GTPase RAC1, leading to cytoskeletal remodeling and loss of cell polarity, ultimately triggering the EMT process(643).

Cells can degrade damaged organelles and proteins by forming autophagosomes that fuse with lysosomes, thereby preventing the accumulation of harmful components and recycling their breakdown products to maintain metabolic homeostasis(644).

In tumor cells, autophagy plays a dual role. On the one hand, as a protective mechanism, autophagy can eliminate damaged components such as dysfunctional mitochondria and oxidized proteins, preventing their toxic accumulation and maintaining genomic stability, thus exerting a tumor suppressive effect in the early stages of carcinogenesis(645). By clearing damaged mitochondria and reducing reactive ROS levels, autophagy helps to lower the risk of DNA damage and chronic inflammation, thereby inhibiting tumor initiation(646). In addition, autophagy can interact with programmed cell death pathways to promote apoptosis or senescence, contributing to the elimination of potentially malignant cells(647).

On the other hand, under metabolic stress or anticancer treatment pressure, tumor cells can utilize autophagy to recycle intracellular resources, enabling them to adapt to unfavorable environments

and support survival and continuous growth(648). In the advanced stages of tumor progression, autophagy becomes a key metabolic adaptation mechanism that allows cancer cells to cope with nutrient deprivation, oxidative stress, or therapeutic interventions(649).

During cancer progression, ox-LDL has been shown to induce autophagy in tumor cells. Mechanistically, ox-LDL can activate the PPARγ signaling pathway, upregulate proline oxidase (POX) expression, and subsequently induce autophagy. Additionally, POX promotes autophagy by generating superoxide anions that regulate the key autophagy protein Beclin 1(650). Another study demonstrated that ox-LDL can also partially induce autophagy by activating microRNA-155 (miR-155)(651). Through autophagy activation, cancer cells undergoing EMT can enhance their resistance to apoptosis and gain survival advantages after detaching from the primary tumor site(652).

Statins, by inhibiting the mevalonate pathway, reduce the synthesis of isoprenoids such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), thereby interfering with the prenylation of small G proteins and suppressing cancer cell proliferation and migration. Multiple studies have shown that statins may help reduce cancer related mortality and recurrence, ultimately improving patient prognosis.

Figure 12. Schematic model of LDL-mediated effects in cancer.

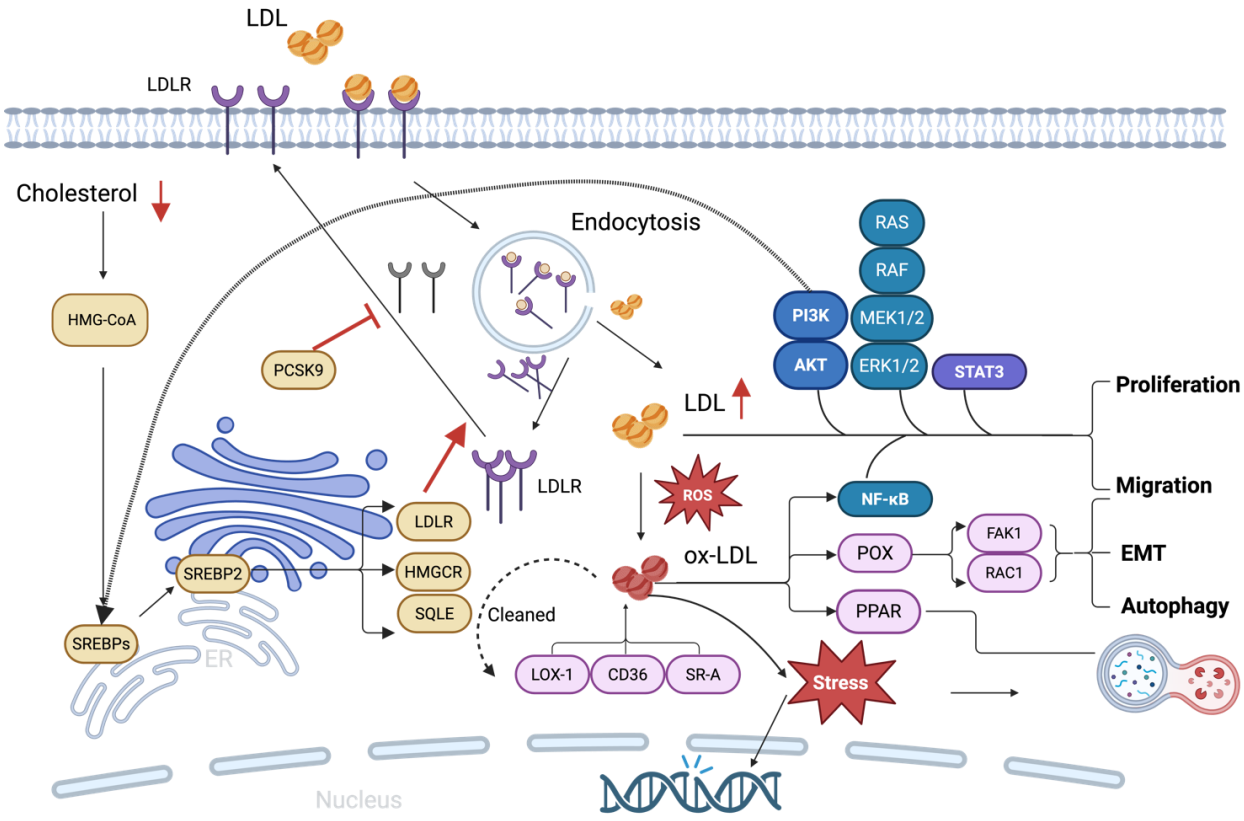


Figure 12. FAO, lipogenesis, and cholesterol uptake are tightly regulated by signaling pathways such as AMPK, AKT/mTOR, and SREBP1/2. Enhanced LDL uptake via LDLR and ox-LDL contributes to tumor proliferation, migration, EMT, and autophagy by activating pathways including PI3K/Akt, STAT3, and NF-κB.

I. 6. Potential Crosstalk between APN and LDL Signaling

As an adipokine with potential clinical significance, APN has garnered widespread attention in clinical settings, with its levels reflecting the state of metabolic dysregulation in the body(653). Studies have shown that levels of VLDL and LDL are negatively correlated with APN, indicating that APN plays an important role in lipid metabolism regulation, including HDLc(654).

In patients with TC, more aggressive tumor types exhibit a higher degree of LDL uptake mediated by LDL receptors(655). Cellular studies further demonstrate that LDL promotes proliferation and migration of thyroid cancer cells through its receptor, which may explain why more invasive thyroid cancers show increased LDL uptake(656). Different thyroid cancer cell lines with various mutations differ in their LDL uptake; notably, in cell lines harboring the BRAF ^{V600E} mutation, LDL uptake leads to overactivation of the RAS/RAF/MAPK (MEK)/ERK signaling pathway, further revealing the close association between thyroid cancer aggressiveness and LDL uptake and metabolism.

In other tumor types, studies have found that APN levels positively correlate with tumor risk, and higher APN levels are associated with poorer survival outcomes(657–660). However, other reports indicate a negative correlation between APN levels and tumor risk(661,662). In the context of TC, the relationship between APN levels and tumor risk remains controversial. Some studies show a negative correlation between APN levels and TC risk, while others suggest that APN alone is insufficient as an independent predictor of TC risk. Subsequent research has suggested an association between APN and metabolic syndrome in the development of TC, implying that APN levels alone may not fully reflect its relationship with thyroid cancer and should be considered alongside other metabolic related factors(663–666).

LDL can promote the release of APN from adipocytes(667), and this mechanism is more related to the regulation of cholesterol transport rather than the direct action of cholesterol itself(668). In breast cancer cells, studies have found that APN inhibits LDL induced cancer cell proliferation by activating the autophagy pathway, which promotes the degradation of LDLR(669). Additionally, regarding oxLDL, APN binds to oxLDL and inhibits oxLDL induced ERK phosphorylation and NF-κB pathway activation(670).

Currently, research on the anticancer mechanisms of APN in thyroid cancer is relatively limited, and its precise role remains to be fully elucidated. Some studies have shown that APN can inhibit the proliferation and migration of papillary thyroid cancer cell lines, although detailed mechanistic studies are still lacking(671). Further research indicates that APN activates autophagy via its receptor ADIPOR2 through an mTOR independent mechanism, thereby suppressing cell proliferation and migration(672). As a novel APN receptor agonist, AdipoRon exerts antitumor effects by inhibiting glucose and amino acid metabolism in thyroid cancer cells and inducing autophagy through the AdipoR2-ULK signaling axis(673).

It is noteworthy that autophagy is not only a key mechanism for cellular homeostasis regulation but also participates in lipid metabolism regulation(674). Studies in breast cancer have shown that AdipoRon maintains cholesterol homeostasis by inducing autophagy to regulate LDLR levels(669). However, whether AdipoRon similarly regulates cholesterol metabolism in thyroid cancer by suppressing LDLR or interfering with its downstream signaling to inhibit tumor growth and migration has not yet been reported. This mechanism remains to be further explored.

II. HYPOTHESES AND AIMS

II.1 Hypotheses

ANP is a major adipokine, plays a key role in lipid metabolism, insulin sensitivity, and anti-inflammatory regulation. It has also been reported to influence the expression and activity of LDL receptors in specific tissues. (675). We hypothesize that APN regulates tumor growth, particularly in BRAF-mutant thyroid cancer, by modulating lipid uptake and metabolism. Moreover, treatment with the adiponectin receptor agonist, AdipoRon, may enhance these effects by promoting apoptosis and/or autophagy.

II.2 Aims

II.2.1 General Aim

This study aims to comprehensively investigate the biological functions and molecular mechanisms of APN signaling in thyroid cancer, with the goal of providing novel theoretical insights and identifying therapeutic strategies for targeted intervention in this disease.

II.2.2 Specific Aims

1. To investigate the gene expression patterns of adiponectin receptor (*ADIPOR1*, *ADIPOR2*) and related genes (*APPL1, CDH13*) in BT, PCT and normal thyroid tissue, using bioinformatics databases (TCGA, GTEx, GEO), and evaluate their correlation with clinicopathological parameters and prognosis.

2. To analyze the secretion profile ability of neck adipose tissue obtained from different histological pattern diagnosis (BT *versus* PTC) to deliver the most important cytokines and adipokines

3. To explore differential expression of ADIPOR1 and ADIPOR2 in a cohort of patients from HSCSP with different histological pattern diagnosis: BT, PCT and normal thyroid tissue.

4. To evaluate the gene expression patterns of *ADIPOR1* and *ADIPOR2* in BRAF mutated and RET/PTC1 rearranged thyroid cancer tissues and cell lines using public databases, in order to predict potential underlying molecular pathways.

5. To investigate the expression of ADIPOR1 and ADIPOR2 in human thyroid cancer cell lines (BCPAP and TPC-1), assess the functional effects of AdipoRon treatment, and explore potential synergistic or antagonistic mechanisms.

6. To determine the gene expression patterns of LDLR and lipid metabolism related genes (*SREBF1, SREBF2, APOE, PCSK9, HMGCR, APOB*) in BT, PCT and normal thyroid tissue, using bioinformatics databases (TCGA, GTEx, GEO), and evaluate their correlation with clinicopathological parameters and prognosis.

7. To investigate the gene expression patterns of *LDLR* in BRAF mutated and RET/PTC1 rearranged thyroid cancer tissues and cell lines using public databases, in order to predict potential underlying molecular pathways.

8. To investigate the combined effect of AdipoRon and LDL on human thyroid cancer cell lines (BCPAP and TPC-1) and explores the possible synergistic or antagonistic mechanisms.

III. MATERIALS AND METHODS

III.1. Reagents and Treatments

Table1. List of Reagent used for cell experiments

Product	**Manufacturer**	**Reference** #
Dimethyl Sulfoxide (DMSO)	Cell Signaling	12611
PRIMI1640	Gibco	11875-093
DMEM	Gibco	11995-065
FBS	Gibco	16000-044
Trypan blue dye,0.4%	BIO RAD	145-0013
Counting slides	BIO RAD	145-0011
L-Glutamine	Gibco	15410314
Penicillin-Streptomycin (P/S)	Gibco	15276355
Trypsin 10x	Biowest	X0930-100
potassium bromide (KBr)	Sigma-Aldrich	P0838
AdipoRon	Cayman Chemical	15941
Vemurafenib (PLX4032)	Cayman Chemical	10618
LDL (Human)	Self-prepared	Custom

* FBS was heat inactivated at 55 °C for 30 minutes in a water bath. During incubation, the serum was gently mixed every 5–10 minutes to ensure even heating and prevent protein precipitation.

Isolation of LDL from Human Serum

1. LDL particles (density: 1.019–1.063 g/mL) were isolated from pooled human serum using sequential density gradient ultracentrifugation with KBr. The density of the serum was adjusted to 1.019 g/mL by adding KBr, calculated using the following formula:

grams of KBr = V (ρ final−ρ initial)/1−(0.312×ρ final).

where V is the total serum volume in mL, ρ is the density in g/mL, and 0.312 is the density of solid KBr.

After mixing gently to avoid bubble formation, the KBr adjusted 100mL serum was evenly distributed into six 20 mL ultracentrifuge tubes (Beckman, maximum 17 mL per tube). Each tube was carefully overlaid with a 3 mL KBr solution (ρ = 1.019 g/mL) to form a sharp density boundary and prevent mixing. The tubes were then balanced to equal weight.

2. Ultracentrifugation was initially performed at 36,000 rpm, 8°C for 20 hours using a fixed-angle rotor. After centrifugation, the upper layer containing VLDL was discarded, and the lower fraction containing LDL was carefully retained and transferred into a 100 mL graduated cylinder. KBr solution with a density of 1.019 g/mL was added to bring the total volume up to 100 mL. Subsequently, solid KBr was added to increase the density to a density of 1.063 g/mL.

3. Each tube was carefully overlaid with 3 mL of KBr solution (ρ = 1.063 g/mL). Following centrifugation at 36,000 rpm, 8 °C for 20 hours using a fixed-angle rotor, the LDL fraction was observed in the upper layer. This LDL-rich layer was carefully collected.

4. To remove KBr and exchange the buffer for downstream cellular experiments, the LDL fraction obtained from ultracentrifugation was subjected to gel filtration chromatography. The sample was loaded onto a pre-equilibrated desalting column previously equilibrated with sterile PBS. The LDL fraction (2.5 mL) was carefully applied to the column, and eluted with 3.5 mL PBS.

5. ApoB from the LDL fraction was measured using an immunoturbidimetric method, employing a commercial kit optimized for the COBAS c501 automated analyzer (Roche Diagnostics, Minato City, Tokyo, Japan).
All solutions and materials used were endotoxin free (LPS-free) to ensure compatibility with subsequent cellular assays.

Experimental Treatments: AdipoRon and Vemurafenib

AdipoRon Preparation and Treatment

AdipoRon (1 mg) was dissolved in 100 µL of DMSO to prepare a 10 mg/mL stock solution. The solution was aliquoted and stored at –20 °C until use.

Medium Preparation

Complete medium: RPMI 1640 supplemented with 1% P/S, 1% L-Glutamine, and 10% FBS.

Control medium: RPMI 1640 with 1% P/S, 1% L-Glutamine, 5% FBS, and 0.1% DMSO.

AdipoRon medium (100 µM): Prepared by adding 100 µL of AdipoRon stock (10 mg/mL) to 23.33 mL of control medium.

For experimental treatments, the AdipoRon stock solution was diluted in control medium to final concentrations of 6.25, 12.5, 25, 50, and 100 µM at 24, 48 and 72 hours).

Vemurafenib Preparation and Treatment

Vemurafenib was prepared as a 1 mg/mL stock solution. For treatment, the stock was diluted at a ratio of 1:2,000 in either control medium or AdipoRon medium to achieve the desired final working concentration (1µM). Cells were treated with the resulting Vemurafenib-containing medium for the MTT assay and Wound healing assay.

LDL Supplementation

LDL was added to either control or AdipoRon medium to reach a final concentration of 200 µg/mL. Cells were incubated with LDL containing medium as per experimental protocols. Control groups received an equal volume of vehicle without LDL.

All working solutions were filtered through a 0.22 µm nylon membrane filter prior to use.

Cell Lines and Cell Culture

The cell lines used were carried out on cell lines derived from human PTC, TPC1 (bearing RET/PTC rearrangement) and BCPAP (bearing the $BRAF^{V600E}$ oncogene).

Both cell lines were provided by Paolo Vigneri of Azienda Ospedaliero Universitaria Policlinico Vittorio Emanuele Catania, Catania, Sicilia, IT.

Cell line maintenance

Aliquots of cell lines (BCPAP, TPC-1), cryopreserved in 10% DMSO in FBS and stored in liquid nitrogen, were rapidly thawed in a 37 °C water bath. Following thawing, residual DMSO was removed by centrifugation at 900 rpm for 5 minutes. Cell viability was assessed using 0.4% trypan blue solution, and cells were counted with the TC20™ automated cell counter (Bio-Rad, #1450102). Cells were then expanded in complete RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS, 100 U/mL penicillin, and 1 µg/mL streptomycin. Cultures were maintained at 37 °C in a humidified incubator with 5% CO_2.

III.2. Publica databases

Sample Collection and Grouping

Human thyroid tissue samples

1. Data Mining from the TCGA-THCA Cohort

Gene expression profiles and clinical information of thyroid cancer (TCGA-THCA) were downloaded from the GDC Data Portal (https://portal.gdc.cancer.gov/) on December 23, 2024.

The cohort included 505 PTC samples from the TCGA-THCA dataset. However, 43 samples were excluded from the analysis due to missing data. Clinical information was extracted from XML files and included gender, age, clinical stage, and TNM classification. For lymph node status, samples were categorized as N0 (n = 236; no lymph node metastasis) or N1 (n = 226; presence of lymph node metastasis)

Clinical and pathological data for 55 pairs of matched tumor and adjacent normal tissues of the PTC samples were retrieved from the GDC portal. Among them, 28 pairs were classified as N0, and 27 as N1.

2. Data Mining from the Genotype Tissue Expression (GTEx) Portal

RNA-Seq expression data for 355 normal thyroid tissue were obtained from the Genotype Tissue Expression (GTEx) Project. The data used for the analyses described in this manuscript were obtained from: the GTEx Portal on 12/23/24 accession number phs000424.vN.pN on 12/23/2024. All data were batch corrected and normalized using the method described by Wang, Q. et al.(676) . Expression levels are presented as log2 transformed Transcripts Per Million (TPM) value.

3. Data Mining from the GSE60542 dataset

Gene expression data were obtained from the GSE60542 dataset, available in the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). This dataset was originally published by Tarabichi et al.,(677)GSE60542 dataset: This GEO dataset (log2 RMA normalized intensity) was reannotated and filtered to exclude lymph node-derived samples. Samples were grouped based on IDs: “-PT” (PTC tumor) and “-N” (adjacent normal tissue). Lymph node status (N0 vs N1) was determined from clinical annotations. Two differential analysis strategies were used:

PTC (N0, n=14) *vs* Normal (N0, n= 14)
PTC (N0, n = 14) *vs* PTC (N1, n = 19)
Mutation grouping: Based on mutation annotations, samples were classified into:
BRAF-mutant (n = 20) *vs* non-BRAF (n = 13)
RET/PTC1 fusion (n = 5) *vs* non-fusion (n = 28)

Human Thyroid Cell Line Samples

RNA-seq data from two human PTC cell lines were obtained from the GEO database (**https://www.ncbi.nlm.nih.gov/geo/**).

- 1. The GSE171483 dataset includes expression profiles (raw counts) from BCPAP cells treated with Vemurafenib (100 μM) or control (n = 3 per group). Sample names were matched, and a design matrix (~0 + group) was constructed for differential expression analysis. This dataset was published by Bonaldi et al.(678)
- 2. The GSE261830 dataset contains RNA-seq data (TPM values) from TPC-1 cells treated with Selpercatinib (48 hours, n = 3) and control (n = 3). These data were used for differential gene expression analysis and visualization. The dataset was published by Katayama et al.(679)

Expression Analysis and Functional Annotation

Differential Expression and Visualization

For TCGA vs GTEx, comparison log2(TPM + 1) expression matrices were processed using the limma package (https://bioconductor.org/packages/devel/bioc/html/limma.html).
Volcano plots were generated to visualize gene expression changes in LDL-related and adiponectin receptor genes. Gene expression boxplots were performed by ggplot2, and statistical significance was evaluated by Wilcoxon test using ggpubr R package (https://rpkgs.datanovia.com/ggpubr/)

Survival Analysis

Kaplan–Meier survival curves were generated by stratifying *ADIPOR1/2* expression by median value. Log rank test was used for significance. Univariate and multivariate Cox regression models were constructed with variables including gene expression, age, gender, and stage. Results were visualized as forest plots.

Correlation Analysis

Pearson correlation coefficients and p-values were calculated between *ADIPOR1/2*, *LDLR* and 15 core pathway genes (including *MTOR*, *AKT1/2/3, MAPK1/3, NFKB1*, autophagy and apoptosis markers) in: GSE171483 (Vemurafenib treated) and GSE261830 (Selpercatinib treated). Results were visualized using dotplot where point size represents –log10(*p* value), and color indicates correlation strength.

Enrichment Analysis

Genes significantly correlated with *ADIPOR1/2* or *LDLR* (*p > 0.3 or < –0.3, FDR < 0.05*) were used for GO Biological Process (BP) and KEGG pathway enrichment.
Enrichment was performed using the clusterProfiler package (Bioconductor.org/packages), with Benjamini Hochberg adjusted p value < 0.05 as the threshold. Pathways related to predefined

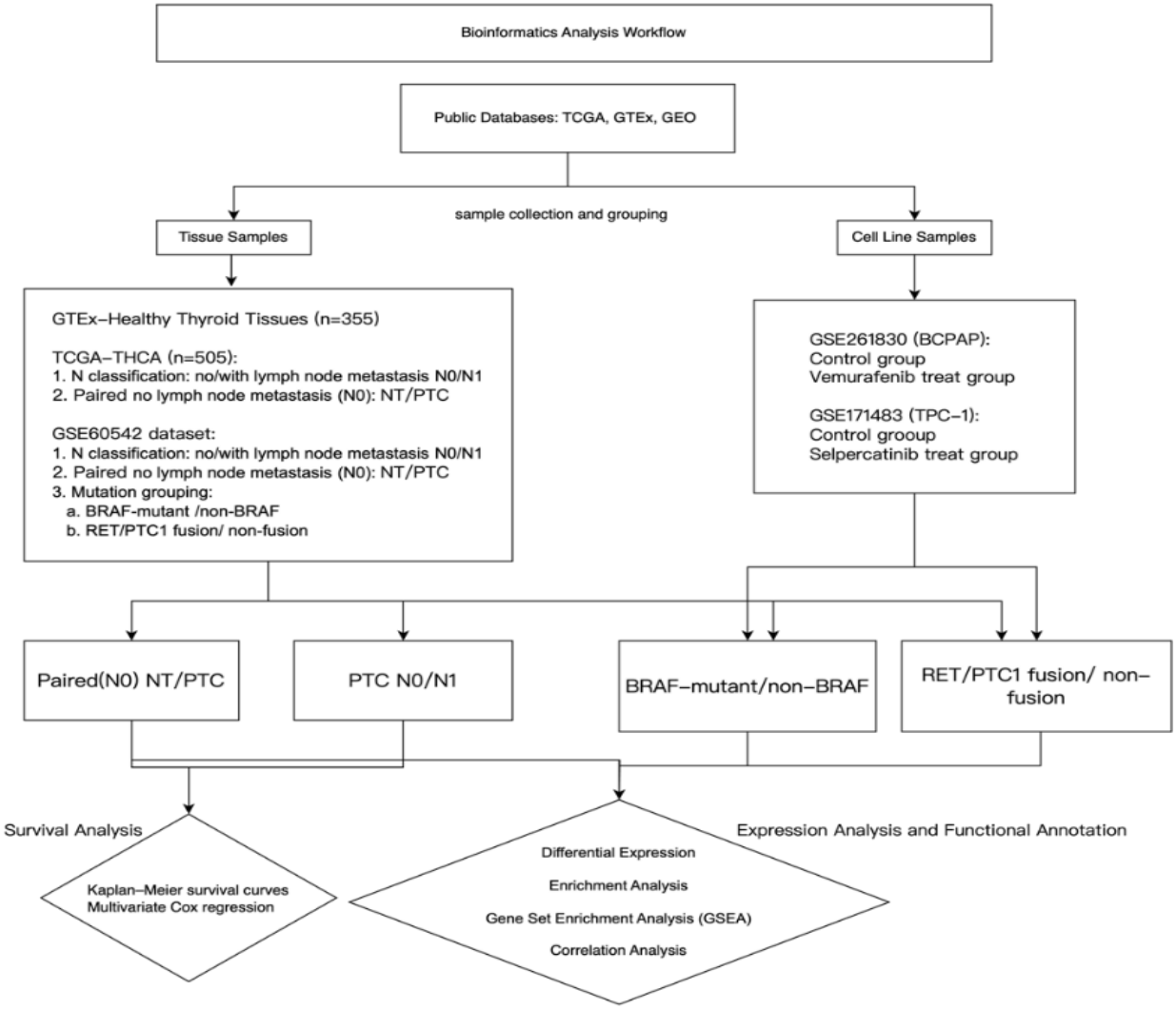
biological keywords (e.g., lipid, autophagy, apoptosis, MAPK, AKT) were highlighted in red on dot plots.

Gene Set Enrichment Analysis (GSEA)

Differential gene ranking (log2FC) from GSE60542, GSE171483 and GSE261830 was used as input for GSEA (GO BP & KEGG) via cluster Profiler.

Parameters: min GSSize = 10, *p* value Cutoff = 0.5. Pathways with adjusted $p < 0.25$ and containing relevant keywords were exported and plotted using gseaplot2.

Figure 13. Bioinformatics analysis workflow used to analyze the different datasets.



III.3. Human tissue and serum samples

Sample Source and Ethics Statement

Surgical snap-frozen specimens (adipose and thyroid) and serum were obtained from patients recruited from January 2009 at Santa Creu i Sant Pau Hospital (HSCSP), as described above. These belonged to the sample collection registered at Instituto de Salut Carlos III (Spain; C.000281). All procedures involving human tissue were approved by the institutional ethics committee of Santa

Creu i Sant Pau Hospital, and written informed consent was obtained from all participants. All this research was carried out in line with relevant European guidelines/regulations.

Patients Included in the Study

A recruitment of a cohort of patients (n = 84) diagnosed with TC was carried out at HSCSP in Barcelona, Spain. The clinical information collected from them was sex, age, weight, height, poorly health habits and no dyslipidemia pathology or statin treatment. Depending on their histopathological diagnosis, tumors were identified, as follows: BT and PTC. Final histological Final diagnosis was conducted by two independent pathologists as WHO 2007 criteria details
Freshly adipose tissue (0.1–0.3 g) was collected in conjunction with the surgical technique of central lymph node dissection (area VI), without additional morbidity to the patients. These tissues were maintained in explant culture in conditioned media (CM), DMEM media supplemented with 1% bovine serum albumin (BSA), overnight (O/N). Afterwards, this media was collected and employed for ELISA analysis.

Table 2. Clinical Characteristics of the Study Cohorts

Characteristic	PTC (n = 52)	BT (n = 32)
Age (years, mean ± SD)	58.2 ± 14.70	60.92 ± 17.54
BMI (kg/m², mean ± SD)	28.4 ± 5.71	27.63 ± 6.81
Sex	24 males / 28 females	7 males / 25 females

III.4. ELISA Assay

Multiplex ELISA Assay Using Luminex xMAP Technology

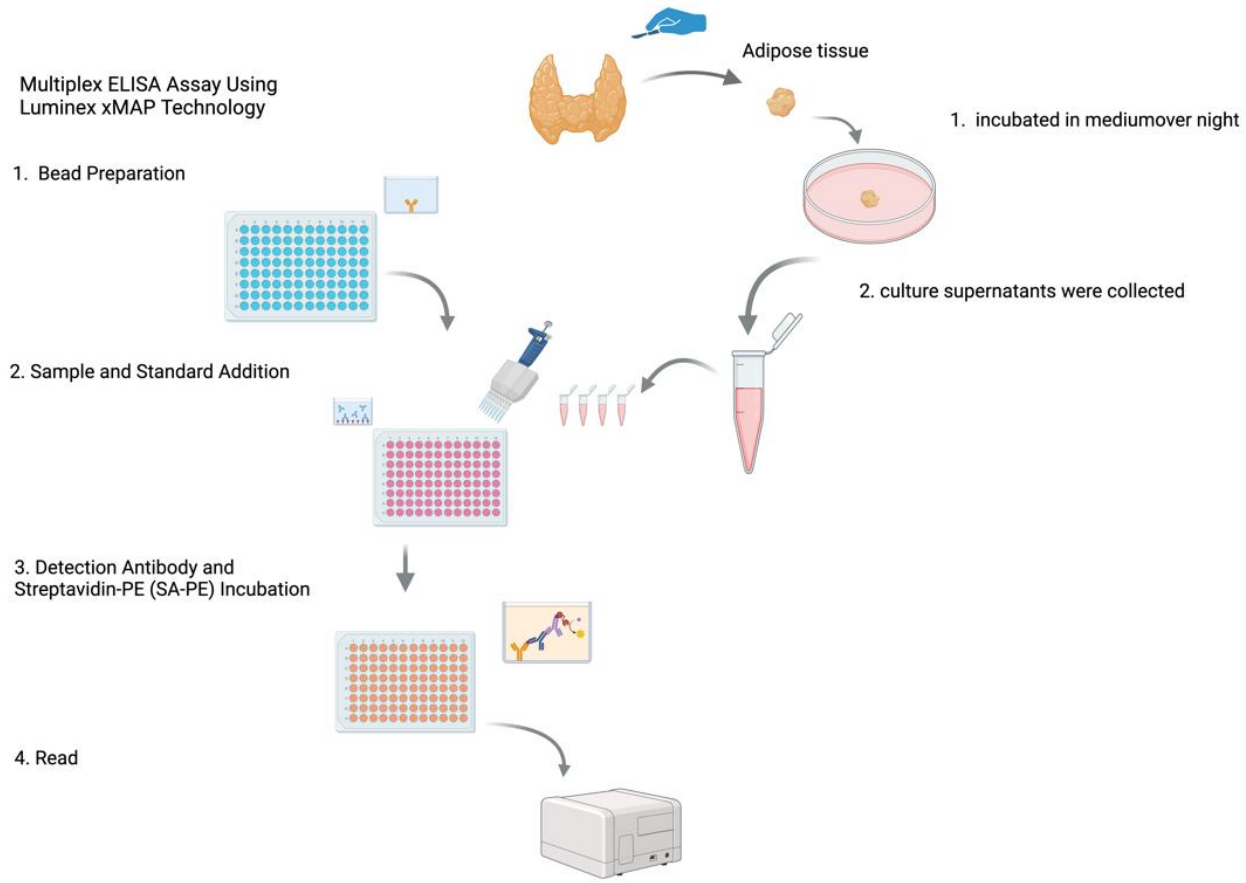
Cytokines and adipokines were analyzed from the CM from NAT explant culture described before (thyroid and adipose tissue) Table 2, by Flexible Bead-Based Multiplex Assays technology.
Human ProcartaPlex Mix&Match 5-plex (PPX-05MX2W929) kit for EGF, IL-6, LEP, Acrp30, resistin and ProcartaPlex (EPX01A-10223-901) kit for TNF-alpha were used for Luminex assay.
Luminex® xMAP® is an immunoassay based on beads that allows to detect a maximum of 100 analytes at in one sample. These beads are microspheres dyed inside in a combination of red and infrared fluorophores corresponding a specific spectral signature, or bead region.

The sample was incubated with unique beads couple to specific antibodies to the target analytes in different areas. Afterwards it was washed to cast aside the unattached molecules. A mixture of biotinylated detection antibodies and a streptavidin phycoerythrin (PE) reporter was prepared and incubated with the sample. The Luminex instrument utilized a single laser to excite the beads and define the region where our target analytes were bound. The number of attached analytes is proportional to the extent of the PE derived signal which is measured by another laser. A considerable number of readings of the same bead region was performed to ensure accuracy in the detection.

Data Acquisition and Processing

Raw fluorescence data were acquired on a Luminex 200 system and analyzed with xPONENT software. Median fluorescence intensity (MFI) values were converted to absolute concentrations (pg/mL) using the standard curve. Final data were exported and processed using R software for statistical analysis, including outlier detection, transformation (if required), and appropriate hypothesis testing with graphical output.

Figure 14. ELISA Analysis Workflow (Create from BioRender)



III.5. MTT assay

To analyze cytotoxicity using the MTT assay, 3,000 TPC-1 cells and 2,000 BCPAP cells were seeded per well in a 96-well microplate media (RPMI 1640) supplemented with 1% FBS and AdipoRon (100μM) at 6.25, 12.5, 25, 50 and 100μM or 0.01% DMSO as control for 24 and 48 hours. To determine the cell viability under this treatment, 20μL MTT solution (5 mg/mL) of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT; Sig-ma-Aldrich) was added. After incubating it at 37ºC for 4h, 100 μL 0.01% DMSO was supplemented, and it was incubated again at 37°C for 10 minutes. Lastly, absorbance was read at 570 nm by using a microplate reader (xMark, Bio-Rad). The growth inhibitory rate was calculated as: [(OD 540 treatment-OD 540 blank)/ OD 540 control-OD 540 blank] ×100%. Each experiment was repeated at least three independent times in sextuplicate.

III.6. Wound healing assay

To analyze the cell migration, both cell lines (TPC1 and BCPAP) were seeded at high densities until 70% confluence was reached. In terms of migration capacity, the cells were scratched using a 10 uL pipette tip and washed with PBS 1X. The wounds were photographed at 0 hours (t = 0) and after 16 hours with or without AdipoRon treatment (50µM,100µM), LDL incubation (200 µg/mL ApoB) and 5% FBS at 37 °C using an inverted microscope and analyzed with Image analysis was performed using ImageJ software (Version 1.53; National Institutes of Health, USA(680). The percentage of wound-healing was obtained from a minimum of three measurements of the wound area, and each result was the mean of three independent experiments.

III.7. DAPI (4′,6-diamidino-2-phenylindole) stain

The cells were washed with PBS to remove any residual media. Cold methanol was then added slowly to ensure complete coverage of the cells, taking care to avoid detaching them. The cells were incubated with methanol at -20°C for 10-15 minutes to permeabilize and fix the cellular structures. After incubation, the methanol was carefully removed, and the cells were washed with PBS to eliminate any excess methanol. Subsequently, the cells were stained with 300nM DAPI and incubated for 1–5 minutes in the dark. The staining solution was then removed, and the cells were washed 2–3 times with PB. Fluorescence images were captured using an Olympus BX51 fluorescence microscope equipped with an Olympus U-RFL-T light source and an Olympus DP72 digital camera (Olympus Corporation, Tokyo, Japan). A DAPI filter set (excitation 358 nm/emission 461 nm) was used to visualize nuclear staining. Image analysis was performed using ImageJ software (version 1.53, NIH, USA).

III.8. Flow cytometry

Sample Preparation: Both cell lines were seeded at a density of 2×10^5 cells/well and maintained for two days in a complete medium for proper growth. After two days, the medium was changed to a basal medium plus 0.1% DMSO (vehicle) as a control or with AdipoRon, and were maintained for 24, 48, or 72 hours, depending on the experiment.

The cells were harvested using trypsin digestion and centrifuged at 900rpm for 5 minutes. After discarding the supernatant, the cells were washed once with 1 mL ice cold 1× PBS. A 10 µL aliquot of the resuspended cell suspension was taken for cell counting using automatic cell counter. Following this, the cells were washed twice with cold PBS at 600 g for 5 minutes each time. Ethanol at 70% cold was then added to fix the cells, and they were left to rest overnight (O/N) at -20 °C. After fixation, a centrifugation was performed at 1000 g for 5 minutes at 4°C. The pellet was resuspended in 1 mL of cold PBS and centrifuged again at 600 g for 10 minutes at 4°C in two rounds to remove any residual ethanol. Following the washes, the Abbkine cell staining kit was used for cell cycle staining.

The staining solution contained RNAse A 100X, propidium iodide 50X, and Assay buffer 10 X; 500 µL of the staining solution was added to the pellet, which was then resuspended and incubated at 37°C for 30 minutes light protected. After incubation, the sample was centrifuged at 900 g for 5 minutes at 4°C, the pellet was resuspended in 200 µL of cold PBS, and the cells were analyzed using a flow cytometer (MACSQuant Analyzer 10) to obtain propidium iodide intensity and cell cycle histograms of the samples, these results were analyzed by the software MACSQuantify.

III.9. Hematoxylin and Eosin (H&E) staining of Cultured Cells

Cultured cells grown on sterile coverslips were gently washed with PBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature. After fixation, cells were rinsed twice with PBS and stained with Harris hematoxylin for 5–7 minutes. Excess hematoxylin was removed by washing under running tap water, followed by differentiation in 1% acid alcohol (1% HCl in 70% ethanol) for a few seconds. Subsequently, eosin staining was performed for 1–2 minutes to visualize cytoplasmic components. After staining, the coverslips were dehydrated through a graded ethanol series (70%, 80%, 95%, and 100%), cleared in xylene, and mounted onto microscope slides using a neutral mounting medium.

III.10. Acridine orange (AO) staining of Cultured Cells

TPC-1 and BCPAP cells (1.0×10^4) were cultured on sterile coverslips and incubated in a CO_2 incubator at 37 °C for 24 hours. After incubation, the culture medium was removed and replaced with the appropriate treatment medium for varying time periods, according to the experimental design. Following treatment, the medium was discarded, and cells were incubated with a solution of AO working solution (2.5 µg/mL) for 15 minutes at 37 °C. After staining, cells were washed three times with PBS to remove excess dye. Fluorescence images were acquired using an Olympus BX51 fluorescence microscope equipped with an Olympus U-RFL-T light source and a DP72 digital camera (Olympus Corporation, Tokyo, Japan). Green fluorescence (excitation ~500 nm, emission ~526 nm) represents cytoplasmic and nuclear RNA, whereas red fluorescence (excitation ~460 nm, emission ~650 nm) indicates the presence of acidic vesicular organelles (AVOs), a hallmark of autophagy. Merged green/red fluorescence images were analyzed using ImageJ software (version 1.53, NIH, USA). The green-to-red fluorescence intensity ratio was calculated as a semi-quantitative measure of autophagic activity. Data were obtained from at least three independent microscopic fields per condition, and the average ratio was used for statistical analysis.

Table 3. Reagents and Consumables Used in Cell Based Assays

MTT assay	Vybrant® MTT Cell Proliferation Assay Kit	Thermo Fisher	V-13154
MTT assay	96-Well Plate	Corning	3599
Wound healing assay	6-Well Plate	Corning	CLS351146
Acridine Orange		Sigma-Aldrich	A6014
Acridine Orange	Culture Slides	BD BioCoat	354632
DAPI		Thermo	D3571
DAPI	Culture Slides	BD BioCoat	354632
Flow Cytometry	PI / Cell Cycle Analysis Kit	Canvax	CA112
H&E		Sigma-Aldrich	318906

III.11. Western blot (WB)

1. Total Protein Extraction

Extraction from Cells and Human Thyroid Tissue

Cells (TPC-1 and BCPAP) and tumoral and normal thyroid tissues protein extractions were executed in a similar fashion. In both conditions, samples were lysed with 1X RIPA lysis buffer (Millipore 20-188) supplemented with 1X protease inhibitor cocktail (P8340-1mL, Sigma-Aldreich), 1mM phenylmethylsulphonyl fluoride (PMSF, Sigma, St. Louis, MO, USA) and 1mM sodium orthovanadate (ORTHO, Sigma). Then, they were incubated at 4ºC for 15 minutes and centrifuge at 15,000 x g at 4ºC. Protein quantification was done following the kit A53225 (Thermo Fisher) kit. Measure absorbance at 480 nm using xMark Microplate Spectrophotometer (BIO-RAD, #1681150). Collect supernatant and store at −80°C until used.

2. Western Blotting

Stain-Free Gel Imaging and Quantification

the protein extracts were mixed with a 4X Laemmli loading buffer and heated at 94 °C for 4 minutes. Then, 20 µg of protein was size-separated on a 10% TGX Stain-Free precast gel (Bio-Rad, Hercules, CA, USA), transferred to a 0.2 µm PVDF membrane (Bio- Bio-Rad, Hercules, CA, USA) and the membrane were blocked with 3% dried milk in Tris-buffered saline containing 0.05% of Tween-20 (TBST buffer) for 15 minutes. Finally, membranes were incubated with optimized dilutions of the primary antibody (Table 4) overnight at 4 °C. Thereafter, the membranes were washed three times for 10 minutes with TBST buffer and re-incubated with the IgG HRP conjugated secondary antibody for 1hours (Table 4). Finally, the membranes were washed three times for 10 minutes with TBST buffer and analyzed using an Immun-Star Western Chemiluminescence Kit (Bio-Rad, Hercules, CA, USA). Imaging and data analysis were performed following the protocol described in Taylor et al. and Neris, R.L.S., et al. TGX Stain-free gels were activated for 1 minutes after SDS-electrophoresis. Images were captured using a ChemiDoc XRS Gel Documentation System (Bio-Rad, Hercules, CA, USA) and Image Lab software (version 6.0.1, Bio-Rad, Hercules, CA, USA). Data normalization analysis for each protein band was performed with the stain-free gel image saved, and the background was adjusted in such a way that the total background was subtracted from the sum of the density of all the bands in each lane(681).

Table 4. List of antibodies used for Western Blot

Antibody	Species	Dilution	Company	catalog#
AdipoR1	Rabbit	1:1000	Bioss Antibodies	bs-0611R
AdipoR2	Rabbit	1:1000	Thermo Fisher	XK3744985D
mTOR	Rabbit	1:1000	Cell Signaling	2972
phosphorylated mTOR	Rabbit	1:1000	Cell Signaling	2971
ERK1/2	Rabbit	1:1000	Cell Signaling	9102
phosphorylated ERK1/2	Rabbit	1:1000	Cell Signaling	9101
AKT	Rabbit	1:1000	Cell Signaling	9272
phosphorylated AKT	Rabbit	1:1000	Cell Signaling	9271
NFKB	Rabbit	1:1000	Cell Signaling	3035
Annexin V	Rabbit	1:1000	GeneTex	GTX103250
AIF	Rabbit	1:1000	GeneTex	GTX113306
Caspase 9	Rabbit	1:1000	Cell Signaling	9502

Caspase 3	Rabbit	1:1000	GeneTex	GTX110543
p62	Rabbit	1:1000	Santa Cruz Biotechnology	sc-28359
LC3B	Rabbit	1:1000	Cell Signaling	2775
LDLR	Mouse	1:500	Proteintech	66414-1-Ig
SREBP-1	Rabbit	1:1000	Cell Signaling	95879
Secondary Anti-mouse		1:10,000	Jackson Immuno	156092
Secondary Anti-rabbit		1:10,000	Jackson Immuno	156914

Table 5. Reagent Formulations for SDS-PAGE Running Buffer 10X

Reagent	Company	Final Concentration	Amount
Tris base	SIGMA	0.250M	30.3g
Glycine	SIGMA	1.924M	144.4g
SDS	SIGMA	0.035M	10g
$_{dd}H_2O$			To 1L

*** adjust the pH to 8.3**

Table 6. Reagent Formulations for SDS-PAGE Transfer Buffer 1X

Reagent	Company	Final Concentration	Amount
Tris base	SIGMA	0.250M	3g
Glycine	SIGMA	1.924M	14.4g
Methanol	SIGMA	20%	200mL
$_{dd}H_2O$			To 1L

*** adjust the pH to 8.3**

Table 7. Reagent Formulations for SDS-PAGE TBST 10X

Reagent	Company	Final Concentration	Amount
Tris base	SIGMA	0.2mM	48g
Nacl	SIGMA	1.5M	176g
$_{dd}H_2O$			To 2L

*** adjust the pH to 7.6**

Table 8. Reagent Formulations for SDS-PAGE Stripping Buffer 1X

Reagent	Company	Final Concentration	Amount
Glycine	SIGMA	100mM	15g
SDS	SIGMA	1%	1g
Tween 20	SIGMA	0.1%	1mL
$_{dd}H_2O$			1L

*** adjust the pH to 2.2**

III.12. Statistical Analysis

All statistical analyses were performed using GraphPad Prism version 10.0 (GraphPad Inc., San Diego, CA, USA). A *p* value of < 0.05 was considered statistically significant. Two-way ANOVA

followed by Sidak's multiple comparisons test was used to evaluate the effects of time and cell type on each dependent variable. For comparisons involving more than two groups, one-way ANOVA was used, and Tukey's post hoc test was applied when appropriate. For comparisons between two groups, an unpaired two tailed Student's t test was employed.

IV.RESULTS

IV.1.1.1 Adiponectin Receptor Expression and Antitumor Potential of AdipoRon in PTC: Insights from Multi-Omics and Tissue Level Validation

Integrated Analysis of TCGA and GTEx Data Reveals Differential Gene Expression Patterns of Adiponectin Receptors in Thyroid Cancer

To investigate the expression characteristics and potential clinical significance of the adiponectin signaling pathway in PTC, we integrated transcriptome data from tumor samples (n = 505) in the Cancer Genome Atlas Thyroid Cancer (**TCGA-THCA**) data collection and normal thyroid tissue samples (n = 355) from the **GTEx** project, both obtained from the UCSC Xena platform (https://xena.ucsc.edu/). Gene expression profiles were recomputed and batch-corrected, as described by Wang et al., enabling direct comparisons across cohorts. After unified normalization describe before, to ensure comparability between tumor and normal tissues, differential expression analysis identified a large number of genes significantly differentially expressed between the two groups (FDR < 0.05, $|\log_2$ fold change$|$ > 1). In **Figure 15A**, a volcano plot illustrating the global expression changes is shown, among the adiponectin pathway related genes, *CDH13* was significantly upregulated in tumor tissues, while *ADIPOR2* and *APPL1*, although not reaching significance, were significantly downregulated (FDR < 0.05), suggesting that these genes may play important regulatory roles in the development of thyroid cancer.

In relation with ***ADIPOR1* and *ADIPOR2* Expression and Their Survival Analysis,** the results showed that *ADIPOR1* expression was significantly higher than *ADIPOR2* (p < 0.0001)), indicating that these two receptors may have distinct regulatory patterns or functional differentiation in thyroid cancer (**Fig15 B**). Moreover, the Kaplan–Meier survival analysis using clinical follow up data, revealed that patients with high *ADIPOR1* expression was significantly associated with shorter overall survival (p = 0.015), while *ADIPOR2* expression was not significantly correlated with prognosis (p = 0.23) (**Fig15 C**). Finally, the multivariate Cox regression analysis indicated that age was the only statistically significant significantly associated with overall survival (HR = 1.13, 95% CI: 1.07–1.20, p < 0.001), whereas *ADIPOR1* and *ADIPOR2* were not independent prognostic factors (HR ≈ 1, p > 0.05) (**Fig15 D**).

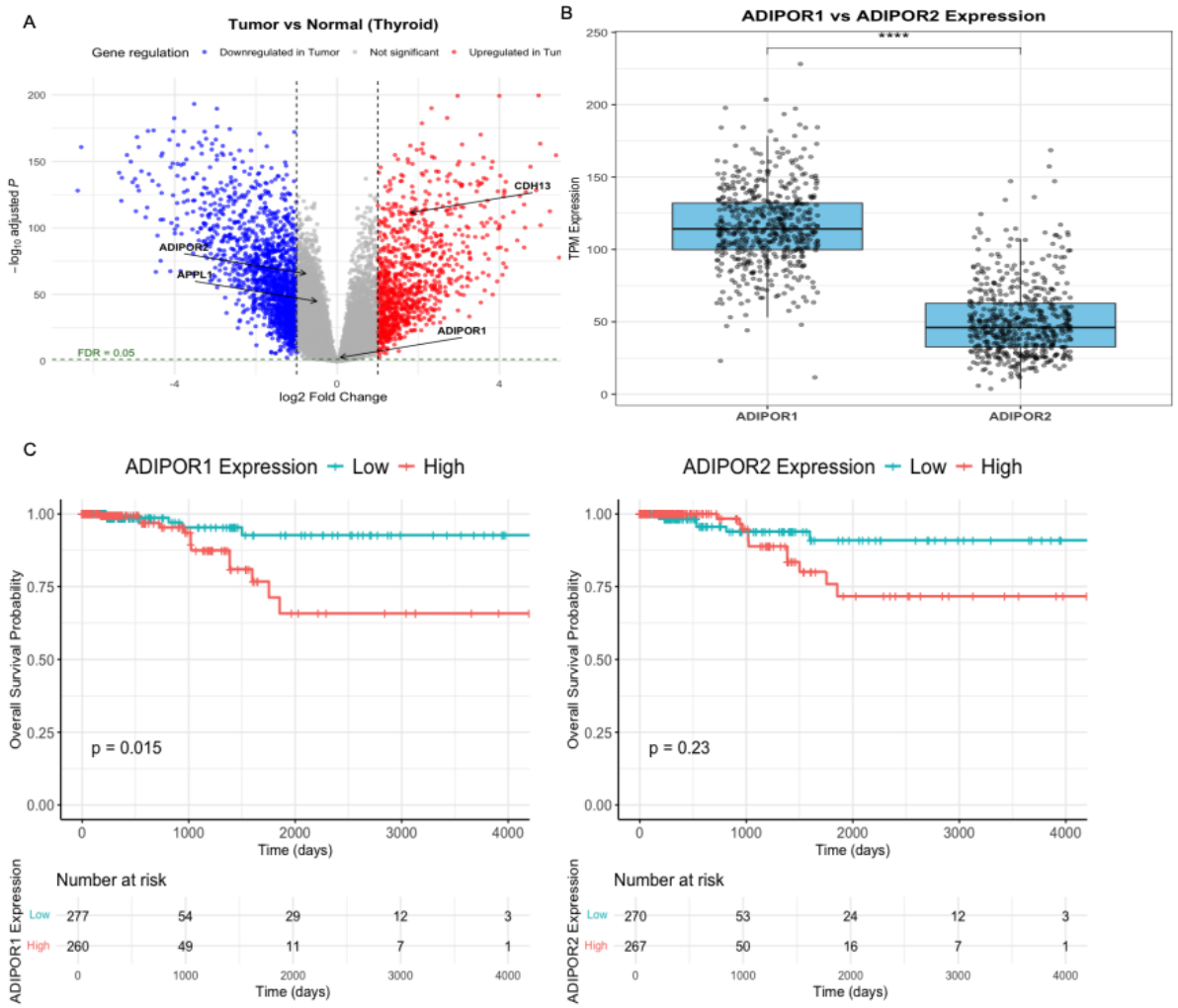


Figure 15. Expression and prognostic significance of *ADIPOR1* and *ADIPOR2* in PTC. (A) Volcano plot showing differentially expressed genes between TCGA-THCA tumor samples (n = 505) and GTEx normal thyroid tissues (n = 355). Significantly upregulated (red) and downregulated (blue) genes were defined by FDR < 0.05 and $|\log_2$ fold change$| > 1$. Key genes related to the adiponectin signaling pathway (*ADIPOR1, ADIPOR2, CDH13, APPL1*) are highlighted. **(B)** Boxplot comparing overall *ADIPOR1* and *ADIPOR2* expression in all thyroid tissue samples. *ADIPOR1* exhibited significantly higher expression than *ADIPOR2* ($p < 0.0001$, Wilcoxon test). **(C)** Kaplan–Meier survival curves based on TCGA-THCA cohort stratified by *ADIPOR1* and *ADIPOR2* expression levels (median split, log-rank test). **(D)** Multivariate Cox regression forest plot showing: hazard ratios (HR) and 95% confidence intervals for *ADIPOR1, ADIPOR2*, clinical stage, gender, and age.

IV.1.1.2 Validation of *ADIPOR1* and *ADIPOR2* gene Expression Differences Using Paired Samples from Multiple Datasets

To validate the above findings, we examined the differential expression of *ADIPOR1* and *ADIPOR2* in 28 paired thyroid tumor and adjacent normal tissue samples (stage N0) from the TCGA-THCA dataset. The analysis revealed no significant differences in the expression levels of either gene between tumor and normal tissues from the same patients ($p = 1$, paired Wilcoxon test) (**Figure 16 A-B**). In contrast, analysis of an independent public dataset, available from Gene Expression Omnibus (GEO) database, GSE60542 (n =14, paired samples), which includes paired PTC and normal samples, *ADIPOR1* and *ADIPOR2* were significantly downregulated in tumor tissues in comparison with their normal counterparts ($p < 0.01$) (**Figure 16 C-D**). The discrepancies between these two cohorts may reflect sample heterogeneity or differences in analytical platforms, underscoring the need for further validation in larger and multicenter cohorts.

IV.1.1.3 Differences in *ADIPOR1* and *ADIPOR2* gene expression According to Lymph Node Metastasis Status of the TCGA-THCA and GEO cohorts

A comparison of gene expression in samples stratified by lymph node status (negative, N0; and positive, N1) revealed that *ADIPOR2* expression was significantly decreased in patients with N1 status compared to N0 samples ($p = 5.5 \times 10^{-8}$). In contrast, *ADIPOR1* expression did not differ significantly between N0 and N1 groups ($p = 0.64$), (**Fig. 17 A–B**). However, this pattern was not replicated in datasets obtained from the GEO database, where no significant association was found neither *ADIPOR1* ($p = 0.32$) nor *ADIPOR2* ($p = 0.87$) in relation with lymph node status. This discrepancy may reflect again the biological heterogeneity, technical variability across cohorts or sample size limitations, underlining the importance of validation in independent sample sets to clarify the biological relevance of these findings in this tumor type (**Fig. 17C–D**).

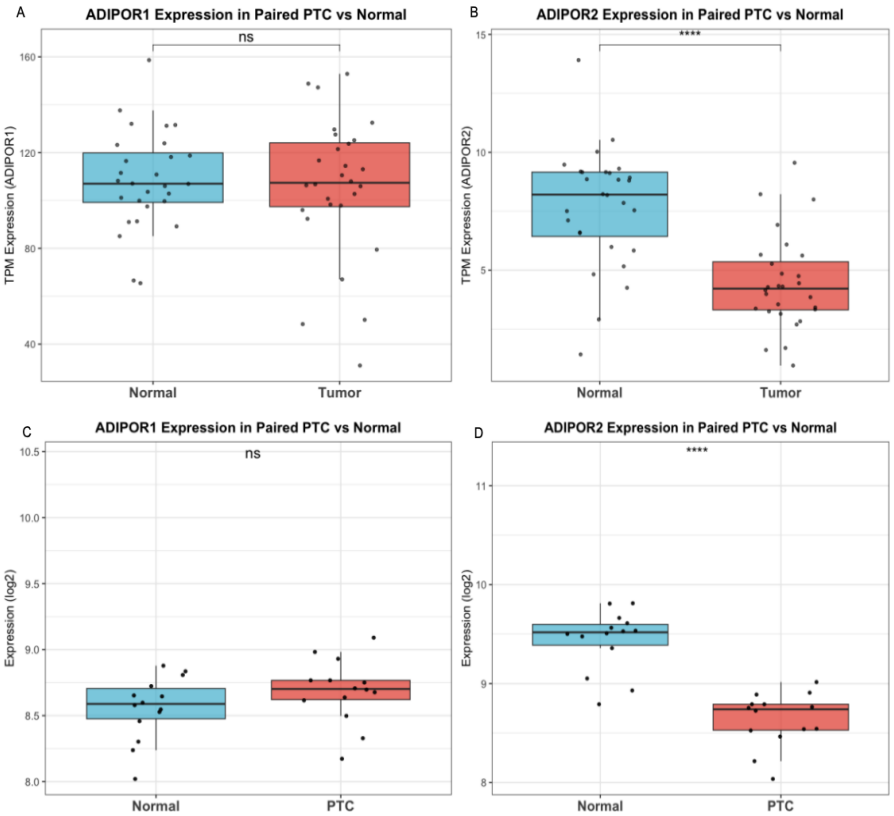


Figure 16. Cross-dataset validation of *ADIPOR1* and *ADIPOR2* expression in paired PTC and adjacent normal thyroid tissues. (A–B) In TCGA-THCA N0 subgroup (n=28 paired samples), expression of *ADIPOR1* and *ADIPOR2*. **(C–D)** Independent GEO dataset GSE60542, which includes paired PTC and normal samples (n = 14). Statistical significance was assessed using the **paired Wilcoxon signed-rank test** for both TCGA-THCA and GSE60542 datasets.

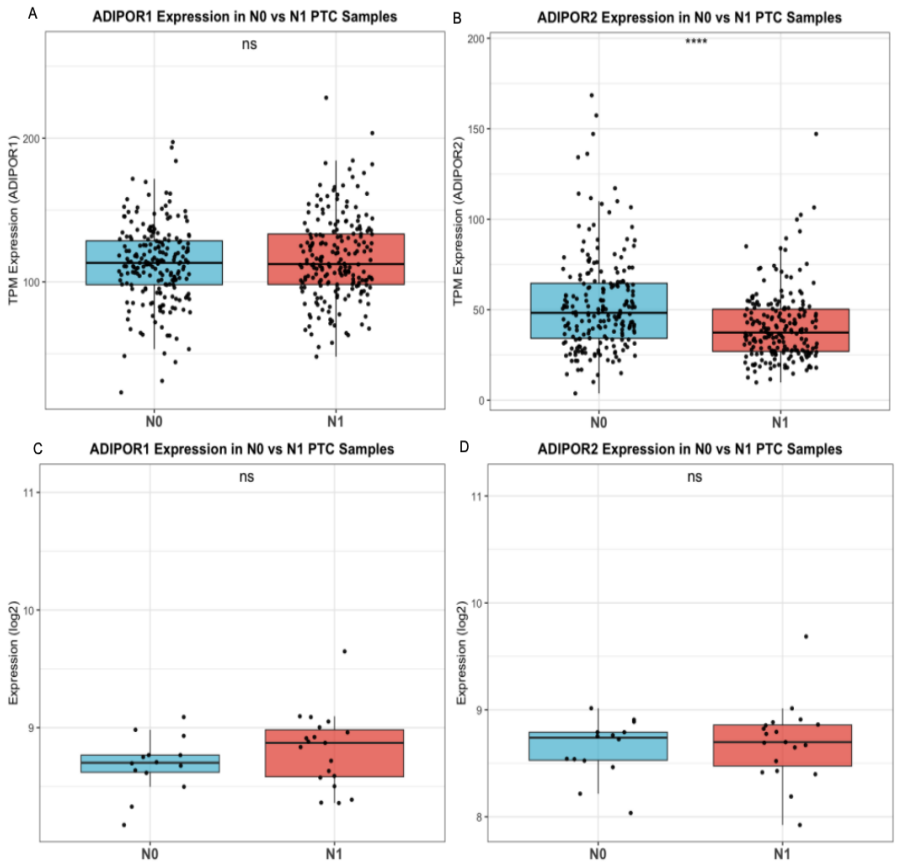


Figure 17. Comparison of *ADIPOR1* and *ADIPOR2* gene expression between lymph node–positive (N1) and lymph node–negative (N0) PTC samples in TCGA and GEO datasets. (A–B) Results from TCGA-THCA dataset. **(C–D)** Results from GSE60542 dataset. For comparison between N0 and N1 groups, statistical significance was assessed using the **Wilcoxon rank-sum test** for both TCGA and GEO datasets.

IV.1.1.4 KEGG Pathway Enrichment Analysis of ADIPOR1/2 Related Genes

Using expression data from N0 and N1 stages samples in TCGA and GEO datasets

We performed KEGG pathway enrichment analyses on genes positively correlated with *ADIPOR1* and *ADIPOR2* gene expression, respectively. *ADIPOR1* associated genes were mainly enriched in pathways related to apoptosis, immune regulation, and cellular senescence, suggesting its involvement in early tumor cell fate decisions and microenvironment modulation (**Fig.18 A-B**). In contrast, *ADIPOR2* associated genes were significantly enriched in pathways related to protein degradation and metabolic processes, indicating a potential role in maintaining metabolic homeostasis (**Fig. 18 C-D**). Further analysis in N1 stage (lymph node-positive) samples revealed that *ADIPOR1* related pathways predominantly involved cell death and proliferation regulation, such as the p53 signaling pathway and cell cycle control (**Fig.19 A-B).** Meanwhile, *ADIPOR2* related genes were closely linked to lipid metabolic reprogramming, including fatty acid metabolism and cholesterol transport pathways (**Fig.19 C-D**). Taken together, *ADIPOR1* and *ADIPOR2* may exhibit functional divergence at different stages of thyroid cancer progression, respectively participating in key processes such as cell death, immune modulation, and metabolic remodeling, highlighting their distinct mechanistic roles in PTC development.

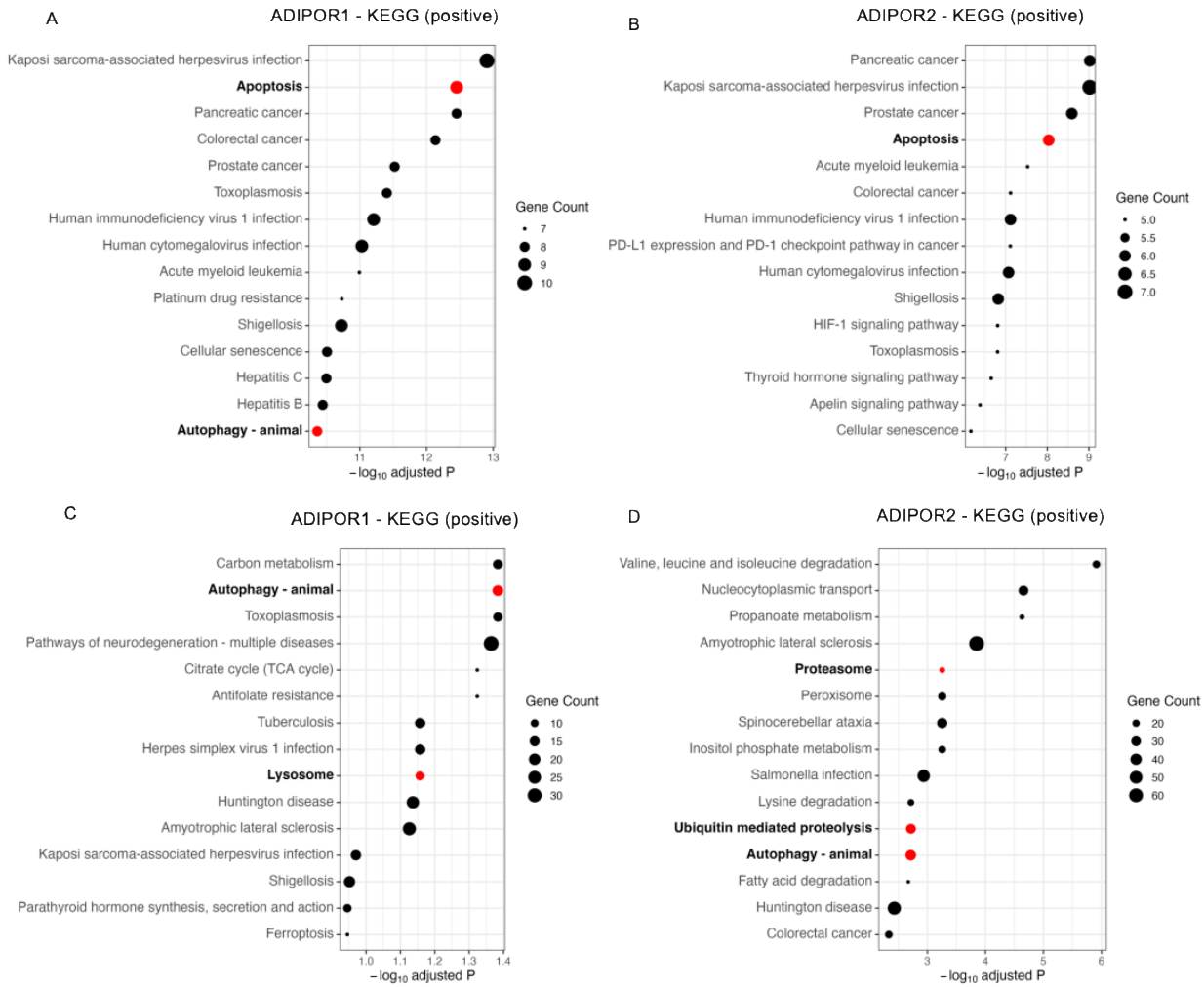


Figure 18. KEGG pathway enrichment analysis of ADIPOR1/2-associated genes in N0-stage PTC from TCGA and GEO datasets. **(A)** KEGG enrichment of genes upregulated in *ADIPOR1*-high samples from TCGA-THCA (N0-stage

PTC)**.** **(B)** In TCGA dataset, genes upregulated in *ADIPOR2*-high. **(C)** In the GEO dataset (GSE60542), genes upregulated in *ADIPOR1*-high **(D)** In the GEO dataset (GSE60542), genes upregulated in *ADIPOR2*-high. **KEGG** pathway enrichment was performed using a hypergeometric test with **Benjamini-Hochberg** correction for multiple comparisons. Only pathways with adjusted *p* values < 0.05 were considered statistically significant and are described in the text. Pathways highlighted in red represent key biologically relevant processes. Dot size indicates the number of enriched genes; the x-axis represents −log10(adjusted *p* value).

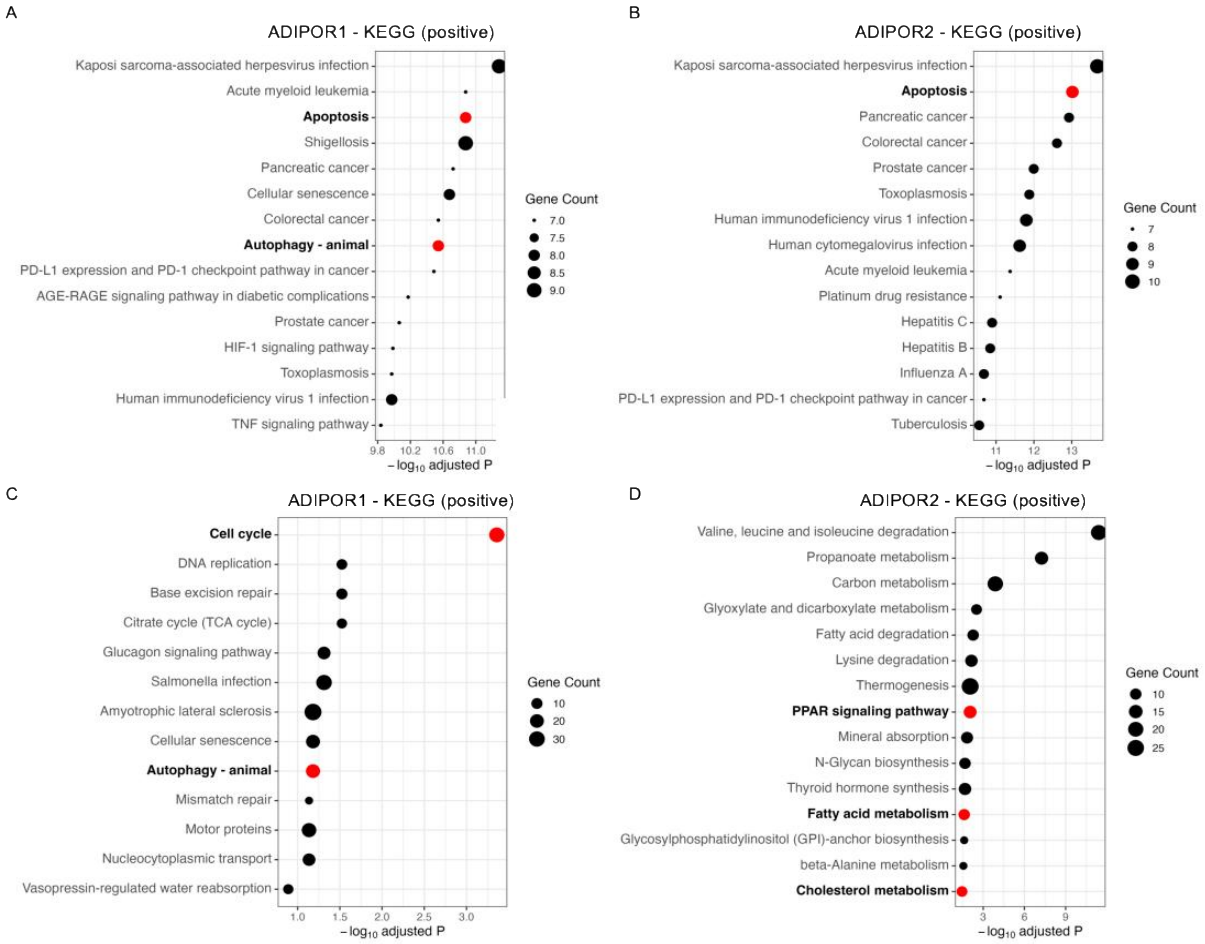


Figure 19. KEGG pathway enrichment analysis of ADIPOR1/2-associated genes in N1-stage PTC from TCGA and GEO datasets. **(A)** In TCGA-THCA, *ADIPOR1*-high samples showed significant enrichment in pathways including apoptosis and Autophagy. **(B)** *ADIPOR2*-high samples from TCGA were significantly enriched in apoptosis, colorectal cancer, and pancreatic cancer pathways, suggesting an association with tumor progression mechanisms. **(C)** GEO dataset (GSE60542) confirmed that *ADIPOR1*-high expression was significantly associated with enrichment in cell cycle supporting roles in cell proliferation. **(D)** In the GEO dataset, *ADIPOR2*-high samples were significantly enriched in fatty acid degradation, PPAR signaling pathway, and carbon metabolism, indicating that *ADIPOR2* may contribute to lipid metabolic regulation in metastatic PTC. **KEGG** pathway enrichment was performed using a hypergeometric test with **Benjamini-Hochberg** correction for multiple comparisons. Only pathways with adjusted *p* values < 0.05 were considered statistically significant and are described in the text. Pathways highlighted in red represent key biologically relevant processes (e.g., autophagy, apoptosis). Dot size indicates the number of enriched genes; the x-axis represents −log10(adjusted *p* value).

IV.1.1.5 Comparison of factors secreted in cervical adipose tissue (CAT) between patients with PTC and BT

To investigate the potential regulatory immunometabolic role of cervical adipose tissue in relation with PTC, secretion levels of adipokines and proinflammatory cytokines were analyzed in cervical adipose tissue (CAT) samples obtained from a cohort of patients diagnosed with PTC (n = 52) and BT (n = 32) in the HSCSP (**see Table 2**). As described in the Materials and Methods section, the samples were maintained in explant culture overnight at 37 °C, and enzyme-linked immunosorbent assays (ELISA) were performed. The results, presented in Figure 6, showed that although none of the cytokines reached statistical significance ($p > 0.05$), several factors, including adiponectin and TNF-α, exhibited a trend toward increased levels in the PTC group. No sex-related differences were observed (data not shown).

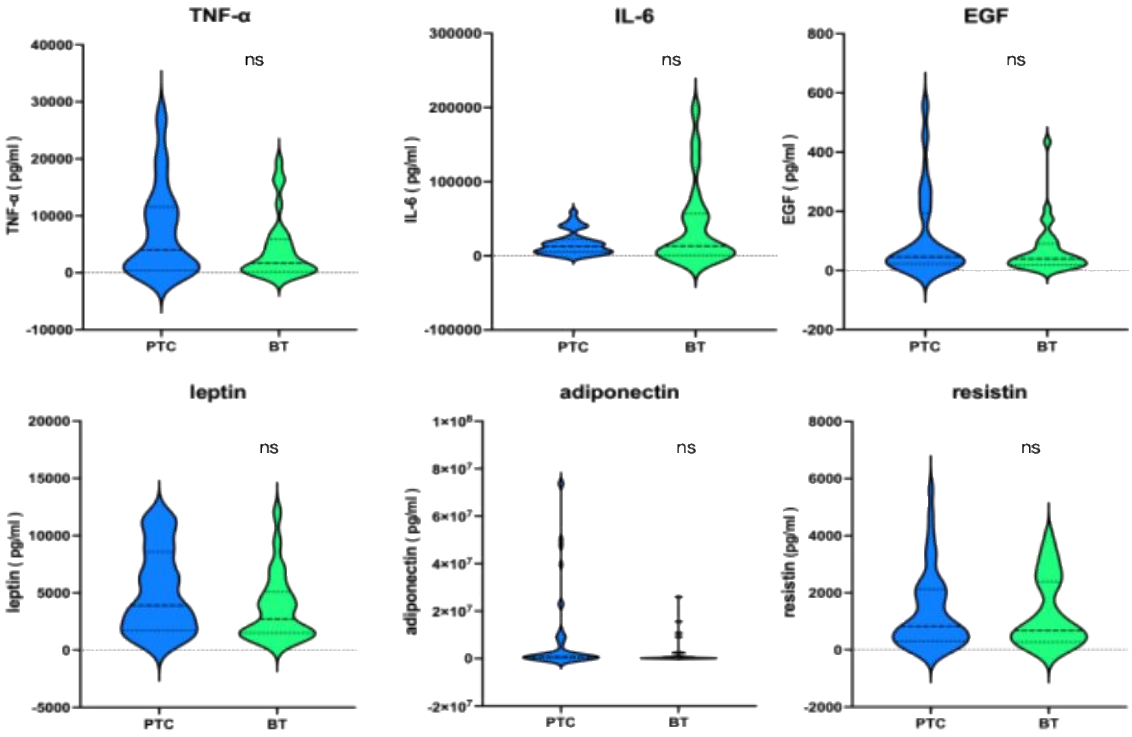


Figure 20. Secretion levels of adipokines and inflammatory cytokines from cervical adipose tissue (CAT) of PTC and BT patients. ELISA was performed to quantify TNF-α, IL-6, EGF, leptin, ANP, and resistin secretion (per mg of CAT) in PTC (n = 52) and BT (n = 32) patient samples. Data are presented as mean ± SD. Statistical significance was assessed using the **Wilcoxon rank-sum test** (ns = not significant).

IV.1.1.6 Differential Expression of AdipoR1 and AdipoR2 in Tumor and Normal Thyroid Tissues of PTC and BT Patients

The AdipoR1 and AdipoR2 analysis of the protein expression by western blot in paired tumor and adjacent normal thyroid tissues from PTC *versus* BT patients diagnosed with PTC (n = 17) and BT (n = 17) at the HSCSP, showed that in PTC patients, both AdipoR1 and AdipoR2 protein levels were significantly higher in tumor tissues compared to paired normal tissues ($p < 0.01$) (**Fig. 21 A)**. In contrast, in BT patients, only AdipoR2 was significantly upregulated in tumor tissues ($p < 0.05$), whereas AdipoR1 expression showed no significant change ($p > 0.05$) (**Fig. 21 B**).

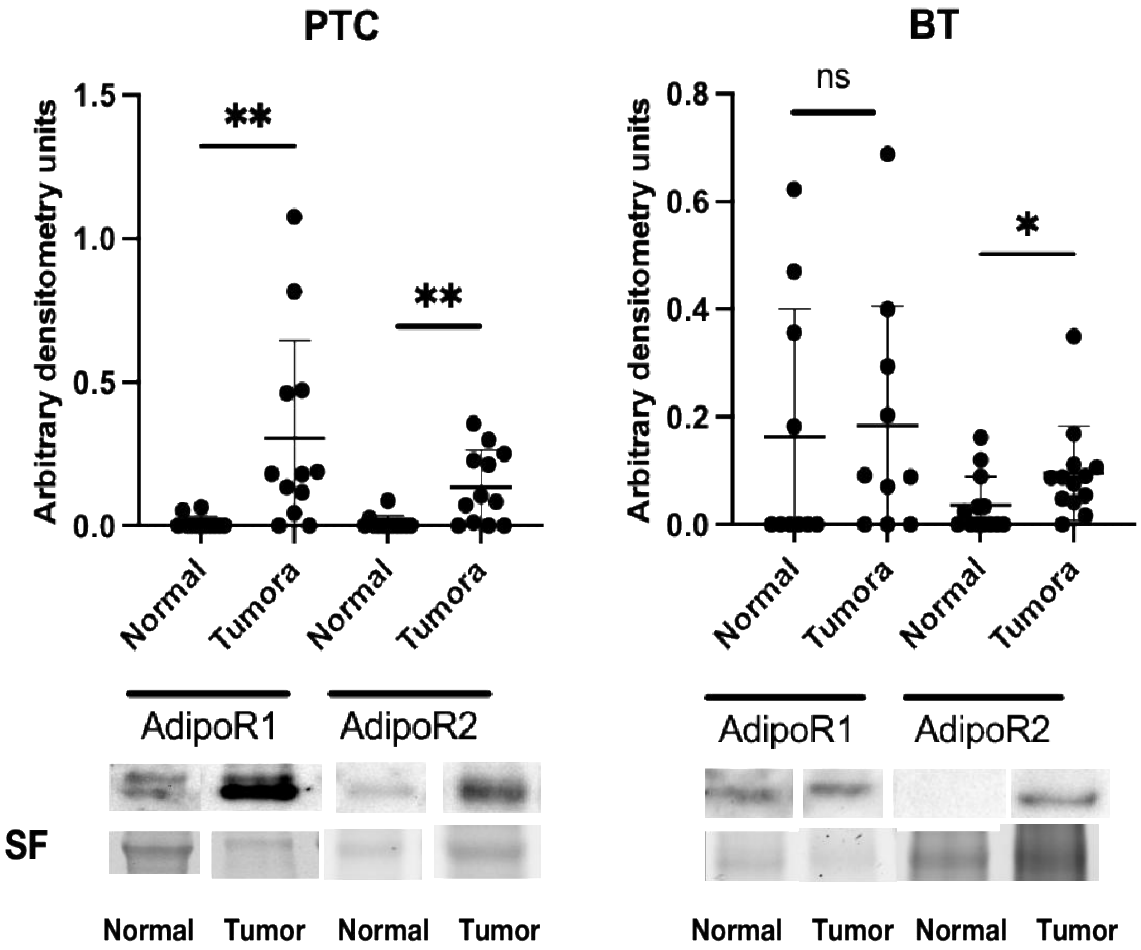


Figure 21. Western blot analysis was performed on paired tumor (Tumoral) and normal (Normal) thyroid tissues from patients with PTC and BT. In PTC tissues, both AdipoR1 and AdipoR2 were significantly upregulated in tumor tissues compared to normal tissues ($p < 0.01$). In BT samples, only AdipoR2 expression was significantly increased in tumors, while AdipoR1 expression showed no significant difference. Representative data are shown as mean ± SD. Statistical significance between paired tumor and normal tissues in both PTC and BT patients was assessed using the **paired Wilcoxon signed-rank** test. *P* values are indicated as * $P < 0.05$; ** $P < 0.01$; ns = not significant.

IV.1.2.1 Expression and Functional Roles of ADIPOR1/2 in Relation to BRAF Mutation in PTC

In orde to investigate whether BRAF mutation status affects the expression levels of adiponectin receptors *ADIPOR1* and *ADIPOR2,* we first analyzed samples in the GEO dataset GSE60542, the samples with different BRAF mutation statuses from PTC tissues. The results showed no significant difference in *ADIPOR1* and *ADIPOR2* expression between the BRAF mutant and wild-type groups (*ADIPOR1: p = 0.645; ADIPOR2: p = 0.5312*), suggesting that at the tissue level, BRAF mutation has a limited impact on adiponectin receptor expression. (**Fig. 22 A-B**). However, in the PTC cell line BCPAP, treatment with the BRAF inhibitor Vemurafenib for 48 hours significantly downregulated the expression of both *ADIPOR1* and *ADIPOR2* (*p < 0.05*). (**Fig. 22 C-D**).

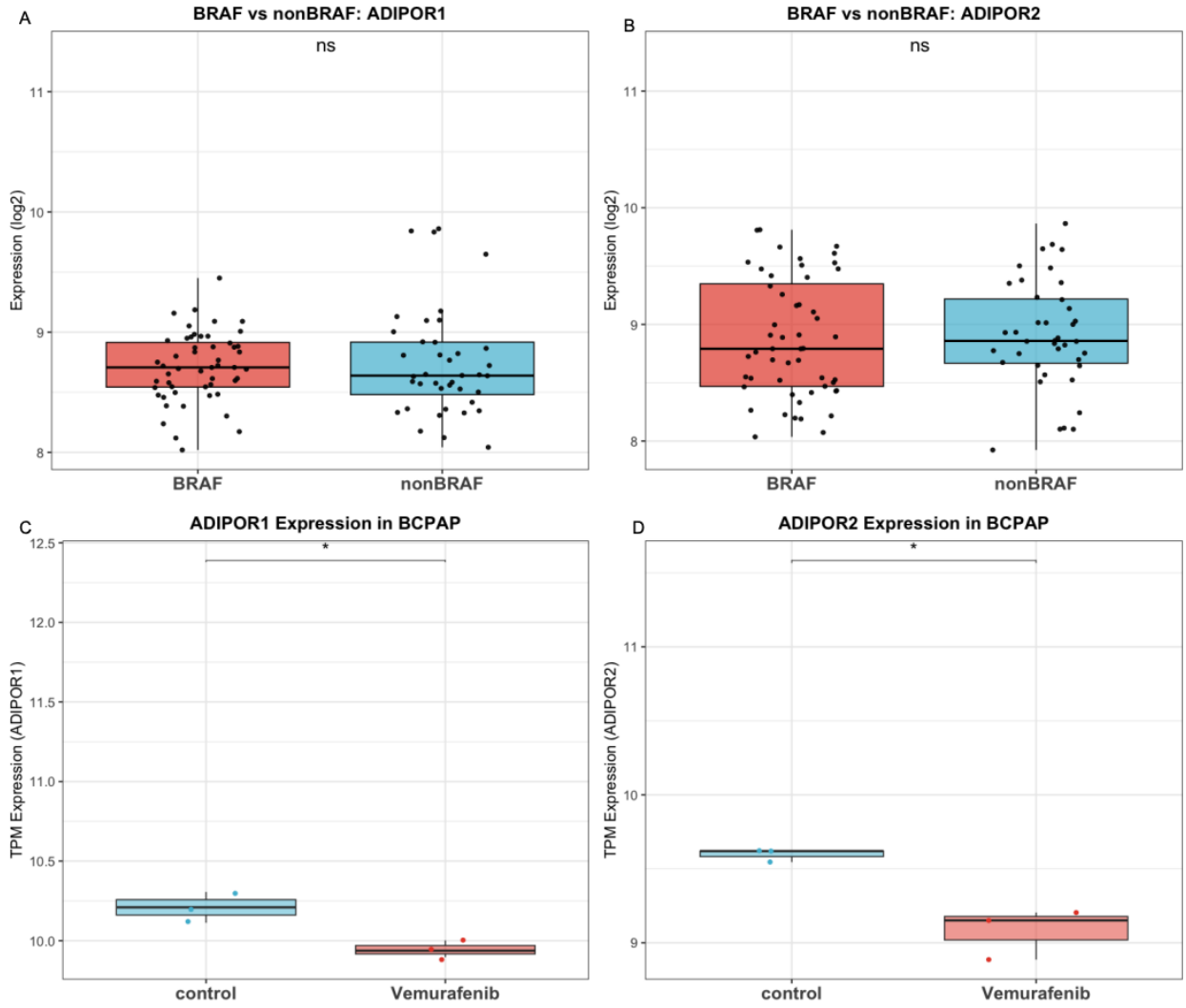


Figure 22. Relationship between BRAF mutation and ADIPOR1/2 expression in PTC tissues and BCPAP cell models. (**A-B**) Boxplots showing expression levels of *ADIPOR1* and *ADIPOR2* in PTC tissues from the GEO dataset (GSE60542), stratified by BRAF mutation status. **(C-D**) Boxplots of *ADIPOR1* and *ADIPOR2* expression in BCPAP cells (GSE171483) treated with BRAF inhibitor Vemurafenib *versus* control. Representative data are shown as mean ± SD. Statistical comparisons between two groups were performed using the **Wilcoxon rank-sum test**. P values are indicated as * $p < 0.05$.

IV.1.2.2 Enrichment Analysis of Apoptosis, Inflammation, and Lysosome Pathways Related to BRAF Mutation Status

To further elucidate the molecular mechanisms associated with BRAF mutation in PTC, gene set enrichment analysis (GSEA) was performed comparing BRAF-mutant and wild-type samples from the GEO dataset GSE60542. Results demonstrated significant positive enrichment of the MAPK signaling pathway, TNF signaling pathway, apoptosis pathway, and lysosome pathway in the BRAF-mutant group. (**Fig. 23 A-D**). Further GSEA analysis was conducted in the BRAF-mutant PTC cell line BCPAP (GSE171483) treated with the BRAF inhibitor Vemurafenib, showing that the apoptosis regulation pathway and intrinsic apoptotic signaling pathways were significantly enriched, indicating that BRAF inhibition *in vitro* can reactivate apoptotic programs, potentially representing a key mechanism of its antitumor effect. (**Fig. 23 E-F).**

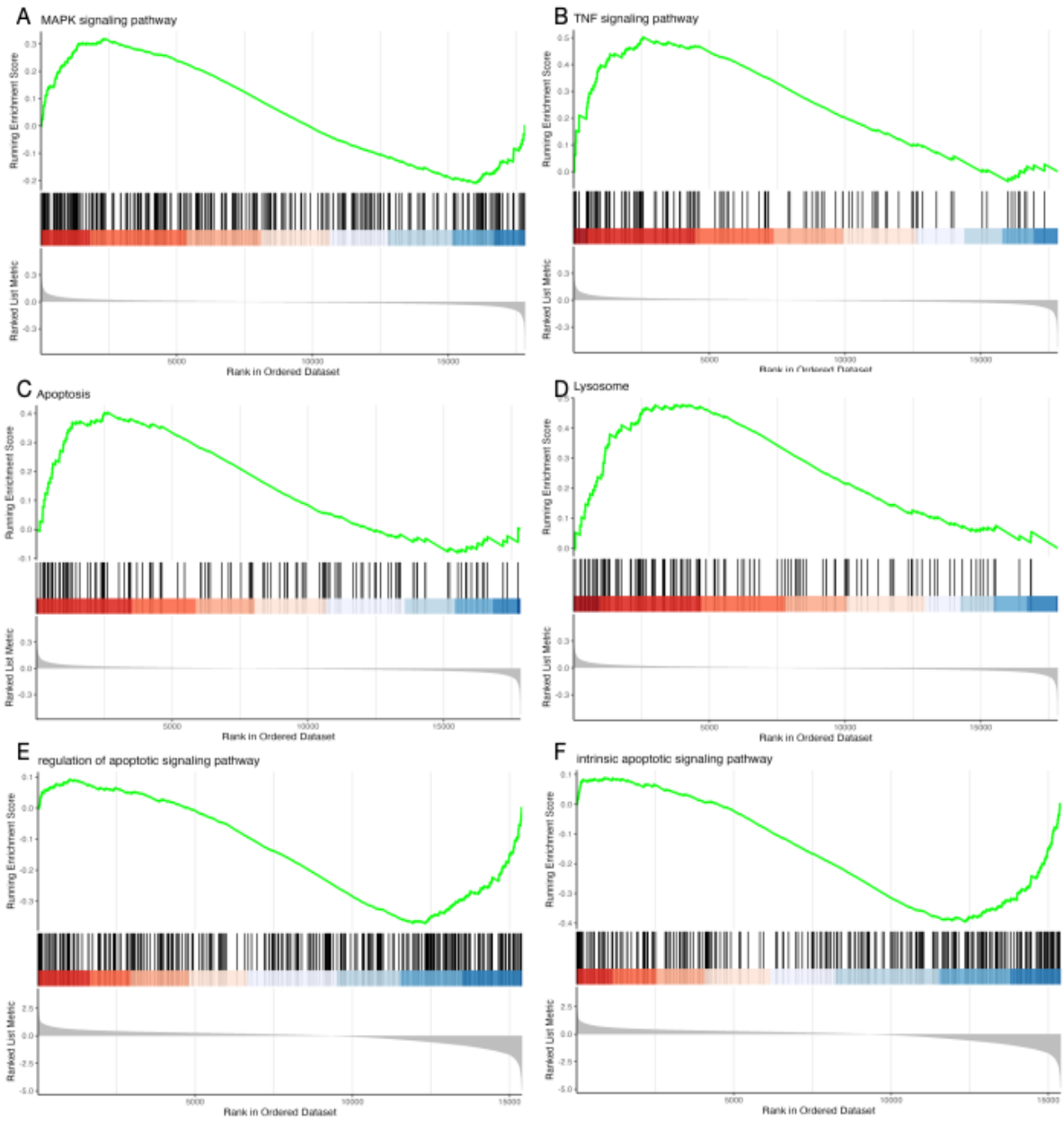


Figure 23. Gene Set Enrichment Analysis (GSEA) of PTC tissues from the GEO dataset (GSE60542) and BCPAP cells (GSE171483). (A–D) Analysis (GSEA) of PTC tissues BRAF-mutant tumors showed positive enrichment of the MAPK signaling pathway **(A),** TNF signaling pathway **(B),** apoptosis **(C),** and lysosome **(D), (E–F) GSEA** of BCPAP cells (GSE171483) treated with the BRAF inhibitor Vemurafenib. The regulation of apoptotic signaling pathway **(E)** and **(F)**

intrinsic apoptotic signaling pathway. **GSEA** was performed using a permutation-based method. Significance was determined by normalized enrichment score (**NES**) and **FDR** adjusted *q*-values, with pathways considered significant at **FDR** < 0.25.

IV.1.2.3 Correlation Analysis of ADIPOR1/2 with Key Apoptosis and Autophagy Genes under BRAF Inhibition in BCPAP cell line

Using the GSE171483 dataset, to explore the potential roles of *ADIPOR1* and *ADIPOR2* against BRAF targeted therapy, we performed Pearson correlation analysis on gene expression profiles in vemurafenib treated BCPAP cells, assessed the relationships between *ADIPOR1/2* and key apoptosis markers (*CASP3, CASP9, AIFM1*) as well as autophagy-related genes (*SQSTM1, MAP1LC3B*). Results demonstrated that expression levels of both *ADIPOR1* and *ADIPOR2* were significantly positively correlated with multiple pro-apoptotic and autophagy genes. Notably, the downstream genes associated with *ADIPOR1* and *ADIPOR2* were not completely overlapping, suggesting potential functional divergence or distinct regulatory mechanisms between the two receptors in modulating cell death pathways (**Fig. 24**).

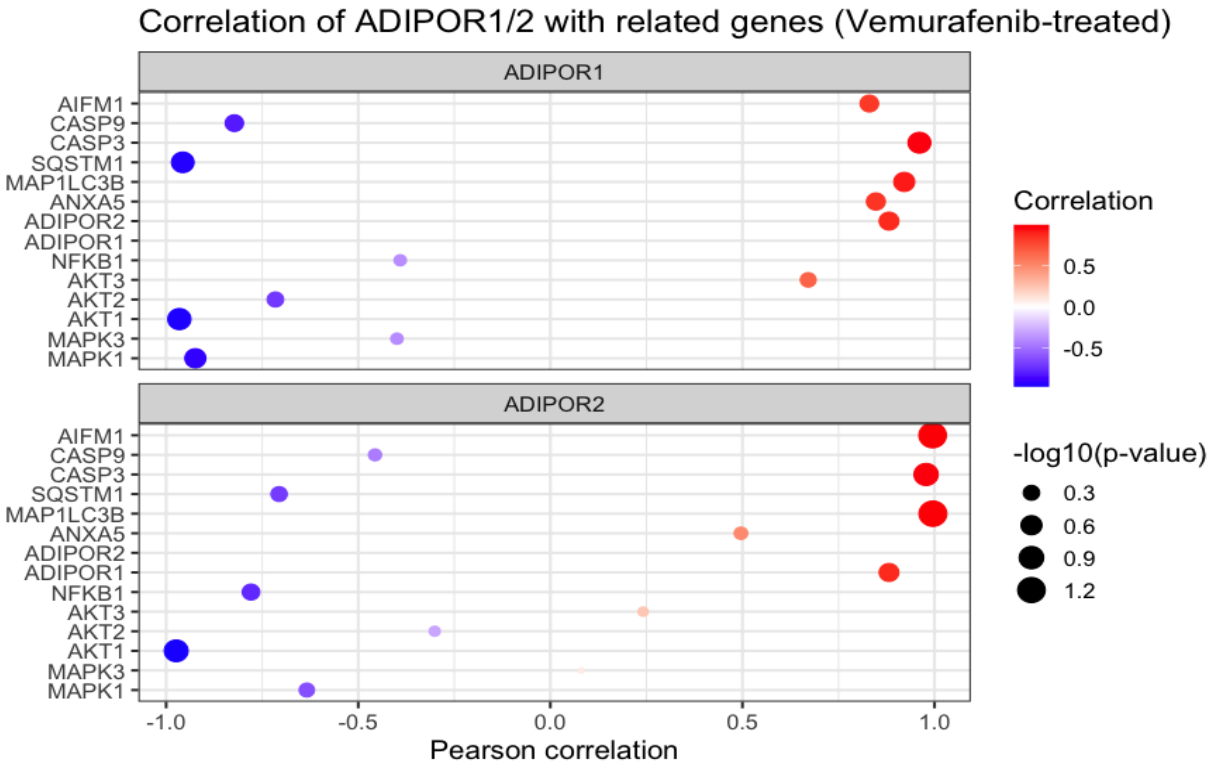


Figure 24. Correlation of ADIPOR1/2 expression with key apoptotic and autophagic genes under BRAF inhibition. Analysis was performed in BCPAP cells treated with the BRAF Inhibition vemurafenib. *ADIPOR1* and *ADIPOR2* expression levels were correlated with apoptosis-related genes and autophagy markers. Correlation coefficients were calculated using Pearson correlation analysis. Positive correlations (in red) suggest potential co-regulation, while negative correlations (in blue) indicate possible inverse relationships. Dot size reflects statistical significance (−log10 of *p* value), and color scale indicates direction and strength of the correlation (from blue: negative, to red: positive).

IV.1.2.4 AdipoRon Inhibits Proliferation and Migration of BCPAP Cell Line

MTT assay results showed that AdipoRon treatment at 25 µM and 100 µM significantly inhibited BCPAP cell proliferation at 24, 48, and 72 hours in a time-dependent manner. Compared with untreated controls, cell viability was markedly reduced (*a: $p < 0.001$; b: $p < 0.0001$*), indicating a sustained inhibitory effect of AdipoRon on cell growth (**Fig. 25 A)**. Treatment with a concentration gradient of AdipoRon (6.25–100 µM) for 48 hours caused a clear dose dependent decrease in BCPAP cell proliferation. Nonlinear regression analysis of the dose response curve further validated the strong pharmacological inhibitory effect of AdipoRon on BCPAP cells **(Fig. 25 B**). Additionally, wound healing assays revealed that AdipoRon significantly suppressed BCPAP cell migration, with the wound closure rate notably lower than that of the control group (*$p < 0.01$*) (**Fig. 25 C**).

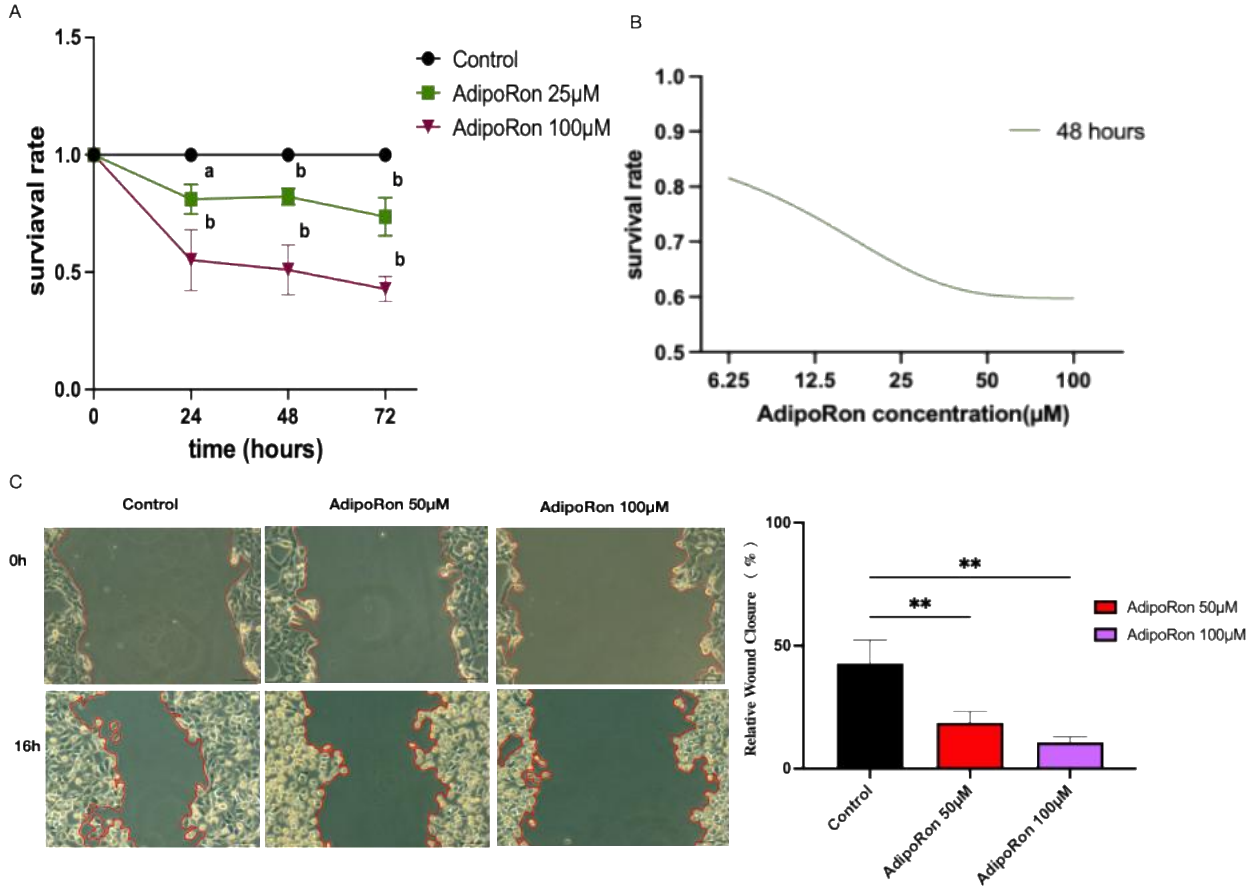


Figure 25. AdipoRon inhibits cell viability and migration in BCPAP cells. (A) Cells were treated with AdipoRon at concentrations of 25 µM and 100 µM for 24, 48, and 72 hours. Cell viability was assessed by the MTT assay and normalized to the corresponding untreated control. Data are presented as mean ± SD (n ≥ 3*).* Statistical comparisons were performed using one-way ANOVA followed by **Dunnett's** post hoc test. a*: $p < 0.001$;* b*: $p < 0.0001$ vs.* control group**. (B)** Cells were treated with increasing concentrations of AdipoRon (6.25–100 µM) for 48 hours. A nonlinear regression model fitted the dose–response curve, showing concentration-dependent inhibition of proliferation. **(C)** Wound healing assay further demonstrated impaired migration capacity after AdipoRon exposure. Data are presented as mean ± SD (n=3). Statistical comparisons were performed using one-way ANOVA followed by **Dunnett's** post hoc test. ** $p < 0.01$ vs. control group.

IV.1.2.5 AdipoRon Induces Changes in the Cell Cycle, Nuclear and Cytoplasmic Morphology in BCPAP Cell Line

Flow cytometry analysis showed that treatment with AdipoRon (25 µM) for 48 hours led to an increase in the sub-G0/G1 cell population, suggesting possible induction of apoptosis. However, this increase did not reach statistical significance (**Fig. 26 A**). DAPI staining demonstrated enhanced nuclear fluorescence intensity in AdipoRon treated cells, indicative of nuclear condensation compatible with apoptosis processes. Nonetheless, differences in fluorescence intensity between treated and control groups were not statistically significant (**Fig. 26 B**). H&E staining revealed morphological changes in AdipoRon treated BCPAP cells, such as cytoplasmic vacuolization and reduced cell volume, suggesting autophagy related structural remodeling in the cells (**Fig. 27 A**). AO staining further confirmed a significant increase in AVOs in AdipoRon treated cells, as evidenced by enhanced red fluorescence, indicating markedly elevated autophagic activity ($p < 0.0001$ vs. control) (**Fig. 27 B**).

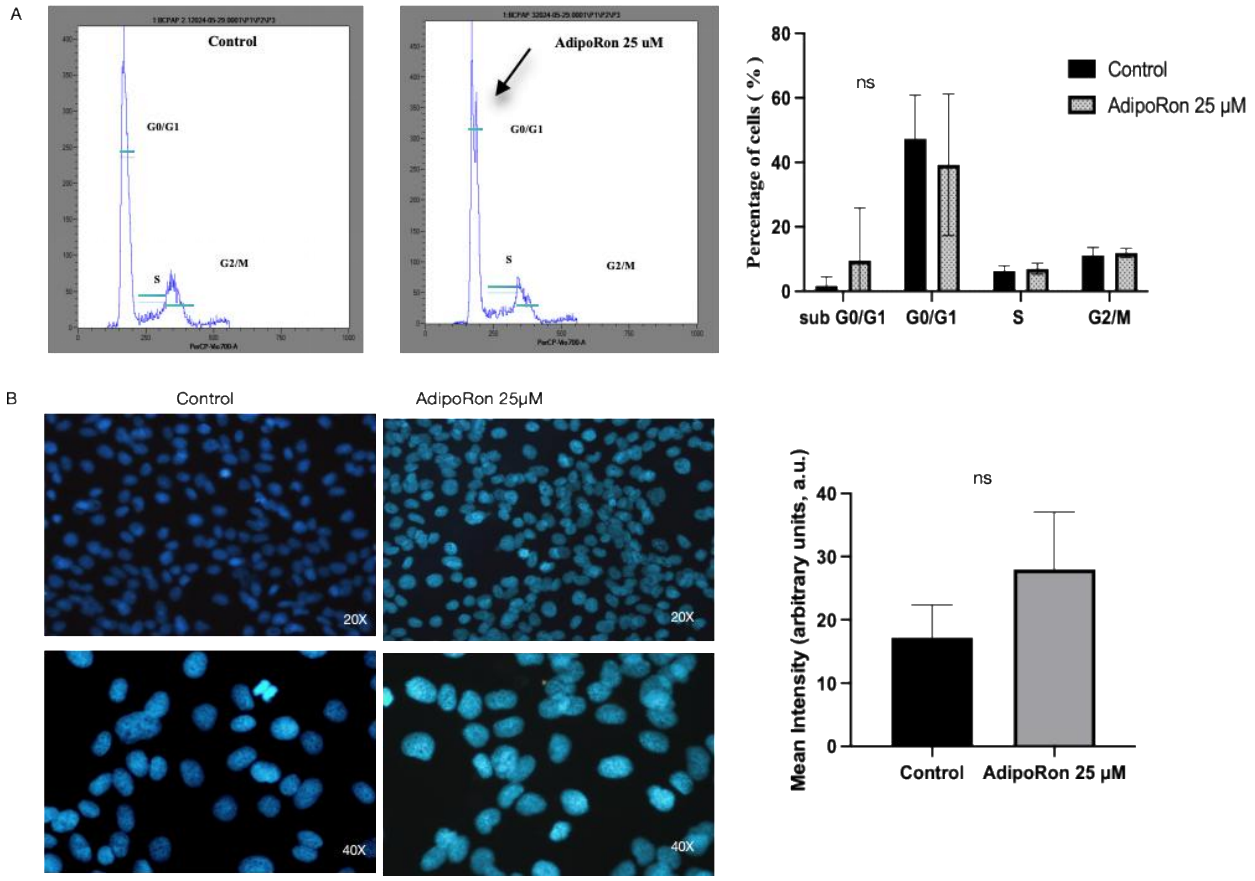


Figure 26. AdipoRon seems to induce apoptotic changes responses in BCPAP cells. **(A)** BCPAP cells were treated with AdipoRon (25 µM) for 48 hours. Flow cytometry after PI staining. **(B)** Fixed cell DAPI staining showed enhanced nuclear fluorescence intensity following AdipoRon treatment, implying nuclear condensation consistent with apoptosis, though without statistically significant change. Data are presented as mean ± SD (n = 3). Statistical comparisons between two groups were performed using unpaired two tailed Student's t test (ns = not significant).

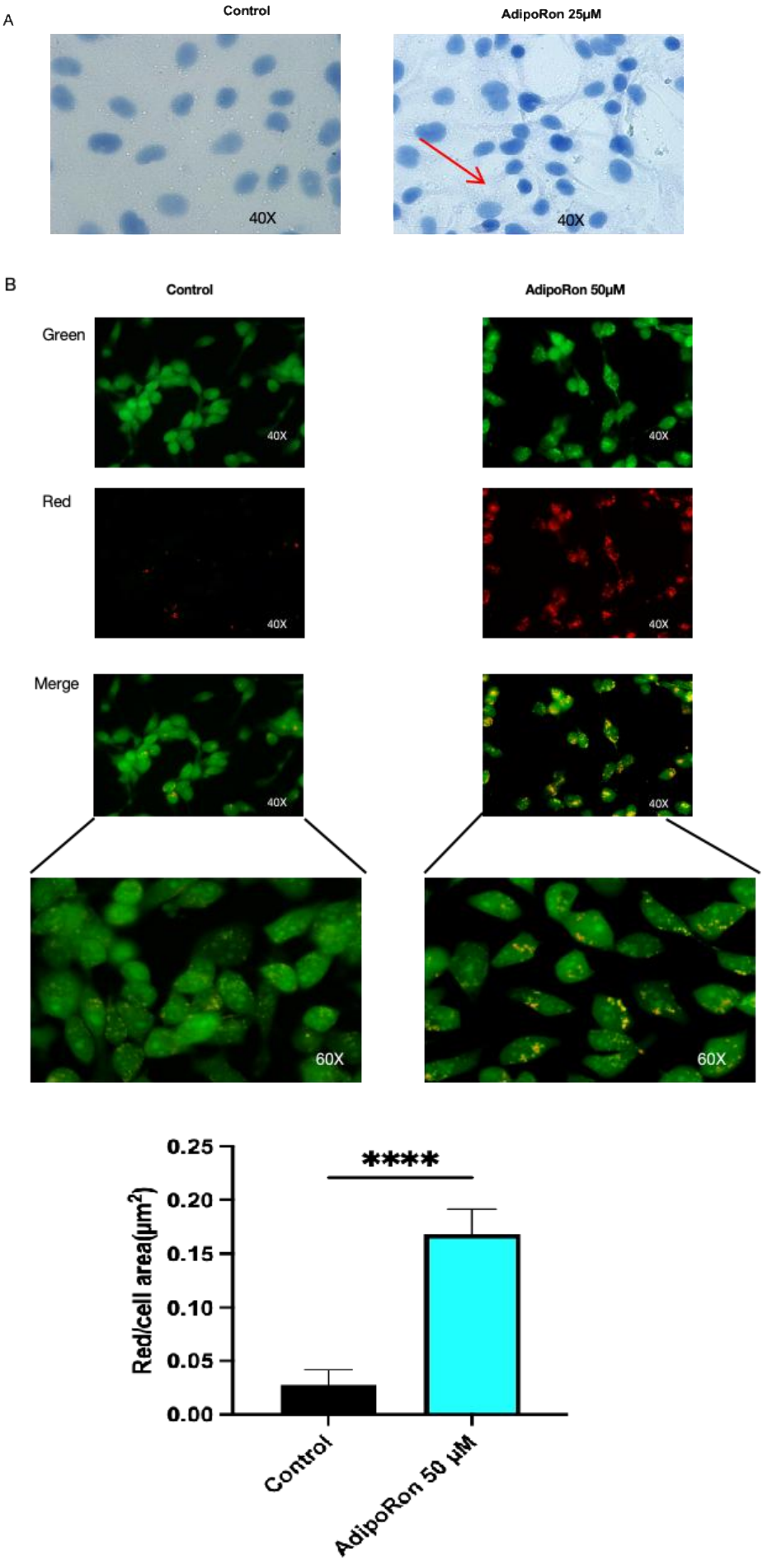


Figure 27. AdipoRon induces changes in the cytoplasm compatible with autophagic responses in BCPAP cells (A) H&E staining revealed a morphological trend toward smaller and more diffuse cytoplasmic vacuoles in AdipoRon treated BCPAP cells, with fewer large vacuoles than in controls. **(B)** AO staining demonstrated increased red fluorescence in AdipoRon treated cells, indicating the accumulation of AVOs, a hallmark of autophagy activation. Data are presented as mean ± SD (n = 3). Statistical comparisons between two groups were performed using unpaired two tailed Student's t test. **** p < 0.0001 vs. control group.

IV.1.2.6 AdipoRon has the ability to modulate autophagy and apoptosis signaling pathways without fully activating their effector mechanisms in the BCPAP Cell Line

Western blot results demonstrated significant alterations in autophagy- and apoptosis-related signaling pathways in AdipoRon treated cells. Specifically, phosphorylated AKT (p-AKT) levels were markedly elevated, indicating activation of survival signaling pathways that typically suppress autophagy. However, phosphorylated mTOR (p-mTOR) and ERK (p-ERK) levels showed no significant changes, suggesting incomplete activation or a blockage within the autophagy-related signaling cascade. In support of this, the autophagy marker p62 accumulated significantly, and the LC3B-II/I ratio remained unchanged, indicating impaired autophagic flux and an overall state of incomplete or suppressed autophagy (**Fig. 28 A-B**). Apoptosis-related proteins showed a significant increase in Annexin V expression ($p < 0.05$) and a non-significant increase in Caspase-3, indicating partial induction of apoptosis. In contrast, Caspase-9 expression was reduced, suggesting that the execution phase of apoptosis was not fully activated. (**Fig. 28 C**).

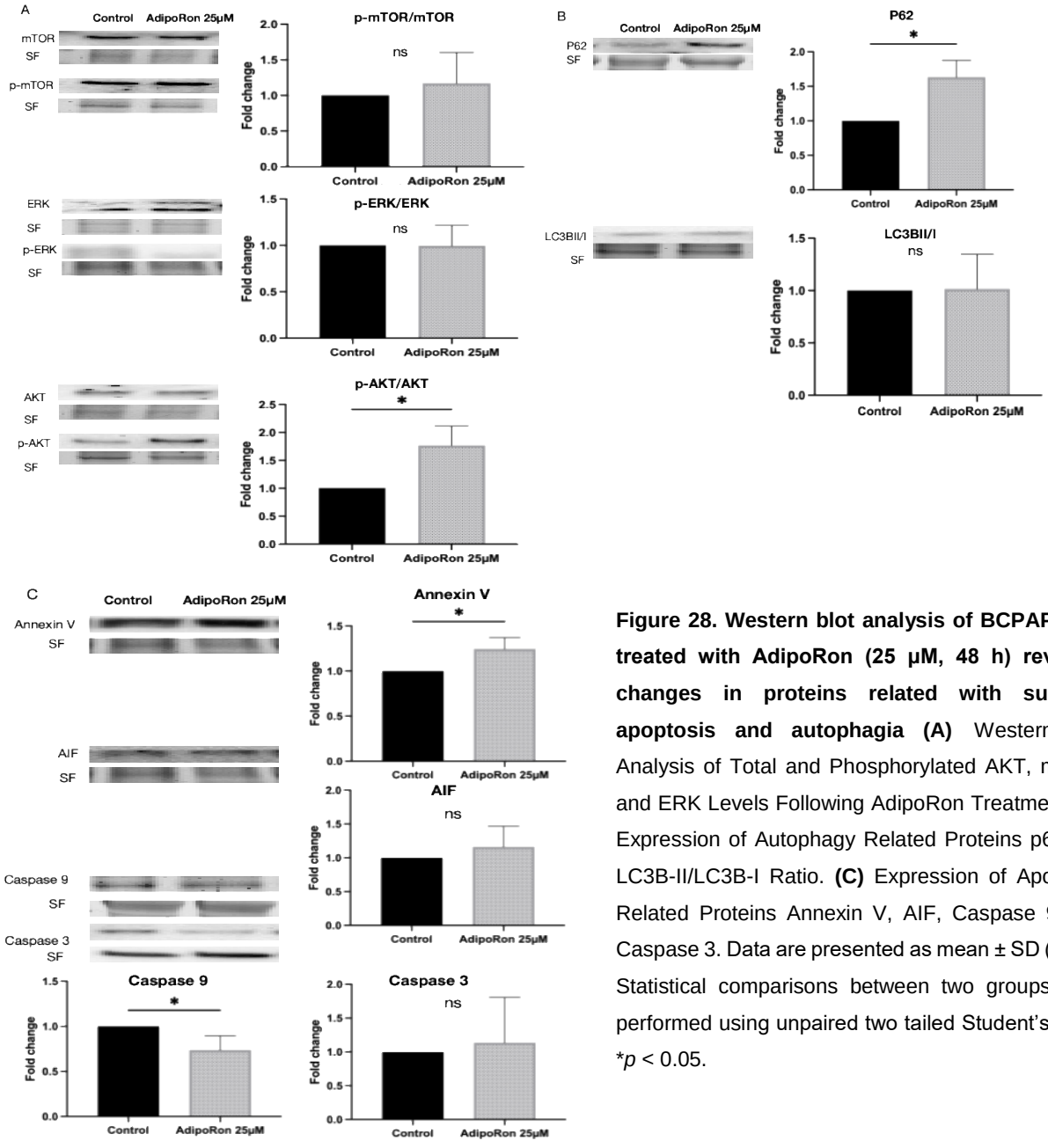


Figure 28. Western blot analysis of BCPAP cells treated with AdipoRon (25 μM, 48 h) revealed changes in proteins related with survival, apoptosis and autophagia (A) Western Blot Analysis of Total and Phosphorylated AKT, mTOR, and ERK Levels Following AdipoRon Treatment. **(B)** Expression of Autophagy Related Proteins p62 and LC3B-II/LC3B-I Ratio. **(C)** Expression of Apoptosis Related Proteins Annexin V, AIF, Caspase 9, and Caspase 3. Data are presented as mean ± SD (n ≥ 3). Statistical comparisons between two groups were performed using unpaired two tailed Student's t test; *$p < 0.05$.

IV.1.2.7 AdipoRon Enhances the Antitumor Effect of Vemurafenib on BCPAP Cell Line

To investigate whether AdipoRon has able to potenciate the antitumor efficacy of the BRAF inhibitor Vemurafenib, combination treatments were performed on BCPAP cells. The MTT assays showed that compared to Vemurafenib alone (1 μM), co-treatment with AdipoRon (50 μM or 100 μM) for 48 hours was able to reduce the cell viability ($p < 0.0001$), indicating that AdipoRon markedly enhances Vemurafenib's inhibitory effect on cell proliferation (**Fig. 29 A**). Furthermore, wound healing assays demonstrated pronounced suppression of cell migration in the combination treatment groups, along with significant cellular loss of adhesion observed after 16 hours ($p < 0.01$) (**Fig. 29 B**).

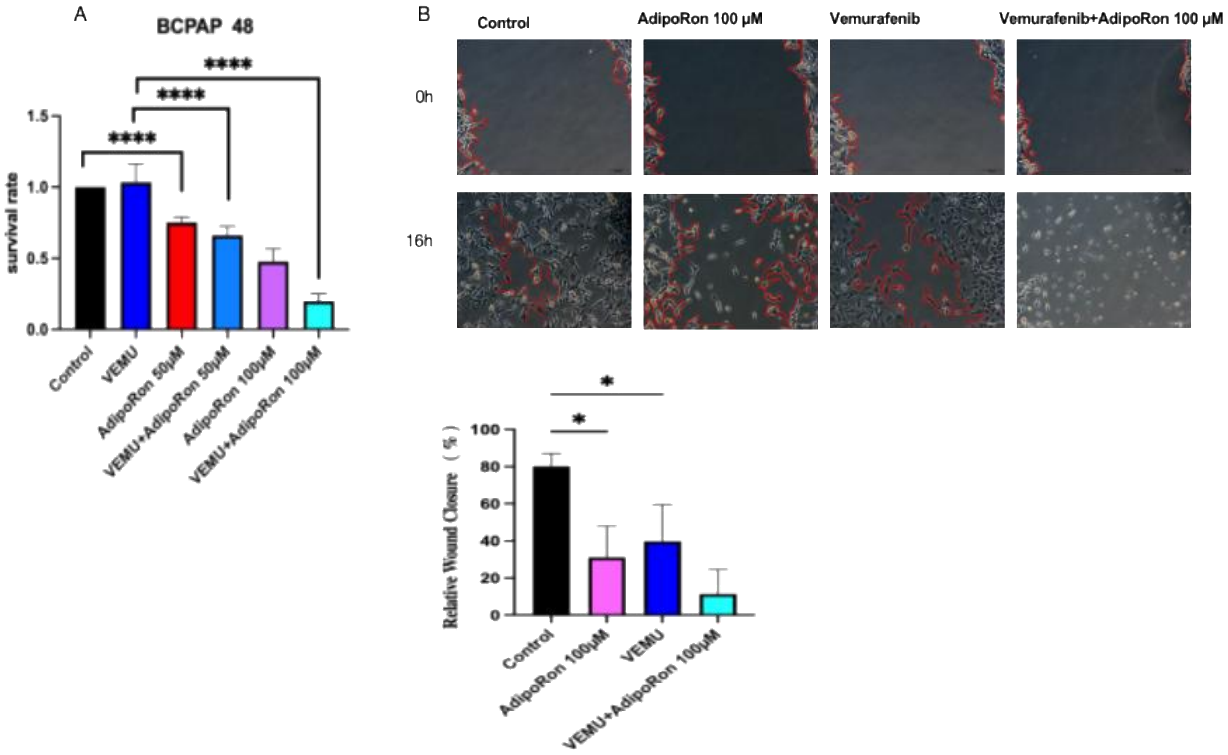


Figure 29. AdipoRon enhances the antitumor effect of Vemurafenib in BCPAP cells. (**A)** BCPAP cells were treated with Vemurafenib (1 μM) alone or in combination with AdipoRon (50μM or 100μM) for 48 hours. Cell viability was assessed by the MTT assay. Data are presented as mean ± SD (n ≥3). Statistical significance was evaluated using one-way ANOVA followed by **Dunnett's** post hoc test; ****$p < 0.0001$ vs Vemurafenib alone. **(B)** Wound healing assay demonstrated that AdipoRon further impaired cell migration in Vemurafenib treated BCPAP cells after 16 hours. Red dashed lines indicate the wound edges. Scale bar = 200 μm. Data are presented as mean ± SD (n=3). Statistical significance was evaluated using one-way ANOVA followed by **Dunnett's** post hoc test; *$p < 0.05$ ； **$p < 0.01$ vs. Control group.

IV.1.3.1 Expression of *ADIPOR1* and *ADIPOR2* and their Impact on the Biological Behavior of PTC Tissue and the PTC Cellular Model (TPC-1) Harboring RET/PTC1 Rearrangement

To investigate the impact of RET/PTC1 rearrangement on adiponectin receptor expression, we analyzed the GEO dataset GSE60542. The results showed that in PTC tissues harboring RET/PTC1 rearrangement, *ADIPOR1* was significantly downregulated in RET/PTC1 rearrangement tissues compared to non-mutant tissues ($p = 0.0027$), whereas *ADIPOR2* showed upregulated ($p = 0.016$). This suggests that RET/PTC1 rearrangement may specifically regulate *ADIPOR1* and *ADIPOR2* expression (**Fig. 30 A-B**). Further, in the TPC-1 cell model treated with the RET tyrosine kinase inhibitor selpercatinib (GSE261830), *ADIPOR1* expression was significantly increased ($p < 0.05$), while *ADIPOR2* expression remained unchanged. This indicates that RET signaling activity may suppress *ADIPOR1* transcriptional expression under *in vitro* conditions, and this inhibitory effect can be reversed by RET-targeted inhibition (**Fig. 30 C-D**). Collectively, these findings imply that the RET signaling pathway may participate in tumorigenesis and progression of PTC by modulating downstream signaling through suppression of *ADIPOR1* expression.

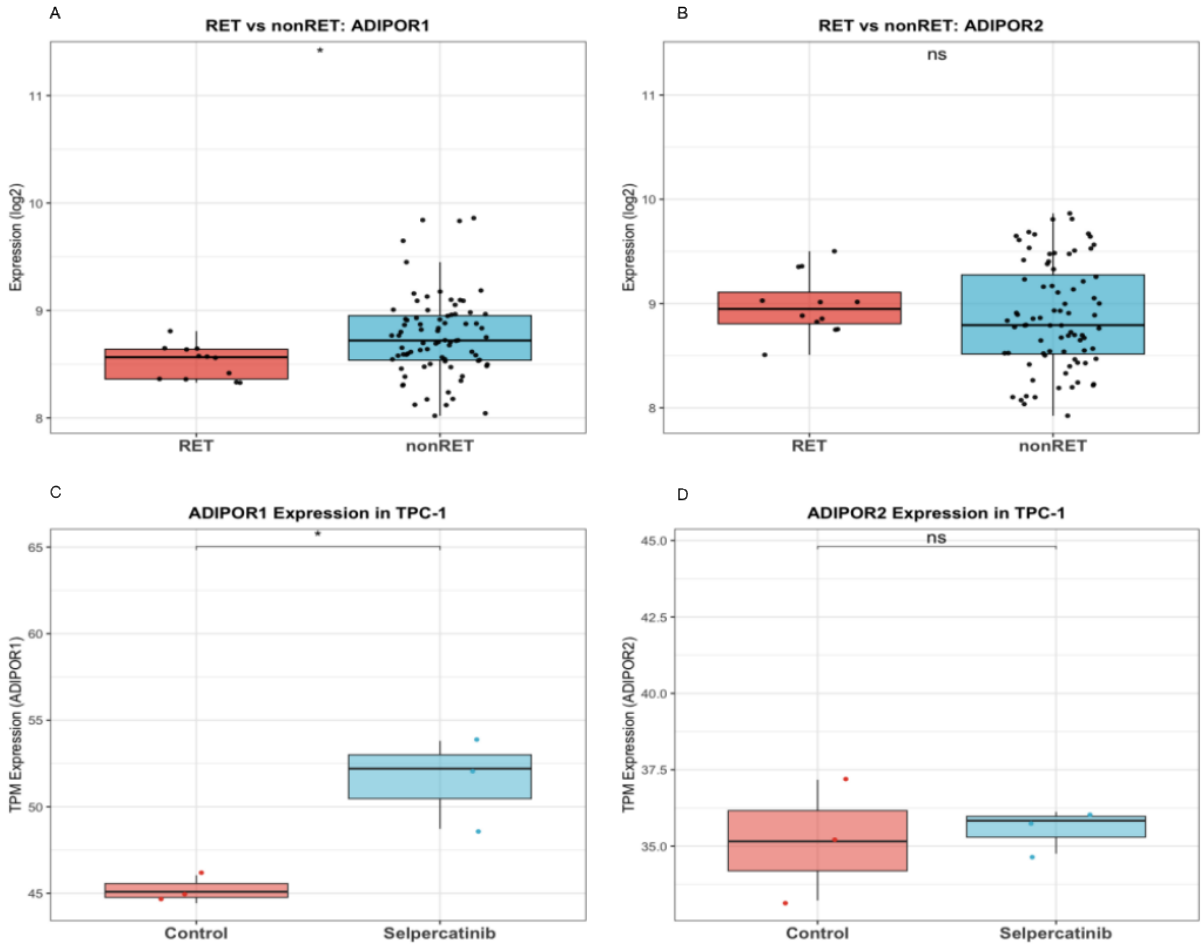


Figure 30. Relationship between RET/PTC1 rearragement and ADIPOR1/2 expression in PTC tissues and TPC-1 cell models. (**A-B)** Boxplots showing expression levels of *ADIPOR1* and *ADIPOR2* in PTC tissues from the GEO dataset (GSE60542), stratified by RET/PTC1 rearrangement status. **(C-D)** Boxplots of *ADIPOR1* and *ADIPOR2* expression in TPC-1 cells (GSE261830) RET- tyrosine kinase inhibitors（selpercatinib) versus control cells (without treatment). Statistical comparisons were performed using the **Wilcoxon rank-sum test**. Significance levels: *$p < 0.05$ ； **$p < 0.01$.

IV.1.3.2 Enrichment Analysis of Apoptosis, Inflammation, and Lysosomal Signaling Pathways Associated with RET/PTC1 Rearrangement Status

Gene Set Enrichment Analysis (GSEA) comparing RET/PTC1-positive and -negative PTC tissue samples revealed significant enrichment of pro-inflammatory TNF signaling and cell cycle regulation pathways in tumors harboring the RET/PTC1 rearrangement. This indicates that this mutation may activate inflammatory responses and promote cellular proliferation. (**Fig. 31 A-B**). In the TPC-1 cell model treated with the RET tyrosine kinase inhibitor selpercatinib, lipid metabolism and atherosclerosis pathways, as well as cytokine-cytokine receptor interaction pathways, were significantly enriched. This suggests that RET signaling inhibition may regulate tumor cell metabolic activities and immune modulation. (**Fig. 31 C-D**).

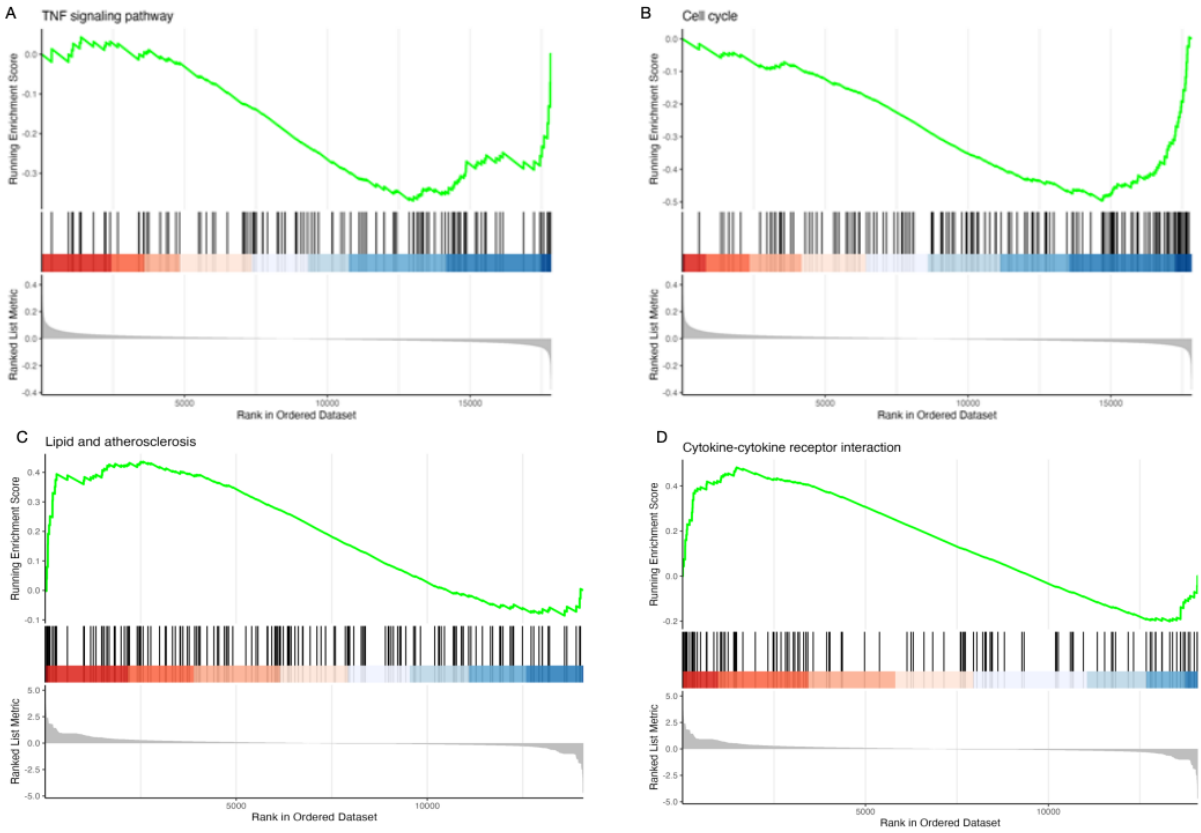


Figure 31. Enrichment of inflammatory, proliferative, metabolic, and immune pathways in RET/PTC1 rearrangement versus non-mutant PTC tissues and TPC-1 cells. (A–B) Gene Set Enrichment Analysis (**GSEA**) of PTC tissues from the GEO dataset (GSE60542), stratified by RET/PTC1 rearragement status. (**A**) TNF signaling pathway and (**B**) Cell cycle pathway were significantly enriched in RET/PTC1 rearragement tumors. **(C–D)** GSEA of TPC-1 cells (GSE261830) treated with the RET- tyrosine kinase inhibitors (selpercatinib). The regulation of (**C**) Lipid and atherosclerosis and (**D**) Cytokine–cytokine receptor interaction. ***GSEA*** was performed using a permutation-based method. Significance was determined by normalized enrichment score (**NES**) and **FDR** adjusted *q* values, with pathways considered significant at **FDR** < 0.25.

IV.1.3.3 Correlation Analysis of *ADIPOR1* and *ADIPOR2* with Key Apoptosis and Autophagy Genes under RET Inhibition

Pearson correlation analysis was performed in TPC-1 cells treated with the RET inhibitor selpercatinib to examine the relationship between ADIPOR1/2 and key apoptosis-related genes (*CASP3, CASP9, AIFM1*), as well as autophagy markers (*SQSTM1, MAP1LC3B*). The results showed that *ADIPOR1* expression was significantly positively correlated with *MTOR*, but negatively correlated with *CASP9*. In contrast, *ADIPOR2* expression was positively correlated with *MAPK1/3* and *AKT3*, but negatively correlated with *AKT1* and the autophagy related genes *SQSTM1* and *MAP1LC3B*. These findings suggest that, under RET signaling inhibition, *ADIPOR1* and *ADIPOR2* may regulate tumor cell apoptosis and autophagy through distinct mechanisms involving the mTOR and AKT pathways. (**Fig. 32**)

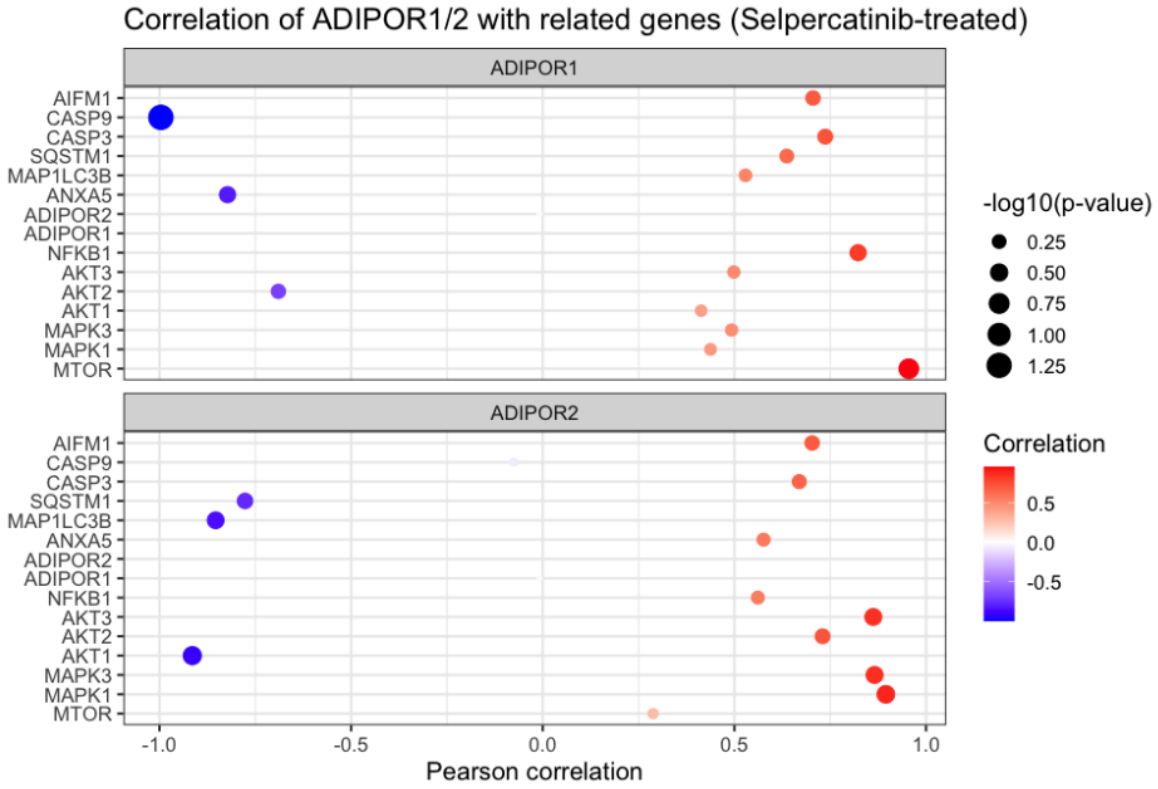


Figure 32. Pearson Correlation of ADIPOR1/2 expression with key apoptotic and autophagic genes under RET/PTC1 inhibition. Analysis was performed in TPC-1 cells treated with the RET inhibitor Selpercatinib. *ADIPOR1* and *ADIPOR2* expression levels were correlated with apoptosis related genes and autophagy markers. Correlation coefficients were calculated using Pearson correlation analysis. Positive correlations (in red) suggest potential co-regulation, while negative correlations (in blue) indicate possible inverse relationships. Dot size reflects statistical significance (−log10 of *p* value), and color scale indicates direction and strength of the correlation (from blue: negative, to red: positive).

IV.1.3.4 AdipoRon Inhibits Proliferation of TPC-1 Cell Line

AdipoRon treatment significantly suppressed TPC-1 cell proliferation and migration. MTT assays revealed that treatment with 25 µM and 100 µM AdipoRon for 24, 48, and 72 hours led to a significant time-dependent decrease in cell proliferation compared to controls ($p < 0.001$ or $p < 0.0001$) (**Fig 33. A**). Dose-response curve analysis further confirmed the concentration dependent inhibitory effect of AdipoRon on TPC-1 proliferation (**Fig. 33 B**). The wound healing assays showed a non-significant trend of slower migration in the AdipoRon treated group, with visibly reduced wound closure rates (**Fig 33. C**). These effects were less pronounced compared to those observed in BCPAP cells.

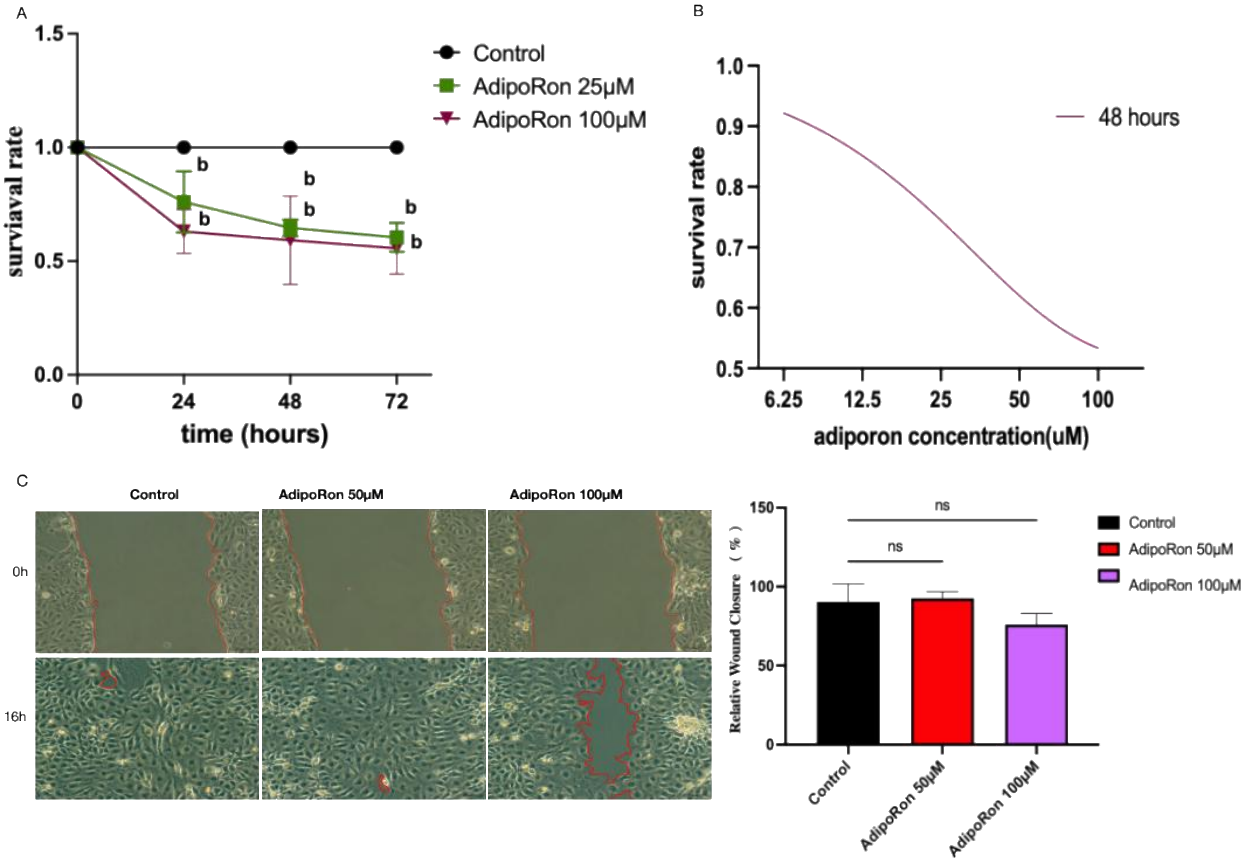


Figure 33. AdipoRon inhibits cell viability and migration in TPC-1 cells. **(A)** TPC-1 cells were treated with AdipoRon at concentrations of 25 µM and 100 µM for 24, 48, and 72 hours. Cell viability was assessed by the MTT assay and normalized to the corresponding untreated control. Data are presented as mean ± SD (n ≥ 3). Statistical comparisons were performed using one-way ANOVA followed by Dunnett's post hoc test. a: $p < 0.001$; b: $p < 0.0001$ vs. control group. **(B)** TPC-1 cells were treated with increasing concentrations of AdipoRon (6.25–100 µM) for 48 hours. A nonlinear regression model fitted the dose response curve, showing concentration-dependent inhibition of proliferation. **(C)** Wound healing assay further demonstrated impaired migration capacity after AdipoRon exposure. Data are presented as mean ± SD (n = 3). Statistical comparisons were performed using one-way ANOVA followed by **Dunnett's** post hoc test. (ns = not significant).

IV.1.3.5 AdipoRon Does Not Significantly Induce Changes in the Cell Cycle, Nuclear and Cytoplasmic Morphology in the TPC-1 Cell Line

AdipoRon treatment failed to significantly induce apoptosis or autophagy related responses in TPC-1 cells. Flow cytometry analysis showed no notable increase in the sub-G0/G1 cell population, indicating absence of apoptotic cell cycle arrest (**Fig. 34 A**). DAPI staining revealed no significant changes in nuclear staining intensity or typical apoptotic nuclear condensation (**Fig. 34 B**). H&E staining showed no obvious morphological alterations except for abundant cytoplasmic vacuolization, suggestive of metabolic stress or paraptosis-like changes. (**Fig. 35 A**). Acridine orange staining indicated no significant accumulation of acidic vesicular organelles, further confirming lack of autophagy activation (**Fig. 35 B**). Overall, AdipoRon did not markedly induce apoptosis or autophagy in TPC-1 cells.

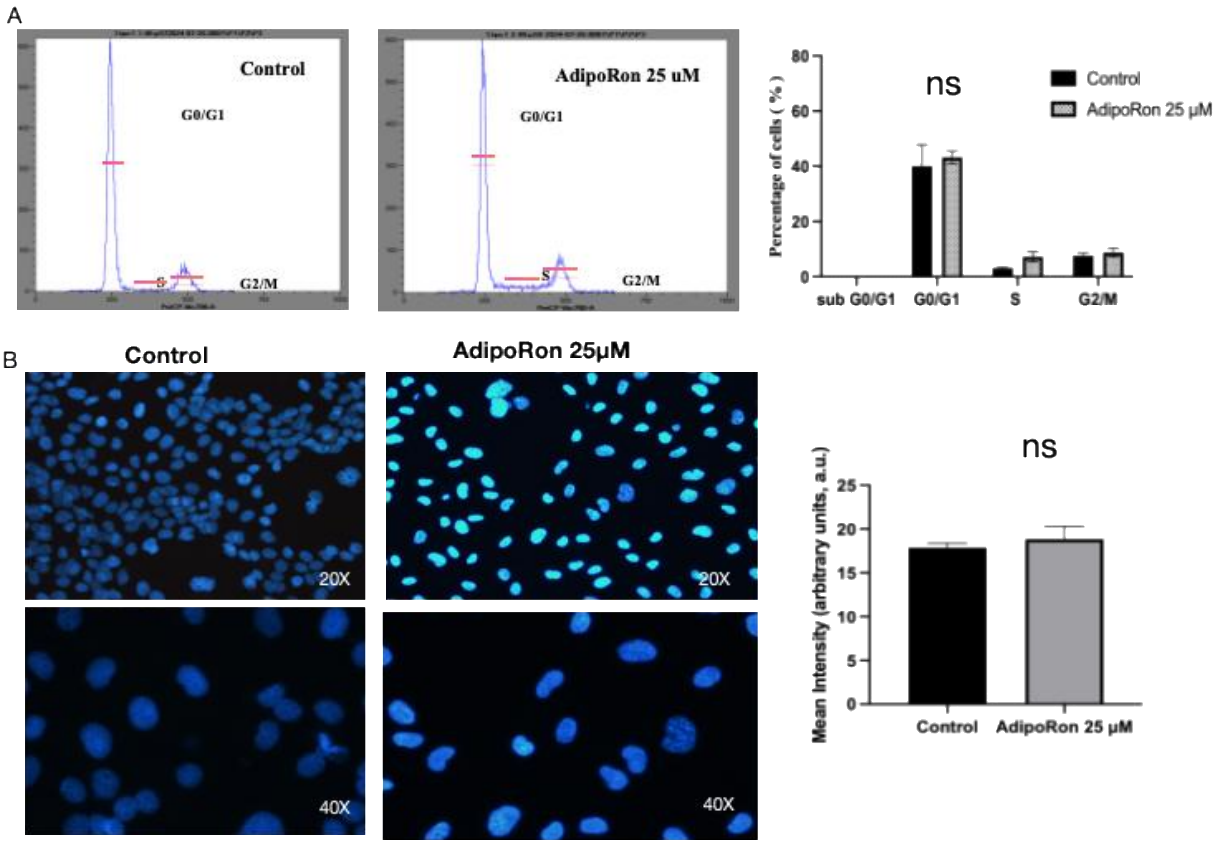
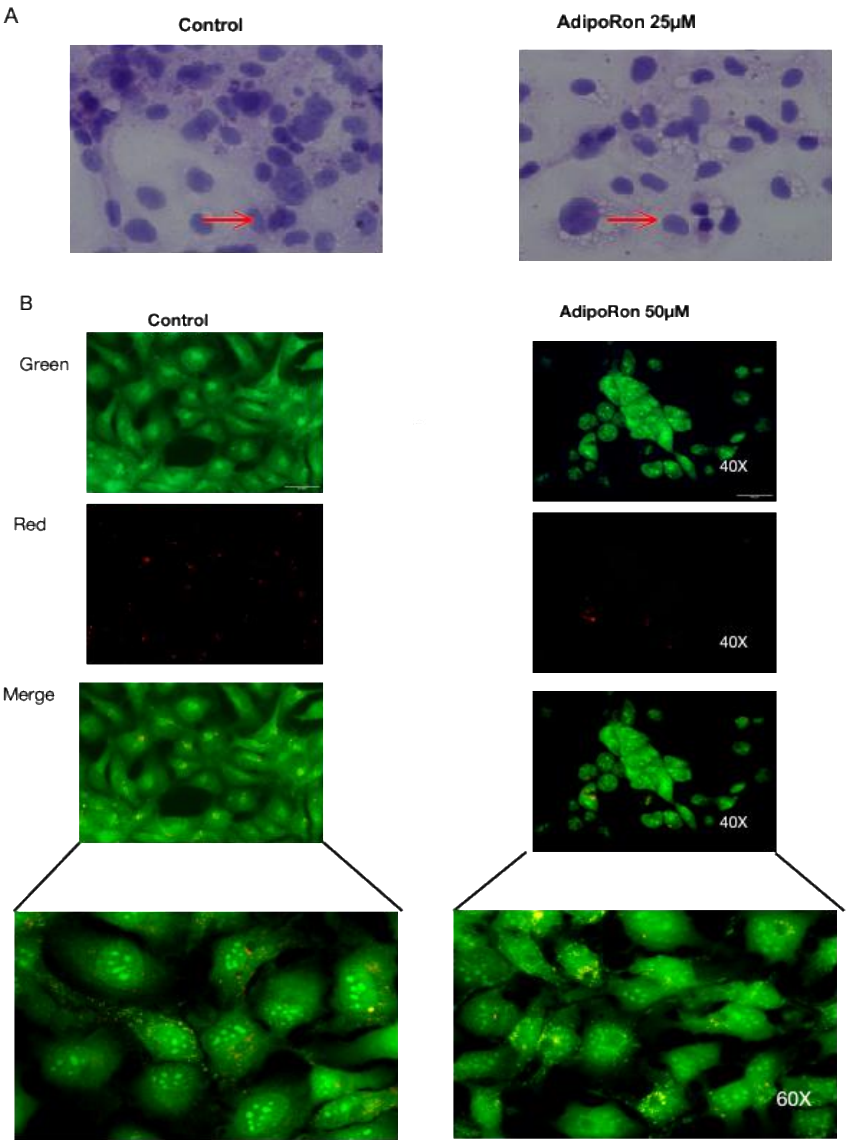


Figure 34. AdipoRon fails to induce apoptosis or autophagy in TPC-1 cells. **(A)** Flow cytometry analysis following PI staining revealed no significant increase in the sub-G0/G1 population in TPC-1 cells after treatment with 25 µM AdipoRon for 48 hours, suggesting the absence of apoptotic cell cycle arrest. **(B)** Fixed-cell DAPI staining showed no notable enhancement in nuclear fluorescence intensity after AdipoRon exposure, further supporting a lack of apoptotic nuclear condensation. Data are presented as mean ± SD (n = 3). Statistical comparisons between two groups were performed using unpaired two tailed Student's t test (ns = not significant).



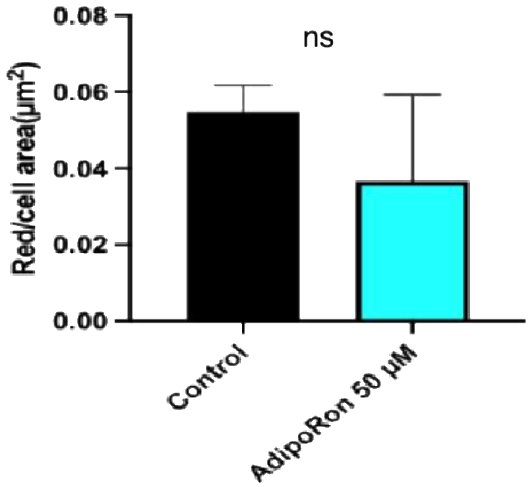


Figure 35. AdipoRon fails to induce apoptosis or autophagy in TPC-1 cells. (A) H&E staining of TPC-1 cells treated with 25 μM AdipoRon revealed no significant morphological alterations compared to control. **(B)** AO staining showed no increase in red fluorescence signal, indicating that AdipoRon did not promote autophagic vacuole formation in TPC-1 cells. Data are presented as mean ± SD (n = 3). Statistical comparisons between two groups were performed using unpaired two tailed Student's t test (ns = not significant).

IV.1.3.6 Survival Pathways Activated and Autophagy Partially Inhibited by AdipoRon in TPC-1 Cell Line

Protein expression results indicated that AdipoRon treatment significantly increased levels of phosphorylated AKT (p-AKT) and phosphorylated mTOR (p-mTOR), suggesting activation of proliferation-associated signaling pathways; however, phosphorylated ERK (p-ERK) levels remained unchanged (**Fig. 36 A**). Regarding apoptosis-related proteins, Caspase-3 expression decreased, while Annexin V, AIF, and Caspase-9 levels showed no significant changes, indicating no effective induction of apoptosis (**Fig. 36 C**). Autophagy markers showed decreased LC3B II/I ratio and unchanged p62 expression, implying autophagy was not activated. In summary, AdipoRon activated proliferation signaling but failed to fully trigger apoptosis or autophagy pathways, potentially leading to impaired cellular functions (**Fig. 36 B**)

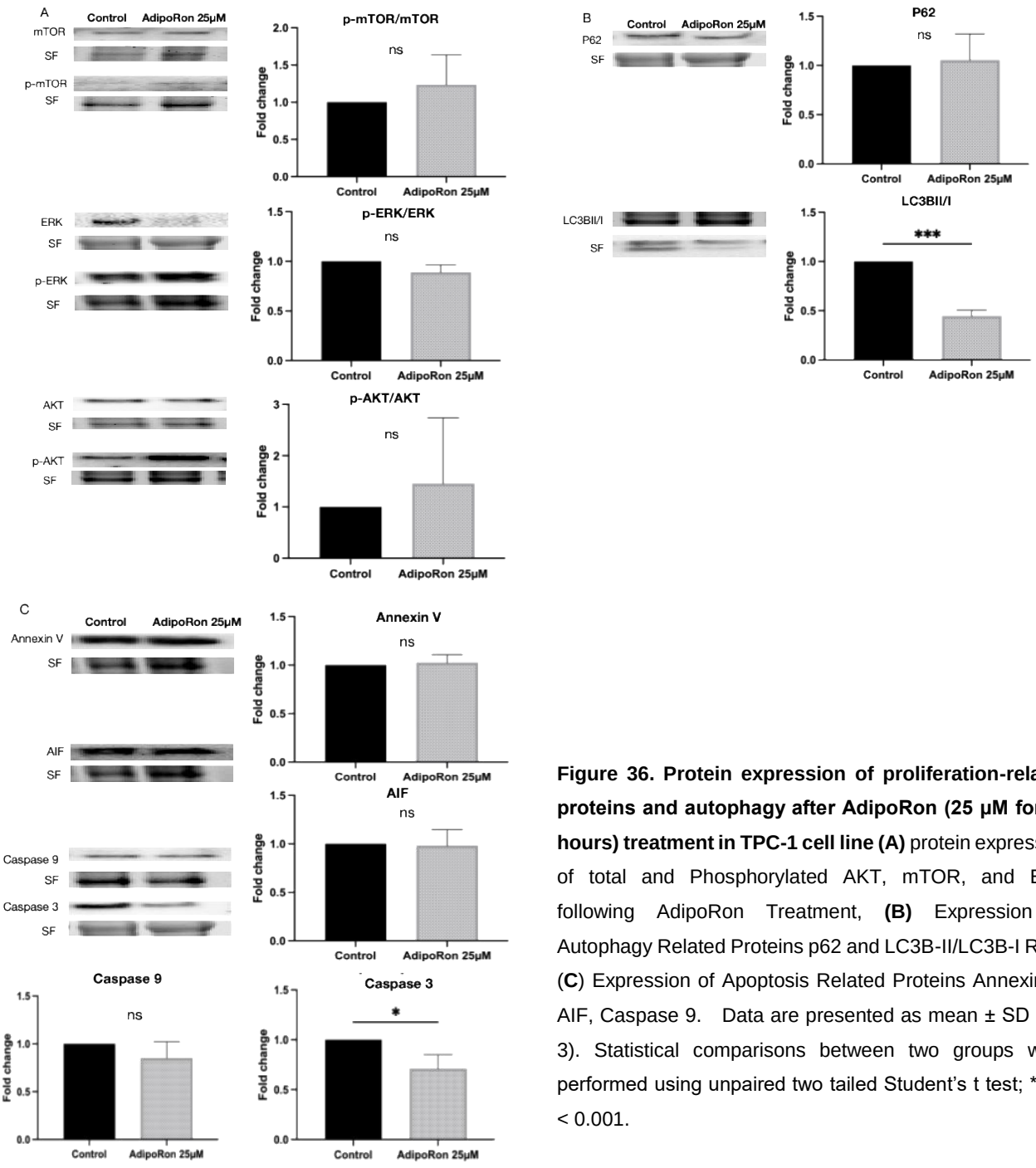


Figure 36. Protein expression of proliferation-related proteins and autophagy after AdipoRon (25 µM for 48 hours) treatment in TPC-1 cell line (A) protein expression of total and Phosphorylated AKT, mTOR, and ERK following AdipoRon Treatment, **(B)** Expression of Autophagy Related Proteins p62 and LC3B-II/LC3B-I Ratio, (**C**) Expression of Apoptosis Related Proteins Annexin V, AIF, Caspase 9. Data are presented as mean ± SD (n ≥ 3). Statistical comparisons between two groups were performed using unpaired two tailed Student's t test; *** $p < 0.001$.

IV.2. Expression Characteristics of LDLR in PTC and the Potential Relationship with AdipoRon

IV.2.1 Transcriptomic Analysis Reveals Differential Expression of *LDLR* and Lipid Metabolism-Related Genes in PTC tissues.

Transcriptome data analysis demonstrated that, compared with normal thyroid tissues, multiple key cholesterol and lipid regulatory genes including *SREBF1, APOE, PCSK9,* and *LDLR* were significantly upregulated in PTC tissues, whereas other genes such as *HMGCR, SREBF2*, and *APOB* were significantly downregulated (**Fig. 37 A-B**). More specifically, in lymph node-negative (N0) patients, *LDLR* expression in tumor tissues was significantly higher than in normal tissues (p = 0.0091) (**Fig. 37 C**). Further comparison between lymph node-positive (N1) and lymph node-negative (N0) groups revealed a markedly elevated *LDLR* expression in the N1 tumor group (p = 4.3×10^{-9}) (**Fig. 37 D**).

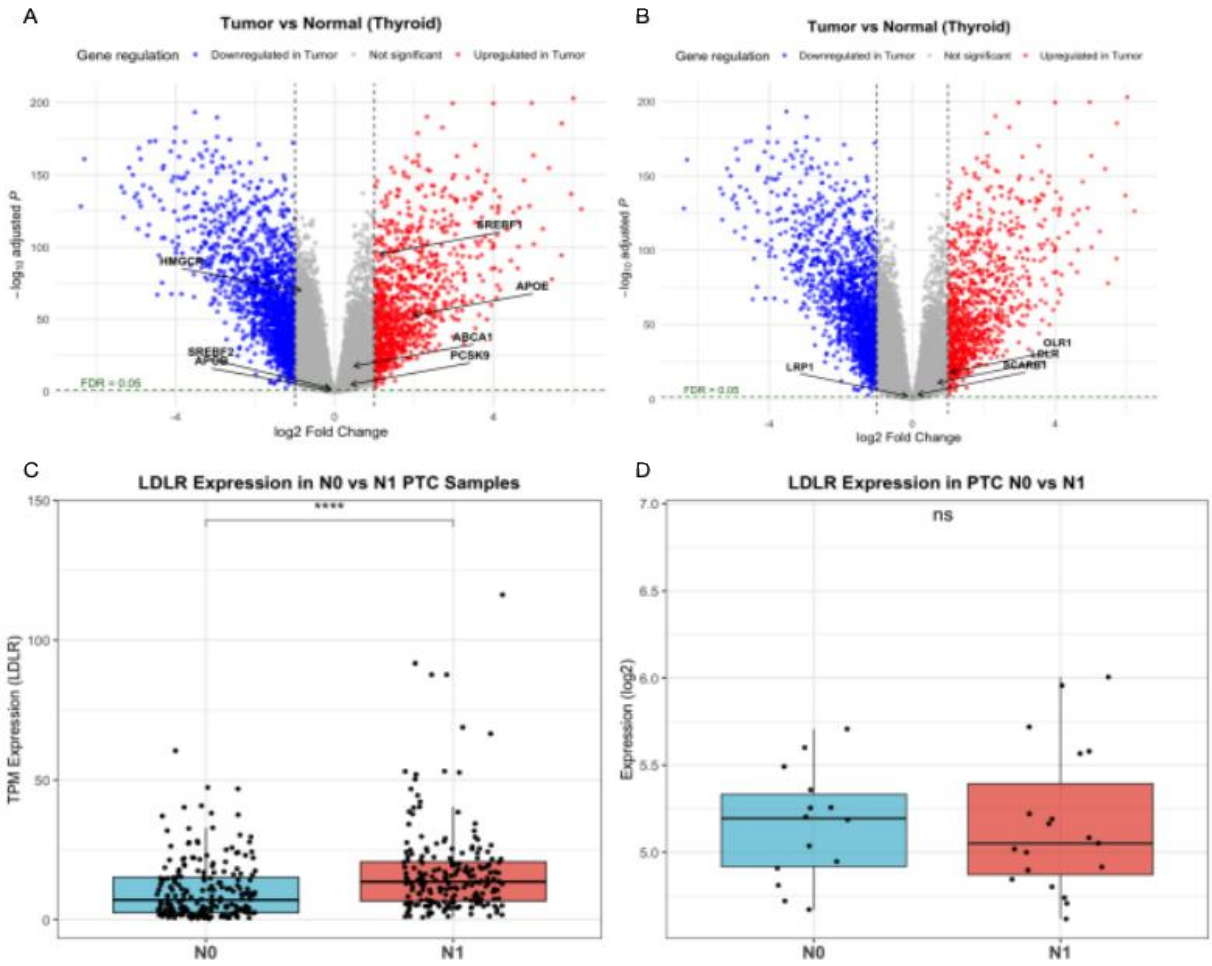


Figure 37. Transcriptomic analysis reveals differential expression of *LDL* related lipid metabolism genes in papillary thyroid cancer (PTC). **(A–B**) Volcano plots show differentially expressed lipid metabolism genes in thyroid tumor vs. normal tissues using transcriptomic data. Key cholesterol/lipid regulators (*SREBF1, APOE, PCSK9, LDLR*) are upregulated in PTC, while others (*HMGCR, SREBF2, APOB*) are downregulated. Significantly upregulated (red) and downregulated (blue) genes were defined by **FDR** < 0.05 and $|\log_2$ fold change$|$ > 1. Key cholesterol/lipid regulators are highlighted. **(C)** Boxplot comparing *LDLR* expression in normal thyroid and PTC tissues (GEO dataset, N0 stage). *LDLR* expression is significantly higher in tumor samples (p = 0.0091 **Wilcoxon test**). **(D)** Boxplot of *LDLR* expression in lymph node-negative (N0) vs. positive (N1) PTC samples (TCGA-THCA cohort), showing a significant increase in N1 tumors (p = 4.3×10^{-9} **Wilcoxon test**).

IV.2.2 *LDLR* Expression Modulated by BRAF Mutation and Its Association with Apoptosis-Related Genes in PTC Tissue and the BCPAP Cell Line.

LDLR expression in PTC may be regulated by the BRAF signaling pathway and linked to apoptosis related gene functions. In the GSE60542 dataset, *LDLR* expression showed an increasing trend in BRAF-mutant PTC tissues compared with wild-type cases, although this difference was not statistically significant (p = 0.645) (**Fig. 38 A**). In the BCPAP cell line, treatment with the BRAF inhibitor vemurafenib (20 µM, 48 hours) significantly downregulated *LDLR* expression (p < 0.01), indicating that *LDLR* may be positively regulated by BRAF signaling (**Fig. 38 B**). Correlation analysis in BCPAP cells (Vemurafenib-treated) revealed that *LDLR, ADIPOR1,* and *ADIPOR2* shared consistent positive correlations with pro-apoptotic genes (*CASP3, CASP8, FAS, TNFRSF1A*) and necroptosis markers (*MLKL, RIPK3*), while exhibiting negative associations with anti-apoptotic regulators such as *BCL2* and *TP53*. These findings support the potential cooperative pro-death role of *LDLR* and adiponectin receptors in BCPAP thyroid cancer cells (**Fig. 38 C**).

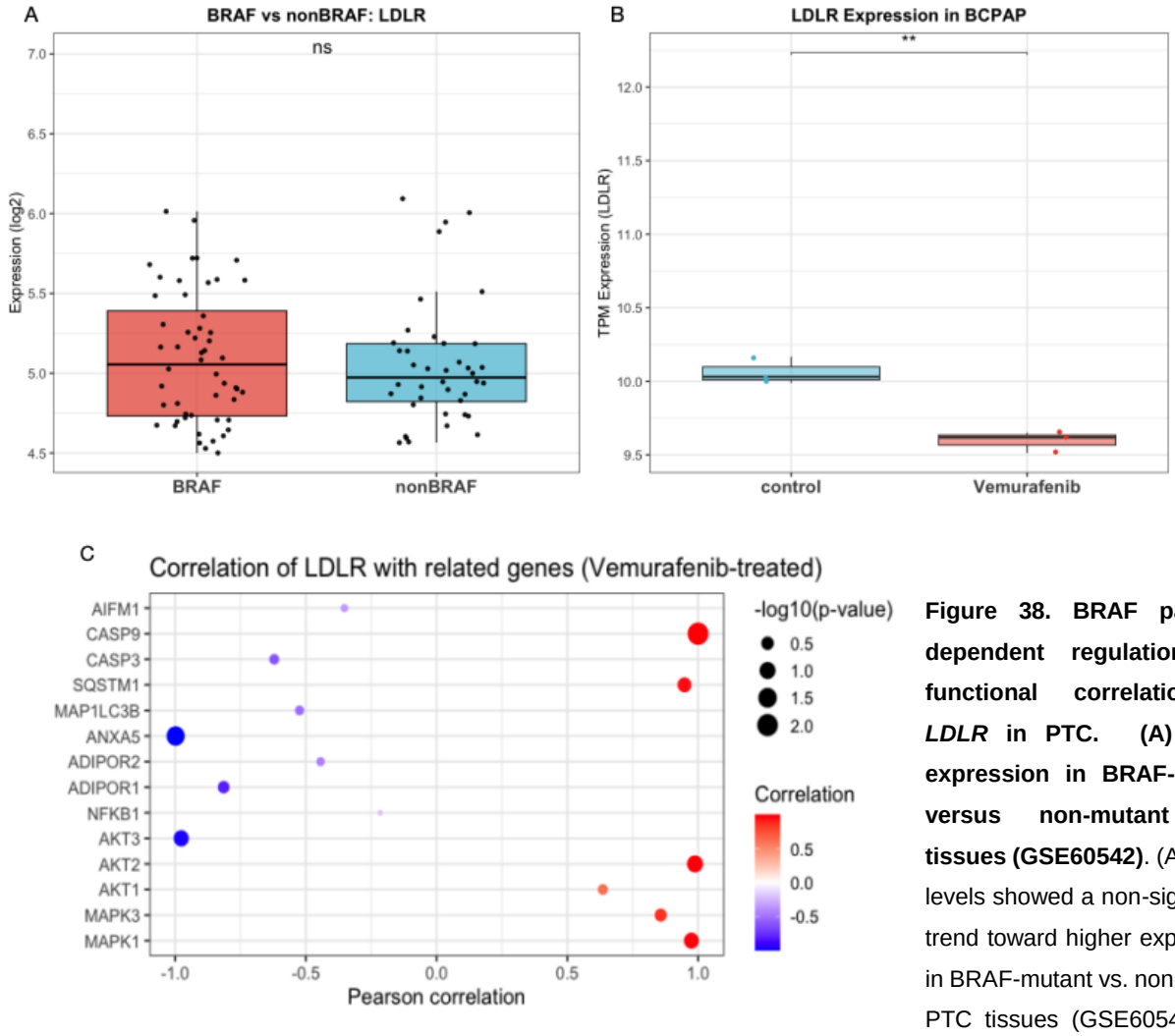


Figure 38. BRAF pathway dependent regulation and functional correlation of *LDLR* in PTC. (A) *LDLR* expression in BRAF-mutant versus non-mutant PTC tissues (GSE60542). (A) LDLR levels showed a non-significant trend toward higher expression in BRAF-mutant vs. non-mutant PTC tissues (GSE60542, p = 0.645). **(B)** Vemurafenib treatment (20 µM, 48 h) significantly reduced LDLR expression in BCPAP cells (p < 0.01), suggesting positive regulation by BRAF signaling (Wilcoxon test; **p < 0.01, ns = not significant). **(C)** In Vemurafenib-treated BCPAP cells, Pearson correlation analysis showed positive (red) and negative (blue) associations between LDLR and other genes. Dot size indicates significance (−log10 p), and color reflects correlation strength and direction

IV.2.3 AdipoRon Suppresses LDL-Induced Proliferation and Migration of BCPAP Cells and Enhances Autophagy Activity

MTT assays showed that under LDL stimulation (200 μg/mL), treatment of BCPAP cells with AdipoRon at 50 μM and 100 μM for 24 and 48 hours especially 100 μM for 48 hours significantly reduced cell viability compared to LDL-only treated cells ($p < 0.01$), demonstrating that AdipoRon effectively inhibits LDL-induced proliferation. (**Fig. 39 A**). Wound healing assays revealed that both AdipoRon alone and combined with LDL reduced cell migration capacity. Although some differences did not reach statistical significance, a clear inhibitory trend was observed (**Fig. 39 B**). Acridine orange staining for autophagy demonstrated a significant increase in red autophagic vesicles in the LDL plus 50 μM AdipoRon group ($p < 0.0001$), indicating that AdipoRon promotes LDL-induced autophagy activation (**Fig. 39 C**).

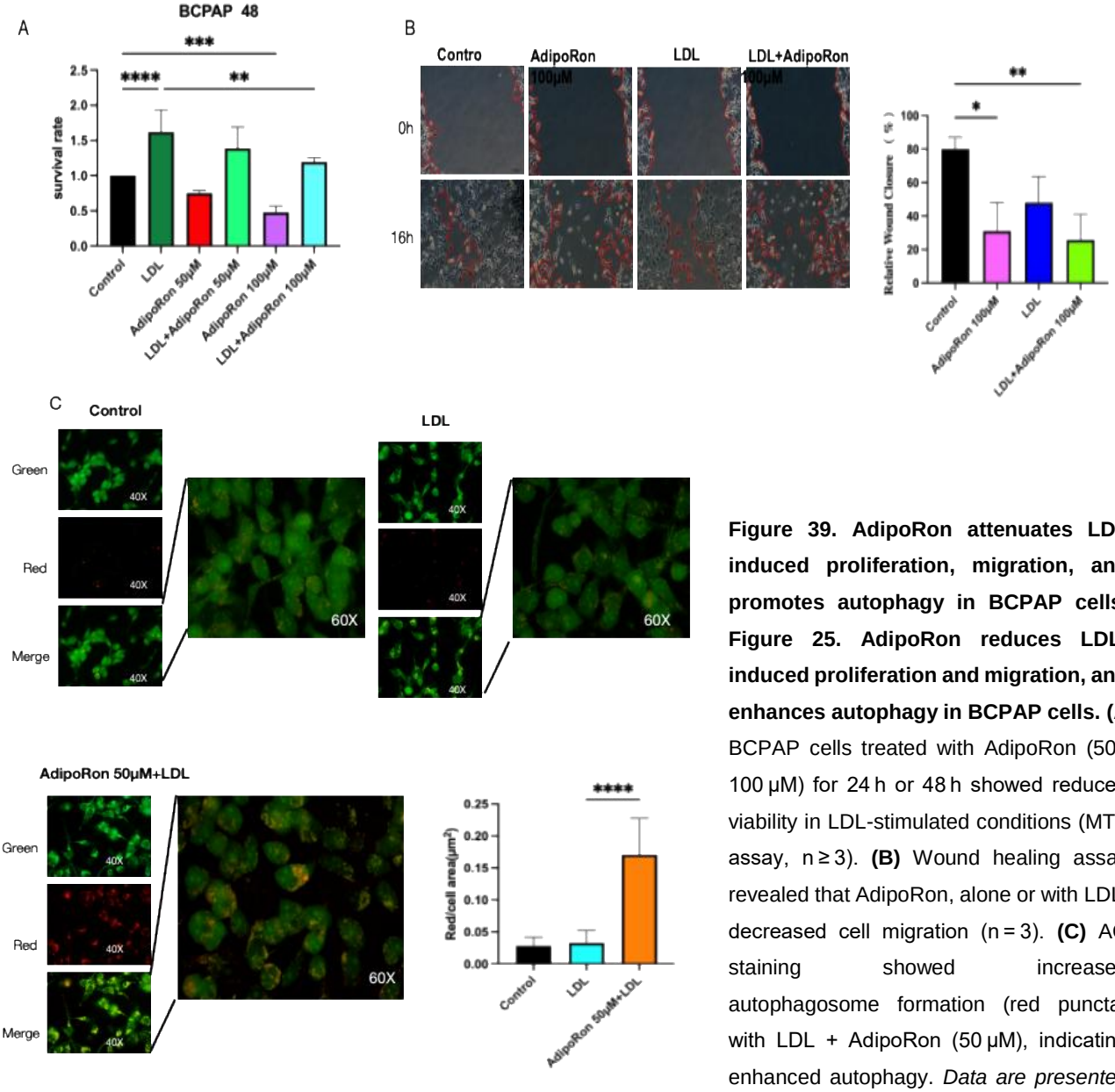
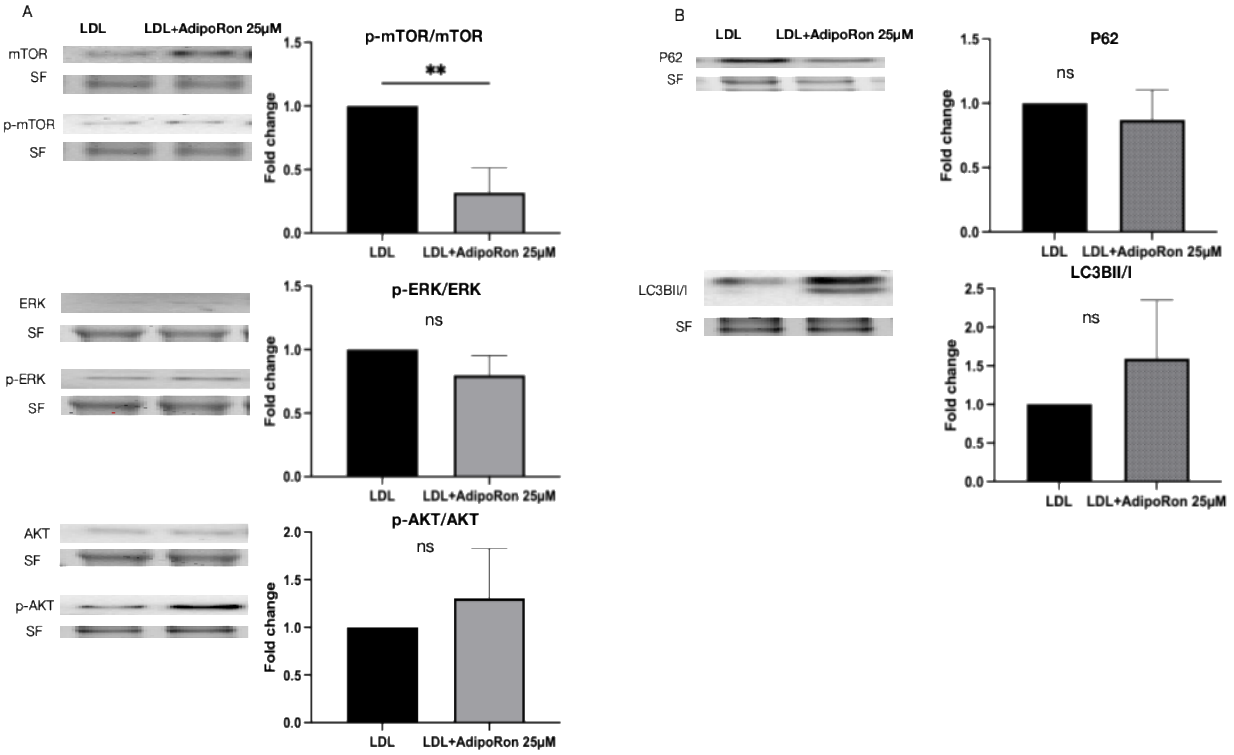


Figure 39. AdipoRon attenuates LDL induced proliferation, migration, and promotes autophagy in BCPAP cells. Figure 25. AdipoRon reduces LDL-induced proliferation and migration, and enhances autophagy in BCPAP cells. (A) BCPAP cells treated with AdipoRon (50–100 μM) for 24 h or 48 h showed reduced viability in LDL-stimulated conditions (MTT assay, n ≥ 3). **(B)** Wound healing assay revealed that AdipoRon, alone or with LDL, decreased cell migration (n = 3). **(C)** AO staining showed increased autophagosome formation (red puncta) with LDL + AdipoRon (50 μM), indicating enhanced autophagy. *Data are presented as mean* ± SD; one-way ANOVA with Dunnett's test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 vs. control or LDL group.

IV.2.4 AdipoRon Restores Signaling Balance and Promotes Apoptotic and Stress Response Pathways in LDL-Treated BCPAP Cell Line.

The LDL treatment, combined AdipoRon application led to a decrease in mTOR phosphorylation, while phosphorylation levels of AKT and ERK were increased, as shown by Western blot analysis (**Fig. 40 A**). Autophagy markers showed a significant increase in the LC3B-II/I ratio and decreased p62 expression, demonstrating effective induction of autophagy (**Fig. 40 B**). Regarding apoptosis related proteins, AIF expression was downregulated, whereas Caspase-9 and Caspase-3 levels were upregulated, indicating activation of intrinsic apoptotic signaling (**Fig. 40 C**). AdipoRon alone significantly inhibited LDLR protein expression, suggesting suppression of cellular LDL uptake, accompanied by activation of intracellular lipid synthesis programs. Although LDL treatment promoted LDLR protein expression, LDLR levels did not significantly change in the combined AdipoRon group. Lipid metabolism regulator SREBP-1 exhibited decreased precursor form (128 kDa) and increased active nuclear form (68 kDa), indicating enhanced nuclear activation of SREBP-1 (**Fig. 40 D**).



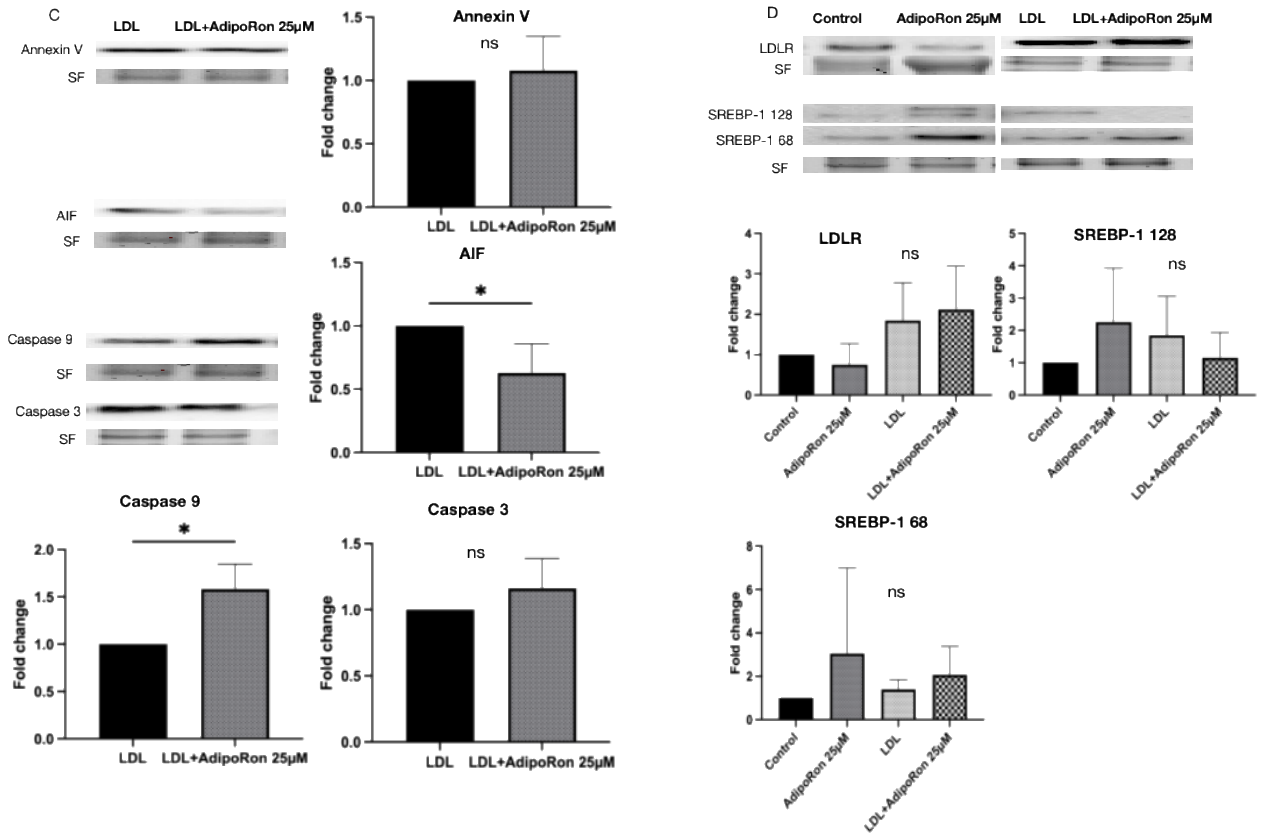


Figure 40. AdipoRon (25 μM, 48 hours) Reverses LDL (200 μg/ml, 48 hours) Induced Signaling Dysregulation and Activates Programmed Cell Death and Stress Responses (**A**) Ratios of phosphorylated to Total and Phosphorylated of AKT, mTOR, and ERK following AdipoRon treatment, (**B**) Protein expression levels of Autophagy Related Proteins p62 and LC3B-II/LC3B-I Ratio, **(C**) Protein expression levels of Apoptosis Related Proteins Annexin V, AIF, Caspase 9 and Caspase 3. *Statistical comparisons between two groups (**A–C**) were performed using unpaired two-tailed Student's t-test. (**D**)* Expression levels of LDLR and SREBP-1 Protein upon AdipoRon and LDL treatment. Statistical significance among multiple groups (**D**) was evaluated using one-way ANOVA followed by **Dunnett's** post-hoc test. Data are presented as mean ± SD (n ≥ 3). *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, ns = not significant.

IV.2.5 *LDLR* Expression Modulated by RET/PTC1 rearrangement and Its Link to Apoptosis-Related Genes in PTC Tissue and TPC-1 Cell Line

To explore the relationship between *LDLR* and the *RET/PTC1* rearrangement gene in PTC, transcriptomic analysis of GEO dataset GSE60542 was conducted. No significant difference in *LDLR* expression was observed between samples with or without RET/PTC1 rearrangement ($p > 0.05$), indicating limited impact of RET/PTC1 status on *LDLR* expression (**Fig. 41 A**). Further analysis based on GSE261830 assessed the effect of the RET-specific inhibitor Selpercatinib (20 μM, 48 h) on *LDLR* expression in RET/PTC1-positive TPC-1 cells. Compared to DMSO control, Selpercatinib slightly reduced *LDLR* expression, though not significantly (**Fig. 41 B**). Correlation analysis under Selpercatinib treatment showed *LDLR* negatively correlated with autophagy markers *SQSTM1* and *MAP1LC3B,* but positively correlated with *ANXA5* and *AKT3*. (**Fig. 41 C**)

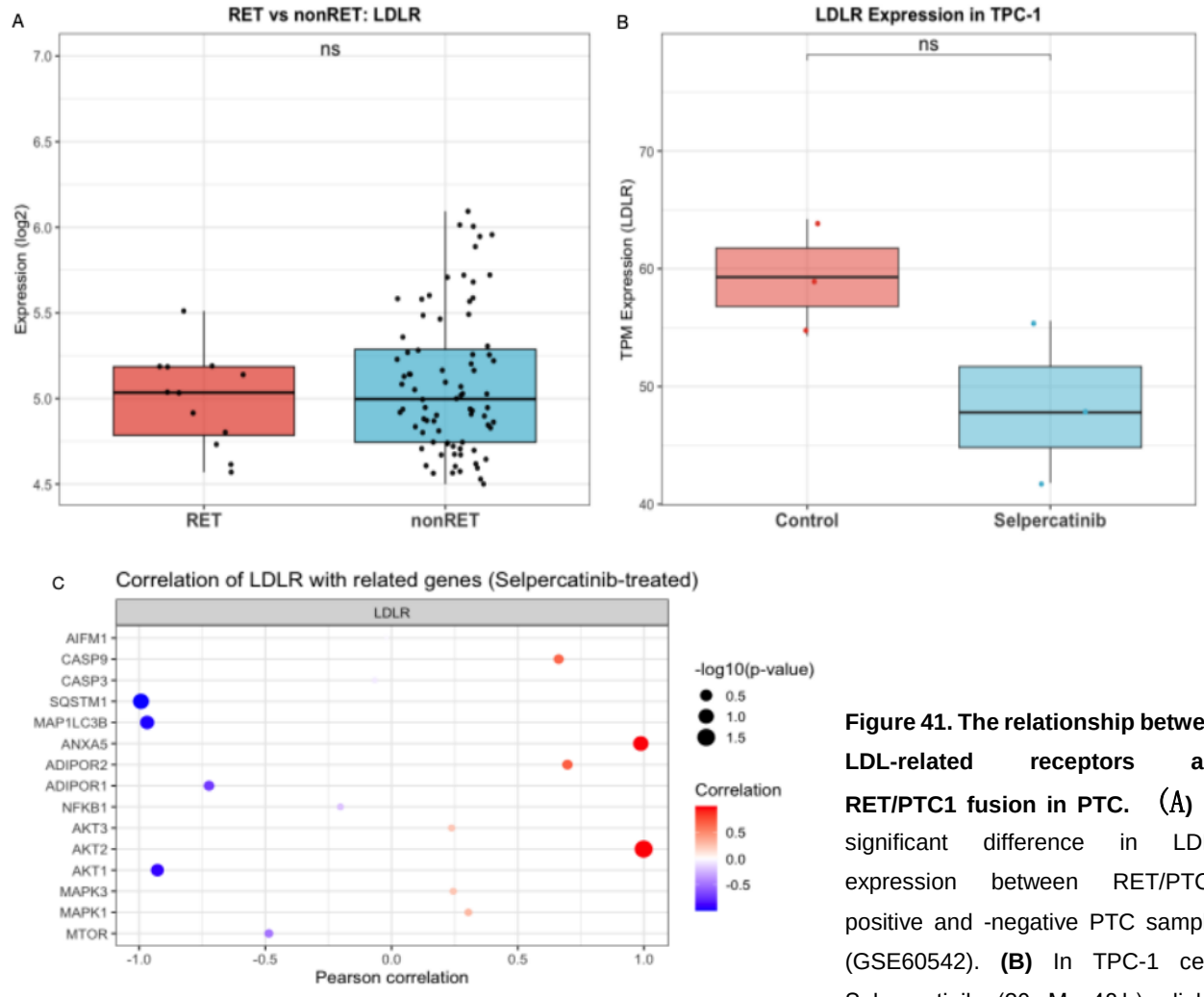


Figure 41. The relationship between LDL-related receptors and RET/PTC1 fusion in PTC. **(A)** No significant difference in LDLR expression between RET/PTC1-positive and -negative PTC samples (GSE60542). **(B)** In TPC-1 cells, Selpercatinib (20 μM, 48 h) slightly reduced LDLR expression vs. DMSO, but not significantly (GSE261830; Wilcoxon test, ns).**(C)** Pearson correlation under Selpercatinib treatment showed positive (red) or negative (blue) associations between LDLR and apoptotic/autophagic markers. Dot size = significance (−log10 of *p* value); color = correlation strength/direction. Dot size reflects statistical significance (−log10 of *p* value), and color scale indicates direction and strength of the correlation (from blue: negative, to red: positive).

IV.2.6 AdipoRon Attenuates LDL-Induced Proliferation in TPC-1 Cells and enhanced autophagy

To verify whether AdipoRon can counteract LDL-promoted proliferation in PTC cells (TPC-1), cells were treated with LDL (200 μg/mL) in the presence of varying concentrations of AdipoRon (50 μM, 100 μM), and cell viability was measured at 24 and 48 hours. MTT assays showed that 100 μM AdipoRon significantly inhibited LDL-induced proliferation at 48 hours ($p < 0.001$) (**Fig. 42 A**). Scratch wound assays indicated trends of reduced migration following AdipoRon alone or combined with LDL treatment, though without statistical significance (**Fig. 42 B**). Acridine orange staining revealed a significant increase in red autophagic vesicles in the LDL plus 50 μM AdipoRon group ($p < 0.001$), indicating enhanced autophagy induced by AdipoRon under LDL stimulation (**Fig. 42 C**)

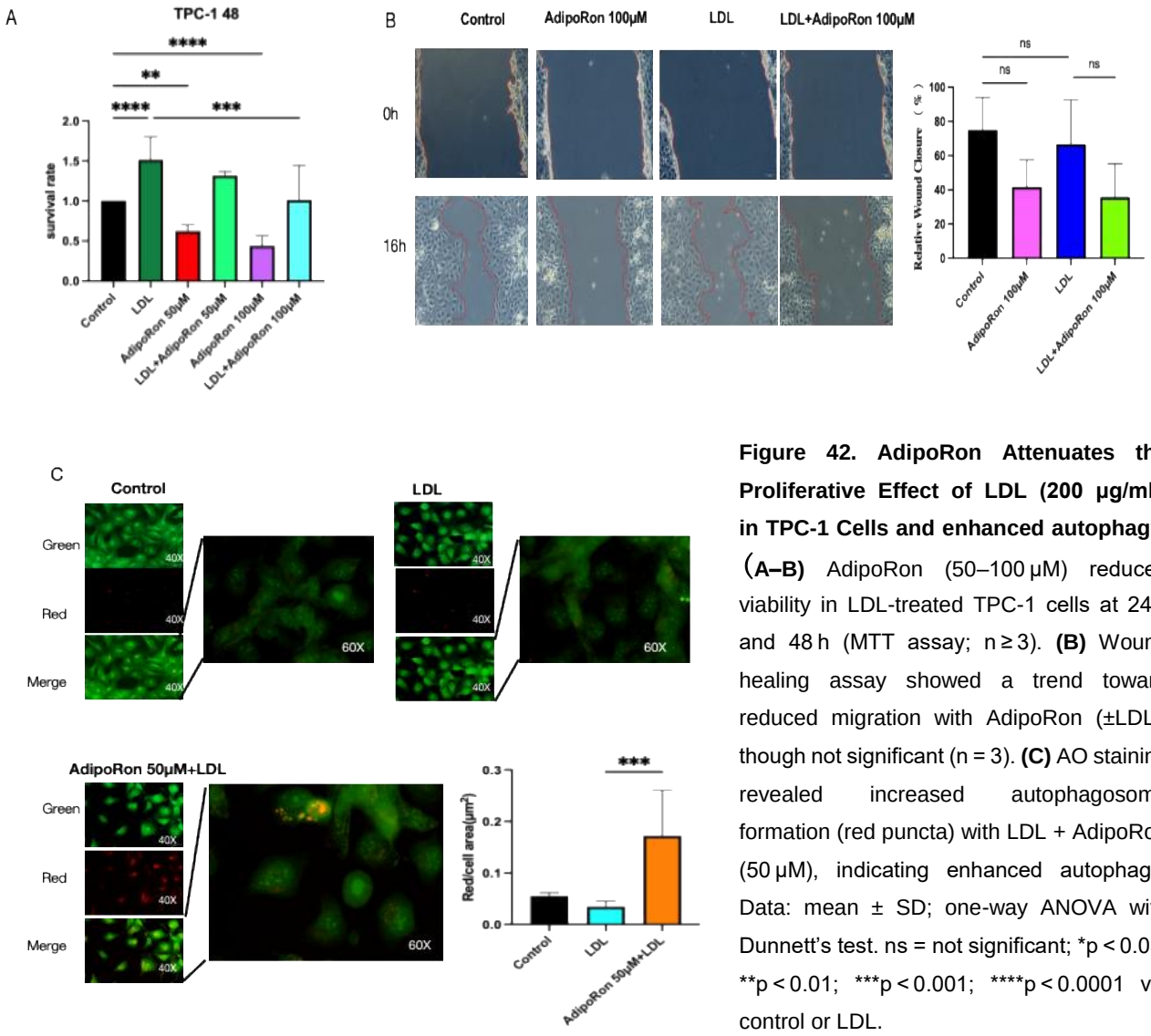
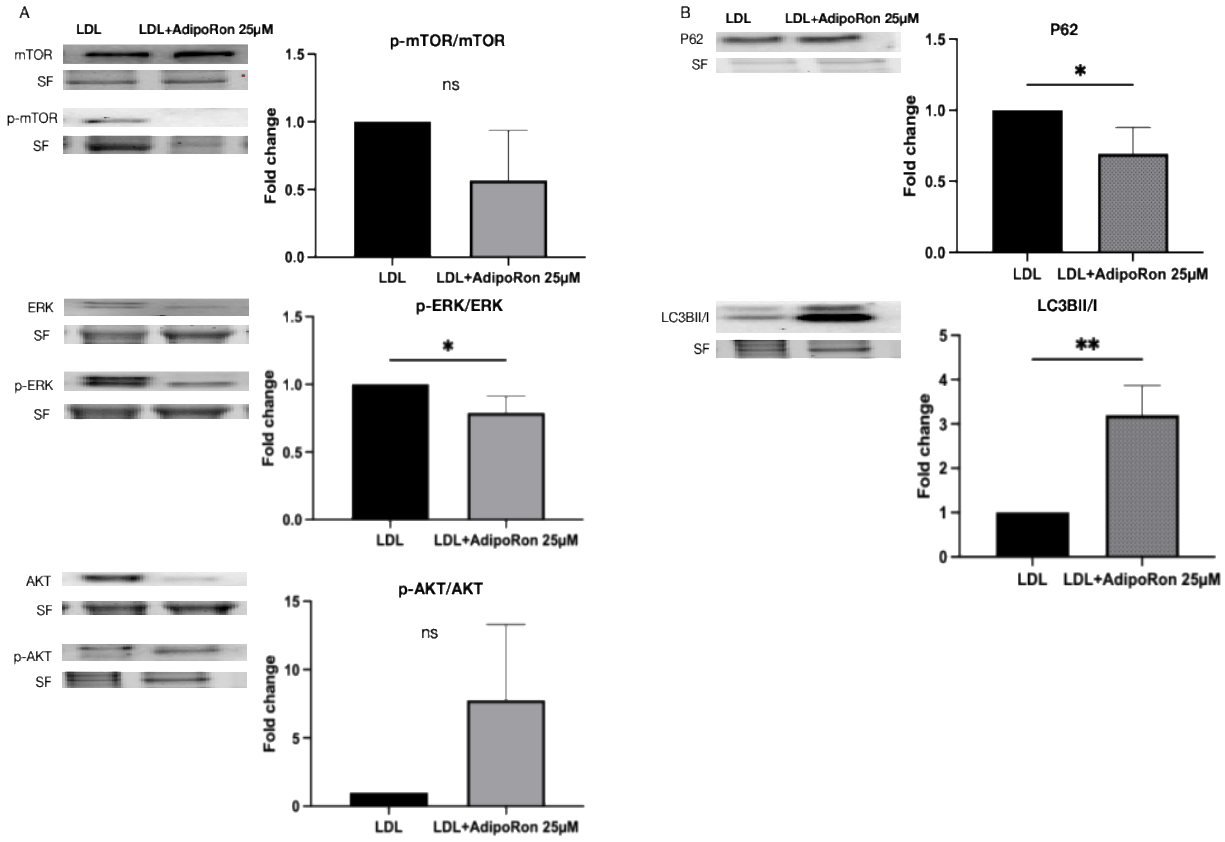


Figure 42. AdipoRon Attenuates the Proliferative Effect of LDL (200 μg/mL) in TPC-1 Cells and enhanced autophagy. (**A–B)** AdipoRon (50–100 μM) reduced viability in LDL-treated TPC-1 cells at 24 h and 48 h (MTT assay; n ≥ 3). **(B)** Wound healing assay showed a trend toward reduced migration with AdipoRon (±LDL), though not significant (n = 3). **(C)** AO staining revealed increased autophagosome formation (red puncta) with LDL + AdipoRon (50 μM), indicating enhanced autophagy. Data: mean ± SD; one-way ANOVA with Dunnett's test. ns = not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 vs. control or LDL.

IV.2.7 AdipoRon Reverses LDL Induced Signaling Aberrations and Activates Programmed Cell Death and Stress Responses in TPC-1 Cell Line

To further elucidate the regulatory mechanisms of AdipoRon in LDL stimulated TPC-1 cells, key signaling pathways and apoptosis/autophagy related proteins were examined by Western blot. Combined LDL and AdipoRon treatment significantly decreased phosphorylation of mTOR and ERK, while phosphorylation of AKT proteins increased, indicating activation of AKT pathways concurrent with suppression of mTOR and ERK signaling (**Fig. 43 A**). Autophagy markers showed elevated LC3B-II/I ratio and reduced p62 levels, reflecting increased autophagy (**Fig. 43 B**). Apoptosis related proteins exhibited downregulated Caspase-9 and AIF, but upregulated Caspase-3, suggesting inhibition of mitochondrial (intrinsic) apoptosis (**Fig. 43 C**). AdipoRon alone significantly decreased LDLR protein expression, implying suppressed LDL uptake and no activation of intracellular lipid synthesis. SREBP-1 precursor (128 kDa) decreased while the nuclear active form (68 kDa) increased, indicating enhanced nuclear localization and activation of SREBP-1. Although LDLR expression tended to decrease under combined treatment, changes were not statistically significant (**Fig. 43 D**)



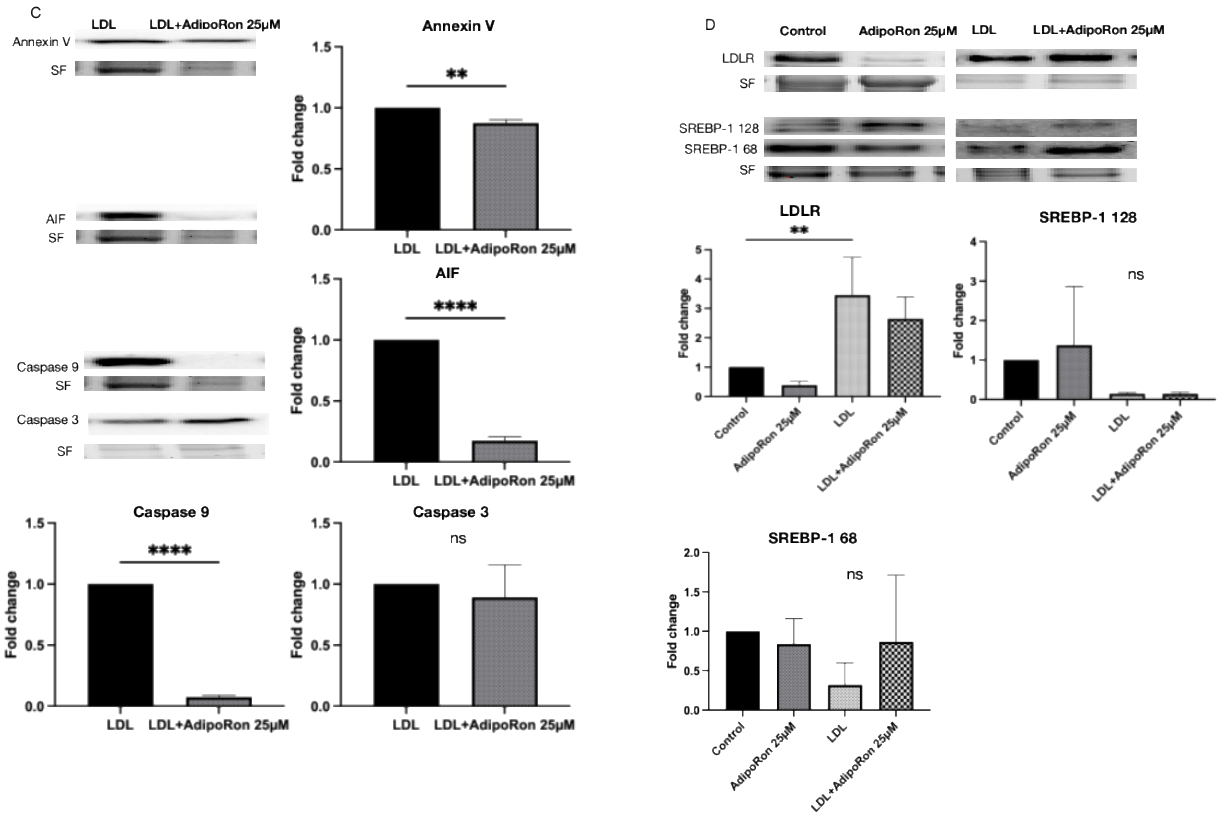


Figure 43. AdipoRon (25 μM, 48 hours) Reverses LDL (200 μg/ml, 48 hours) Induced Signaling Aberrations and Activates Programmed Cell Death and Stress Responses in TPC-1 Cells (**A**) Expression levels of Total and Phosphorylated AKT, mTOR, and ERK following AdipoRon treatment, (**B**) Expression levels of Autophagy Related Proteins p62 and LC3B-II/LC3B-I Ratio, (**C**) Expression levels of Apoptosis Related Proteins Annexin V, AIF, Caspase 9 and Caspase 3. Statistical comparisons between two groups (**A–C**) were performed using unpaired two tailed Student's t test. (**D**) Expression levels of LDLR and SREBP-1 Protein upon AdipoRon and LDL treatment. Statistical significance among multiple groups (**D**) was evaluated using one-way ANOVA followed by **Dunnett's** post-hoc test. Data are presented as mean ± SD (n ≥ 3). * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, ns = not significant.

V. DISCUSSION

V. 1 Exploring the Relationship Between APN and Its Receptors in Thyroid Cancer

In this study, the secretion levels of adipokines and inflammatory cytokines in cervical adipose tissue obtained from a cohort of patients with PTC and BT showed significantly elevated levels of APN and TNF-α in the PTC group, while IL-6 levels were markedly reduced. These findings support the hypothesis of a possible reciprocal regulatory relationship between APN with other inflammatory factors, highlighting the potential role of adipose tissue in the malignant tumor microenvironment. In line with our findings, previous studies have reported the roles of inflammatory factors such as ROS, TNF-α, and IL-6, as well as various vascular factors, can negatively regulate APN expression by suppressing the activity of its promoter in overweight/obese conditions (682–687). Conversely, APN can indirectly modulate the expression or function of other adipokines (leptin and resistin), a variety of inflammatory cytokines (TNF-α and IL-6), extracellular matrix components, pro-angiogenic factors (VEGF), and metabolic regulators. Through these interactions, APN is involved in a wide range of biological processes, including the maintenance of metabolic homeostasis, immune regulation, and inhibition of tumor angiogenesis(688,689).

These biological effects are exerted by binding to its membrane receptors, AdipoR1 and AdipoR2, which are expressed predominantly expressed in skeletal muscle, and liver respectively, though both are also expressed at lower levels in other organs. Our results revealed differential expression patterns of AdipoR1 and AdipoR2 across thyroid tumor types (PTC *versus* BT), as well as between tumoral and adjacent normal thyroid tissue in PTC patients. Notably, AdipoR1 was specifically upregulated in PTC, suggesting a potential role in malignant progression. In contrast, AdipoR2 expression was elevated in both benign and malignant lesions, implying its involvement in broader metabolic regulatory processes. This differential expression could be indicating distinct roles for these receptors in the cellular signaling pathways involved such as cell proliferation, cell cycle and apoptosis.

The analysis of public database (TCGA and GTEx), confirm a differential expression pattern of *AdipoR1* and *AdipoR2* in PTC samples. Interferingly, these receptors did not exhibit synchronized expression trends, suggesting distinct regulatory mechanisms. Further analysis of the TCGA-THCA dataset revealed that *ADIPOR1* expression was significantly higher than *ADIPOR2*, supporting the hypothesis of functional divergence between the two receptors in thyroid cancer development. However, after validation these differences and as well as between lymph node metastasis positive and negative tumor tissues using paired samples using GEO datasets, we can detect heterogeneity and platform specific variation, with inconsistent expression trends observed between them with inconsistent expression trends. These findings highlight potential biological differences and technical biases across cohorts, suggesting that further validation using independent sample sets is necessary to clarify the biological significance of APN receptors in thyroid cancer. Interestingly was to detected in the Survival analysis (Kaplan–Meier) that high *ADIPOR1* expression was significantly associated with reduced overall survival, whereas *ADIPOR2* expression showed no significant correlation with patient prognosis. Nevertheless, further multivariate Cox regression analysis identified age as the only statistically significant independent prognostic factor, and neither *ADIPOR1* nor *ADIPOR2* retained independent prognostic value after adjusting for other clinical variables. Our receptors expression findings could be in consistent with the survival analysis results, in which high *ADIPOR1* expression was associated with a malignancy, but also could be further support the hypothesis of functional divergence between the two receptors in thyroid cancer development. However, our analysis has a limitation in the number of the samples analyzed, we can further analyze

the receptors expression profiles in samples with N0 stage in TCGA and GEO datasets and performed KEGG pathway enrichment analysis on genes positively correlated with high expression of each receptor. The results of these analysis showed that *ADIPOR1* associated genes were mainly enriched in pathways related to apoptosis, immune regulation, and cellular senescence, suggesting that *ADIPOR1* may play a role in early tumorigenesis by modulating cell fate and the tumor microenvironment. In contrast, *ADIPOR2* associated genes were significantly enriched in pathways involving protein degradation and metabolism, indicating a preference for regulating metabolic homeostasis. Further analysis of N1 stage (lymph node metastasis positive) samples revealed that *ADIPOR1* related genes were significantly enriched in pathways closely associated with cell growth and death, such as the p53 signaling pathway and cell cycle regulation. Meanwhile, *ADIPOR2* related pathways were concentrated on lipid metabolic reprogramming, including fatty acid metabolism and cholesterol transport, suggesting its involvement in adaptive metabolic regulation in advanced stages. In summary, *ADIPOR1* and *ADIPOR2* may mediate distinct biological processes at different stages of thyroid cancer, with *ADIPOR1* primarily participating in cell death and immune modulation, and *ADIPOR2* involved in metabolic remodeling. This supports their functional differentiation and complementary roles in the development and progression of PTC.

V. 2 Exploring the Relationship Between AdipoRon and BRAF mutation in Thyroid Cancer

AdipoRon, orally active APN receptors agonist with a favorable stability and bioavailability having considered an ideal candidate for APN replacement and the treatment of metabolic disorders. This agonist, has demonstrated multiple beneficial effects, including anti-diabetic and anti-obesity properties, primarily through improving insulin resistance and chronic inflammation. Nevertheless, in the last years, the antitumoral potential of AdipoRon has garnered increasing attention and some reports suggest its ability to regulate different pathways involved in the proliferation, apoptosis, and autophagy by the by activating AMPK and inhibiting the PI3K/Akt/mTOR pathway(690,691). Moreover, its capacity to improve the tumor microenvironment has been reported in various cancers such as breast cancer(543), ovarian cancer(544), and pancreatic cancer(545), their capacity to improve the tumor microenvironment by suppressing pro-inflammatory cytokines and angiogenesis, thereby inhibiting tumor growth and metastasis.

In thyroid cancer, the therapeutic application of AdipoRon is still in preclinical studies. In 2024, it was reported that *in vitro* treatment with AdipoRon suppressed the proliferation, migration, and invasion of PTC cells and also was the ability to enhance autophagic activity and promote apoptosis by upregulating the expression and phosphorylation of ULK1, a key autophagy related protein, potentially mediated via AdipoR2, thereby inhibiting tumor growth(548).

In our study, we detected the expression of AdipoR1 and AdipoR2 in both human PTC cell lines used (BCPAP and TPC-1). Although not statistically significant was found, there was a trend toward higher expression levels of both receptors in BCPAP compared to TPC-1. A key difference between these cell lines lies in their mutational profiles: Notably, BCPAP carries the $BRAF^{V600E}$ mutation, the most frequent and PTC specific oncogenic alteration linked to tumor aggressiveness, which leads to constitutive activation of the classical RAS/MAPK pathway, enhanced ERK signaling, and promotes abnormal proliferation and malignant transformation of cancer cells.

To determine whether $BRAF^{V600E}$ status influences *ADIPOR1/2* expression, we analyzed the expression of APN receptors in PTC tissues with different BRAF mutation statuses using the GEO dataset GSE60542. The results did not confirm this hypothesis, suggesting that BRAF mutations may have limited influence on APN receptor expression at the tissue level. However, the analysis

conducted in the $BRAF^{V600E}$ mutated PTC cell line BCPAP, treatment with the BRAF inhibitor (vemurafenib) for 48 hours significantly reduced the expression levels of *ADIPOR1* and *ADIPOR2*. This indicates that inhibition of BRAF activity *in vitro* can markedly affect the expression of APN receptors, suggesting that *ADIPOR1/2* may be directly regulated by the BRAF-MAPK pathway. The discrepancy between tissular samples and cell line results may reflect the influenced of the heterogenicity of data set samples, the role of the tumor microenvironment and the complexity of intracellular signaling networks.

To further elucidate the molecular mechanisms of BRAF mutations in PTC, we performed Gene Set Enrichment Analysis (GSEA) on BRAF mutant *versus* wild type samples in dataset GSE60542. The analysis revealed significant enrichment of pathways including the MAPK signaling, TNF signaling, apoptosis, and lysosome related pathways in the BRAF mutant group. These findings indicate that BRAF driven PTC activates multiple signaling cascades associated with tumor progression, stress response, inflammation, and autophagy, reflecting a complex network of compensatory and survival mechanisms under oncogenic stress.

Furthermore, in the BRAF mutated PTC cell line BCPAP (GSE171483), we conducted GSEA following Vemurafenib treatment. The results demonstrated significant enrichment of pathways related to apoptotic regulation and intrinsic apoptotic signaling, suggesting that BRAF inhibition *in vitro* may reactivate apoptotic programs, which could be a key mechanism underlying its antitumor effects.

Both tissue and cell-based analyses indicate that BRAF mutations in PTC are closely associated with the activation of multiple oncogenic and stress related signaling pathways. Targeted inhibition of BRAF can partially reverse these effects, notably enhancing apoptotic signaling.

To assess the potential role of *ADIPOR1*/*2* under BRAF mutated conditions, we preformed Pearson correlation analyses on expression profiles from Vemurafenib treated BCPAP cells. The expression of *ADIPOR1*/*2* was significantly positively correlated with several key pro-apoptotic genes (e.g., *CASP3, CASP9, AIFM1*) and autophagy-related genes (e.g., *SQSTM1, MAP1LC3B*). Notably, the downstream genes associated with *ADIPOR1*/*2* showed partially distinct patterns, suggesting that these two receptors may regulate cell death through differentiated mechanisms. Taken together, under BRAF inhibition, *ADIPOR1*/*2* may modulate apoptosis and autophagy via cooperative or independent signaling pathways, thereby contributing to tumor cell death. Based on these findings, we treated BRAF mutant PTC cells (BCPAP) with AdipoRon. The results showed significant suppression of cell proliferation and migration. Subsequent experiments assessed apoptosis and autophagy. Although apoptotic tendencies were observed, the changes did not reach statistical significance. Among apoptosis-related proteins, Annexin V and Caspase-9 were significantly elevated, indicating partial activation of early apoptotic signals. However, Caspase-3 expression was reduced, suggesting that the execution phase of apoptosis may be incomplete or suppressed.

Interestingly, p-AKT levels were significantly increased, while p-ERK levels remained unchanged, indicating that AKT-mediated antiapoptotic signaling was active, potentially interfering with the effective transmission or execution of pro-apoptotic signals.

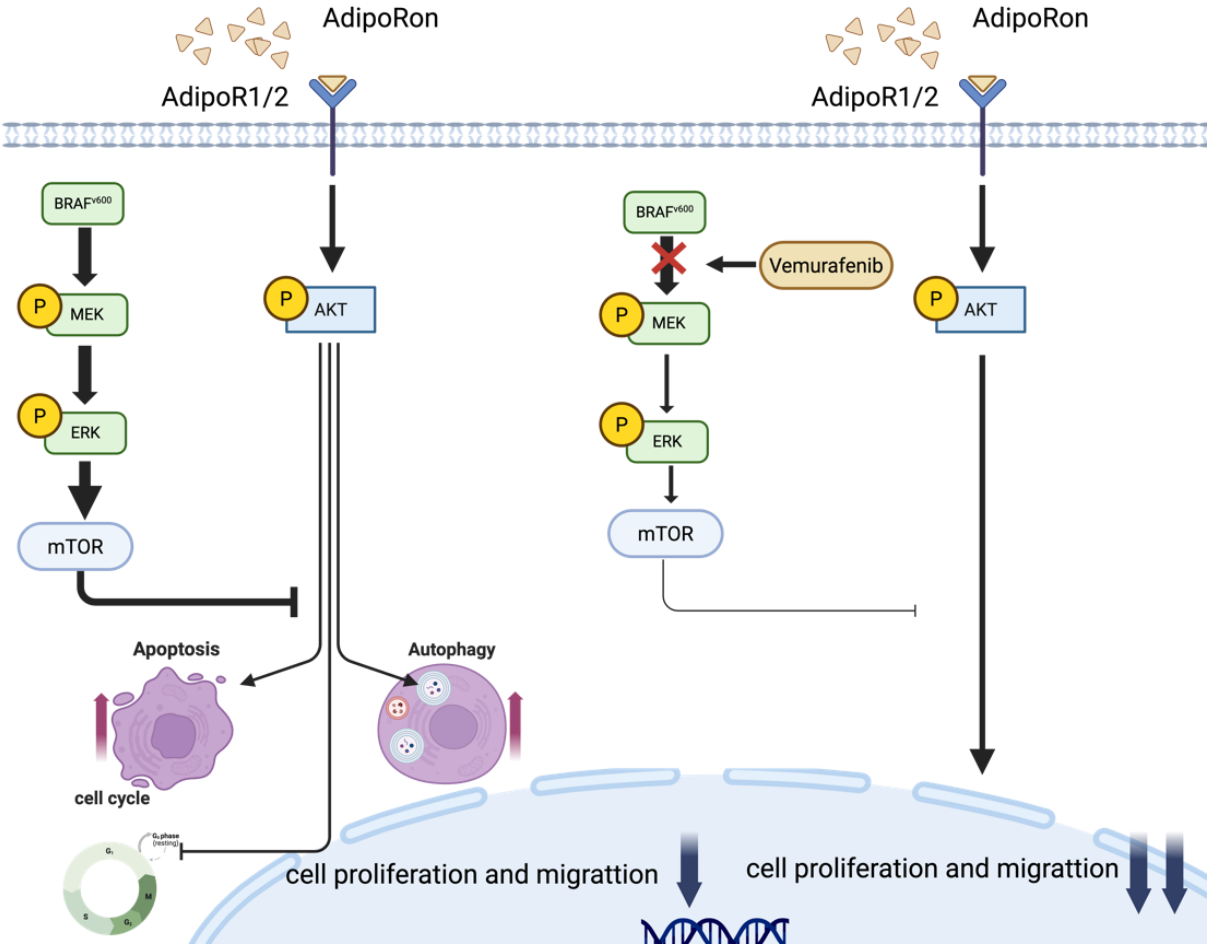
In autophagy related analyses, AdipoRon treatment led to a notable accumulation of AVOs, evidenced by enhanced red fluorescence, compatible with upregulated autophagic activity. The increase in p-AKT further suggests that AKT may be involved in the regulation of autophagy. However, no significant changes were observed in p-mTOR or p-ERK, implying that AdipoRon induced autophagy may proceed through an mTOR-independent mechanism, or that classical signaling routes may be obstructed. In terms of autophagy markers, p62 was markedly accumulated, while the LC3B-II/I ratio remained unchanged, indicating that autophagic flux was incomplete or

blocked at a late stage(692–694). These findings suggest that although autophagy could be not fully activated, AdipoRon may still induce autophagy via mTOR independent mechanisms, potentially involving the AKT signaling axis.

Subsequent co-treatment of BCPAP cells with Vemurafenib and AdipoRon significantly enhanced the inhibitory effects on cell proliferation and migration, outperforming either monotherapy. These results suggest that while AdipoRon's antitumor effects may not be entirely dependent on the BRAF-MAPK pathway, its efficacy is markedly enhanced when BRAF signaling is suppressed, revealing a potential synergistic interaction between the two treatments.

We further speculate that the persistently activated MAPK pathway in BRAF mutated tumors may exert pro-autophagic and anti-apoptotic effects, forming a protective feedback loop that counters AdipoRon induced cell death. In other words, BRAF mutant cells may employ MAPK mediated survival mechanisms to limit the pro-death effects of AdipoRon, thereby diminishing its therapeutic efficacy. When BRAF signaling is blocked by Vemurafenib, this protective mechanism may be disrupted, allowing AdipoRon induced apoptosis and autophagy to proceed more effectively. Through coordinated regulation of MAPK and AMPK signaling pathways, the combination therapy may further modulate AKT activity and its downstream targets, thereby amplifying cell death and achieving a more potent antitumor effect (see Figure 44).

Figure 44. Schematic model of the Relationship Between AdipoRon and BRAF mutation in Thyroid Cancer. (BioRender.com)



V. 3 Exploring the Relationship Between AdipoRon and RET/PTC1 rearragment in Thyroid Cancer

RET/PTC rearrangement is a specific chromosomal alteration commonly found in PTC, occurring in about 25% of adult and up to 50% of pediatric cases(695). The most prevalent subtypes are RET/PTC1 and RET/PTC3(696). This rearrangement leads to the formation of fusion genes combining the RET tyrosine kinase domain with proteins like CCDC6 or NCOA4, resulting in constitutive RET activation(697). This persistent activity drives oncogenic pathways such as RAS/MAPK and PI3K/AKT, promoting uncontrolled proliferation, resistance to apoptosis, and increased invasiveness of thyroid cells(698). These features highlight the distinct biological and clinical role of RET/PTC in PTC. While $BRAF^{V600E}$ driven tumors may resist MAPK targeted therapies via feedback activation of pathways like PI3K/Akt, RET/PTC1 driven tumors co-activate both MAPK and PI3K/Akt pathways from the start. Thus, evaluating the antitumor potential of agents like AdipoRon in RET/PTC driven PTC is important, as it may offer new therapeutic strategies for genetically distinct thyroid cancer subtypes (699).

Based on these findings, the impact of RET/PTC rearrangement on the expression of APN receptors using the GEO dataset GSE60542 confirm the differential pattern in the *ADIPORs* gene expression, suggesting this type of mutation rearrangement may specifically regulate *ADIPOR1*. Further analysis using dataset GSE261830, where TPC-1 cell line was treated with the RET tyrosine kinase inhibitor selpercatinib revealed a negatively regulate of *ADIPOR1* transcription *in vitro*, and this repression can be effectively relieved by targeted RET inhibition. Taken together, although *ADIPOR1* expression responded differently to RET/PTC rearrangement in tissue samples and cell models, the overall trend suggests that the RET signaling pathway may suppress *ADIPOR1* expression, thereby modulating downstream signaling activity and contributing to PTC tumorigenesis. This observation provides a potential explanation for the biological mechanism by which RET/PTC rearrangements drive tumor development, as well as for the differential sensitivity of such tumors to AdipoRon.

Moreover, GSEA comparing pathway activity between RET/PTC1 positive and negative PTC tissue samples revealed significant enrichment of the TNF signaling and cell cycle regulation pathways, suggesting that this gene rearrangement may promote tumorigenesis and progression by activating inflammatory responses and proliferation-related signaling pathways. Interestingly, it was observed that selpercatinib treatment *in vitro* led to significant enrichment of pathways related to lipid metabolism, atherosclerosis, and cytokine–cytokine receptor interactions. These findings suggest that inhibition of RET signaling may modulate tumor cell metabolic activity and immune regulation. Taken together, RET/PTC1 rearrangement may contribute to tumor progression not only by downregulating *ADIPOR1* expression and activating inflammatory and proliferative pathways, but also by reshaping metabolic and immune microenvironments. Targeted RET inhibition may exert antitumor effects by reversing these aberrant pathways.

Further analysis revealed that following selpercatinib treatment in TPC-1 cells, *ADIPOR1*/*2* exhibited distinct patterns of correlation with cell death related genes. Specifically, *ADIPOR1* expression was associated with apoptosis related genes such as *CASP3, CASP9*, and *AIFM1,* while *ADIPOR2* was more closely correlated with autophagy related genes including *SQSTM1* and *MAP1LC3B*. These results suggest that *ADIPOR1* and *ADIPOR2* may regulate cell death through different signaling mechanisms.

Experimental validation revealed that AdipoRon significantly inhibited the proliferation and migration of TPC-1 cells; however, it did not markedly induce apoptosis or autophagy, suggesting that its antitumor effects may not depend on classical cell death mechanisms. Protein expression analysis further supported this observation, as no substantial activation of apoptotic or autophagic pathways

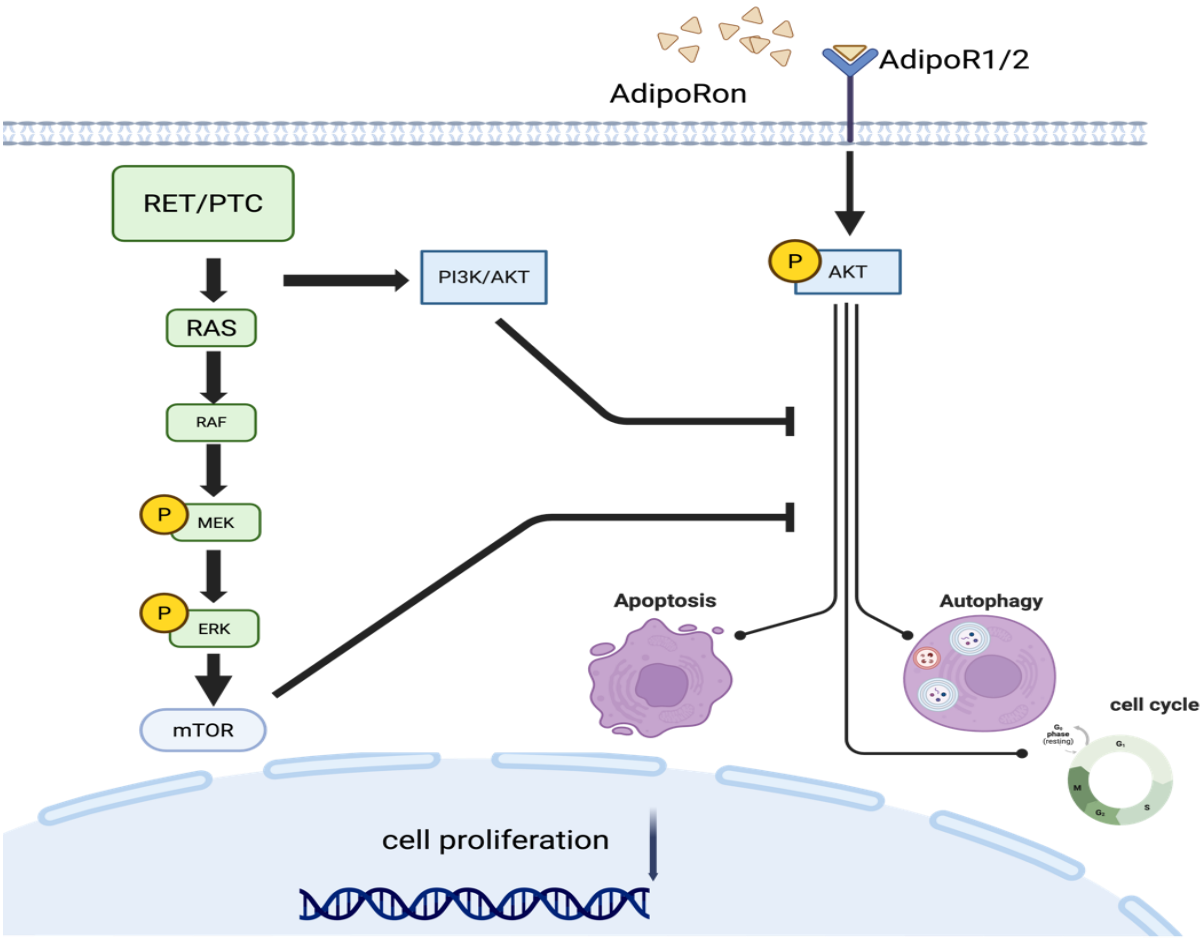
was detected, indicating that AdipoRon may influence RET/PTC1 driven cellular behavior through non-canonical mechanisms.

Our results, showed partial activation of proliferation related signaling upon AdipoRon treatment, but failed to demonstrate complete induction of apoptosis or autophagy, which may limit its overall antitumor efficacy. Compared to BCPAP cells, TPC-1 cells exhibited greater resistance to AdipoRon induced migration inhibition and higher tolerance in terms of apoptotic and autophagic responses. This differential sensitivity may stem from the presence of RET/PTC1 rearrangement in TPC-1 cells, wherein constitutive activation of RET kinase not only triggers the MAPK pathway but may also modulate multiple other signaling axes involved in cell death regulation, thereby enhancing resistance to AdipoRon mediated cytotoxic stress.

Some studies have shown that AdipoRon can exert antitumor effects through non-classical cell death mechanisms such as cell cycle arrest and necroptosis(700). In our study, we observed that AdipoRon treatment induced G0/G1 phase cell cycle arrest in BCPAP cells, indicating its significant activity in regulating the cell cycle. Meanwhile, AdipoRon treatment was accompanied by marked activation of ADIPOR2. Given that ADIPOR2 regulates lipid metabolism and antioxidant responses via the PPAR-α pathway(701,702), it is speculated to play a role in cell death regulation.

We further hypothesize that APN or AdipoRon alone may simultaneously activate ADIPOR1 and ADIPOR2, initiating distinct signaling pathways such as AMPK and PPAR-α, respectively. There may be a functional balance between these pathways that maintains cell survival. Only when the signaling of one receptor is significantly enhanced or inhibited, disrupting this balance, might cells initiate apoptosis or autophagy. This mechanism provides a new perspective on the antitumor effects of APN related drugs and suggests that targeted modulation of different APN receptor pathways could potentially enhance their anticancer efficacy (see Figure 45).

Figure 45. Schematic model of the Relationship Between AdipoRon and RET/PTC1 rearragment in Thyroid Cancer. (BioRender.com)



V. 4 Exploring the Relationship Between AdipoRon and LDL in Thyroid Cancer

Previously results for our group identified that the cholesterol is involved in the aggressive behavior PTC, through intratumorally accumulation of its metabolite 27-hydroxycholesterol (27-HC) and also with a LDLR dysregulation, suggesting a potential therapeutic target in high-risk thyroid cancers(655,656). On the other hand, in breast cancer cells, APN has been shown to promote the degradation of LDL receptor (LDLR) by activating the autophagy pathway, thereby inhibiting LDL induced cancer cell proliferation(703). Additionally, APN can bind oxidized LDL (oxLDL) and suppress oxLDL induced ERK phosphorylation and NF-κB pathway activation(704).

AdipoRon has been reported to exert anti-tumor effects in thyroid cancer cells by suppressing glucose and amino acid metabolism and inducing autophagy via the AdipoR2–ULK signaling axis(705). Notably, autophagy is not only a key mechanism for maintaining cellular homeostasis but also plays an essential role in lipid metabolism regulation(706). Studies in breast cancer have demonstrated that APN regulates LDLR levels through autophagy to maintain intracellular cholesterol balance(703).

However, whether AdipoRon regulates cholesterol metabolism in thyroid cancer through a similar mechanism possibly by inhibiting LDLR or interfering with its downstream signaling remains unknown. In this sense, Transcriptomic analysis revealed significant alterations in the expression of key genes involved in cholesterol and lipid metabolism in PTC tissues compared to normal thyroid tissues.

Notably, genes such as *SREBF1, APOE, PCSK9*, and *LDLR* were significantly upregulated, whereas *HMGCR, SREBF2*, and *APOB* were markedly downregulated, suggesting a potential reprogramming of lipid metabolic pathways in these types of tumors.

Our *in vitro* experiments demonstrated that treatment with AdipoRon significantly inhibited LDL-induced PTC cell proliferation in both cell lines analyzed (BCPAP and TPC-1) and that both AdipoRon alone and in combination with LDL also reduced cell migration capacity. Furthermore, AO staining revealed that treatment with 50 μM AdipoRon combined with LDL markedly enhanced autophagic activity, which may indicate that AdipoRon can enhances LDL-induced autophagy mechanism.

At the mechanistic level, *LDLR* expression in PTC may be regulated by the BRAF signaling pathway and may be functionally associated with apoptosis related genes. In the GSE60542 dataset, *LDLR* expression was higher in PTC tissues harboring BRAF mutations than in wild type samples, although the difference did not reach statistical significance ($p = 0.645$). However, in BCPAP cells carrying the $BRAF^{V600E}$ mutation, treatment with the BRAF inhibitor Vemurafenib (20 μM, 48 h) significantly downregulated *LDLR* expression ($p < 0.01$), supporting the hypothesis that LDLR is positively regulated by BRAF signaling(655).

Correlation analysis further revealed that after Vemurafenib treatment, *LDLR* expression was positively correlated with pro-apoptotic genes such as *CASP9, AKT2,* and *MAPK1,* and negatively correlated with *Annexin V* and *AKT3*, suggesting a potential role of LDLR in the regulation on the tumoral cell survival. Further analysis demonstrated that in lymph node-negative (N0) patients, *LDLR* expression in tumor tissues was significantly higher than in adjacent normal tissues. Moreover, comparison between lymph node-positive (N1) and N0 patients revealed a further increase in *LDLR* expression in N1 tumor tissues. These findings suggest that *LDLR* and related lipid metabolism receptors may play important roles in the initiation, progression, and lymphatic metastasis these tumors. In relation with these data, protein expression analysis shown that AdipoRon under LDL stimulated conditions may induce in BCPAP cells, both Caspase-dependent apoptosis and autophagy via activation of the AKT signaling pathway. Furthermore, in relation with the metabolism lipid, the *LDLR* expression was consistently upregulated, and AdipoRon failed to significantly reverse the LDL-induced upregulation of *LDLR*, suggesting that its suppressive effect on LDLR is limited in a high-lipid environment. This implies a possible compensatory mechanism by which cells enhance cholesterol uptake to maintain lipid homeostasis. At the same time, the treatment significantly enhanced the nuclear activation of the lipid metabolism associated transcription factor SREBP-1, as evidenced by a reduction in the precursor form (128 kDa) and increase in the active nuclear form (68 kDa). Our results, are align with the studies that shown that AdipoRon can activate the AdipoR2–PPARα signaling axis(707). This enhancement of lipid catabolism may result in an imbalance between lipid supply and demand, leading to compensatory activation of the SREBP-1 mediated fatty acid synthesis pathway to restore lipid homeostasis. This phenomenon highlights the dynamic adaptability of the lipid metabolic network via self-regulatory feedback mechanisms (see Figure 46).

Figure 46. Schematic model of the Relationship Between AdipoRon LDL in BCPAP cells. (BioRender.com)

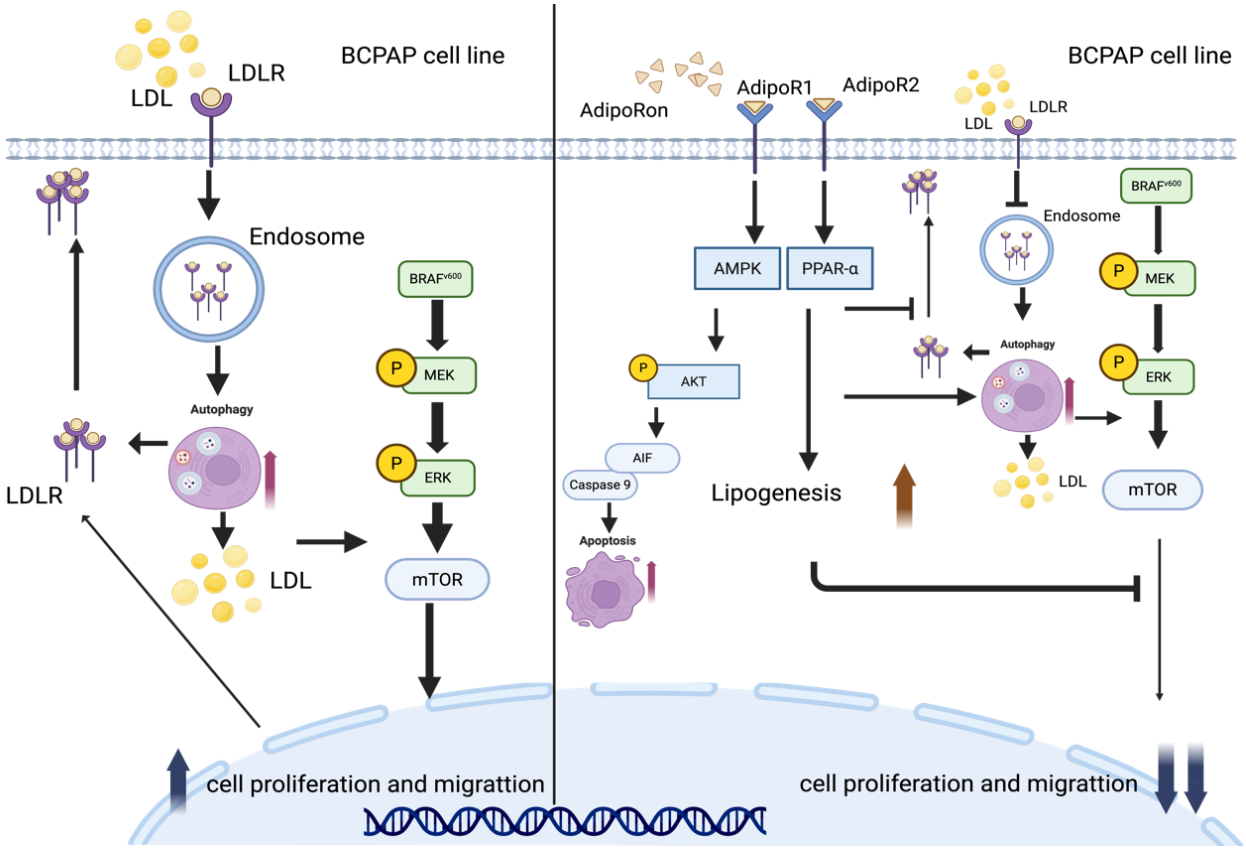


Figure 46. AdipoRon suppresses LDL-induced proliferation and migration of BCPAP cells and enhances autophagic activity, potentially through the AdipoR2–ULK axis. Despite increased LDLR expression under high lipid conditions, AdipoRon fails to significantly reverse LDLR upregulation, suggesting a compensatory mechanism to maintain cholesterol homeostasis.

In the absence of BRAF harboring mutation, and in the presence or absence of the RET/PTC1 rearrangement, the transcriptomic analysis from GEO dataset GSE60542, showed no significant difference between both conditions, suggesting that RET/PTC1 fusion status has limited impact on *LDLR* expression. Although *LDLR* expression was slightly reduced when Selpercatinib drug was used the change was not statistically significant. Under conditions of RET pathway inhibition, correlation analysis between *LDLR* and key apoptosis/autophagy-related genes revealed that *LDLR* expression was negatively correlated with autophagy markers *SQSTM1* (p62) and *MAP1LC3B* (LC3B), but positively correlated with *ANXA5* and *AKT3*. These findings suggest that under RET pathway suppression, reduced *LDLR* expression may be associated with decreased autophagic activity and partial activation of apoptotic signaling. Our data, further revealed that AdipoRon effectively attenuated LDL induced proliferation of TPC-1 cells. Moreover, despite of, both AdipoRon alone and in combination with LDL showed a decreasing trend in cell migration, the changes did not reach statistical significance. The APN in presence of the LDL in the media was able to enhanced the autophagic activity.

To further elucidate the regulatory mechanism of AdipoRon under LDL stimulated conditions in TPC-1 cells, we examined the expression of key signaling proteins and markers related to apoptosis and autophagy. Western blot analysis revealed that AdipoRon alone significantly downregulated LDLR protein levels, suggesting that it can suppress cellular LDL uptake and prevent cholesterol overload

and subsequent activation of lipid synthesis pathways. Under LDL stimulation, combined AdipoRon treatment resulted in reduced phosphorylation of mTOR and ERK, while p-AKT levels increased, indicating activation of the AKT pathway alongside inhibition of mTOR and ERK signaling. With regard to apoptotic markers, Caspase-9 and AIF levels were downregulated, while Caspase-3 expression was elevated, suggesting partial inhibition of mitochondrial-mediated intrinsic apoptosis. For autophagy markers, the LC3B-II/I ratio increased and p62 expression decreased, supporting the conclusion that autophagy was effectively activated(694,708,709). Collectively, these results indicate that under LDL stimulation, AdipoRon activates AKT signaling and suppresses mTOR, thereby enhancing autophagic activity, along with a complex modulation of apoptotic pathways (see Figure 47).

Importantly, these findings differ from those observed in the BCPAP cell line, where ERK signaling was not significantly affected. This differences as a response can be support our hypothesis that while BCPAP cells harbor the $BRAF^{V600E}$ mutation, which constitutively activates the MAPK pathway, making ERK signaling resistant to AdipoRon modulation, TPC-1 cells carry the RET/PTC1 fusion, which activates upstream tyrosine kinases and it is less sensitive to the AdipoRon action. Moreover, under LDL condition, this upstream positioning allows for more efficient negative regulation by AdipoRon, resulting in simultaneous suppression of ERK and mTOR pathways. In TPC-1 cells, we observed a phenomenon similar to that in BCPAP cells: under basal conditions, AdipoRon downregulated LDLR expression, whereas exogenous LDL stimulation generally led to upregulated LDLR expression. Notably, combined treatment demonstrated that AdipoRon could partially reverse the LDL induced upregulation of LDLR, although LDLR levels remained relatively high compared to AdipoRon treatment alone. Additionally, AdipoRon treatment significantly enhanced the nuclear activation of the key lipid metabolism transcription factor SREBP-1, characterized by a decrease in its precursor form and an increase in the active nuclear form; this trend persisted under LDL stimulation. Integrating the findings of enhanced autophagic activity, we propose that under LDL treatment, AdipoRon may activate the AMPK signaling pathway via AdipoR1, leading to AKT activation and mTOR inhibition, thereby promoting autophagy. The enhanced autophagy facilitates intracellular cholesterol clearance and LDLR degradation, modulating cellular LDL handling capacity. Meanwhile, AdipoRon may also activate the PPAR-α pathway via AdipoR2, promoting fatty acid β-oxidation and increasing lipid catabolism. These two mechanisms synergistically affect intracellular lipid metabolism: on one hand, autophagy promotes LDLR degradation and cholesterol processing; on the other hand, accelerated fatty acid metabolism enhances lipid efflux, together, these processes resulting in a lipid supply-demand imbalance within the cell. To maintain lipid homeostasis, cells activate a compensatory feedback loop mediated by SREBP-1-driven fatty acid synthesis (see Figure 46/47).
This phenomenon can be suggested a reveal an adaptive lipid metabolic mechanism induced by AdipoRon under high-lipid conditions, reflecting its potential dual-pathway regulation of lipid metabolism and cellular homeostasis.

Figure 47. Schematic model of the Relationship Between AdipoRon LDL in TPC-1 cells. (BioRender.com)

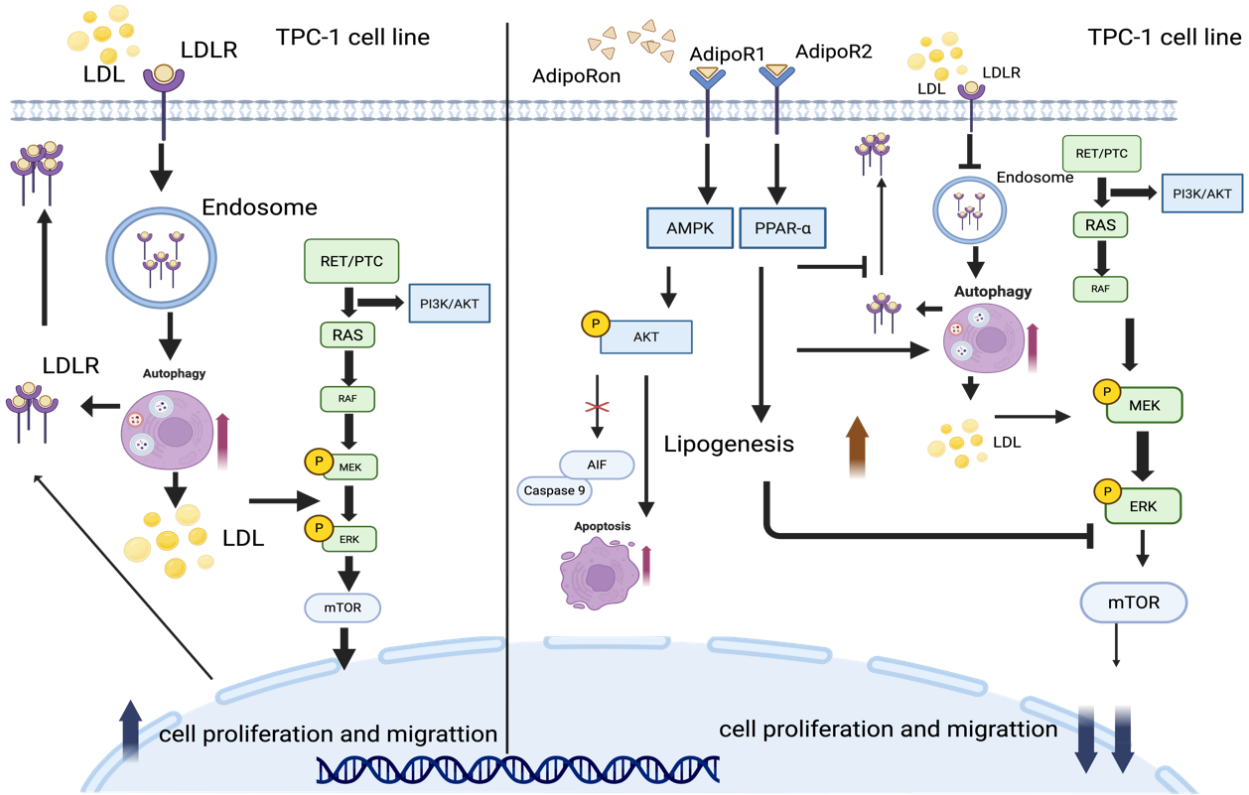


Figure 47. Mechanistic model illustrating the regulatory effects of AdipoRon on lipid metabolism, autophagy, and apoptosis in TPC-1 thyroid cancer cells under LDL-stimulated conditions. AdipoRon downregulates LDLR expression, inhibits ERK and mTOR signaling, and activates AKT, thereby enhancing autophagic flux. The elevated LC3B-II/I ratio and decreased p62 expression indicate effective autophagy induction. Concurrently, AdipoRon partially suppresses mitochondrial apoptosis while engaging the AdipoR1–AMPK–AKT and AdipoR2–PPARα pathways.

VI. CONCLUSIONS

The main conclusions are as follows:

1. APN related genes are differentially expressed in PTC tissues compared to BT, suggesting their involvement in PTC tumorigenesis and progression.

2. The secretion level of APN in adipose tissue was higher in PTC patients than in BT patients, and was associated with altered levels of inflammatory cytokines. This suggests that APN may participate in PTC malignant transformation by modulating the local inflammatory microenvironment.

3. The expression levels of APN receptors, ADIPOR1 and ADIPOR2, were both elevated in PTC tissues compared to BT. Bioinformatics analysis based on public datasets further revealed that ADIPOR1 is primarily involved in regulating cell death and immune modulation, whereas ADIPOR2 is more closely associated with metabolic remodeling. These findings indicate distinct biological functions of the two receptors in PTC progression.

4. The proliferation and migration of two PTC cell lines with different genetic backgrounds (BCPAP and TPC-1) were inhibited by AdipoRon treatment. The antitumor effects of AdipoRon may be mediated through the modulation of apoptotic and autophagic pathways.

5. In BCPAP cells, co-treatment with the BRAF inhibitor Vemurafenib enhanced the suppressive effects of AdipoRon, whereas TPC-1 cells showed relatively lower sensitivity to AdipoRon.

5. AdipoRon reduced LDLR expression in BCPAP cells and inhibited LDL-induced proliferative effects. Under high LDL conditions, AdipoRon activated the AKT signaling pathway and induced apoptosis and autophagy via an mTOR-independent mechanism.

6. AdipoRon downregulated LDLR expression in TPC-1 cells and inhibited LDL-induced proliferation. However, under high LDL conditions, AdipoRon activated AKT while inhibiting mTOR and ERK signaling pathways, resulting in decreased apoptosis but enhanced autophagy.

VII. BIBLOGRAPHY

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