

# Dopachrome tautomerase is a retinoblastoma-specific gene, and its proximal promoter is preferentially active in human retinoblastoma cells

Kanya Moolsuwan,<sup>1</sup> Tiravut Permpoon,<sup>2</sup> Chanachai Sae-Lee,<sup>3</sup> Mongkol Uiprasertkul,<sup>4</sup> Viroj Boonyaratanakornkit,<sup>5</sup> Pa-thai Yenchitsomanus,<sup>6</sup> Naravat Pongvarin,<sup>2</sup> La-ongsri Atchaneeyasakul<sup>7</sup>

<sup>1</sup>Graduate Program in Molecular Medicine, Faculty of Science, Mahidol University, Bangkok, Thailand; <sup>2</sup>Clinical Molecular Pathology Laboratory, Department of Clinical Pathology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand; <sup>3</sup>Research Division, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand; <sup>4</sup>Department of Pathology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand; <sup>5</sup>Faculty of Allied Health Sciences, Chulalongkorn University, Bangkok, Thailand; <sup>6</sup>Division of Molecular Medicine, Department of Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand; <sup>7</sup>Department of Ophthalmology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

**Purpose:** Retinoblastoma (RB) is a malignant childhood intraocular tumor. Current treatment options for RB have undesirable side effects. A comprehensive understanding of gene expression in human RB is essential for the development of safe and effective new therapies.

**Methods:** We reviewed published microarray and RNA sequencing studies in which gene expression profiles were compared between human RB and normal retina tissues. We investigated the expression of genes of interest using quantitative reverse transcription PCR. We examined the activities of cloned promoter DNA fragments with luciferase assay.

**Results:** *Dopachrome tautomerase (DCT)* was among the most overexpressed genes in RB in published studies. We found that *DCT* was highly expressed in six of 13 samples microdissected from Thai RB tissues. Expression of *DCT* was absent or barely detected in retina tissues, various human ocular cells, and major organs. We also demonstrated that the -657 to +411 *DCT* promoter fragment efficiently directs RB cell-specific transcription of the luciferase reporter gene in cell lines.

**Conclusions:** The present work highlights that *DCT* is one of the most RB-specific genes. The regulatory elements required for this cell-specific gene expression are likely located within its proximal promoter.

Retinoblastoma (RB) is the most frequent primary cancer of the eye in childhood and predominantly affects young children under the age of 5 years [1,2]. The global incidence of RB is 1 in 15,000 to 1 in 20,000 live births per year. Most affected individuals live in underdeveloped countries. In Thailand, the estimated incidence of this eye cancer is about 3.1 per 1 million people [3]. The most common initial sign of RB is leukocoria, an abnormal white reflection from the retina in an eye with RB. Other signs of RB include redness of the eye and painful or bulging eye.

Untreated RB can be life-threatening because of its capability to spread outside the eye or to other parts of the body, including lymph nodes, bones, bone marrow, brain, spinal cord, or liver. Early diagnosis and prompt treatment can

save the eyes and lives of children with RB. RB-associated mortality rates are relatively high in developing countries [4]. The 5-year overall survival rate is 73% for Thai children with RB [3].

The current standard treatment for RB can cause eye problems and other side effects. Cataracts, dry eye, facial asymmetry, and dental problems may occur after radiotherapy. Patients treated with chemotherapy may develop complications in the eye, kidney, or hearing. Enucleation is considered the primary treatment for advanced RB. Although this surgery is the best way to save these children's lives, it can be a stressful experience for pediatric patients and their families. The surgery also affects the quality of life of RB survivors [5].

Almost all RB is caused by either germline or somatic mutations in the *RB transcriptional corepressor 1 (RBI; Gene ID: 5925, OMIM 614041)* gene, which encodes a tumor suppressor protein. Children born with germline mutations tend to have bilateral tumors. RB could also be caused by

Correspondence to: La-ongsri Atchaneeyasakul, Department of Ophthalmology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand; Phone: +66893138367; FAX: +6624111906; email: atchaneec@hotmail.com

amplification of the *MYCN proto-oncogene, bHLH transcription factor* (*MYCN*; Gene ID: 4613, OMIM 164840) gene. Other somatic genomic alterations are rare [6].

Genetic changes in RB consequently lead to alteration of the gene expression pattern. Comparisons between the patterns of genes expressed in RB and normal retina tissues have been previously demonstrated with microarray and RNA sequencing analyses [7-12]. Proposed dysregulated pathways in RB include, but are not limited to, the PI3K/AKT/mTOR pathway, DNA damage-response pathways, aryl hydrocarbon receptor signaling, polo-like kinase and mitosis, and purine metabolism pathways.

Studies on RB gene expression profiling not only help us understand RB biology but also allow us to further unravel potential candidate genes that could be therapeutic targets for RB treatment. In this study, we aimed to identify the most RB cell-specific gene. Its promoter may be useful in controlling gene expression in RB cells for expression-targeted gene therapy.

## METHODS

*Human specimens and cell lines:* The protocol for this study was approved by the Siriraj Institutional Review Board (SIRB), Faculty of Medicine Siriraj Hospital, Mahidol University (approval number Si 075/2016). Thirteen retinoblastoma specimens submitted to the Department of Pathology, Faculty of Medicine Siriraj Hospital, Mahidol University during the years 2013 to 2018 were included in this study. The data were analyzed anonymously. We excluded samples from analysis based on the availability of an adequate amount of tissue and extracted RNA.

Nine human ocular RNA samples were purchased from 3H Biomedical (Uppsala, Sweden). They were total RNA samples prepared from early passage human corneal epithelial cells, retinal astrocytes, ocular choroid fibroblasts, trabecular meshwork cells, keratocytes, RPE cells, lens epithelial cells, conjunctival fibroblasts, and nonpigment ciliary epithelial cells.

All human cancer cell lines were purchased from American Type Culture Collection (ATCC; Manassas, VA). ATCC-formulated RPMI 1640 Medium (Gibco; Waltham, MA) was used to culture the Y79 cell line supplemented with 20% fetal bovine serum (FBS, Biochrom, Berlin, Germany). The NCI-H1975 and HCT-15 cell lines were cultured in 10% FBS-supplemented RPMI-1640 media (Gibco). The ARPE-19 cells (a generous gift from Associate Professor Kanokpan Wongprasert, Faculty of Science, Mahidol University, Bangkok, Thailand), a human RPE cell line, were cultured

in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM:F12; Thermo Scientific, Waltham, MA). All cell lines were maintained at 37 °C in a humidified incubator in an atmosphere of 5% CO<sub>2</sub> in air. Twenty-five short tandem repeat (STR) loci plus the gender-determining locus, amelogenin, were performed using Investigator 26plex QS kit. Data were analyzed using [ATCC STR Profiling Analysis software](#). Appropriate positive and negative controls were run and confirmed for each sample submitted. The STR analyses are presented in Appendix 1. All cell lines were authenticated using short tandem repeat (STR) analysis with the Investigator 26plex QS kit (QIAGEN, Hilden, Germany).

*Bioinformatic analysis:* Microarray differential expression profiles for retinoblastoma and normal retina tissue were retrieved from a previous study [8-10]. Sequence read archive (SRA) data for retinoblastoma and retina tissue were collected from previous transcriptomic studies [11,12]. SRA data were converted to FASTQ using the SRA tools (<https://github.com/ncbi/sra-tools>). The resulting data were then subjected to FASTQC for quality control. Low-quality reads and technical sequences were removed using Trimmomatic version 0.39 [13]. The cleaned read sequences were mapped to the human genome sequence (GRCh38) with HISAT2 (v 2.1.0) [14]. Samples with an overall mapping rate of less than 70% were not included in further analysis.

For gene expression quantification, a raw read count was performed using the HTseq Python script for baseline expression. Differential expression analysis of retinoblastoma versus normal retina tissue was calculated using the [edgeR R package](#) [15]. Differential expression genes (DEGs) were filtered with the criteria  $|\log_{2}FC| > 1$  and a false discovery rate (FDR)  $< 0.01$ . Genes with significant upregulation were further analyzed with Gene Set Enrichment Analysis (GSEA) software [16]. To evaluate gene expression abundance, we calculated the transcript per million (TPM) of each aligned read with [TPMcalculator](#) [17].

*Laser capture microdissection:* The formalin-fixed paraffin-embedded (FFPE) tissues from patients with retinoblastomas were cut in 5 µm thickness using a microtome and placed on the MMI membrane slides. Slides were incubated in xylene and absolute ethanol before cresyl violet staining [18]. Areas of interest were microscopically identified. Target cells from the slides were excised using a laser beam from the MMI CellCut Plus (Molecular Machines & Industries; Eching, Germany) and collected on the MMI isolation caps.

*RNA isolation and cDNA synthesis:* Total RNA was isolated from microdissected tissues using an FFPE-T RNA extraction kit (Roche, Basel, Switzerland). The RNA concentrations were determined using a spectrophotometer (FLUOstar

Omega, BMG Labtech; Ortenberg, Germany). cDNA was synthesized with the Sensiscript Reverse Transcription Kit (QIAGEN).

The RNeasy Mini Kit (QIAGEN) was used for RNA extraction from the cell lines. Cell line RNA and human ocular RNA samples were converted to cDNA using the Omniscript reverse transcription kit (QIAGEN).

**Quantitative PCR (qPCR):** Quantitative real-time PCR for the gene expression study was performed on the LightCycler 480 System (Roche) using FastStart SYBR Green Master (Roche) according to the manufacturer's instructions. The qPCR protocol began with one cycle of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. The melting curve step (72 °C to 95 °C) occurred after the amplification step. PCR products were used for gel electrophoresis to determine specific product amplification using 2% agarose gel. The *18s rRNA* (Gene ID: 106631781, OMIM 180450) gene was used as a housekeeping gene for the gene expression analysis. The delta-deltaCt method was used to normalize the expression levels. Oligonucleotide sequences are shown in Appendix 2.

**Plasmid constructs:** We amplified human *DCT* promoter fragments from Y79 genomic DNA using *Taq* DNA polymerase (Thermo Scientific). The sequences for the amplification primers are shown in Appendix 2. We replaced the EcoRI-BamHI fragment of the pGLuc-Basic vector (New England Biolabs, Ipswich, MA) with the promoter fragments. Plasmid sequences were verified using MacroGen Sanger (Seoul, South Korea) sequencing services.

**Luciferase reporter assay:** We transiently transfected two plasmid DNA constructs, 210 ng of *Gaussia* luciferase construct containing the promoter of interest and 40 ng of cytomegalovirus (CMV) promoter-driven *Cypridina* luciferase construct, to cells in 96-well plates using Lipofectamine 2000 (Life Technologies, Carlsbad, CA). We collected the culture media 48 h after transfection. We then sequentially detected secreted luciferases using *Gaussia* luciferase and *Cypridina* luciferase assay kits (New England Biolabs). Luciferase bioluminescence was measured using a FLUOstar Omega plate reader (BMG Labtech). Luciferase expressed from the *Gaussia* luciferase reporter plasmid was normalized by the activity of *Cypridina* luciferase.

## RESULTS

**Commonly upregulated genes in RB are mainly related to the cell cycle:** We first performed a literature search in PubMed and found four microarray studies on the gene expression

profile of RB published between 2007 and 2013 (Table 1) [7–10]. The number of RB tissues in each study varied from three to 21 samples. Gene expression levels in RB were compared with those in normal retinas (normal retinas in the same patient, normal adult retinas, or fetal retinas). Significant overexpression was seen in 1,004–2,106 genes (2.3–7.3% of genes contained on the microarray chip used in each study). We excluded one microarray data set published by Chakraborty et al. [7] who examined the smallest number of genes (about 19,000 genes), from further analysis.

In addition, we reanalyzed RNA sequencing data from ten RB and six retina samples available in two transcriptome data sets (accession numbers PRJNA436090 and PRJNA517916) deposited in the NCBI Gene Expression Omnibus (GEO; Table 1 and Appendix 3) [11,12]. Four data sets (three RB: SRR8507306, SRR8507307, and SRR8507308; and one normal retina: SRR8507309) were excluded from further analysis because they had a low percentage of reads that mapped to the human reference genome.

Overlap between gene sets is demonstrated in Figure 1A. A total of 116 genes were commonly upregulated in RB samples among the RNA-Seq data and three microarray data sets. To gain mechanistic insight into this gene list, we performed gene ontology (GO) enrichment analysis and found that the top three significant GO terms were related to the cell cycle (Figure 1B), i.e., GO\_CELL\_CYCLE, GO\_CELL\_CYCLE\_PROCESS, and GO\_MITOTIC\_CELL\_CYCLE.

***DCT* is the most-expressed gene in RB tissues and is undetectable in normal retinas:** To identify genes with high expression in RB and low expression in retina, we first classified 116 commonly upregulated genes based on the level of expression in reanalyzed RNA-Seq data from seven RB samples. None of these genes had a high level of expression (>1,000 TPM). The median expression level of 17 genes in RB was medium (11 to 1,000 TPM), including *TMSB15A* (Gene ID: 11013, OMIM 300939), *MCM7* (Gene ID: 4176, OMIM 600592), *TMEM97* (Gene ID: 27346, OMIM 612912), *NT5DC2* (N/A), *ZWINT* (Gene ID: 11130, OMIM 609177), *UBE2C* (Gene ID: 11065, OMIM 605574), *MYCN* (Gene ID: 4613 OMIM 164840), *FANCG* (Gene ID: 2189, OMIM 602956), *CENPV* (Gene ID: 201161 OMIM 608139), *TYMS* (Gene ID: 7298, OMIM 188350), *REC8* (Gene ID: 9985 OMIM 608193), *DCT* (Gene ID: 1638, OMIM 191275), *HMGB3* (Gene ID: 3149 OMIM 300193), *CDC20* (Gene ID: 991; OMIM 603618), *KPNA2* (Gene ID: 3838 OMIM 600685), *TCF19* (Gene ID: 6941, OMIM 600912), and *RXRG* (Gene ID: 6258 OMIM 180247). Interestingly, *dopachrome tautomerase (DCT)* had an expression level below the 0.5 TPM cut-off value in four of the five retina samples (Figure 2). The TPM for *DCT* in one

retina sample was 0.85, which is considered a low expression level.

We next examined laser-microdissected formalin-fixed paraffin-embedded tissues collected from 13 Thai patients with RB to investigate whether RB tissues from Thai patients showed similar *DCT* expression patterns. The characteristics of these patients with RB are described in Table 2. We

performed quantitative reverse transcription PCR (qRT-PCR) and demonstrated that six of the 13 RB tissues (46.2%) from the patients had high *DCT* expression (Figure 3A). The expression levels of *DCT* varied across these samples. The levels of *DCT* expression in five RB samples, that is, samples 2, 3, 9, 12, and 13, were higher than those in the Y79 retinoblastoma cell line. Due to the limited amount and quality of extracted FFPE RNA from normal retinas in the same

**TABLE 1. SUMMARY OF PUBLISHED DATA FOCUSED ON GENE EXPRESSION PROFILING IN RETINOBLASTOMA TISSUES.**

	Microarray				RNA sequencing	
	Chakraborty S. et al., 2007 [7]	Ganguly A. and Shields C., 2010 [8]	Kapatai G et al., 2013 [9]	Nalini V. et al., 2013[10]	Chai P. et al., 2018 Bioproject number PRJNA436090 [11]	Rajasekaran S. et al., 2019 Bioproject number PRJNA517916 [12]
Platform	Human 19K cDNA microarray (University Health Network) ( 19,000 genes)	GeneChip Human U133 V2.0 microarray (Affymetrix) (38,500 genes)	Human Gene 1.0ST (Affymetrix) (28,869 genes)		Illumina TruSeq RNA Sample Prep Kit, HiSeq 2000	Illumina TruSeq RNA Library Prep Kit, HiSeq 2500
<b>Retinoblastoma tissues</b>						
Number of tissues	3	6	21	3	3	7
Age of patients (years)	N/A	0.9 to 5 (0.9, 1, 1.1, 1.3, 2, 5)	N/A	1 to 3 (1, 2.5, 3)	N/A	0.8 to 6 (0.8, 1, 1, 3, 3, 6)
Treatment	N/A	enucleation	enucleation	2 patients with chemotherapy and 1 without chemotherapy	N/A	1 with chemotherapy, 6 without chemotherapy
<b>Normal control tissues</b>						
Number of tissues	10 (normal retina)	6 (retina in opposite quadrant to RB)	N/A (adult retina and 96-day human fetal retina)	2 (adult retina)	3 (para-tumor)	3 (frozen retina)
Age of donors/patients (years)	N/A	same patients	N/A	N/A	N/A	(5 to 22) 5, 12, 22
<b>Results</b>						
Number of significantly upregulated genes	1,004	1,116	2,106	1,672	N/A	2,168
Top ten genes with highest expression in RB tissue	DYNC1I2, TOR1AIP1, ZNF767, MBOAT5, RBM5, LRRF58, PCSK5, GNPTAB, ZNF551, SPATA21	NUF2, PBK, TOP2A, E2F7, CASC5, MELK, EZH2, UHRF1, KIF11, PPP4R4	SNORD41, HIST1H3I, DCT, SNORD29, SNORA23, SNORA71D, HIST1H3B, SNORA3, DTL, TOP2A	DCT, TOP2A, PTCH2, C18orf56, CLUL1, C8orf46, NT5E, DTL, ASPM, CENPF	N/A	DEPDC1, NDC80, NEIL3, KIF20A, GATA4, IGF1, IGFBP1, CKAP2L, AL451127.1, MNX1-AS1, AC112777.1

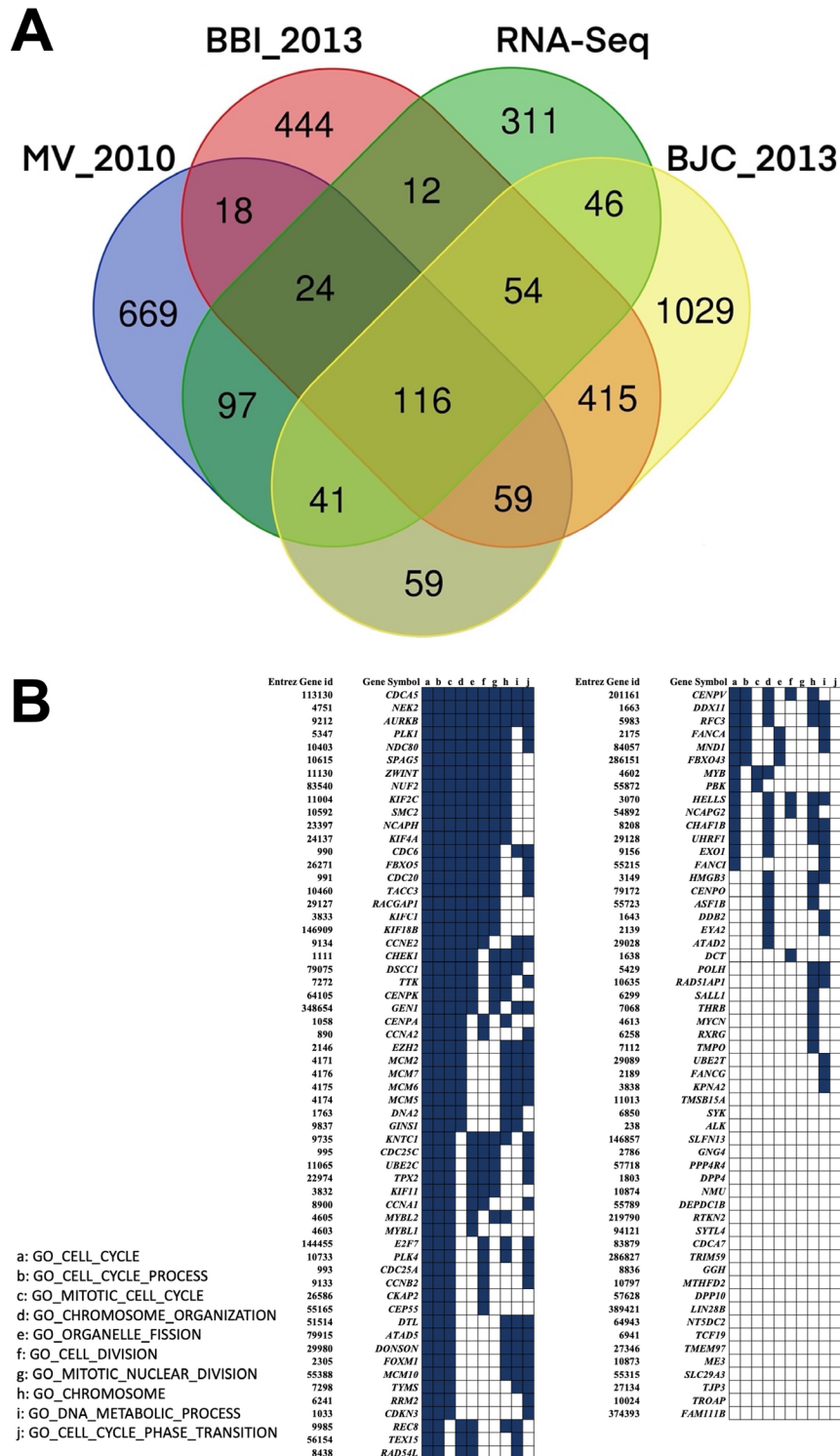


Figure 1. Overlap of gene sets and GO enrichment analysis. **A:** Venn diagram showing overlap in the number of genes identified as upregulated genes in RB from 3 microarray and reanalyzed RNA-seq data. **B:** Gene/Gene Set Overlap Matrix of 116 commonly upregulated genes. Overlaps between the list of 116 commonly upregulated genes and the top 10 most significantly enriched Gene Ontology terms (FDR q-value less than 0.05) was demonstrated using Gene Set Enrichment Analysis (GSEA) software.

**TABLE 2. CLINICAL CHARACTERISTICS OF 13 THAI RETINOBLASTOMA PATIENTS INCLUDED IN GENE EXPRESSION ANALYSIS OF RB AND RETINA TISSUES.**

Patient number	Age (months)	Gender	Type	Tumor classification	Eye	Enucleation	RB tissues	Retina tissues
1	14	Female	Unilateral RB	E	Right	Primary enucleation	Y	Y
2	36	Male	Unilateral RB	E	Right	Primary enucleation	Y	Y
3	6	Female	Bilateral RB	E	Left	Primary enucleation	Y	N
4	16	Male	Bilateral RB	D	Right	Secondary enucleation	Y	N
5	39	Male	Bilateral RB	E	Right	Secondary enucleation	Y	N
6	36	Female	Bilateral RB	E	Right	Primary enucleation	Y	N
7	5	Male	Bilateral RB	E	Left	Secondary enucleation	Y	N
8	24	Male	Unilateral RB	E	Left	Primary enucleation	Y	N
9	24	Male	Bilateral RB	E	Right	Secondary enucleation	Y	N
10	25	Male	Unilateral RB	E	Right	Primary enucleation	Y	N
11	40	Male	Unilateral RB	D	Left	Secondary enucleation	Y	N
12	3	Female	Bilateral RB	E	Left	Primary enucleation	Y	N
13	18	Male	Unilateral RB	E	Left	Primary enucleation	Y	N

Gene id	Gene symbol	Retinoblastoma							Retina tissue				
		RB-A1	RB-A2	RB-A3	RB-A4	RB-B1	RB-B2	RB-B3	R-A1	R-A2	R-B1	R-B2	R-B3
ENSG00000080166	<i>DCT</i>	129.45	35.58	134.81	26.91	15.30	14.86	13.55	0.35	0.85	0.46	0.43	0.42
ENSG00000137310	<i>TCF19</i>	123.99	26.56	180.80	26.03	11.03	11.34	9.84	3.36	3.08	0.78	0.75	0.69
ENSG00000158164	<i>TMSB15A</i>	191.46	108.36	213.41	103.45	5.47	5.29	4.86	0.28	0.61	0.64	0.67	0.67
ENSG00000122952	<i>ZWINT</i>	239.08	52.66	238.10	99.76	7.19	7.29	5.92	2.46	3.61	1.67	1.52	1.40
ENSG00000175063	<i>UBE2C</i>	148.78	46.05	370.87	97.05	5.35	5.71	4.66	0.18	0.45	1.45	1.37	1.30
ENSG00000134323	<i>MYCN</i>	67.91	43.36	58.64	837.12	3.30	3.29	3.07	1.61	3.20	0.25	0.22	0.24
ENSG00000221829	<i>FANCG</i>	124.97	42.37	41.64	112.70	5.01	5.10	4.33	10.01	10.57	1.24	1.06	1.14
ENSG00000176890	<i>TYMS</i>	129.85	39.98	78.15	82.15	3.61	3.65	3.31	4.65	7.38	0.57	0.55	0.60
ENSG00000100918	<i>REC8</i>	79.50	37.90	72.79	100.82	3.00	3.04	2.94	6.01	6.57	0.24	0.21	0.24
ENSG00000029993	<i>HMGB3</i>	67.62	26.78	109.99	64.54	2.75	2.68	2.28	2.36	6.40	0.62	0.62	0.63
ENSG00000117399	<i>CDC20</i>	106.02	26.39	209.80	65.78	4.14	4.53	3.90	1.11	3.57	0.99	0.90	0.81
ENSG00000143171	<i>RXRG</i>	59.66	24.71	110.51	52.66	4.61	4.58	3.98	6.61	7.88	0.27	0.26	0.24
ENSG00000145386	<i>CCNA2</i>	91.07	21.85	141.66	43.49	4.26	4.16	3.65	7.99	7.33	1.11	1.16	1.03
ENSG00000077152	<i>UBE2T</i>	92.76	21.08	145.67	33.13	2.22	2.22	1.96	1.43	1.01	0.26	0.26	0.31
ENSG00000073111	<i>MCM2</i>	40.98	19.56	68.47	64.52	4.11	4.12	3.71	1.93	5.26	0.71	0.63	0.64
ENSG00000076003	<i>MCM6</i>	36.88	17.73	45.73	28.94	2.07	2.11	1.89	0.97	1.82	0.33	0.30	0.28
ENSG00000136108	<i>CKAP2</i>	61.49	15.43	113.66	17.58	2.67	2.58	2.39	3.63	3.35	0.56	0.50	0.44
ENSG00000065911	<i>MTHFD2</i>	49.36	14.46	25.73	50.34	2.09	2.13	1.95	8.02	6.16	0.59	0.55	0.53
ENSG00000178999	<i>AURKB</i>	71.49	14.28	89.89	41.46	1.41	1.60	1.28	0.06	0.32	0.68	0.44	0.50
ENSG00000100297	<i>MCM5</i>	30.10	12.24	41.02	32.85	2.72	2.67	2.46	1.58	4.25	0.55	0.54	0.48

Figure 2. Gene expression levels of 116 commonly upregulated genes. The results are displayed as transcripts per million (TPM) in seven retinoblastoma (RB) and five retina (R) samples, collected from two publicly available RNA-sequencing data sets (designated as **A** and **B**). Medium blue box: medium expression, light blue box: low expression, gray box: expression level below 0.5 TPM.

patients with RB, we performed qRT-PCR in only two retina tissues (designated as N1 and N2). Expression of the *DCT* gene was not detected in either retina sample (Figure 3B).

*Expression of DCT is barely detectable in various human ocular cells and major organs:* We performed RT-qPCR to investigate *DCT* expression in nine human ocular cells and demonstrated that *DCT* was minimally detected in the ocular choroid fibroblasts, RPE cells, and nonpigment ciliary epithelial cells (Figure 3C). The expression of *DCT* in ocular choroid fibroblasts, RPE cells, and nonpigment ciliary epithelial cells was 12.61-, 89.33-, and 86.00-fold lower than in Y79 cells, respectively. No *DCT* expression was detected in six human ocular cells: retinal astrocytes, lens epithelial cells, corneal epithelial cells, trabecular meshwork cells, keratocytes, and conjunctival fibroblasts.

To further investigate the expression of *DCT* genes in other cell types in the human body, we explored the large-scale database of gene expression in human tissues from the NIH-funded Genotype-Tissue Expression (GTEx) project [19]. We found that this gene is not detectable (<0.5 TPM) in major organs, including brain, kidneys, liver, pancreas, stomach, intestine, and lungs (Figure 3D,E).

*Proximal DCT promoter is active in retinoblastoma cells:* To determine whether the proximal promoter of the *DCT* gene could drive RB-specific transgene expression, we performed luciferase assays in the Y79 and ARPE-19 RPE cell lines. We found that the -657/+411 *DCT* promoter fragment was active in the Y79 cells (Figure 4A). This promoter activity in the Y79 cells was about ten times higher than that in the ARPE-19 cells (Figure 4B).

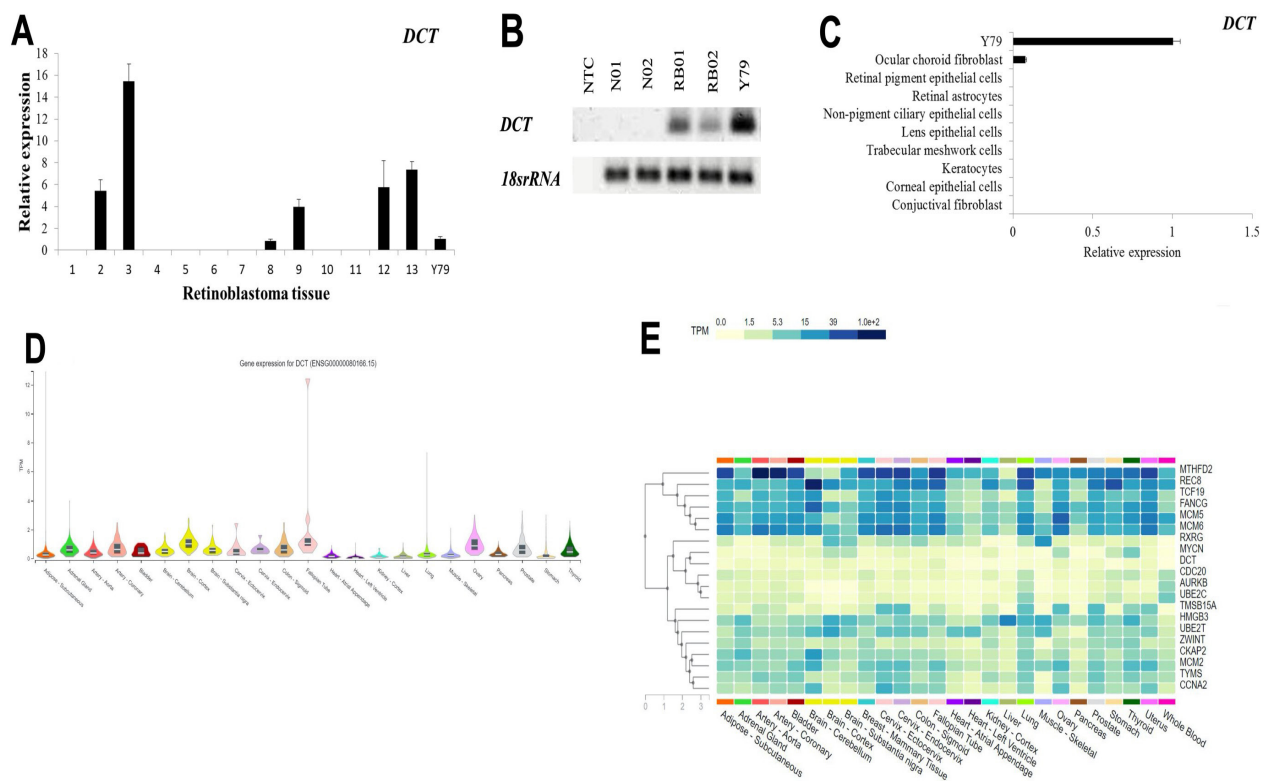


Figure 3. *DCT* gene expression in retinoblastoma (RB) tissues, normal retinas, ocular cells, and other tissues. **A:** *DCT* expression normalized to *18s rRNA* in 13 Thai RB tissues. The quantitative polymerase chain reaction (qRT)-PCR results are shown as relative expression compared to *DCT* expression in Y79 cells. **B:** *DCT* and *18s rRNA* expression in two retinas and two RB tissues from the same patients. The PCR products were demonstrated with gel electrophoresis. **C:** *DCT* expression in nine normal human ocular cells. The qRT-PCR results are shown as relative expression compared to *DCT* expression in Y79 cells. **D:** Expression of *DCT* in normal tissues from the GTEx database. **E:** Relative *DCT* expression in normal tissues in relation to 16 upregulated genes with medium expression levels in RB.

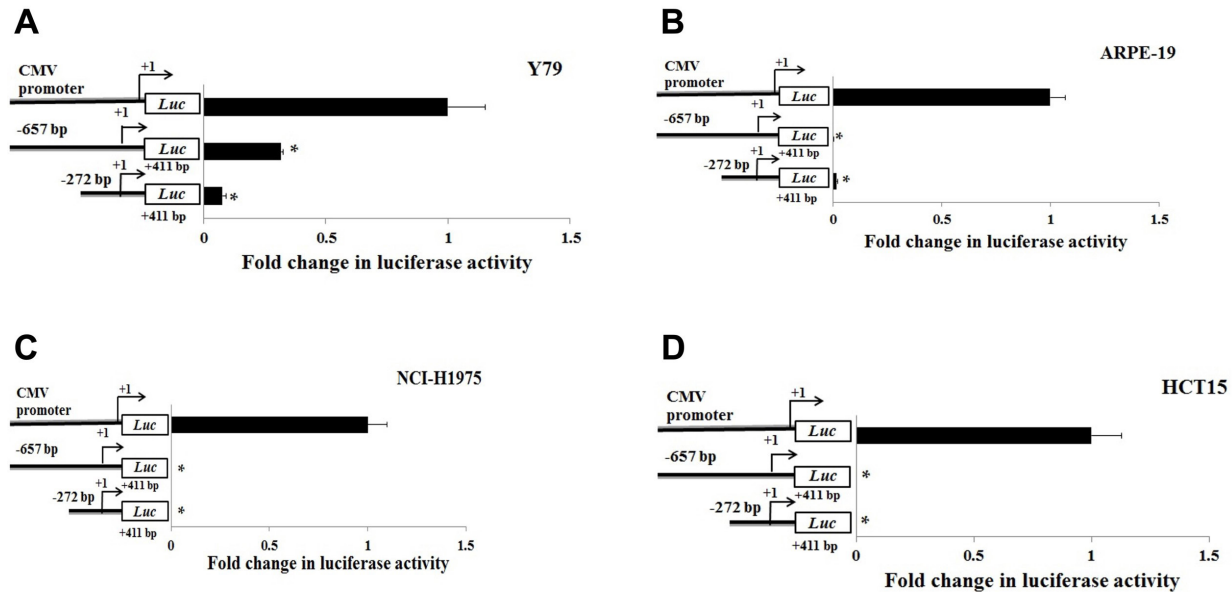


Figure 4. Analysis of *DCT* promoter activities using dual-luciferase assay. The activity of -657 to +411 and -272 to +411 *DCT* promoters was compared to CMV promoter activity in Y79 (A), ARPE-19 (B), NCI-H1975 (C), and HCT15 (D) cell lines. The bar graphs show the means and standard deviations for three independent experiments for Y79 and ARPE-19 cell lines.

We also performed a promoter study in NCI-H1975 (non-small cell lung cancer) and HCT15 (colorectal adenocarcinoma) cell lines. The activity of the proximal *DCT* promoter fragment was not detected by the luciferase assay in these lung and colorectal cancer cells (Figure 4C,D).

**Statistical analysis:** We presented all data as the mean  $\pm$  standard deviation (SD) or the standard error of the mean (SEM). We compared sample groups with Student's two-tailed *t* test. A *p* value of less than 0.05 was considered statistically significant.

## DISCUSSION

The gene expression profile of retinoblastoma was first reported in 2007. A human 19K cDNA microarray was used to analyze gene expression in the retinoblastoma and normal retina samples at that time. Later, three studies used Affymetrix microarray chips, which cover larger numbers of human genes, to identify the lists of upregulated and downregulated differentially expressed genes and the altered pathways in retinoblastoma [7-10]. It should be emphasized that these studies used different control tissues, including normal retinas from donors' eyes, normal retinas from the same patients, and fetal retinas. Today, high-throughput sequencing technologies

are routinely applied in numerous transcriptome studies. Our reanalysis of two recently published RNA-Seq data sets from RB and normal retina tissues [11,12], in combination with information from three previously reported microarray experiments, suggested that *DCT* is the most RB-specific gene.

The *DCT* gene, also known as *tyrosinase-related protein 2 (TYRP2)*, encodes an enzyme required for the melanin biosynthesis pathway. The *DCT* enzyme catalyzes the tautomerization of dopachrome to 5,5-dihydroxyindole-2-carboxylic acid (DHICA) [20]. *DCT* expression was previously examined in human melanocytes, melanocyte stem cells, and melanoma cells [21]. To the best of our knowledge, only one study has focused on *DCT* expression in RB [22]. Japanese researchers performed an RT-PCR experiment and successfully demonstrated that *DCT* mRNA was highly expressed in three excised RB specimens and Y79 cells in study that has been conducted in 2001. The present results confirmed and extended previous findings regarding *DCT* expression in RB. The levels of expression in most *DCT*-expressed RB are greater than those in Y79 cells (Figure 3A). However, *DCT* was not expressed in all Thai RB tissues in the present study. This finding may be explained in part by retinoblastoma



tumor heterogeneity and heterogeneous study samples. Tumor heterogeneity in retinoblastoma has been reported in several studies [9,23-25]. The microdissected samples used in the present study contained only a small fraction of the tumor and thus, may not be a true representation of all the subclones present in retinoblastoma.

The present study highlighted that the expression levels of *DCT* in normal human cells and tissues are clearly lower than those in RB cells and tissues, if not undetectable. A previous report showed the presence of *DCT* expression in ARPE-19 cells [26]. However, the expression levels of *DCT* in RB appeared much higher than those detected in these cells. Instead of using RNA from the ARPE-19 cell line, in the present qRT-PCR experiments, we used RNA samples prepared from early passage human RPE cells and obtained similar results. *DCT* mRNA expression in RB cells was much higher than that in RPE cells (Figure 3C). We additionally reported two more human ocular cells with minimal expression of *DCT*, i.e., ocular choroid fibroblasts and nonpigment ciliary epithelial cells. No *DCT* expression was detected in the rest of the normal human ocular cells included in the present experiments and in two microdissected normal retina tissues from patients with RB. The RNA-Seq results available from the GTEx database provide more insight into the expression levels of genes in human tissues (Figure 3D,E). These findings underline the fact that *DCT* is absent or barely expressed in normal tissues.

*DCT* is also expressed in other cancers derived from melanocytes and neuronal cells, such as melanoma, glioma, and glioblastoma [27,28]. Little is known about the role of *DCT* in retinoblastoma and these cancers. *DCT* overexpression in a melanoma cell line resulted in increased activity of the extracellular signal-regulated kinases (ERK)/mitogen-activated protein kinase (MAPK) pathway, which may play an important role in radiation and drug resistance [29]. *DCT* functions have been investigated in other cell types, including promoting proliferation of neural progenitor cells and regulating reactive oxygen species levels, DNA damage, and the cell cycle in human epidermal keratinocyte cell lines [30,31].

More experimental research is needed to determine whether *DCT* plays similar roles in retinoblastoma. Cell-specific promoters are beneficial to basic science and gene therapy studies. Use of the RB cell-specific promoter is desirable in the expression cassette to control the expression of the therapeutic gene in RB cells and restrict unwanted expression in other cells. It has been shown that the human *DCT* promoter construct containing the 3.6 kb 5'-flanking region efficiently directed the expression of the luciferase reporter gene in Y79 cells [22]. Our promoter studies provide

preliminary evidence that the -657/411 *DCT* promoter fragment is likely an RB-specific promoter because it is active in Y79 RB cells, not in normal RPE ARPE-19 cells or other tested cancer cells, that is, NCI-H1975, and HCT15 cells. Further studies on other ocular cells and cancer cells are needed to confirm the specificity of this promoter fragment.

Taken together, the study results suggest that the most RB-specific gene is *DCT*. Its proximal promoter fragment is possibly useful as an RB cell-specific promoter. These findings enhance the ability to develop novel therapies that can specifically eliminate retinoblastoma cells without many side effects.

### APPENDIX 1. STR ANALYSIS

To access the data, click or select the words “[Appendix 1.](#)”

### APPENDIX 2. OLIGONUCLEOTIDES USED IN THIS STUDY.

To access the data, click or select the words “[Appendix 2.](#)”

### APPENDIX 3. OVERALL MAPPING RATE OF SEQUENCE READ ARCHIVE (SRA) DATA OF RETINOBLASTOMA AND NORMAL RETINA TISSUE.

To access the data, click or select the words “[Appendix 3.](#)”

### ACKNOWLEDGMENTS

This work was supported by the Royal Golden Jubilee Ph.D. Scholarship (PHD/0179/2554) from Thailand Research Fund (K. Moolsuwan) and Siriraj research fund (R015934004) from the Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. The authors express their gratitude to Pichpisith Pierre Vejvisithsakul and Nunthawut Chat-uthai for their helps in optimization of gene expression analysis. Naravat Pongvarin (naravat.pou@mahidol.ac.th) is a co-corresponding author.

### REFERENCES

1. Ray A, Gombos DS, Vats TS. Retinoblastoma: an overview. *Indian J Pediatr* 2012; 79:916-21. [PMID: 22421935].
2. Meel R, Radhakrishnan V, Bakhshi S. Current therapy and recent advances in the management of retinoblastoma. *Indian J Med Paediatr Oncol* 2012; 33:80-8. [PMID: 22988349].
3. Jain M, Rojanaporn D, Chawla B, Sundar G, Gopal L, Khetan V. Retinoblastoma in Asia. *Eye (Lond)* 2019; 33:87-96. [PMID: 30385881].

4. Kivela T. The epidemiological challenge of the most frequent eye cancer: retinoblastoma, an issue of birth and death. *Br J Ophthalmol* 2009; 93:1129-31. [PMID: 19704035].
5. Zhang L, Gao T, Shen Y. Quality of life in children with retinoblastoma after enucleation in China. *Pediatr Blood Cancer* 2018; 65:e27024-[PMID: 29528176].
6. Kooi IE, Mol BM, Massink MP, Ameziane N, Meijers-Heijboer H, Dommering CJ, van Mil SE, de Vries Y, van der Hout AH, Kaspers GJ, Moll AC, Te Riele H, Cloos J, Dorsman JC. Somatic genomic alterations in retinoblastoma beyond RB1 are rare and limited to copy number changes. *Sci Rep* 2016; 6:25264-[PMID: 27126562].
7. Chakraborty S, Khare S, Dorairaj SK, Prabhakaran VC, Prakash DR, Kumar A. Identification of genes associated with tumorigenesis of retinoblastoma by microarray analysis. *Genomics* 2007; 90:344-53. [PMID: 17604597].
8. Ganguly A, Shields CL. Differential gene expression profile of retinoblastoma compared to normal retina. *Mol Vis* 2010; 16:1292-303. [PMID: 20664703].
9. Kapatai G, Brundler MA, Jenkinson H, Kearns P, Parulekar M, Peet AC, McConville CM. Gene expression profiling identifies different sub-types of retinoblastoma. *Br J Cancer* 2013; 109:512-25. [PMID: 23756868].
10. Nalini V, Segu R, Deepa PR, Khetan V, Vasudevan M, Krishnakumar S. Molecular Insights on Post-chemotherapy Retinoblastoma by Microarray Gene Expression Analysis. *Bioinform Biol Insights* 2013; 7:289-306. [PMID: 24092970].
11. Chai P, Jia R, Jia R, Pan H, Wang S, Ni H, Wang H, Zhou C, Shi Y, Ge S, Zhang H, Fan X. Dynamic chromosomal tuning of a novel GAU1 lncing driver at chr12p13.32 accelerates tumorigenesis. *Nucleic Acids Res* 2018; 46:6041-56. [PMID: 29741668].
12. Rajasekaran S, Nagarajha Selvan LD, Dotts K, Kumar R, Rishi P, Khetan V, Bisht M, Sivaraman K, Krishnakumar S, Sahoo D, Campbell MJ, Elchuri SV, Miles WO. Non-coding and Coding Transcriptional Profiles Are Significantly Altered in Pediatric Retinoblastoma Tumors. *Front Oncol* 2019; 9:221-[PMID: 31058073].
13. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 2014; 30:2114-20. [PMID: 24695404].
14. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. *Nat Methods* 2015; 12:357-60. [PMID: 25751142].
15. McCarthy DJ, Chen Y, Smyth GK. Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. *Nucleic Acids Res* 2012; 40:4288-97. [PMID: 22287627].
16. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* 2005; 102:15545-50. [PMID: 16199517].
17. Vera Alvarez R, Pongor LS, Marino-Ramirez L, Landsman D. TPMCalculator: one-step software to quantify mRNA abundance of genomic features. *Bioinformatics* 2019; 35:1960-2. [PMID: 30379987].
18. Kolijn K, van Leenders GJ. Comparison of RNA extraction kits and histological stains for laser capture microdissected prostate tissue. *BMC Res Notes* 2016; 9:17-[PMID: 26744212].
19. Consortium GT. The Genotype-Tissue Expression (GTEx) project. *Nat Genet* 2013; 45:580-5. [PMID: 23715323].
20. Tsukamoto K, Jackson IJ, Urabe K, Montague PM, Hearing VJ. A second tyrosinase-related protein, TRP-2, is a melanogenic enzyme termed DOPAchrome tautomerase. *EMBO J* 1992; 11:519-26. [PMID: 1537333].
21. Itakura E, Huang RR, Wen DR, Paul E, Wunsch PH, Cochran AJ. RT in situ PCR detection of MART-1 and TRP-2 mRNA in formalin-fixed, paraffin-embedded tissues of melanoma and nevi. *Modern pathology: an official journal of the United States and Canadian Academy of Pathology Inc.* 2008; 21:326-33. .
22. Udono T, Takahashi K, Yasumoto K, Yoshizawa M, Takeda K, Abe T, Tamai M, Shibahara S. Expression of tyrosinase-related protein 2/DOPAchrome tautomerase in the retinoblastoma. *Exp Eye Res* 2001; 72:225-34. [PMID: 11180971].
23. Liu H, Zhang Y, Zhang Y-Y, Li Y-P, Hua Z-Q, Zhang C-J, Wu KC, Yu F, Zhang Y, Su J, Jin ZB. Human embryonic stem cell-derived organoid retinoblastoma reveals a cancerous origin. *Proc Natl Acad Sci USA* 2020; 117:33628-38. [PMID: 33318192].
24. Norrie JL, Nityanandam A, Lai K, Chen X, Wilson M, Stewart E, Griffiths L, Jin H, Wu G, Orr B, Tran Q, Allen S, Reilly C, Zhou X, Zhang J, Newman K, Johnson D, Brennan R, Dyer MA. Retinoblastoma from human stem cell-derived retinal organoids. *Nat Commun* 2021; 12:1-13. [PMID: 34315877].
25. LiuHuiHuaZi-QiJinZi-BingModeling human retinoblastoma using embryonic stem cell-derived retinal organoids. *STAR protoc.* 2021;2:100444.
26. Takeda K, Yokoyama S, Yasumoto K, Saito H, Udono T, Takahashi K, Shibahara S. OTX2 regulates expression of DOPAchrome tautomerase in human retinal pigment epithelium. *Biochem Biophys Res Commun* 2003; 300:908-14. [PMID: 12559959].
27. Chi D, Merchant RE, Rand R, Conrad AJ, Garrison D, Turner R, Morton DL, Hoon DS. Molecular detection of tumor-associated antigens shared by human cutaneous melanomas and gliomas. *Am J Pathol* 1997; 150:2143-[PMID: 9176405].
28. Saikali S, Avril T, Collet B, Hamlat A, Bansard J-Y, Drenou B, Guegan Y, Quillien V. Expression of nine tumour antigens in a series of human glioblastoma multiforme: interest of EGFRvIII, IL-13R $\alpha$ 2, gp100 and TRP-2 for immunotherapy. *J Neurooncol* 2007; 81:139-48. [PMID: 17004103].
29. Pak BJ, Lee J, Thai BL, Fuchs SY, Shaked Y, Ronai Ze, Kerbel RS, Ben-David Y. Radiation resistance of human melanoma analysed by retroviral insertional mutagenesis reveals a

- possible role for dopachrome tautomerase. *Oncogene* 2004; 23:30-8. [PMID: 14712208].
30. Jiao Z, Zhang ZG, Hornyak TJ, Hozeska A, Zhang RL, Wang Y, Wang L, Roberts C, Strickland FM, Chopp M. Dopachrome tautomerase (Dct) regulates neural progenitor cell proliferation. *Dev Biol* 2006; 296:396-408. [PMID: 16857183].
31. Aksoy P, Meneses PI. The role of DCT in HPV16 infection of HaCaTs. *PLoS One* 2017; 12:e0170158-[PMID: 28095444].

Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 7 August 2022. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.