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Molecular Mechanisms of Melatonin for Treating Medullary Thyroid Cancer Using *in silico* Analysis

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Abstract Melatonin is a neuroendocrine hormone that regulates various biological functions. The objective of this work was to conduct a thorough assessment of the molecular targets of melatonin for the treatment of medullary thyroid carcinoma (MTC) via bioinformatics methods. The identification of target proteins for melatonin and MTC was accomplished through the utilization of many databases. A total of 359 gene targets were acquired, then subjected to gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses. The GO subjects indicated a strong association in kinase activity and receptor-ligand activity between the target genes. On the other hand, the KEGG enrichment analysis indicated a significant involvement of the target genes in the PI3K/AKT and MAPK signaling pathways. To identify key genes, an initial step involved the construction of a protein-protein network using the intersecting targets. Subsequently, Cytoscape software unveiled TP53, STAT3, AKT1, JUN, and IL6 as hub genes within the network. The interaction of melatonin and hub genes was validated by molecular docking analysis. The results indicated that melatonin had high binding affinities towards all of the hub genes, with the exception of JUN. The interaction of melatonin and AKT1 had -10.54 kcal/mol binding free energy with several H-bonds and hydrophobic interactions. Also, the melatonin/p53 complex revealed low binding free energy (-9.95 kcal/mol) with strong H-bond interactions. All the outcomes suggest that melatonin induces apoptosis in MTC cells through the PI3K/AKT and MAPK signaling pathways. Therefore, melatonin has the potential to serve as a powerful therapeutic drug for the treatment of MTC.

Keywords Network Pharmacology, Target Prediction, p53, AKT1, JUN, STAT3, TP53, IL6

1. Introduction

Thyroid carcinoma, or thyroid cancer (TC), refers to a malignant neoplasm originating from the thyroid gland. This neoplasm represents the prevailing malignancy within the endocrine system, while its occurrence remains few, constituting around 1% of all malignant tumors. Nevertheless, there has been a notable upward trend in its prevalence in recent times [1]. Thyroid cancer has many subtypes, such as differentiated, anaplastic, and medullary thyroid cancer, originating from distinct cellular populations inside the thyroid gland [2]. Medullary thyroid carcinoma (MTC) is an infrequent neoplastic growth of neuroendocrine origin, characterized by the ability of parafollicular cells to synthesize and secrete calcitonin. Hence, the diagnostic and treatment approaches for MTC exhibited variations as compared to those employed for well-differentiated TC originating from follicular cells [3].

Melatonin, also known as N-acetyl-5-methoxytryptamine, is a kind of indole amine that is synthesized by many sources inside the human body. Melatonin is primarily synthesized by the pineal gland in response to periods of darkness. Additional organs involved in the synthesis of melatonin are the skin, bone marrow, lymphocytes, retina, and gastrointestinal system [4]. The suprachiasmatic nucleus (SCN), located in the

hypothalamus, serves as the central pacemaker that governs the synthesis and production of melatonin during a 24-hour period. Increased concentrations of melatonin during the nocturnal period induce target organs to adopt appropriate homeostatic metabolic patterns, therefore safeguarding the body against the onset of many ailments [5]. Presently, melatonin is recognized as possessing cell-protective properties in addition to its role as a hormone. Numerous studies have documented the significant effects of melatonin on several biological processes, such as oxidative stress, immunological regulation, and hematopoiesis [6]. Melatonin has been proposed to possess remarkable *in vitro* effects on diverse tumor cell types and their apoptotic processes [7]. The protective effects of melatonin against various cancer types, including TC, have been reported [8]. Melatonin has been shown to induce impairment in tumor growth in TC [9]. Lately, the cytotoxic effects of melatonin on anaplastic TC cell lines have been shown [10]. However, up to now, no studies have been reported on the effects of melatonin against MTC.

The utilization of computational approaches has the potential to bring significant advances to the field of drug development. Currently, there are several networks that facilitate the identification of proteins and disease targets. Conducting a systematic investigation aimed at constructing a network that connects compounds, proteins, genes, and diseases in a high-throughput way might serve as a promising initial step in the field of drug design [11]. In addition, molecular docking serves as a valuable technique for assessing the efficacy of receptor-ligand interactions, thereby enabling the prediction of receptor-ligand binding patterns, affinities, and the correct orientation [12]. Molecular docking provides information on whether the ligand and receptor can form a stable complex [13]. In this study, the molecular mechanisms of melatonin in the treatment of MTC were evaluated via an *in silico* approach. The validation of target protein/melatonin interactions was carried out via the molecular docking method.

2. Materials and Methods

2.1. Screening of Melatonin-related Protein Targets

Figure 1 shows an illustration of the study. All targets of melatonin were collected by using the following four databases: Drugbank (https://www.drugbank.com) [14], Swiss target prediction database (http://www.swisstargetprediction.ch) [15], Superpred target prediction (https://prediction.charite.de/subpages/target_prediction.php) [16], and Genecards (https://www.genecards.org) [17]. Gene score >1 was chosen for the Genecards database. *Homo sapiens* was limited as the selected organism.

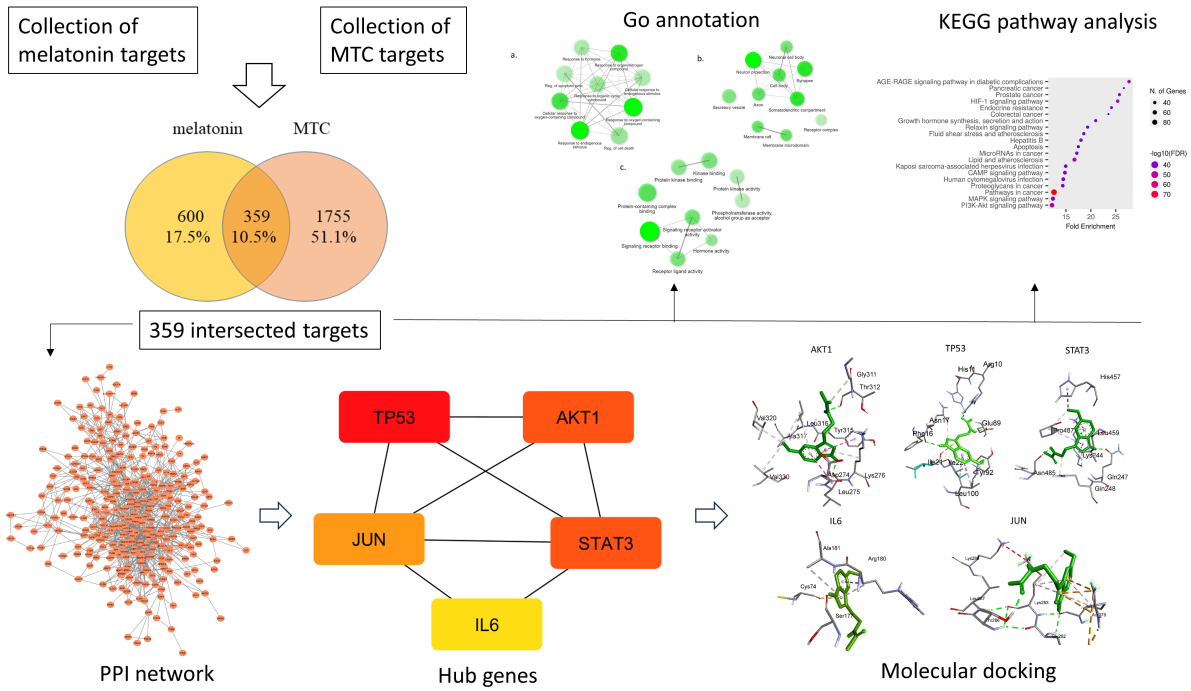

Figure 1. The flow chart of melatonin in the treatment of medullary thyroid cancer

2.2. Screening of MTC-related Protein Targets

Genecards [17] and DisGeNet (https://www.disgenet.org) [18] databases were used for the screening of potential MTC-related targets. A gene-disease association score >0.1 was selected from the DisGeNet database. The duplicate protein targets of melatonin and MTC were removed.

2.3. Construction of Protein-protein Interaction (PPI) Network and Determination of Hub Genes

The intersected targets of MTC-related and melatonin-related genes were obtained, and a Venn diagram was constructed. The intersected proteins were imported to STRING database version 12.0 (https://string-db.org) [19]. The species selection was limited to "*Homo sapiens*" and the confidence score was set to ≥0.9 (the highest confidence score). The file was then exported to Cytoscape software version 3.10.1 [20] to illustrate the protein-protein interaction figure and also to detect hub genes. The three important topological parameters (degree, betweenness centrality, and closeness centrality) were used to screen core targets. The CytoNCA plug-in was used to find out the hub genes. CytoNCA offers comprehensive calculations, analyses, and evaluations for biological networks. CytoNCA uses important topological parameters to screen the core targets [21]. The CytoHUBBA plug-in was used to illustrate the top nodes ranked by degree, and to validate the results obtained by CytoNCA [22]. The mCODE plug-in was used to identify protein clusters.

2.4. Go and KEGG Enrichment Analyzes

Intersecting genes were evaluated by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses using the ShinyGO 0.77 database (http://bioinformatics.sdstate.edu/go). *Homo sapiens* was used for the matching species. FDR <0.05 and p <0.05 were set as cut-off values. The top 10 enriched terms of GO-biological processes (GO-BP), GO-molecular functions (GO-MF), and GO-cellular components (GO-CC) were selected. 20 KEGG pathways were selected by FDR and sorted by fold enrichment, then a dot-plot chart was constructed.

2.5. Molecular Docking Verification Analysis

The protein 3D structures were deposited at the Protein Data Bank (PDB) (https://www.rcsb.org). The PDB codes are as follows: 3DCY for TP53, 6NUQ for STAT3, 1JUN for JUN, 2IL6 for IL6, and 6S9X for AKT1.

The 3D structure of the ligand (melatonin) was collected from the PubChem database (https://pubchem.ncbi.nlm.nih.gov) [23] in SDF format (compound cID:896). Openbabel GUI was used to convert the sdf format of melatonin to pdf format [24]. Autodock tools were used for the melatonin and protein preparation and also for the molecular docking analysis. The preparation of proteins was carried out as follows: The ligands and water molecules were deleted, polar hydrogens and Kollman charges were added and then the proteins were saved in pdbqt format. The grid boxes were constructed according to the binding sites of the target proteins, separately. The genetic algorithm was used as search parameter.

Autodock4 was used for the docking analysis (100 runs) and estimating the free binding energies [25]. Binding free energies were determined as follows: Final intermolecular energy + final total internal energy + torsional free energy - unbound system's energy. Molecular interactions were evaluated via the Protein-Ligand Interaction Profiler (PLIP) [26] and illustrated by Discovery Studio Visualizer.

3. Results

3.1. Screening of Melatonin and MTC Targets

Our results reveal that the Drugbank, SuperPred, Gene Cards, and Swiss target prediction databases had a total of 1023 melatonin targets. After removing duplicate targets, 959 targets were finally obtained. In the meantime, GeneCards and DisGeNet revealed 2114 MTC-related targets after removing repeated genes. 359 intersected gene targets were obtained, constructing 10.5% of the total targets. A Venn diagram was constructed to visualize the data in Figure 2.

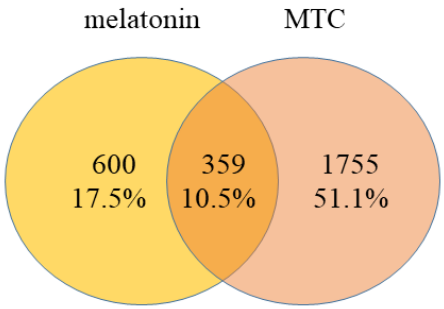

Figure 2. Venn diagram of melatonin, MTC, and intersected targets. The yellow circle represents the gene targets of melatonin, while the orange circle represents the gene targets of MTC based on various databases. The intersection represents the targets of melatonin for MTC

3.2. Construction of PPI Network and Identification of Key Targets

Fig 3 shows the PPI network constructed via the STRING database, which reveals the number of nodes as 355, the number of edges as 1217, the average node degree as 6.86, and the average local clustering coefficient as 0.398. Due to the high interaction score, many genes were found to have no interaction. Therefore, after these non-interacted genes were removed, 301 nodes and 1216 edges were obtained. Each node represents a gene, while the functional association (interaction) between genes is defined as an edge. The CytoNCA plug-in in Cytoscape revealed the top 5 genes as TP53, STAT3, AKT1, JUN, and IL6 based on degree values.

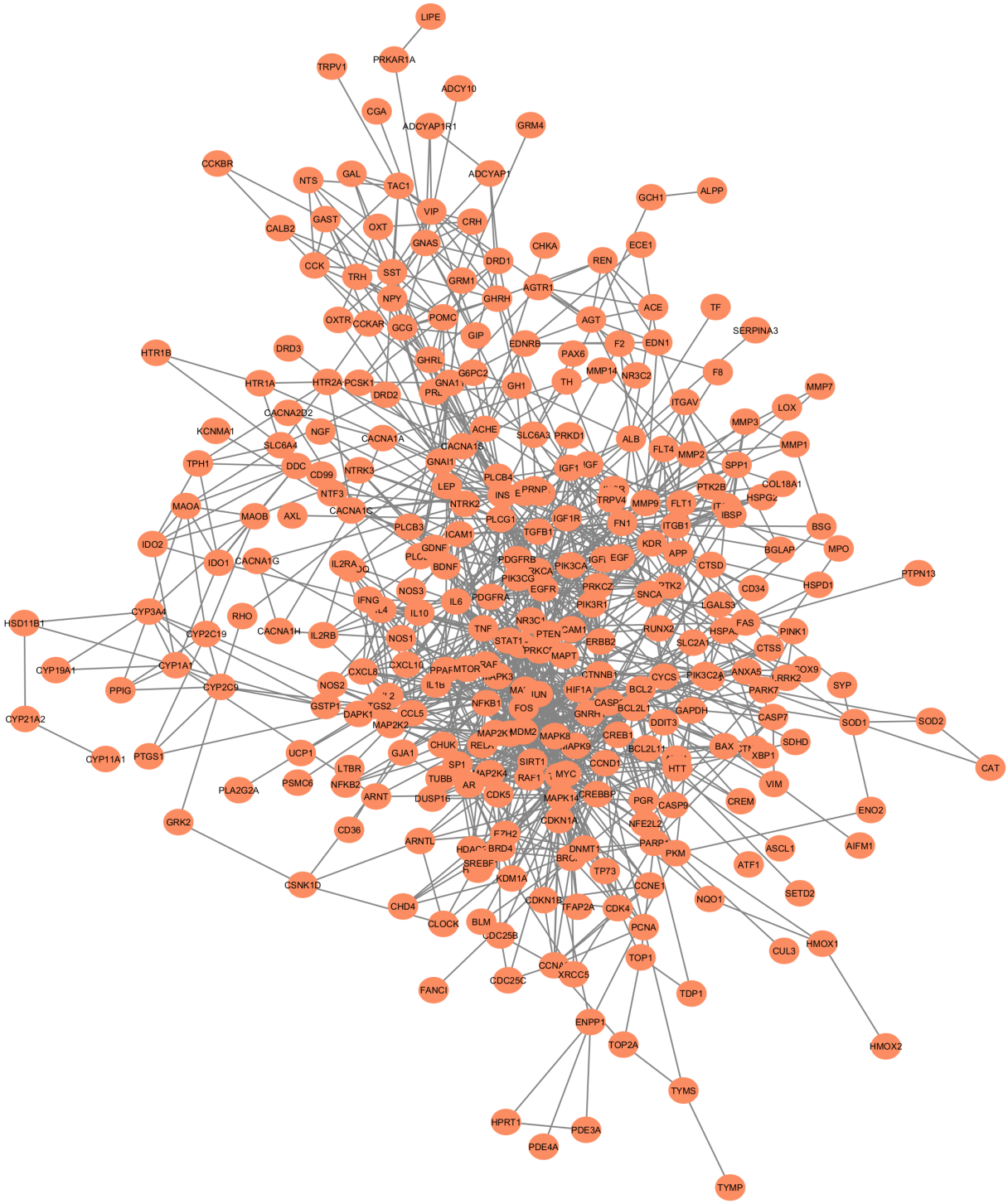

Figure 3. Protein-protein network of the intersected targets constructed by STRING database. The nodes represent potential gene targets of melatonin against MTC

The degree values, betweenness centrality, and closeness centrality values of hub genes are shown in Table 1. Furthermore, MCODE revealed 13 protein clusters. The top 4 clusters are shown in figure 4 TP53, STAT3, and JUN were included in cluster 1, which has 24 nodes, 95 edges, and a score of 8.261. IL6 was included in the enlarged version of Cluster1 (data not shown). Cluster 2 has 5 nodes, 10 edges, and a score of 5.0. Cluster 3 has 4 nodes, 6 edges, and a score of 4.0. Cluster 4 revealed 23 nodes, 43 edges, and a score of 3.909. On the other hand, AKT1 was included in cluster 7, which has 17 nodes, 25 edges, and a score of 3.125 (data not shown). The results were verified by the CytoHUBBA plug-in. Figure 5 shows the network of hub genes.

3.3. GO Enrichment Analysis

GO enrichment analysis of 359 target proteins revealed 1032 total GO terms, of which 480 were biological processes (BP), 212 were cellular components (CC), and 340 were molecular functions (MF). Figure 6 displays the top 10 terms from each category. BP terms suggest that the targets regulate hormone response, endogenous response, and apoptosis. For MF, the targets are mostly involved in kinase activity, hormone activity, and receptor-ligand activity. CC terms included secretory vesicle, membrane raft, receptor complex, neuron projection, and synapse.

Table 1. Topological features of hub genes

No	Gene	Degree	Betweenness cent.	Closeness cent.
1	TP53	59.0	12235.525	0.23000762
2	STAT3	42.0	7217.2334	0.22948328
3	AKT1	42.0	7160.841	0.22913505
4	JUN	33.0	4217.7603	0.22140762
5	IL6	31.0	4864.192	0.22189566

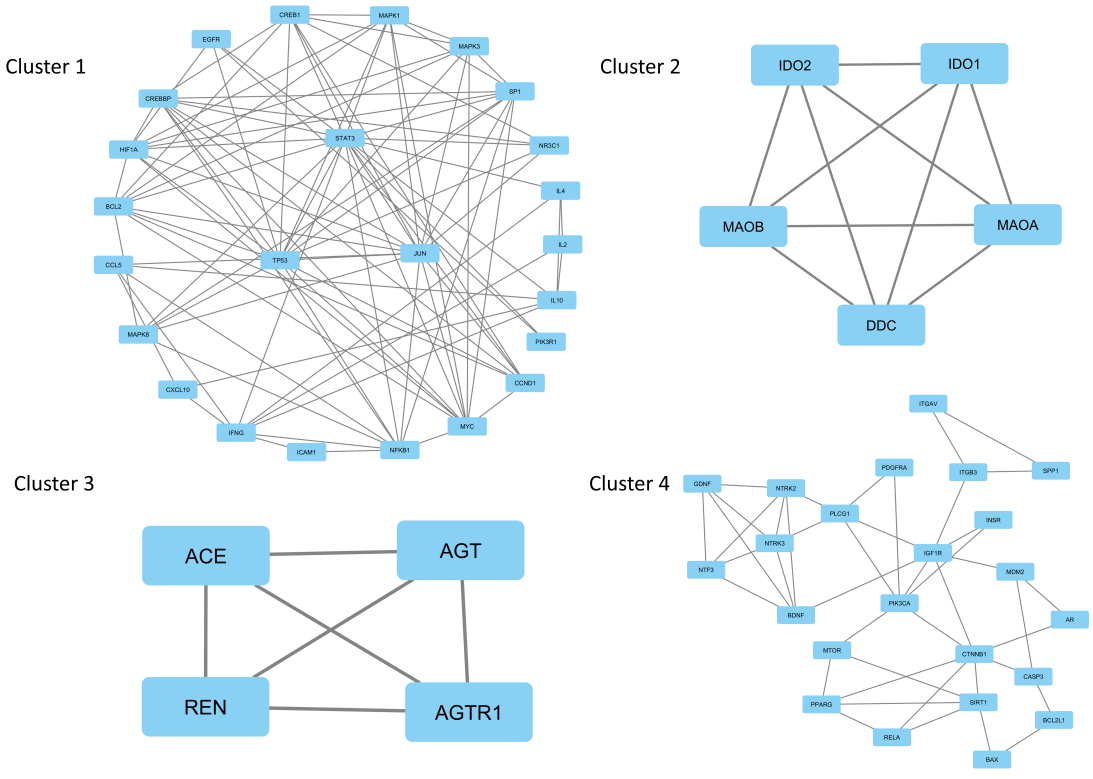

Figure 4. Protein clusters constructed by Cytoscape with MCODE plug-in. a) Cluster 1 (score: 8.261), b) Cluster 2 (score: 5.0), c) Cluster 3 (score: 4.0), d) Cluster 4 (score: 3.909)

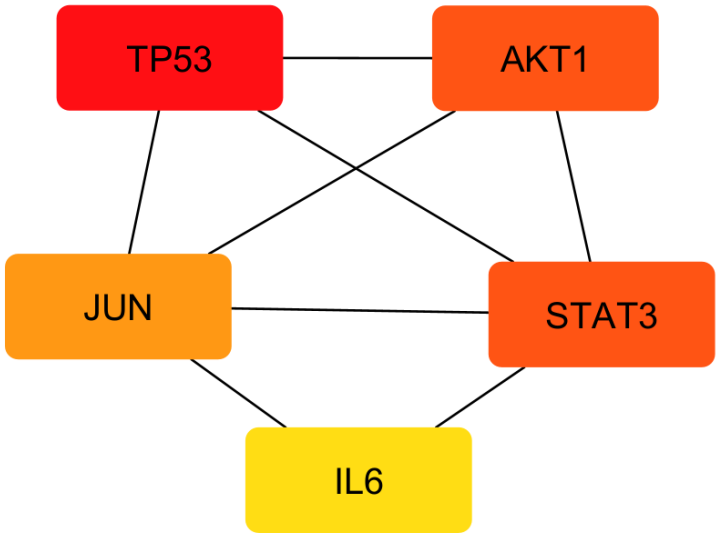

Figure 5. Protein-protein network of hub genes. The darker colors represent higher degree values, while the lighter colors represent lower degree values. The arrangement in descending order from the highest to the lowest degree value is as follows: TP53, STAT3, AKT1, JUN, and IL6

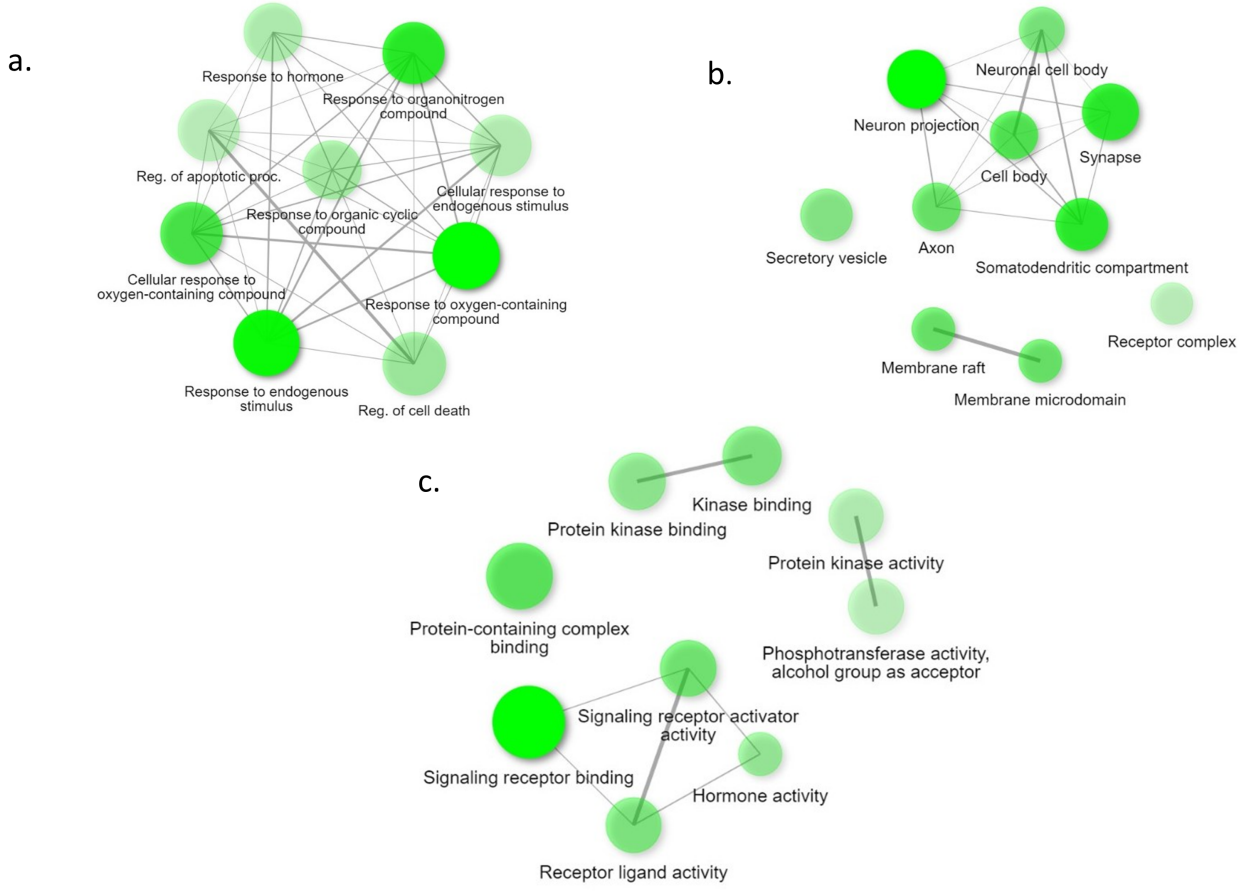

Figure 6. GO network of intersected protein targets. Gene sets are considerably enriched in darker nodes. Bigger nodes correspond to bigger gene sets. More overlapping genes are represented by thicker edges. a) biological processes, b) cellular components, c) molecular functions

3.4. KEGG Enrichment Analysis

The KEGG enrichment analysis revealed 237 pathways. The top 20 results are shown in Figure 7. The targets were found to be closely related to pathways in cancer, apoptosis, MAPK signaling pathway, PI3K/AKT signaling pathway, AGE-RAGE signaling pathway, HIF-1 signaling pathway, endocrine resistance, growth hormone activity, relaxin signaling pathway, microRNAs in cancer, cAMP signaling pathway, and proteoglycans in cancer. Pathways in cancer are related to 99 target genes, which include all of the hub genes. On the other hand, the MAPK signaling pathway is found to be one of the key pathways that involves 54 target genes, including TP53, AKT1, and JUN. Also, the PI3K-AKT signaling pathway is related to 64 target genes, including TP53, IL6, and AKT1. cAMP is found to be related to 48 genes involving AKT1 and JUN. The HIF-1 signaling pathway is related to 41 target genes, including STAT3, IL6, and AKT1.

3.5. Confirmation of Hub Genes by Molecular Docking

The RMSD results, including the binding energies, molecular interactions, and inhibition constant values of melatonin/hub gene complexes, are shown in Table 2. The Melatonin/AKT1 complex revealed the lowest binding free energy (-10.54 kcal/mol) among all complexes. The lower the binding free energy, the higher the binding affinity. Three hydrogen bonds and several hydrophobic interactions were identified between AKT1 and melatonin. Also, the lowest inhibition constant (0.02 μM) among all complexes has been determined in the AKT1/melatonin complex. On the other hand, the inhibition constant between TP53 and melatonin was found to be 0.05 μM. The TP53/melatonin complex was determined to have -9.95 kcal/mol free binding energy with 7 H bonds and several hydrophobic interactions. Molecular docking results revealed low binding energy (-8.70 kcal/mol) between STAT3 and melatonin with 4 H bonds, many hydrophobic interactions, and one *pi*-cation interaction. The Melatonin/IL6 complex was also demonstrated to have significant binding affinity (-8.14 kcal/mol), facilitated by the formation of 2 H-bonds. Figure 8 shows the interactions between melatonin and hub genes.

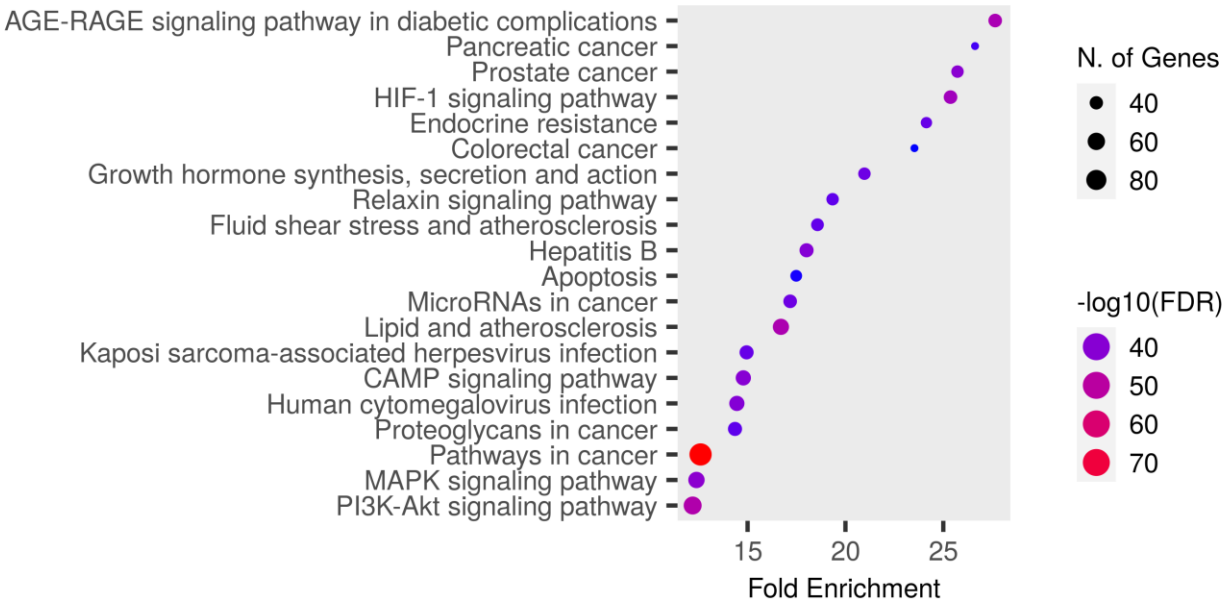

Figure 7. KEGG pathway dot-plot of the intersected proteins ranked by fold enrichment values. Top 20 pathways are shown in the figure. Larger dots in the graph indicate a greater number of genes involved

Table 2. Molecular docking results of melatonin and target proteins

No	Target	Run	Binding energy (kcal/mol)	RMSD[a]	Inh. constant (μM)	H bonds	Hydrophobic int.	*pi*-cation int.
1	AKT1	90	**-10.54**	9.905	0.02	275LEU 312THR 317ALA	18TYR 315TYR 316LEU 316LEU	none
2	TP53	88	**-9.95**	43.212	0.05	10ARG 10ARG 11HIS 16PHE 17ASN 61ARG 89GLU	20LYS 23GLN 89GLU 100LEU 100LEU 198HIS	none
3	STAT3	95	**-8.70**	26.729	0.22	244LYS 247GLN 326GLN 457HIS	244LYS 244LYS 248GLN 459LEU 487PRO	244LYS
4	IL6	67	**-8.14**	15.461	1.08	177SER 184GLN	75PHE 79PHE 79PHE 176GLN 180ARG 184GLN	none
5	JUN	28	**-4.79**	18.160	308.28	279ARG 279ARG 286THR	279ARG 283LYS 283LYS 286THR	279ARG 283LYS

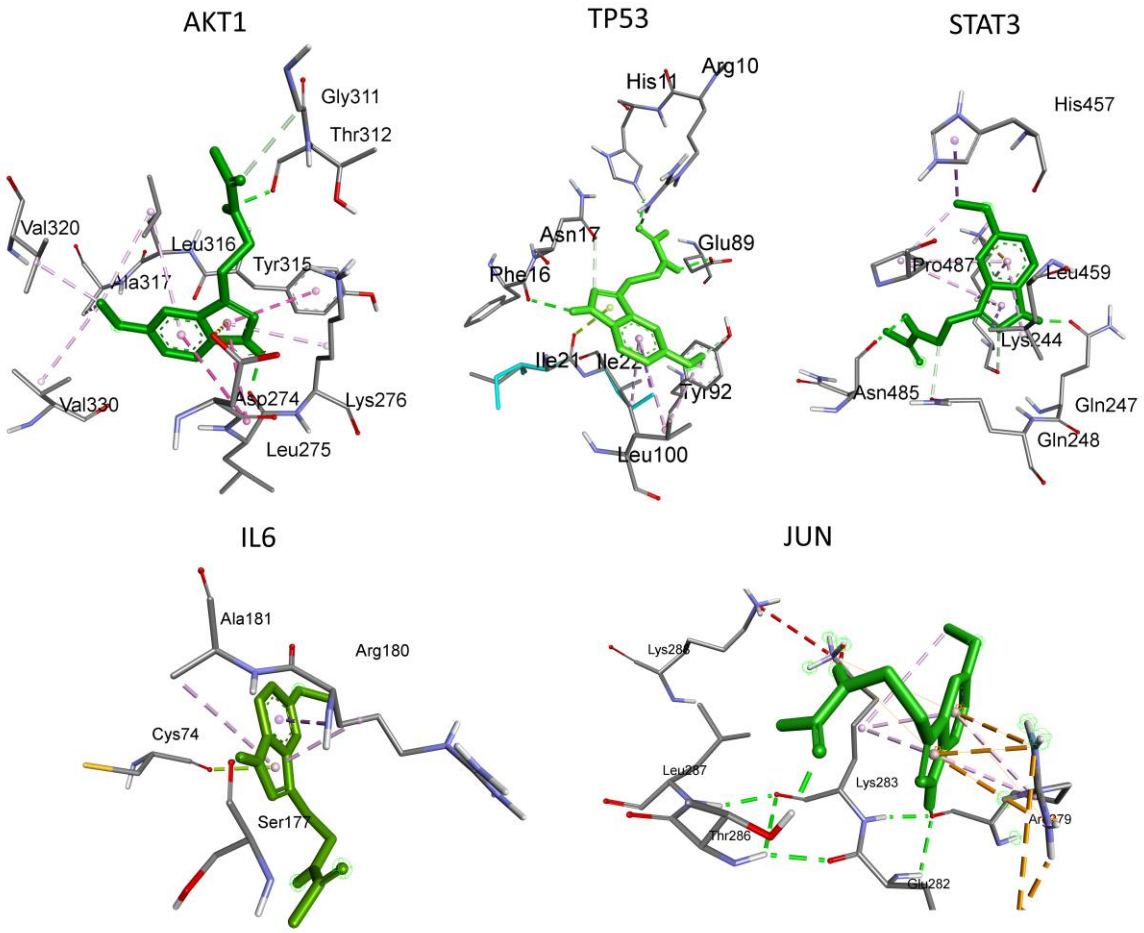

Figure 8. The molecular interactions between hub proteins and melatonin. Melatonin is shown in green wire-frame model. Green color dotted lines indicate hydrogen bonds

4. Discussion

The PPI network is used to identify the interaction between genes in the studied system and also to find out the key genes called hub genes, which are significantly related to each other. The genes in a PPI network do not have to be physically interacted with; however, they influence the functionality of other genes. The interacting genes in a PPI network generally cause the same or a similar disease. Cytoscape is an open-source software package for visualizing, modelling, and analyzing molecular and genetic interaction networks. Various plug-ins are integrated into Cytoscape with diverse functions, such as identifying hub genes in a PPI network [27]. In this study, three different plug-ins for Cytoscape have been used to identify hub genes in the PPI network. The plug-ins use centrality measures such as degree to assess the relative importance of proteins in PPI networks. The degree is the number of interacting partners of a protein [28]. In this study, the hub genes were ranked based on degree values. The genes with the highest degree values were identified as hub genes, which were TP53, STAT3, AKT1, JUN, and IL6.

AKT is a serine/threonine kinase, participating in the key role of the PI3K/AKT signaling pathway. Once activated, AKT modulates the function of many downstream proteins, ultimately resulting in increased proliferation and a loss of apoptosis signaling. AKT is the central node of the pathway, and it is frequently deregulated in many types of human cancer. Increased AKT kinase activity has been reported in several cancer types [29]. In this study, molecular docking results indicated a strong interaction between melatonin and AKT1. Also, KEGG enrichment analysis revealed that melatonin is significantly related to the PI3K/AKT pathway. Therefore, melatonin is suggested to deactivate AKT and reduce the activities of downstream proteins in the PI3K/AKT pathway, which eventually decrease cell proliferation in MTC.

RET proto-oncogene mutations are highly related to MTC. Although less common, RAS mutations can also lead to thyroid cancer [33]. However, in this study, RET and RAS were not found within the target proteins. Probably, melatonin does not directly interact with RET and RAS proteins. RAS is known to initiate several signaling pathways, such as the RAF-MEK-ERK/MAPK cascade, which facilitates the transmission of signals in a

downstream manner. Consequently, this signaling cascade leads to the transcription of genes responsible for regulating various cellular processes, including cell proliferation, differentiation, and survival [34]. The RAS pathway also activates PI3K/AKT and MAPK signaling cascades.

The activation of the PI3K pathway has been demonstrated to occur as a result of the deletion or downregulation of PTEN, a frequently mutated tumor suppressor gene in numerous kinds of human cancers [35] [36]. Constitutive RAS activation caused by a mutation in MTC further activates MAPK and PI3K/AKT signaling pathways, reprogramming tumour cells to result in uncontrolled growth [37] [38]. The KEGG enrichment analysis revealed that target proteins are significantly related to MAPK and PI3K/AKT signaling cascades. These results suggest the effects of melatonin on these pathways, which could result in changes in transcription and translation processes and aberrations of proliferation in MTC cells. This study argues that melatonin has significant efficacy in the therapy of MTC through modulating the MAPK and PI3K/AKT signaling pathways.

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway, which involves over fifty cytokines and growth factors, is considered one of the most important communication nodes in cell function. The JAK/STAT pathway influences cell division, proliferation, and metastasis. Multiple types of cancer are associated with the constitutive activation of STAT3 [30]. In this study, molecular docking results showed a strong interaction between melatonin and STAT3, which may deactivate STAT3 and lead to a decrease in cell proliferation.

Interleukin-6 (IL-6), a significant cytokine present in the microenvironment of tumors, is a critical element that is notoriously dysregulated in cancer and is present in high concentrations. There have been reports of its overexpression in virtually all types of tumors. [31]. A strong interaction between melatonin and IL-6 suggests deactivation of IL-6, which may lead to reduced MTC cell survival.

GO terms suggest that the target proteins are highly related to kinase activity, hormone activity, and receptor-ligand activities. GO-cellular component analysis revealed that target proteins are found in secretory vesicles and receptor complexes. It is evident that all of these findings are consistent with each other, indicating that melatonin affects signaling pathways and establishes multiple ligand-receptor relationships. GO and KEGG analyses suggest that the target genes are involved in apoptotic processes, which are significantly related to cancer development. Also, KEGG analyses revealed that a significantly high number of target genes of melatonin are involved in cancer pathways, with many targets being related to microRNAs or proteoglycans in cancer.

Melatonin is more than only a hormone; it also functions as a cell-protective agent. Melatonin has also been referred to as “nature’s most versatile biological signal” [39]. Multiple investigations have provided confirmation of the anticancer and oncostatic properties of melatonin, which are facilitated by many mechanisms of action. Melatonin has been identified as a promising option for the prevention and treatment of several types of cancer, including breast cancer, prostate cancer, gastric cancer, and colorectal cancer [40]. The antioxidant activity of melatonin has an essential role in maintaining DNA against the damaging effects of oxidative stress [41]. Melatonin has been reported to suppress autophagy in colorectal cancer cells [42]. Melatonin has also been reported to induce an anti breast cancer effect through the MAPK signaling cascade [43]. Furthermore, the protective effects of melatonin against ovarian cancer have been shown via the ROS-YTHDF2-MAPK-NF-κB pathway [44]. In addition, melatonin has been reported to induce apoptosis in gallbladder cancer cells by suppressing the PI3K/AKT/mTOR signaling pathway [45]. Melatonin has been shown to induce apoptosis in TC cells via increased expression of PTEN, which is the main negative regulator of the PI3K/AKT pathway [46]. The findings of this study align with the studies mentioned above, providing validation for the argument that melatonin enhances apoptosis in TC cells by suppressing the PI3K/AKT pathway. The present study demonstrated a substantial quantity of target proteins (64 proteins) associated with the PI3K/AKT pathway.

TP53 mutations have been reported to be involved in many cancers, including thyroid cancer [32]. This study shows a strong interaction between melatonin and TP53, which may lead to the apoptotic death of MTC cells. Although not commonly observed, p53 mutations may cause sporadic MTC [48]. Cells expressing mutant variants of p53 can fail to undergo cell cycle arrest or apoptosis in response to DNA damage [49]. GO annotations in this study revealed that several target genes are related to the regulation of apoptotic processes. Recently, melatonin has been reported to induce anti-apoptotic effects in mouse cells through the RORα/p53 pathway [50]. Melatonin, therefore, may activate p53 to trigger apoptotic processes in MTC cells.

The molecular docking analysis of melatonin and MTC yielded remarkable findings. The results of the study demonstrated that melatonin had high binding affinities towards four hub genes (p53, AKT1, STAT3, and IL6) while displaying a comparatively lower binding affinity towards JUN. The molecular docking outcomes indicated that melatonin had strong binding affinities to p53, AKT1, STAT3, and IL6 in low concentrations. A commonly recognized criterion for significance in binding free energy is a value below -6.0 kcal/mol. Melatonin had a strong affinity for AKT1, as evidenced by its low binding energy (-10.54 kcal/mol) and the lowest inhibition constant among the hub genes. Hydrogen bonding plays a crucial role in maintaining the stability of protein-ligand interactions, serving as an intermolecular force that facilitates the

interaction of two or more molecules [47]. The melatonin\AKT1 complex was found to interact with 3 H-bonds. The binding interaction of melatonin and AKT1 in low concentration suggests significant effects on the PI3K/AKT signaling pathway. This study suggests melatonin interacts with and reduces the activity of AKT1, which would lead to a decrease in MTC cell proliferation. Therefore, melatonin could be used as a therapeutic agent against MTC via AKT1 inhibitory properties.

This study evaluates the possible therapeutic effects of melatonin on MTC via network pharmacology and molecular docking studies. Further studies are necessary to overcome these limitations, such as conducting molecular dynamic simulations between melatonin and the hub genes, especially AKT1, p53, STAT3, and IL-6.

5. Conclusions

This study proposes that melatonin plays a significant role in the regulation of the MAPK by *in vitro* analyses. This study speculates that melatonin has inhibitory effects on AKT1, STAT3, and IL-6 while having an activatory effect on p53. However, *in vivo* studies are urgently needed. Also, the melatonin levels of MTC patients and the expression levels of the aforementioned genes should be investigated in clinical studies to better understand the importance of melatonin against MTC. Furthermore, more studies may be conducted to investigate the potential protective effects of melatonin pills against MTC and PI3K/AKT signaling pathways in MTC cells. The administration of melatonin has the potential to trigger apoptosis in MTC cells. Strong binding affinities of melatonin with p53, STAT3, IL6, and AKT1 should be studied.

Abbreviations

BP: Biological processes; CC: Cellular components; GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; MF: Molecular functions; MTC: Medullary thyroid carcinoma; MTC: Thyroid carcinoma; PDB: Protein Data Bank; PPI: Protein-protein interaction; TC: Thyroid cancer.

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