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Evidence of chelonid herpesvirus 5 infection in green turtle (*Chelonia mydas*) indicated a possible tumorigenesis activation by transcriptome analysis

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Chelonia mydas (green turtles) are being threatened worldwide by fibropapillomatosis (FP), which has seriously affected their survival. The presence of FP on the body surface and visceral organs of green turtles found dead was confirmed, causing obstruction of the gastrointestinal tract, changes in foraging behavior, and reduction of visceral functions. The etiology of FP has not yet been elucidated, and previous research generally considers that the occurrence of FP is related to the chelonid alphaherpesvirus 5 (ChHV5), associated with low animal immunity, and also with marine environmental factors, such as poor water quality and eutrophication. However, there is no evaluation on the induction of FP pathogenesis associated with the green turtle. In this study, we evaluated blood samples from green turtles with and without FP using *de novo* transcriptome assembly. Results indicated that 3,090 differentially expressed genes (DEGs) ($p < 0.05$) were identified, including 1,357 upregulated genes and 1,733 downregulated genes in turtles with or without FP. We observed that DEGs, which are significantly upregulated, are found in cancer development, namely, *MAPK1IP1L* and *APAF1*. Furthermore, the infected green turtle indicated that the greater number of DEGs was contributed by the NOD-like receptor signaling pathway, which can be activated through an endocytosis of the viral particle by the immune system cells, and the Wnt signaling pathway, which is believed to have played a role in FP tumorigenesis. We validated the more upregulated/downregulated DEGs in cancer development and immunization, and DEGs such as *LEF1*, *BTRC*, and *FOSL1* participating in the NOD-like receptor signaling pathway, as well as *ERBIN*, *TRAF6*, and *NFKB1* in the Wnt signaling pathway, using real-time quantitative polymerase chain reaction (RT-qPCR). Altogether, this study provided some genes as potential markers during FP infection and a further evidence of FP in endangered green turtles in Taiwan.

KEYWORDS

marine turtle, fibropapillomatosis, *de novo* transcriptome assembly, differentially expressed genes, NOD-like receptor signaling pathway, pathogenesis

1 Introduction

There are a total of seven species of sea turtles in the world, five of which have been found in the sea off the coast of Taiwan, namely, green turtles (*Chelonia mydas*), hawksbill turtles (*Eretmochelys imbricate*), olive ridley turtles (*Lepidochelys olivacea*), loggerhead turtles (*Caretta caretta*), and leatherback turtles (*Dermochelys coriacea*) (Cheng and Chen, 1997; Kuo et al., 2017). The green turtle is the most common species and the only one that nests in Taiwan (Cheng, 2000; Cheng et al., 2018). In fact, the green turtle has been listed as a globally endangered species and is listed in the Red List of the IUCN (International Union for Conservation of Nature; IUCN, 2016). Furthermore, anthropogenic impacts, including artificial coastal development, fisheries by-catch, global environmental change, illegal egg harvesting, marine debris, environmental pollution, and diseases, pose a major threat to green turtle populations all over the world (Aguirre and Lutz, 2004; Hamann et al., 2010; Shigenaka, 2010; Jones et al., 2016; Li et al., 2017; Perrault et al., 2017; Godoy and Stockin, 2018; Ng et al., 2018; Li and Chang, 2020). Among the different threats to turtles, as a consequence of the aforementioned circumstances, the recent study focused on threats from the diseases of marine turtles, which is a priority research area for sea turtle's conservation (Hamann et al., 2010; Rees et al., 2016). Fibropapillomatosis (FP) is a tumor-forming disease that globally affects marine turtles (Herbst, 1994; Alfaro-Nunez et al., 2014). However, the reports of this disease in Asia are still very limited (Hargrove et al., 2015; Li et al., 2017; Li and Chang, 2020; Dujon et al., 2021; Li et al., 2022). This disease was first discovered in Florida in 1938 (Smith and Coates, 1938); there are still many cases until the present day and the geographic distribution of the disease was moving northward from the equatorial region (Duffy et al., 2018). Together with multiple anthropogenic pressures impacting sea turtles such as ingestion of marine litter and interaction with fishing gear (Dimitriadis et al., 2022; Jo et al., 2022; Rodríguez et al., 2022; Mghili et al., 2023), FP is one of the main causes of stranding in sea turtles (Foley et al., 2005; Chaloupka et al., 2008; Da Silva-Júnior et al., 2019). FP could occur in all these seven species of sea turtles, but it is more common in green turtles (Jones et al., 2016; Dujon et al., 2021). FP cases were more likely observed in immature turtles and less common in adults (Jones et al., 2016; Lawrance et al., 2018). In sea turtles with FP, tumors can appear on the body surface (such as the eyes, oral cavity, skin, and carapaces) or in internal organs such as lungs and kidney (Herbst, 1994; Aguirre and Lutz, 2004; Rossi et al., 2021), preventing foraging, reducing sea turtle mobility and visceral function of sea turtles, and causing death from infirmity (Work et al., 2001).

A higher prevalence of FP has been reported in highly contaminated marine environments or environments with poor water quality (Formia et al., 2007; Van Houtan et al., 2010; Guimarães et al., 2013; Van Houtan et al., 2014; Baines et al., 2021; Manes et al., 2022). Furthermore, a recent global meta-analysis study further indicated that a high prevalence of sea turtle FP is thought to be associated with exposure to toxic phytoplankton bloom events (Dujon et al., 2021). In contrast, a relationship between sea turtle FP prevalence and water quality indices rankings at each site along the Queensland coastline could not be quantified (Jones et al., 2022). A study conducted in Taiwan on FP in green turtles described that all green turtles with FP were discovered in eastern Taiwan (Li and Chang, 2020). However, the study by Liu et al. (2015) indicated that the highly problematic region of coastal eutrophication is located in southwestern Taiwan. Hence, the exact etiology of FP is not well understood. Thus, Li et al. (2017) also concluded that for the conservation of endangered sea turtles, more studies of this nature are recommended for Asian marine turtle populations to improve our understanding of the infection. Marine environmental factors or infectious agents, among which Chelonid herpesvirus 5 (ChHV5), have been hypothesized to be associated with sea turtle FP (Jones et al., 2016; Duffy et al., 2018). A strong association between sea turtle FP and the presence of ChHV5 have been reported. For example, ChHV5 DNA has been distinguished molecularly from diseased tissues of sea turtles with FP (Lackovich et al., 1999; Page-Karjian et al., 2012; Alfaro- Nunez and Gilbert, 2014; Li et al., 2017). However, sea turtles have been diagnosed with the occurrence of FP, and ChHV5 nucleic acid can also be detected in tumor-free (asymptomatic) sea turtles (Page-Karjian et al., 2020a; Loganathan et al., 2021; Li et al., 2022). Nevertheless, the mode of transmission route of ChHV5 among wild sea turtles is still unknown (Dujon et al., 2021). *Ozobranchus* spp. (marine leeches) were commonly found in sea turtles with FP, and the large amount of ChHV5 DNA was detected in *Ozobranchus* leech parasite in green turtles that died from FP (Greenblatt et al., 2004) while leeches removed from healthy sea turtles did not test positive for ChHV5 (Farrell et al., 2021). Therefore, the *Ozobranchus* spp. was suggested to be a possible vector of ChHV5 transmitting between sea turtles (Greenblatt et al., 2004). Nowadays, several studies suggested that FP in sea turtles is mainly caused by the interaction of ChHV5 and environmental factors such as water quality and autoimmunity (Aguirre et al., 1995; Alfaro-Nunez et al., 2016; Bruno et al., 2021; James et al., 2021; Sposato et al., 2021; Nash & Ryan, 2023). Sea turtles with FP were shown to have immunosuppression and reduced activity of natural killer cells (Work et al., 2001; Li and Chang, 2020; Perrault et al., 2021). Hence, some concern further exists regarding FP in sea turtle rehabilitation facilities (Page-Karjian et al., 2014; Li and Chang, 2020; Page-Karjian et al., 2020b). Jones et al. (2016) also concluded that molecular investigations on ChHV5 from different areas are critical to improve our understanding of the epidemiology and pathogenesis of this virus. As a consequence of the aforementioned circumstances, it is extremely important and timely to clarify the pathogenesis of this viral disease.

Previous studies have shown that the prevalence of FP is higher in juvenile green turtles than in other size/age groups (Jones et al., 2016; Perrault et al., 2021; Manes et al., 2023). So far, there is evidence that green turtles develop FP only after recruitment to nearshore habitats (Jones et al., 2016). This is supported by the fact that juvenile green turtles, the most vulnerable individuals to FP, are

coastal foragers living in coastal areas (Work et al., 2005; Hazel et al., 2009) that have undergone significant anthropogenic change in recent decades (Dujon et al., 2021; Jones et al., 2022; Manes et al., 2023). In fact, in the same feeding ground, there were more than one sea turtle at the same time, and ChHV5 could be transmitted horizontally through seawater (Farrell et al., 2021) to other sea turtles in the same feeding area. Hence, the migratory habit of sea turtles may also play an important role in distributing ChHV5 worldwide (Jones et al., 2016).

Few studies from European and American regions (Duffy et al., 2018; Blackburn et al., 2021; Yetsko et al., 2021) used *C. mydas* skin samples to perform transcriptome profiling and discovered that there were similar signaling pathways between green sea turtles FP and human basal cell carcinoma (BCC). Currently, there is no research on transcriptome analysis of FP in *C. mydas* in the Asian context; the results of this study may provide additional information of this disease. In the present study, blood samples of FP-afflicted and clinically healthy *C. mydas* were used to evaluate immune-related gene expression and tumor-related gene expression using transcriptome analysis. This study aims to evaluate and understand the possible pathogenesis of the formation of FP with the gene expression results of transcriptome analysis. Detection of risk genes may help early identification of FP in green turtles, improve the management of infected sea turtles, and further develop effective treatment method against green turtle FP in the future.

2 Materials and methods

2.1 Blood sample collection from green sea turtles

C. mydas, including eight green sea turtles with FP tumors (n = 1 with tumor score 1; n = 1 with tumor score 2; n = 6 with tumor score 3) was used in this study (Work and Balazs, 1999). FP tumors were detected through visual inspection of external masses by veterinarians (Figure 1). The green turtles analyzed showed a size range of 29.4–86.5 cm of curved carapace length (Figure 1).

FIGURE 1
Green turtle (*Chelonia mydas*) with fibropapillomatosis (FP).

2.2 Ethics

All individuals in this study were discovered and rescued through the official reporting system of the Marine Animal Rescue Network (established by the Ocean Conservation Administration) and transported to the National Museum of Marine Biology and Aquarium (NMMBA) for further rehabilitation/medical care. Therefore, blood samples were necessarily collected under humane procedures from an external jugular vein (Dutton, 1996; Day et al., 2010; Li et al., 2015) by our licensed veterinarians (Dr. Tsung-Hsien Li or Dr. I-Chun Chen) for routine medical care. Hence, no individuals of green turtles were specifically captured from the wild for the purpose of this study. As a consequence of the aforementioned circumstances, the excess of blood was obtained and utilized. The scientific permit numbers for sample use were obtained from the Ocean Conservation Administration (Permit nos. 1090009887 and 1100012644).

2.3 Detection of ChHV5 DNA by PCR

Total DNA was extracted from whole blood samples of 24 green turtles using the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The chelonid alphaherpesvirus 5 (ChHV5) infection status of all 24 green turtles was determined by polymerase chain reaction (PCR) using three genes associated with ChHV5. Three independent primers sets were designed and targeted on UL18 (capsid protein gene), UL22 (glycoprotein H gene), and UL27 (glycoprotein B gene) regions of the ChHV5 (Alfaro-Nunez and Gilbert, 2014). PCR amplifications were prepared in a total reaction volume of 50 µl. The reactions included 1 µl of the template DNA, 1 µl of each primer (10 µM), 22 µl of distilled water (DDW), and 25 µl of enzyme of AmpliTaq Gold® 360 Master Mix (Life Technologies, Valencia, CA, USA). The reaction conditions were as follows: Initial denaturing at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 60 s, and a final extension step of 72°C for 7 min. Amplified PCR products were then visualized by gel electrophoresis (2% agarose) with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA).

2.4 Isolation of total RNA and cDNA synthesis from blood

Total RNA was extracted using TRIzol® LS Reagent according to the manufacturer's protocol. RNA samples were subjected to removal of DNA contamination using DNase I (BioLab, London, UK) and 5 µg of total RNA was used for cDNA synthesis using the iScript® cDNA Synthesis Kit (Bio-Rad, California, USA) according to the manufacturer's instructions.

2.5 *De novo* transcriptome assembly and annotation

Total RNA samples were sent to sequencing company Genomics Bioscience Technology Co Ltd. (Taipei, Taiwan). Total RNA was used for library construction using the TrueSeq library construction kit. The library was sequenced on the Illumina HiSeq™4000 (Illumina, Inc., San Diego, USA) platform. After that, "Trimmomatic" was used for read-quality filtering, and low-quality bases and adaptors were removed from Illumina sequences. Trinity (Illumina, Inc) was used to assemble the RNA sequence data, and *de novo* assembly was later performed (Haas et al., 2013; Bolger et al., 2014). The data after *de novo* transcriptome assembly was used for annotation (Supek et al., 2011). Owing to the lack of detailed genetic data on green sea turtles, the human genetic database was chosen for alignment. Aligning human genetic sequence to assembly result evaluated the gene expression according to the number of sequences aligned. Differential expression calculated by the software "edgeR v3.5" and functional annotation of Gene Ontology (GO) term (Kanehisa and Goto, 2000; Kanehisa, 2019) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment (Mi et al., 2018) were also conducted. Differentially expressed genes (DEGs) were filtered with the parameter false discovery rate (FDR), in which the *p*-adjusted value (p_{adj}) should be smaller than 0.05.

2.6 Real-time quantitative polymerase chain reaction

DEGs with a large difference in expression between tumor and healthy green sea turtles were selected. Upregulated genes related to cancer-forming and other DEGs participate in the Wnt signaling pathway, the NOD-like receptor signaling pathway was selected, and *β-actin* was used as control. Primer sequences were designed using Pimer3 Input (Reference or weblink) and the NCBI primer designing tool. The details of the primers tested, including *MPAK1IP1L*, *TBRG4*, *NFIA*, *APAF1*, *ZBTB41*, and *LATS2*, are mentioned in Table 1. Real-time quantitative polymerase chain reaction (RT- qPCR) was performed using iQ™ SYBR® Green Supermix (Bio-Rad, California, USA), and each sample was performed in quadruplicate. Cycling parameters are as follows: 95°C for 2 min, 45 cycles of 95°C for 10 s, 60°C for 20 s, 72°C for 20 s, and a melting curve (Duffy et al., 2018). The fold expressions of target genes were calculated using the $2^{\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

3 Results

From the PCR result for the detection of ChHV5 DNA in blood samples (Table 2), eight green sea turtles involved in this study afflicted with FP were all positive for ChHV5 (viremia); the positive rate was 100.0% (8/8). For those who were clinically healthy (without FP), the positive rate of viral infection was 0.0% (0/16).

3.1 Differentially expressed genes

Four blood samples, two from green turtles with FP and two from green turtles without FP, were included in transcriptome analysis. In total, 3,090 DEGs (DEGs were calculated using edgeR v3.22.5.; $p < 0.05$) (Robinson et al., 2010; Britt et al., 2020), 1,357 upregulated genes and 1,733 downregulated genes, were identified (Figure 2). In reference to human orthologs, comparing gene expression between two sample groups, the 10 most expressed differential genes of up- and downregulation were shown (Table 3). Upregulated genes related to tumor formation and immunity were selected and quantified using RT-qPCR.

From the annotation result, functions of DEGs mainly included cancer formation and immunity. Among the top 10 upregulated genes, with higher performance in blood samples of green sea turtles with FP, more than half of the DEGs took part in tumorigenesis or associated with some other type of cancer, namely, *MAPK1IP1L*, *NFIA*, *APAF1*, *PDZD11*, *ZBTB41*, and *LATS2*. Other than promoting tumor growth, *PDZD11* has also a correlation with immune cell infiltrating levels. For downregulated genes, with a higher performance in blood samples of clinically healthy green sea turtles, the top three have a connection with the development of cancer, namely, *EIF3A*, *STAB2*, and *HIPK3*.

3.2 Gene Ontology annotation

Dot plots of significant GO ontology enriched among DEGs in three domains—cellular component (Figure 3A), molecular function (Figure 3B), and biological process (Figure 3C)—were conducted.

In the cellular component, the most enriched ontology was the cell leading edge. In molecular function, the most enriched ontology was transcription coregulator activity. Lastly, in the domain of biological process, covalent chromatin modification and histone modification were the first and second enriched ontology in green sea turtles with tumor (Figure 3C).

3.3 KEGG enrichment

KEGG pathway was conducted, and as shown in Figure 4, the top 20 KEGG pathway permutations according to their gene ratio took part in the specific pathways in green sea turtles with tumors. The NOD-like receptor signaling pathway was the most enriched pathway. The second is Epstein–Barr infection and endocytosis in the third.

In the NOD-like receptor signaling pathway, genes *NFKB1* and *TRAF6* have the highest upregulation whereas the gene *ERBIN* has the highest expression in downregulation (Figure 5). In the Wnt signaling pathway, genes *BTRC* and *FOSL1* have the highest expression in upregulation, whereas *LEF1* has the highest expression in downregulation (Figure 6).

TABLE 1 Primer sequences used in this study for performing qPCR reaction.

Name	Sequence	Product size	Reference
β-actin F	TGGTA CAGTC TCCCA TTCCA	232 bp	Duffy et al., 2018
β-actin R	AGGCA TACAG GGACA ACACA		
MAPK1IP1L F	TCCAG AGCTT CCAAG ACCAT	118 bp	In this study
MAPK1IP1L R	ATACT GCCCT CCCAT TGTTG		
TBRG4 F	TGTTA GCACC AGCAT CCAAG	205 bp	
TBRG4 R	TGTTC ACCCA CGTTC AAGAG		
NFIA F	CATGC AACAC CATCG ACTCT	228 bp	
NFIA R	CAACA TTGGG GTAGG AAGGA		
APAF1 F	ATCTG ACTGA AGCCC AGGAA	241 bp	
APAF1 R	TCTGA CTGCC AATTC CACAC		
ZBTB41 F	ACTCT CTCAC CCCAG TCTCA	202 bp	
ZBTB41 R	TTGGA GCTGT ATCAA GCCCT		
LATS2 F	TTAAC AACCA CCAGC AGCAG	137 bp	
LATS2 R	CATGC TCTTC ACTGG TTGGA		
ERBIN F	TCAAA GGATG GGTGG AAGAC	237 bp	
ERBIN R	CTCGG TTGCC CAGAC ATTAT		
TRAF6 F	TGGGA AACCA GGCTA CAAAC	195 bp	
TRAF6 R	TCGTG ATTCT GCCTT ACGTG		
NFKB1 F	ATGCA GGACC GAAGG ATATG	98 bp	
NFKB1 R	TGTCA TTCGT GCTTC CAGAG		
LEF1 F	AGAGC CGAAA AGACC TCACA	221 bp	
LEF1 R	AGCCT GGGTA AAGCT GCATA		
BTRC F	AAAGG AAGCT GTCTG CAAGC	243 bp	
BTRC R	ATTTG GCATC CAGGT ACGAC		
FOSL1 F	AGCTA ACGGA CTGTC TGCAA	105 bp	
FOSL1 R	GCTCA AACCT CTCCT TCTGC T		

The table includes name of the primer, sequence, and product size.

TABLE 2 Morphometric measurements and ChHV5 testing data for green turtle (*Chelonia mydas*).

Turtle number	CCL(cm)[a]	FP disease status	FP tumor score[b]	Viremia status[c]
1	66.0	With FP	3	Positive
2	53.3	With FP	2	Positive
3	58.1	With FP	1	Positive
4	54.9	With FP	3	Positive
5	61.0	With FP	3	Positive
6	45.6	With FP	3	Positive
7	45.5	Without FP	0	Negative
8	37.0	Without FP	0	Negative

(Continued)

TABLE 2 Continued

Turtle number	CCL(cm)[a]	FP disease status	FP tumor score[b]	Viremia status[c]
9	53.5	With FP	3	Positive
10	44.7	Without FP	0	Negative
11	86.5	Without FP	0	Negative
12	43.1	Without FP	0	Negative
13	68.3	With FP	3	Positive
14	48.2	Without FP	0	Negative
15	61.7	Without FP	0	Negative
16	43.2	Without FP	0	Negative
17	40.9	Without FP	0	Negative
18	66.5	Without FP	0	Negative
19	48.6	Without FP	0	Negative
20	29.4	Without FP	0	Negative
21	54.2	Without FP	0	Negative
22	52.3	Without FP	0	Negative
23	45.3	Without FP	0	Negative
24	59.1	Without FP	0	Negative

[a]Curved carapace length (CCL).
[b]The green sea turtle tumor score system (Work and Balazs, 1999). 0, non-afflicted; 1, lightly afflicted; 2, moderately afflicted; 3, heavily afflicted.
[c]Determined by PCR using specific primer sets (UL18, UL22, or UL27). Any of the results that showed positive were considered as PCR-positive ones and with viremia status.

3.4 Real-time quantitative polymerase chain reaction

Expressions of genes selected from upregulated DEGs, NOD-like receptor signaling pathway, and Wnt signaling pathway were validated through RT-qPCR. Because of the small sample size, non-parametric statistical tests (Mann–Whitney *U* test) (Mansfield et al., 2017; Tanabe et al., 2022; Fumero-Hernández et al., 2023) were used in this study. There were no major significant findings (using Mann–Whitney *U* test) for the comparison of gene expression profiles between green turtles with and without FP. As shown in Figure 7, the quantification of *ERBIN, MAPK1P1IL, APAF1*, and *LATS2* in green turtles with FP was much higher than in green turtles without FP, though not showing statistical significance. The

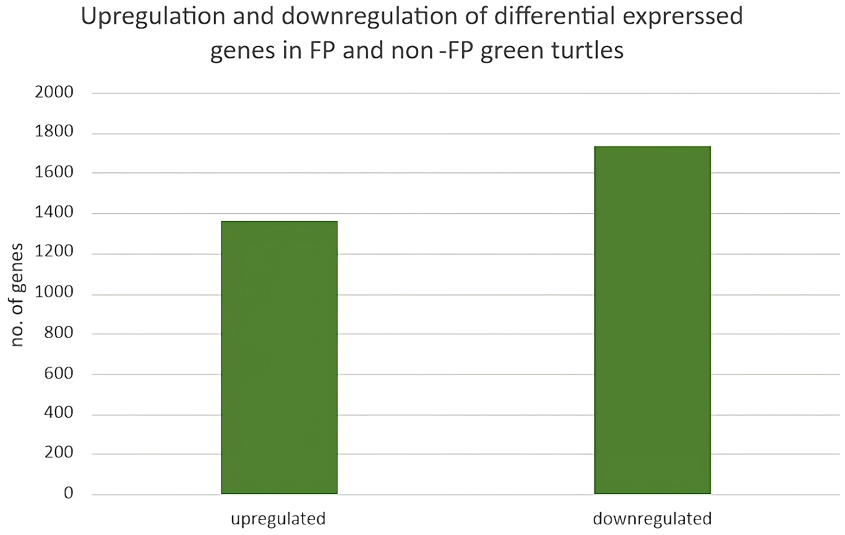

FIGURE 2
Upregulation and downregulation of differentially expressed genes in FP and non-FP green turtles.

TABLE 3 Top 10 most up- and downregulated differentially expressed genes of clinically healthy green sea turtles and those with tumors.

Current turtle gene symbol	Human gene symbol	log_2 FC	FDR (p_{adj})
Upregulation[a]			
MAPK1IPIL	MAPK1IPIL	12.70289	2.54E−14
TBRG4	TBRG4	12.43844	1.22E−13
NFIA	NFIA	12.42088	1.22E−13
APAF1	APAF1	12.31789	2.03E−13
LOC102939502	GIMAP7	11.99484	1.55E−12
PDZD11	PDZD11	11.96182	1.68E−12
SNX17	SNX17	11.89342	2.33E−12
ZBTB41	ZBTB41	11.88704	2.33E−12
LATS2	LATS2	11.77053	4.44E−12
EIF3L	EIF3L	11.74958	4.85E−12
Downregulation[b]			
EIF3A	EIF3A	−13.4631	3.25E−16
STAB2	STAB2	−13.1358	2.13E−15
HIPK3	HIPK3	−13.0961	2.13E−15
ESD	ESD	−12.4236	1.22E−13
ATP2B4	ATP2B4	−11.7919	4.18E−12
TANGO2	TANGO2	−11.7794	4.35E−12
LOC102931868	RNF213	−11.6788	7.55E−12
RBM23	RBM23	−11.6653	7.78E−12
BTBD9	BTBD9	−11.4537	2.80E−11
NCOA7	NCOA7	−11.4299	3.00E−11

[a]FDR < 0.05, log_2 FC ≥ 0;
[b]FDR < 0.05, log_2 FC ≤ 0.

quantification of *ZBTB41* and *NFKB1* was slightly higher in green turtles with FP when compared with green turtles without FP. The results of the other two genes, *BTRC* and *NFIA*, were similar between turtles with and without FP.

The relative quantifications of DEGs in the NOD-like receptor signaling pathway as well as the Wnt signaling pathway are shown in Figure 7. Relative quantification of *TRAF6* and *LEF1* was much higher in clinically healthy green turtles. The quantification of

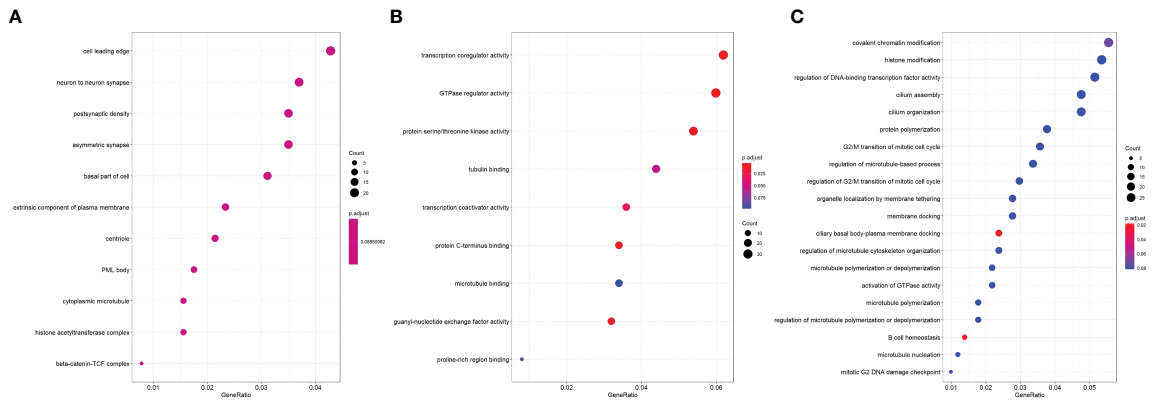

FIGURE 3
Dot plot of significant GO ontology enriched among DEGs. **(A)** Cellular component; **(B)** molecular function; **(C)** biological process.

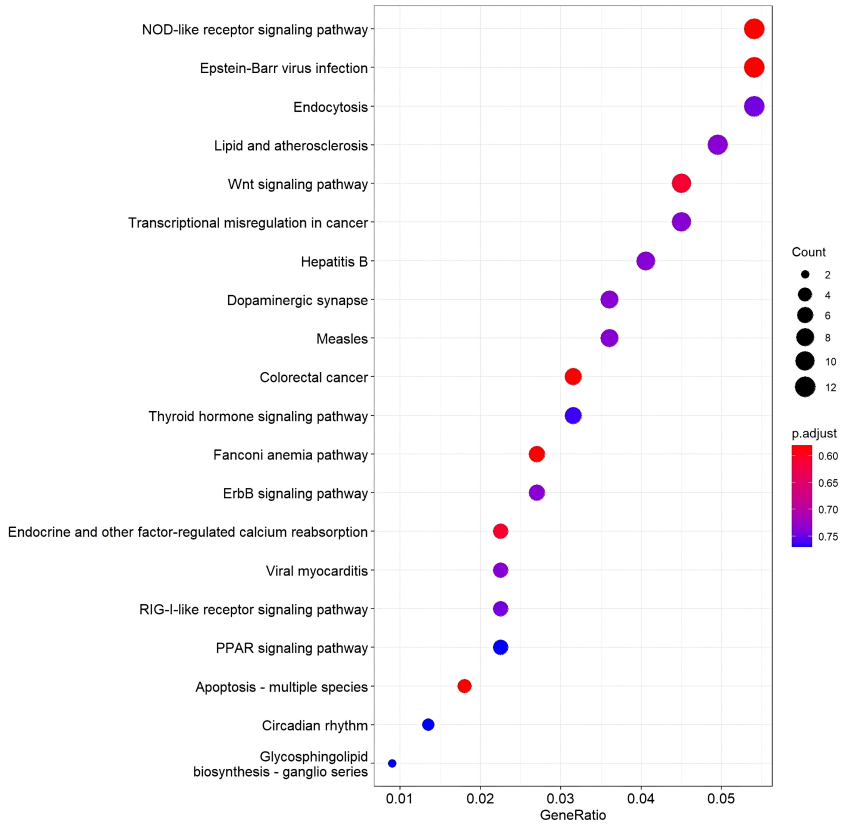

FIGURE 4
A dot plot of top 20 KEGG pathway permutations according to gene ratio. Dot size shows the number of DEG, and the color shows the adjusted *p*-value of the gene set enrichment.

TBRG4 was slightly higher in green turtles without those afflicted with FP.

4 Discussion

Green turtles involved in this study were detected with ChHV5 using PCR (Table 2); the positive rate of green turtles afflicted with FP and green turtles without FP was 100.0% (8/8) and 0.0% (0/16), respectively. Several studies have previously reported the low ChHV5 detection rate in green turtles without external FP, including wild, rehabilitating, and nesting green turtles (Page-Karjian et al., 2020a; Page-Karjian et al., 2020b; James, 2020; Perrault et al., 2021). Among 24 green sea turtles, 8 developed FP: 12.5% (1/8) was lightly afflicted, 12.5% (1/8) was moderately afflicted, and 75% (6/8) was heavily afflicted. Sixteen of them were not afflicted with FP.

In this study, blood samples of green turtles with and without FP were evaluated using *de novo* transcriptome assembly. From the annotation result, functions of DEGs mainly included cancer formation and immunity. The most enriched KEGG pathway was the NOD-like receptor signaling pathway. The Wnt signaling pathway, which ranked fifth, indicates a potential role in *C. mydas* while regulating FP (Duffy et al., 2018; Blackburn et al., 2021; Yetsko et al., 2021). However, there were no statistically significant results in KEGG enrichment. The DEGs in the NOD-like receptor signaling pathway and Wnt signaling pathway are shown in Figures 5 and 6; genes *NFKB1* and *TRAF6* have the highest upregulation whereas gene *ERBIN* has the highest expression in downregulation in the NOD-like receptor signaling pathway. Genes *BTRC* and *FOSL1* have the highest expression in upregulation whereas *LEF1* has the highest expression in downregulation in the Wnt signaling pathway. The ability of tumorigenesis or expression as a tumor suppressor gene which may have, the presence of *LEF1* indicating clinical value to become a biomarker of FP (Santiago et al., 2017).

In the DEGs' annotation result, over half of the upregulated DEGs took part in tumorigenesis or were associated with some other type of cancer. The first three most downregulated genes have a connection or are associated with the development of cancer. *MAPK1IP1L*, encoded for mitogen-activated protein kinase 1 interacting protein 1 like the most expressed genes in green sea turtles with FP, has the ability to develop as an auxiliary diagnostic tool for lung cancer detection in human as it significantly overexpressed in the lung cancer group (Zhang et al., 2018). In our qPCR result, this gene has a significant increase in green turtles with FP, which we believe is worthy of further investigation. *APAF1* encoded apoptotic peptidase activating factor 1, which appeared to

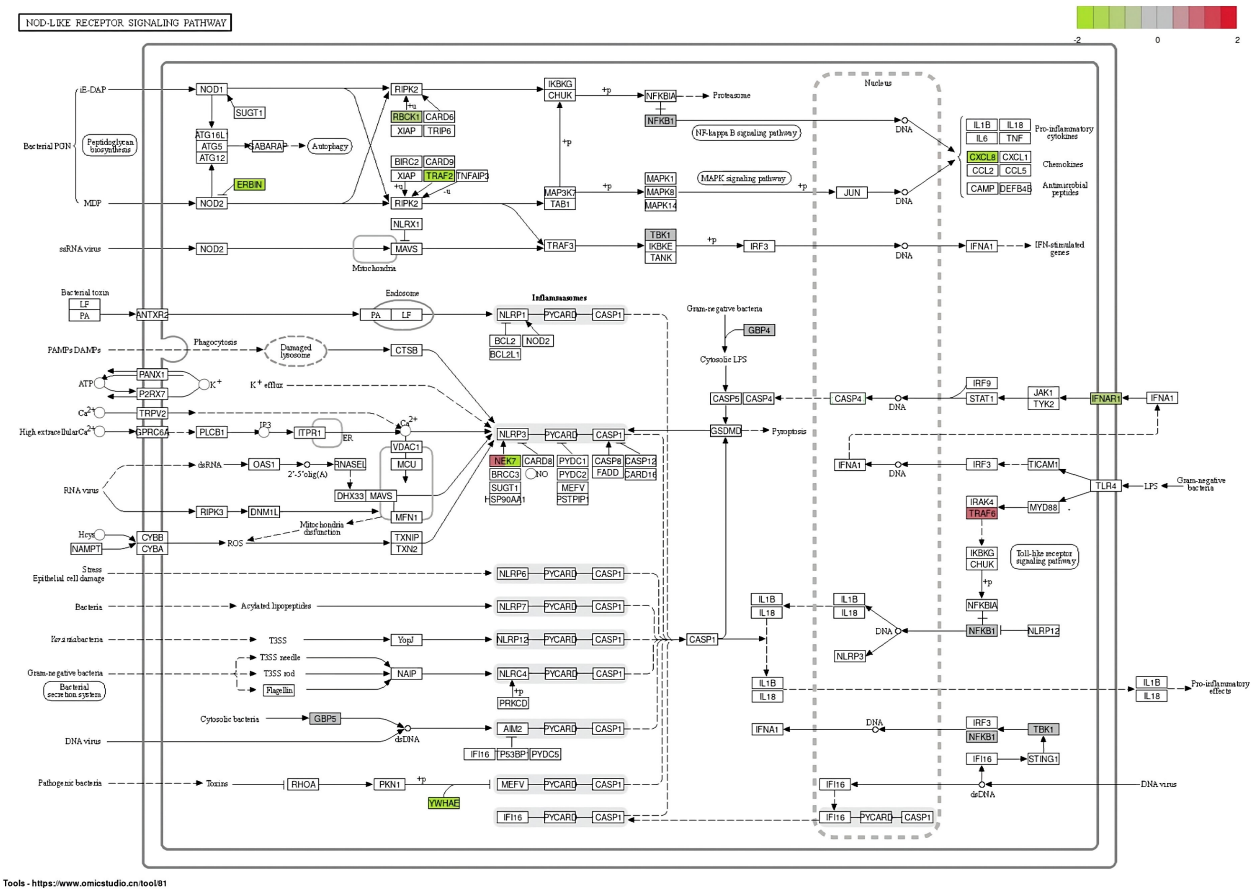

FIGURE 5
Differentially expressed genes in the NOD-like receptor signaling pathway.

be a tumor-suppressor gene, and research reported that the expression of *APAF1* has an inverse correlation with the progression of tumor (Zlobec et al., 2007); however, we have a quite different result in our qPCR quantification: the expression of APAF1 was higher in green sea turtles with FP. *LATS2* encoded for large tumor suppressor kinase 2, similar to *APAF1*, and it played a role in tumor suppression by inhibiting cell proliferation (Li et al., 2003); at the same time, it has the same quantification result as *APAF1*. We believed that it has a connection with the health status or tumor progression of green sea turtles and further research was needed.

Relative quantification of DEGs annotated in the NOD-like receptor signaling pathway and Wnt signaling pathway was evaluated using RT-qPCR. Expression of *ERBIN* was higher in green sea turtles with FP; this result did not agree with the annotation result in which *ERBIN* has a higher expression in clinically healthy green sea turtles. *ERBIN* was the most downregulated DEG in the NOD-like signaling pathway; loss of ERBIN expression increases cell migration and invasion, which is an early event in tumorigenesis (Stevens et al., 2018). *TRAF6* has a higher expression, and this result did not agree with the annotation result in which *TRAF6* has a higher expression in green sea turtles with FP. Overexpression of *TRAF6* may result in NF-κB activation in human lung cancer and promoted proliferation of colon cancer cells (Starczynowski et al., 2011; Sun et al., 2014). NF-κB was a pathway that regulated cancer cell proliferation, survival, and angiogenesis, and it also regulated immune responses and inflammation (Dolcet et al., 2005; Tilborghs et al., 2017; Soleimani et al., 2020); the expression of NF-κB reflected the potential risk of developing malignant tumors (Xia et al., 2018). Another activated NF-κB gene is *TRAF6*, which is also involved in angiogenesis and one of the key elements of tumorigenesis. *TRAF6* was suggested to serve as a therapeutic target suppressing cancer formation for cancer patients (Sun et al., 2014).

Relative quantification of *LEF1* in the Wnt signaling pathway was the highest in green sea turtles without FP. This agreed with the annotation where *LEF1* is expressed in downregulated DEGs, meaning it was more significantly expressed in clinically healthy green turtles. Santiago et al. (2017) stated that the presence of *LEF1* may have clinical value to be a potential biomarker of FP.

Understanding the cause of FP in endangered sea turtles is crucial for sea turtle conservation works. Sea turtles at rehabilitation facilities provide us with an opportunity to advance our knowledge and understanding of FP from wild green turtles. Moreover, with

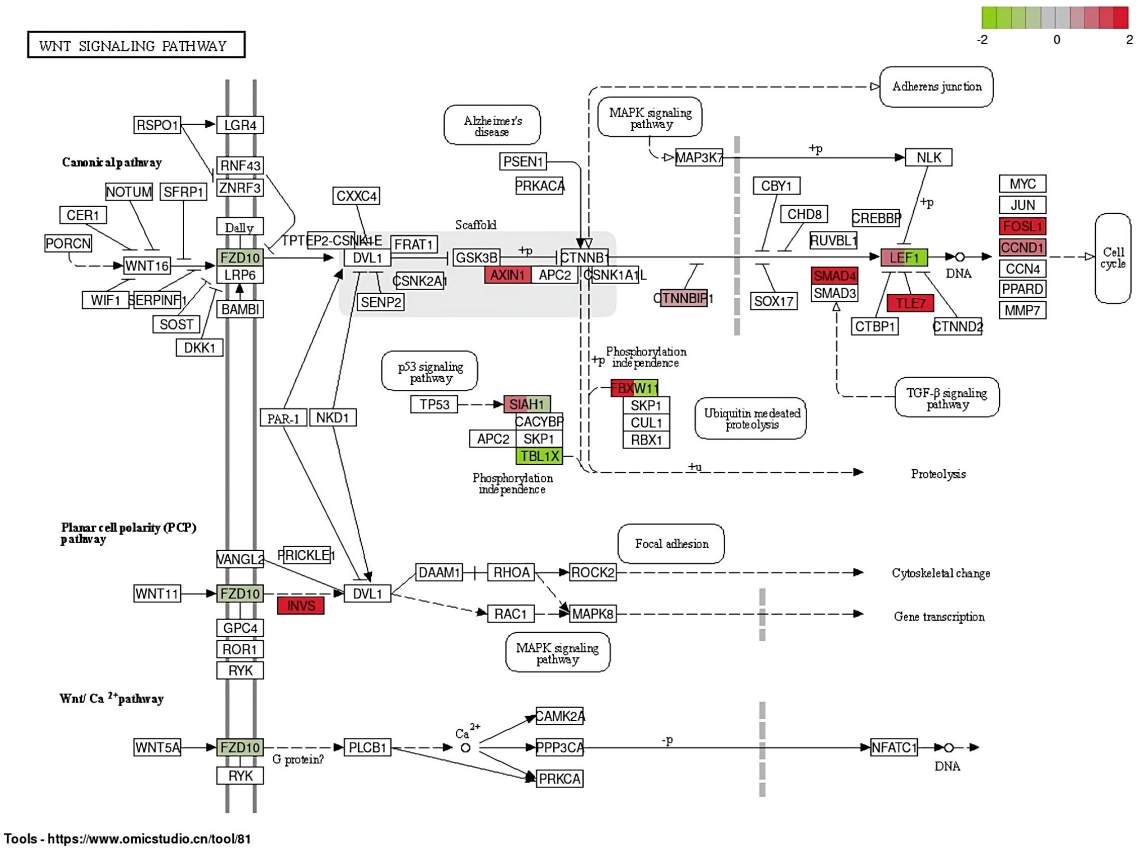

FIGURE 6
Differentially expressed genes in the Wnt signaling pathway.

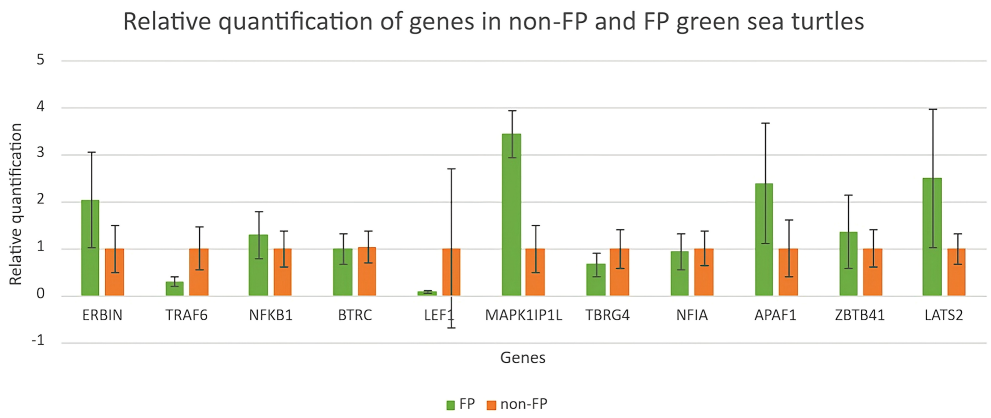

FIGURE 7
Relative quantification of genes in green turtles with and without FP. Data indicate mean ± SD from n = 8 (FP) and n = 16 (non-FP).

the rise in FP in recent years within the Taiwan waters, and based on the findings, green turtles with FP can be identified and thus can be rehabilitated before releasing them back to the wild. In this study, we have listed some of the risk genes that may participate in the occurrence and established possible pathogenesis of FP in green turtles according to the transcriptome analysis result. In conclusion, most of the DEGs annotated participated in the NOD-like receptor and Wnt signaling pathway. Genes expressed in these two pathways may cause either tumor formation (tumorigenesis) or suppression. Among these genes, the presence of LEF1 may have a clinical value

as a biomarker of FP (Santiago et al., 2017). Additionally, studies of FP are recommended for marine turtle populations in Asia to improve our understanding of the infection.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

Ethical review and approval was not required for the animal study because All individuals in this study were discovered and rescued through the official reporting system of the Marine Animal Rescue Network (established by the Ocean Conservation Administration) and transported to the National Museum of Marine Biology and Aquarium (NMMBA) for further rehabilitation/medical care. Therefore, blood samples were necessarily collected by our licensed veterinarians (Dr. Tsung-Hsien Li or Dr. I-Chun Chen) for routine medical care. Hence, no individuals of green turtles were specifically captured from wild for the purpose of this study. As a consequence of the aforementioned circumstances, the excess of the blood was obtained and utilized from blood samples taken for routine medical care by our veterinarians. The scientific permit numbers for sample use were obtained from the Ocean Conservation Administration (Permit no.1090009887 and 1100012644).

Author contributions

M-AT and T-HL conceived the ideas and designed the methodology. T-HL and I-CC collected the data. I-IL, OB, and M-AT analyzed the data. M-AT, T-HL, and I-IL led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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