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Original Article

Activation of integrin-ERBB2 signaling in undifferentiated thyroid cancer

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Abstract: Undifferentiated thyroid carcinoma is one of the most aggressive human cancers. Although genetic changes underlying this aggressive cancer remain to be elucidated, RAS mutations have been frequently identified in it. Mice harboring a mutant thyroid hormone receptor $Thrb^{PV}$ ($Thrb^{PV/PV}$) spontaneously develop differentiated follicular thyroid carcinoma similar to human thyroid cancer. We recently demonstrated that targeting a RAS mutation ($Kras^{G12D}$) to the thyroid of $Thrb^{PV/PV}$ mice ($Thrb^{PV/PV}Kras^{G12D}$ mice) promotes initiation and progression of undifferentiated thyroid cancer. To uncover genes destined to drive the aggressive cancer phenotype, we used cDNA microarrays to compare the gene expression profiles of thyroid cells of $Kras^{G12D}$ mice and thyroid tumor lesions of $Thrb^{PV/PV}$ and $Thrb^{PV/PV}Kras^{G12D}$ mice. Analyses of microarray data identified 14 upstream regulators that were significantly altered in thyroid tumors of $Thrb^{PV/PV}$ and $Thrb^{PV/PV}Kras^{G12D}$ mice. Most of these genes with altered expression function as key regulators in growth factor-induced signaling. Further analysis identified gene expression profiles of markedly elevated integrin levels, acting as upstream activators to stimulate ERBB2-mediated downstream signaling in thyroid tumors of $Thrb^{PV/PV}Kras^{G12D}$ mice. The present studies uncovered integrin-activated ERBB2 signaling as one of the mechanisms in synergy between TRβPV and KRASG12D signaling to promote aggressive tumor growth in undifferentiated thyroid cancer.

Keywords: Growth regulation, thyroid cancer, ERBB2, integrins, microarrays, gene expression

Introduction

Thyroid cancer is the most common malignancy of the endocrine organs. There are 4 follicular cell-derived thyroid cancers: well-differentiated papillary and follicular carcinomas, poorly differentiated carcinoma, and undifferentiated carcinoma. Although well-differentiated thyroid cancer often has a favorable outcome, the10-year survival rate of undifferentiated thyroid cancer is less than 10% owing to distant metastasis and lack of effective treatment [1].

The point mutations in *RAS* genes together with the mutations in *TP53* and *CTNNB1*genes are among prevalent genetic alterations identified in undifferentiated thyroid cancer [1]. Previously we demonstrated that mice with a mutant thyroid hormone receptor *ThrbPV* ($Thrb^{PV/PV}$) spontaneously develop well-differentiated follicular thyroid cancer with pathological progression and metastasis frequency similar to human thyroid cancer [2, 3]. The PV mutation was originally identified in a patient with resistance to thyroid hormone (RTH) [4]. The PV mutant has completely lost thyroid hormone (T3) binding activity and transcription capacity. It acts to abnormally regulate the expression of the T3 target genes via dominant negative activity.

Recently, we genetically introduced the $Kras^{G12D}$ mutation to express specifically in the thyroids of the $Thrb^{PV/PV}$ mice. We found that double mutant $Thrb^{PV/PV}Kras^{G12D}$ mice have much worse survival than the mice with a single mutation in either the *Kras* or the *Thrb* gene as a result of markedly aggressive thyroid tumors [5]. Capsular invasion, vascular invasion, and distant metastases to the lung occur at an earlier age and at a higher frequency than in $Thrb^{PV/PV}$ mice. We identified the occurrence of anaplastic foci with a high frequency in the thyroid of

Thrb$^{PV/PV}$*Kras*^{G12D} mice [5]. These anaplastic foci have lost normal thyroid follicular morphology as well as the expression of the paired box 8 gene (*Pax8*). Importantly, the protein level of PAX8 is inversely correlated with MYC in the undifferentiated thyroid tumors of *Thrb*$^{PV/PV}$*Kras*^{G12D} mice. Thus, our recent study established a mouse model of undifferentiated thyroid cancer and identified MYC as a potential target for treatment [5].

Moreover, we showed that synergistic signaling of oncogenic actions of TRβPV and Kras^{G12D} in thyroid tumors of *Thrb*$^{PV/PV}$*Kras*^{G12D} mice led to the aggressive thyroid tumor growth resulting from rapid cell proliferation [5]. However, the mechanisms underlying the increased cell proliferation remain to be elucidated. In the present studies, we used cDNA microarray analysis to compare gene expression profiles of thyroid cells of *Kras*^{G12D} mice and thyroid tumors of *Thrb*$^{PV/PV}$ and *Thrb*$^{PV/PV}$*Kras*^{G12D} mice. We uncovered increased integrins-ERBB2 signaling as a novel pathway resulting from the synergistic signaling of oncogenic actions of TRβPV and Kras^{G12D}, thereby promoting the aggressive tumor growth of undifferentiated thyroid cancer.

Materials and methods

Experimental animals

All animal experiments were performed according to the protocols approved by the Animal Care and Use Committee at the National Cancer Institute. The *Thrb*$^{PV/+}$, *Kras*$^{LSL\text{-}G12D/+}$, *TPO-Cre (Cre)* mice and the mice with four different genotypes were previously described [2, 5-7]. Thyroids and other tissues were harvested from the mice and wild-type littermates for weighing, histological analysis, and biochemical studies.

Microarray analysis

Biotinylated-aRNA samples from three individual mice of each group were used in hybridization of the GeneChip Mouse Exon 1.0 ST Array (affymetrix, Santa Clara, CA) and scanned on an Affymetrix GeneChip scanner 3000. Data were collected using Affymetrix GCOS software. Data processing and analysis were done by affy, limma, xps, et al R/Bioconductor packages (http://www.bioconductor.org). Briefly, the robust multichip average (RMA) method was used for computing expression measures, the Benjamini and Hochberg method [8] was used for calculating the adjusted *p* values. Top differentially expressed genes were selected by the adjusted *p* values with minimum 1.5 fold change. The differentially expressed genes were further analyzed for enrichment of pathways and functions using the DAVID bioinformatics database [9], Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Inc., Redwood City, CA) and the Gene Set Enrichment Analysis (GSEA) by the Broad Institute [10]. The GEO array data submission is in progress.

RNA extraction and real time RT-PCR validation of microarray data

Total RNA from thyroids was isolated using TRIzol (Invitrogen, Carlsbad, CA) as indicated by the protocol of the manufacturer. Selected genes from microarray data were chosen for real time RT-PCR validation. A total 50-200 ng of RNA extracted from thyroids of wild-type, *Kras*^{G12D}, *Thrb*$^{PV/PV}$, and *Thrb*$^{PV/PV}$*Kras*^{G12D} mice was used in the real-time RT-PCR. The reactions were performed with the QuantiTect SYBR RT-PCR kit (Qiagen, Germantown, MD) on an ABI 7900HT system. In each group, four to six samples with triplicates were tested on the target genes. Data were analyzed using Prism V5 (GraphPad Software, Inc., La Jolla, CA). Primers were as follows: for the endogenous control gene mouse glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), forward, 5'-cgtcccgtagacaaaatggt-3'; reverse, 5'-gaatttgccgtgagtggagt-3'. For Itga6, forward, 5'-CAGGTTGTGGAACAGCACAT, reverse, 5'-AAGAACAGCCAGGAGGATGA-3'. For Itgb4, forward, 5'-GAGGGGCCCTATAGCTCACT-3'; reverse, 5'-GTTGTCCACGAGCACCTTCT3'. For Itgb1, forward, 5'-TCGTGCATGTTGTGGAGACT-3'; reverse, 5'-CACAGTTGTCACGGCACTCT-3'. For Itgb3, forward, 5'-TGACATCGAGCAGGTGAAAG-3'; reverse, 5'-GAGTAGCAAGGCCAATGAGC-3'. For Itgav, forward, 5'-GATAGAGGCAAGAGCGCAAT-3'; reverse, 5'-AATGCCCCAGGTGATGTTAG-3'. For Itga5, forward, 5'-GCCAAGAGAGCCGTAGTCTG-3'; reverse, 5'-CCTTCTGCCTTGGTCCACT-3'. For Fibronectin, forward, 5'-GATCGGCAGGGAGAAAATG-3'; reverse, 5'-CAGGTCTACGGCAGTTGTCA-3'.

Western blot analysis

The Western blot analysis was carried out as described by Furumoto *et al.* [11]. Primary antibody for GAPDH (#2118) was purchased from

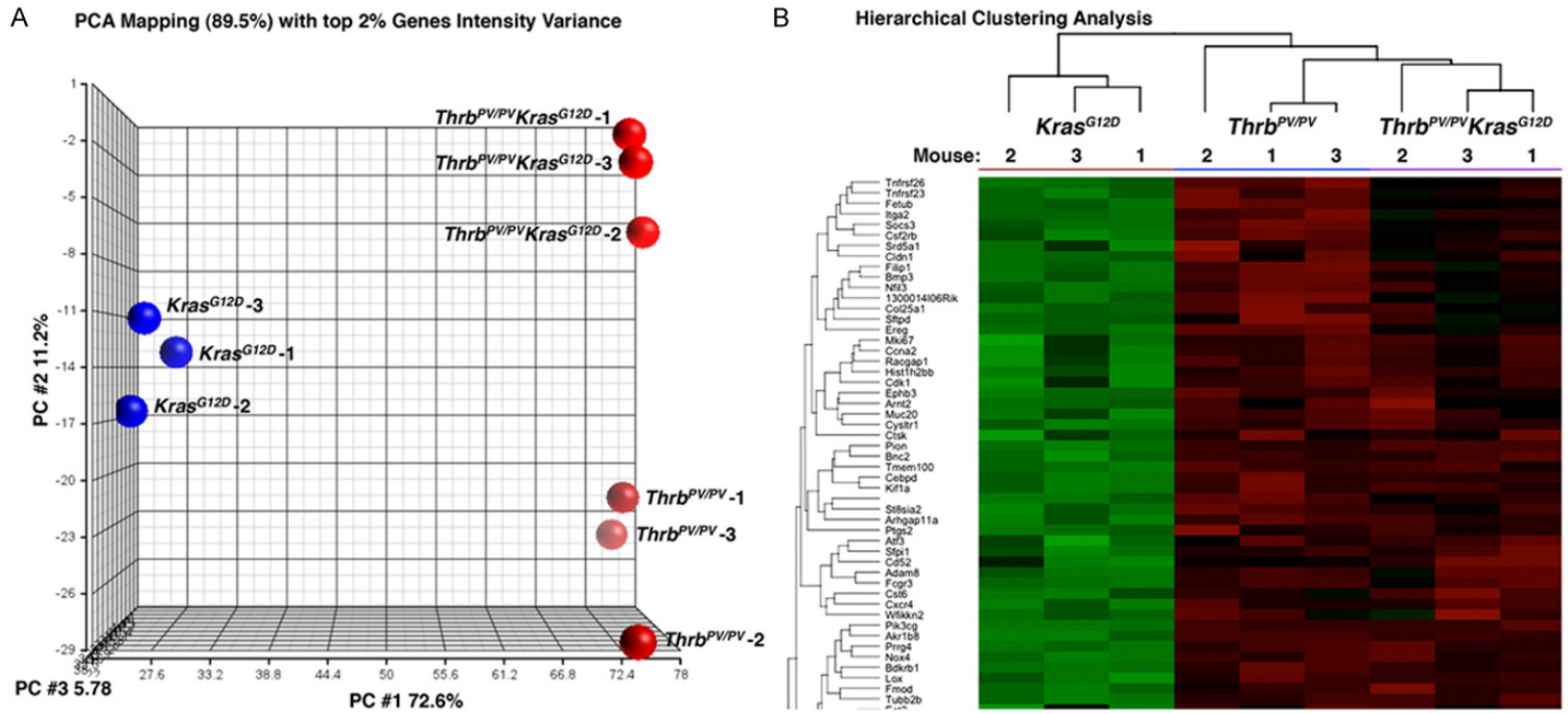

Figure 1. Principal component analysis (PCA) and heat-map of hierarchical clustering analysis of the top 2% of genes selected by variance in gene expression of follicular cells of *Kras*^{G12D} mice and thyroid tumor cells of *Thrb*$^{PV/PV}$ and *Thrb*$^{PV/PV}$*Kras*^{G12D} mice. A. Three-dimensional projection of the top 3 principal components of PCA in the figure, which captures 89.5% of total variance, shows clear separation of the 3 mouse groups. B. A heat-map (cropped) presentation of hierarchical clustering analysis (average of Euclidean distance) of the top 2% of genes selected by variance in gene expression of the 3 groups of mice. The clustering in the figure shows the 3 groups of mice form 3 clusters, but at the top level, the *Kras*^{G12D} mice remain 1 cluster and the *Thrb*$^{PV/PV}$ and *Thrb*$^{PV/PV}$*Kras*^{G12D} mice form another cluster, which indicates the *Thrb*$^{PV/PV}$ mice might closer to *Thrb*$^{PV/PV}$*Kras*^{G12D} mice in the gene expression profiling.

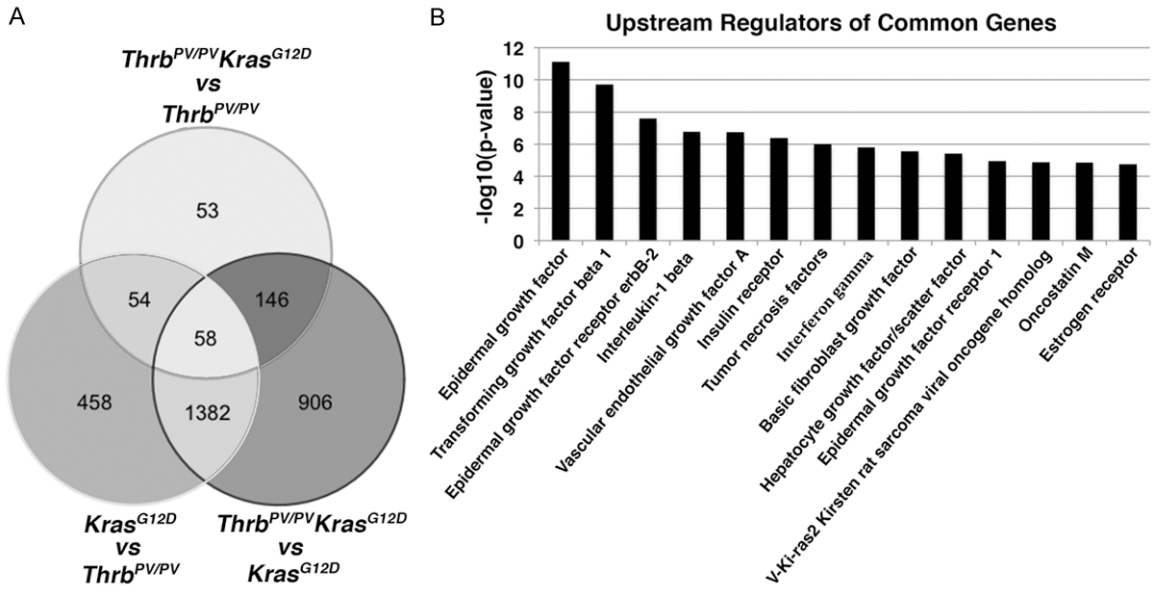

Figure 2. A. Differential and common expression genes among *Kras*^{G12D}, *Thrb*$^{PV/PV}$, and *Thrb*$^{PV/PV}$*Kras*^{G12D} mice. B. Top 14 upstream regulators derived from common genes by comparison of *Thrb*$^{PV/PV}$ vs *Thrb*$^{PV/PV}$*Kras*^{G12D} mice and *Kras*^{G12D} *vs Thrb*$^{PV/PV}$*Kras*^{G12D} mice.

Cell Signaling Technology (Danvers, MA). Primary antibodies for Integrin β1 (sc-8978), Integrin β3 (sc-14009), Integrin α5 (sc-10729), Integrin αV (sc-10719), Fibronectin (sc-6952), Integrin β4 (sc-9090), and Integrin α6 (sc-10730) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies were used at the concentration recommended by the manufacturers. For control of protein loading, the blot was probed with the antibody against GAPDH.

Statistical analysis

All data are expressed as means ± standard errors. Statistical analyses were preformed performed and $p < 0.05$ was considered significant unless otherwise specified. GraphPad Prism version 5.0 for Mac OS X was used to perform analysis of variances.

Results

Differential gene expression profiles in the thyroid of Kras^{G12D} *mice and thyroid tumors of Thrb*$^{PV/PV}$ *and Thrb*$^{PV/PV}$*Kras*^{G12D} *mice*

In our previous studies, we demonstrated that *Kras*^{G12D}, *Thrb*$^{PV/PV}$, and *Thrb*$^{PV/PV}$*Kras*^{G12D} mice had different outcomes of tumorigenesis as the mice aged. *Kras*^{G12D} mice have normal morphology up to 10 months old. During the same observation period, *Thrb*$^{PV/PV}$ mice develop follicular thyroid carcinoma. *Thrb*$^{PV/PV}$*Kras*^{G12D} mice develop aggressive undifferentiated thyroid carcinoma.

Here we obtained array data from thyroid samples of age-matched *Kras*^{G12D}, *Thrb*$^{PV/PV}$, and *Thrb*$^{PV/PV}$*Kras*^{G12D} mice (n=3 for each type of mice). **Figure 1A** shows principal component analysis (PCA) of the gene expression profiles from the mice with 3 different genotypes. The 3-dimensional projection of the top 3 principal components of PCA, capturing 89.5% of total variance, shows clear separation of the 3 groups.

A hierarchical clustering analysis shows that the expression of *Thrb*$^{PV/PV}$*Kras*^{G12D} mice was closer to that of *Thrb*$^{PV/PV}$ mice than that of *Kras*^{G12D} mice (**Figure 1B**). However, it is also clear that the gene expression profile in *Thrb*$^{PV/PV}$*Kras*^{G12D} mice was distinct from that in *Thrb*$^{PV/PV}$ mice. Comparison analysis of the array data between *Thrb*$^{PV/PV}$*Kras*^{G12D} and *Thrb*$^{PV/PV}$ mice showed that 311 genes were differentially expressed (>1.5-fold change, adjusted $p < 0.1$). Of those 311 genes, 150 were upregulated and 161 were downregulated. Comparison between *Thrb*$^{PV/PV}$*Kras*^{G12D} and *Kras*^{G12D} mice displayed 2492 genes (>1.5-fold change, adjusted $p < 0.1$); among them, 1436 genes were upregulated and 1056 were downregulated. Com-

Table 1. Upstream regulators of the common genes

Upstream Regulator	Molecule type	*p*-value of overlap
EGF	Growth factor	7.48E-12
TGFB1	Growth factor	1.90E-10
ERBB2	Kinase receptor	2.55E-08
IL1B	Cytokine	1.73E-07
VEGFA	Growth factor	1.82E-07
INSR	Kinase receptor	4.18E-07
TNF	Cytokine	1.04E-06
IFNG	Cytokine	1.58E-06
FGF2	Growth factor	2.72E-06
HGF	Growth factor	3.88E-06
EGFR	Kinase receptor	1.13E-05
KRAS	Growth factor	1.33E-05
OSM	Cytokine	1.39E-05
ESR	Nuclear receptor	1.75E-05

parison between *Thrb*$^{PV/PV}$ and *Kras*^{G12D} mice identified 1952 differentially expressed genes (>1.5-fold change, adjusted p < 0.1), of which 1143 were upregulated and 809 were downregulated.

Growth factor and growth factor receptor signaling profiles of thyroid tumors in Thrb$^{PV/PV}$*Kras*^{G12D} *mice*

The distinct clusters of data derived from *Kras*^{G12D}, *Thrb*$^{PV/PV}$, and *Thrb*$^{PV/PV}$*Kras*^{G12D} mice enabled us to compare the changes in gene expression due to either activated TRβPV signaling (compare *Thrb*$^{PV/PV}$*Kras*^{G12D} mice with *Kras*^{G12D} mice), activated Kas^{G12D} signaling (compare *Thrb*$^{PV/PV}$*Kras*^{G12D} mice with *Thrb*$^{PV/PV}$ mice) or synergistic signaling of both TRβPV and Kras^{G12D} signaling in *Thrb*$^{PV/PV}$*Kras*^{G12D} mice. Accordingly, we used a Venn diagram to analyze the relationship of gene expression profiles among the 3 comparisons. Interestingly, only 53 genes were unique to the comparison of *Thrb*$^{PV/PV}$ vs *Thrb*$^{PV/PV}$*Kras*^{G12D}. Furthermore, 204 genes were common between the comparison of *Thrb*$^{PV/PV}$ vs *Thrb*$^{PV/PV}$*Kras*^{G12D} and *Kras*^{G12D} vs *Thrb*$^{PV/PV}$*Kras*^{G12D}. We found 906 genes were specific to the comparisons of *Kras*^{G12D} vs *Thrb*$^{PV/PV}$*Kras*^{G12D} and 458 were specific to the comparison of *Kras*^{G12D} vs *Thrb*$^{PV/PV}$. In addition, 1440 genes were common between comparisons of *Thrb*$^{PV/PV}$*Kras*^{G12D} vs *Thrb*$^{PV/PV}$ and *Thrb*$^{PV/PV}$ vs *Kras*^{G12D} mice. Interestingly, only 58 genes were common to all in the 3 comparisons (**Figure 2A**).

To identify the pathways specifically involved in the aggressive thyroid tumor growth of *Thrb*$^{PV/PV}$*Kras*^{G12D} mice, we examined the upstream regulators present in the differential gene expression profiles. To do this, we uploaded common differentially expressed genes between 2 comparisons—*Thrb*$^{PV/PV}$ vs *Thrb*$^{PV/PV}$*Kras*^{G12D} and *Kras*^{G12D} vs *Thrb*$^{PV/PV}$*Kras*^{G12D}—into Ingenuity Pathway Analysis (IPA, http://www.ingenuity.com). The 14 top potential upstream regulators thus retrieved are listed with *p*-values in **Table 1**. These 14 upstream regulators could potentially drive differential expression of genes between *Thrb*$^{PV/PV}$*Kras*^{G12D} and *Thrb*$^{PV/PV}$ mice (**Figure 2B**). Among the factors analyzed, 9 of 14 genes were related signaling mediated by growth factors and their receptors (**Table 1** and **Figure 2B**). Among these factors, five were in the category of cytokines (transforming growth factor beta 1, interleukin-1 beta, tumor necrosis factors, interferon gamma, and oncostatin M). In our previous studies, we identified transforming growth factor beta 1 (TGFβ) as a critical factor driving thyroid carcinogenesis [12]. Another 8 factors [epidermal growth factor (EGF), epidermal growth factor receptor erbB2 (ERBB2), vascular endothelial growth factor A (VEGFA), insulin receptor (INSR), basic fibroblast growth factor 2 (FGF2), hepatogrowth factor/scatter factor (HGF), epidermal growth factor receptor (EGFR) and V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS)] are related to growth factor- and growth factor receptor-promoted cell signaling.

Because of the overwhelming enrichment of growth factors and growth factor receptors, we explored the mechanisms by which these upstream regulators could contribute to the aggressive growth of thyroid tumors in *Thrb*$^{PV/PV}$*Kras*^{G12D} mice. Among 4 upregulated growth factor receptors (ERBB2, INSR, EGFR, and estrogen receptor), 2 of them (ERBB2 and EGFR) belong to the EGFR family. EGF is a ligand for EGFR family members. As shown in **Figure 2B**, ERBB2 was the most upregulated growth factor receptor in *Thrb*$^{PV/PV}$*Kras*^{G12D} mice. Therefore, we further explored whether ERBB2 could play a major role in driving aggressive thyroid tumor growth in *Thrb*$^{PV/PV}$*Kras*^{G12D} mice.

Table 2. Expression of Integrins

Symbol	Accession	Probeset_id	Total_probes	Mean	Percentile of Mean
Itga1	NM_001033228	10412298	29	8.911	87.482
Itga10	NM_001081053	10494467	32	5.909	44.463
Itga11	NM_176922	10586079	30	6.289	50.023
Itga2	NM_008396	10412267	28	7.617	69.311
Itga2b	NM_010575	10391697	34	6.758	56.42
Itga3	NM_013565	10390117	29	10.078	96.342
Itga4	NM_010576	10473125	32	9.349	91.7
Itga5	NM_010577	10433114	30	7.775	71.786
Itga6	NM_008397	10472820	25	10.54	98.019
Itga7	NM_008398	10367440	30	6.347	50.821
Itga8	NM_001001309	10480090	30	6.198	48.755
Itga9	NM_133721	10590031	28	7.456	66.802
Itgad	NM_001029872	10557928	22	4.84	26.113
Itgae	NM_008399	10378286	32	5.551	39.004
Itgal	NM_001253872	10557591	32	6.267	49.634
Itgam	NM_001082960	10557862	32	7.458	66.844
Itgav	NM_008402	10473281	30	10.566	98.082
Itgax	NM_021334	10557895	29	7.388	65.942
Itgb1	NM_010578	10576661	36	10.542	98.023
Itgb2	NM_008404	10364262	34	8.343	80.074
Itgb3	NM_016780	10381809	31	7.172	62.677
Itgb4	NM_001005608	10382713	42	8.375	80.49
Itgb5	NM_001145884	10435305	30	10.401	97.529
Itgb6	NM_001159564	10483000	44	9.102	89.444
Itgb7	NM_013566	10432957	28	5.833	43.315
Itgb8	NM_177290	10403229	28	9.188	90.233

Integrin-ERBB2 signaling in thyroid tumors of $Thrb^{PV/PV}Kras^{G12D}$ mice

As 1 member of the EGFR family, ERBB2 is a receptor tyrosine kinase whose activity depends on dimerization with another ligand-binding ERBB receptor [13]. Binding of ligands such as EGF or neuregulins to the extracellular domain of ERBB receptors induces formation of dimerization and activation of the intrinsic receptor kinase activity, ultimately leading to stimulation of intracellular signaling cascades [14, 15]. The ERBB2 signaling pathway has been shown to increase several other signaling pathways including mitogen-activated protein kinase (MAPK). Overexpression of the *ERBB2* gene occurs in 15-30% of breast cancers [16]. It is strongly associated with increased breast cancer recurrence and a poor prognosis [17, 18]. Overexpression is also detected in several cancers including some differentiated and undifferentiated thyroid cancer [19-21].

To confirm the role of ERBB2 in thyroid carcinogenesis, we examined whether its expression was upregulated in thyroid tumors of $Thrb^{PV/PV}Kras^{G12D}$ mice (**Table 2**). As shown in **Figure 3A**, real-time quantitative mRNA analysis indicated that the mRNA level of the *Erbb2* gene in the thyroid of $Kras^{G12D}$ mice (bar 2) and $Thrb^{PV/PV}$ mice (bar 3) was not significantly different from that in wild-type mice (bar 1). Strikingly, the mRNA level in the thyroid tumors of $Thrb^{PV/PV}Kras^{G12D}$ mice (bar 4) was significantly higher than that in the thyroid of wild-type, $Kras^{G12D}$ mice, and thyroid tumors of $Thrb^{PV/PV}$ mice. These increases in the *Erbb2* mRNA levels indicated that the *Erbb2* gene might be critical for the aggressive thyroid tumor growth in $Thrb^{PV/PV}Kras^{G12D}$ mice.

To further validate the role of ERBB2 in thyroid carcinogenesis, we looked into other factors contributing to ERBB2 signaling. Integrins are a large family of heterodimeric transmembrane

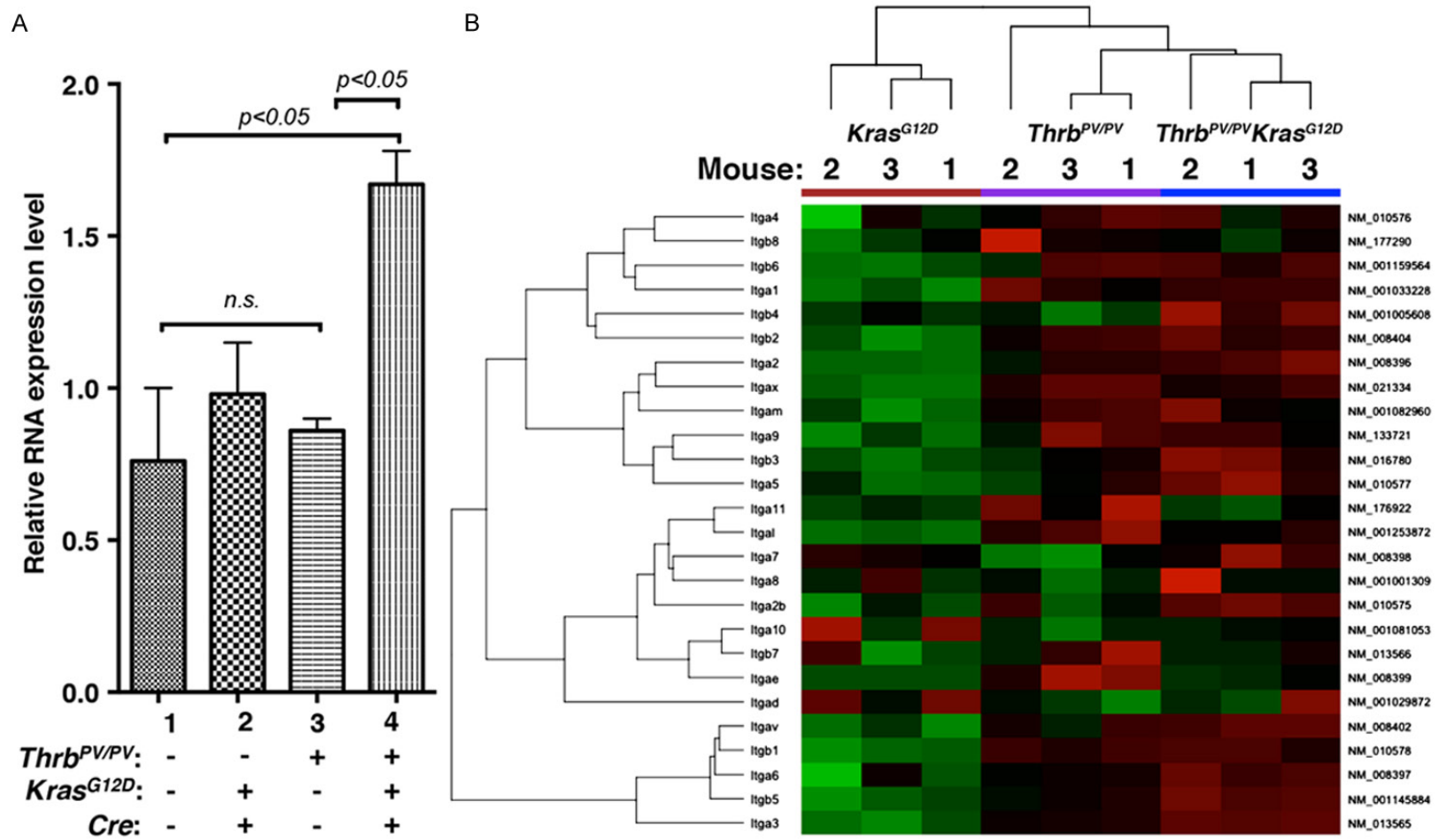

Figure 3. A. An increased mRNA expression of the *Erbb2* gene in the thyroid of $Thrb^{PV/PV}Kras^{G12D}$ mice. Total RNA was extracted from thyroids of *wild-type*, $Kras^{G12D}$, $Thrb^{PV/PV}$, and $Thrb^{PV/PV}Kras^{G12D}$ mice and analyzed by quantitative real-time RT-PCR. B. A heat-map presentation of hierarchical clustering (average of Euclidean distance) analysis of the integrins gene expression in mice with the genotypes indicated.

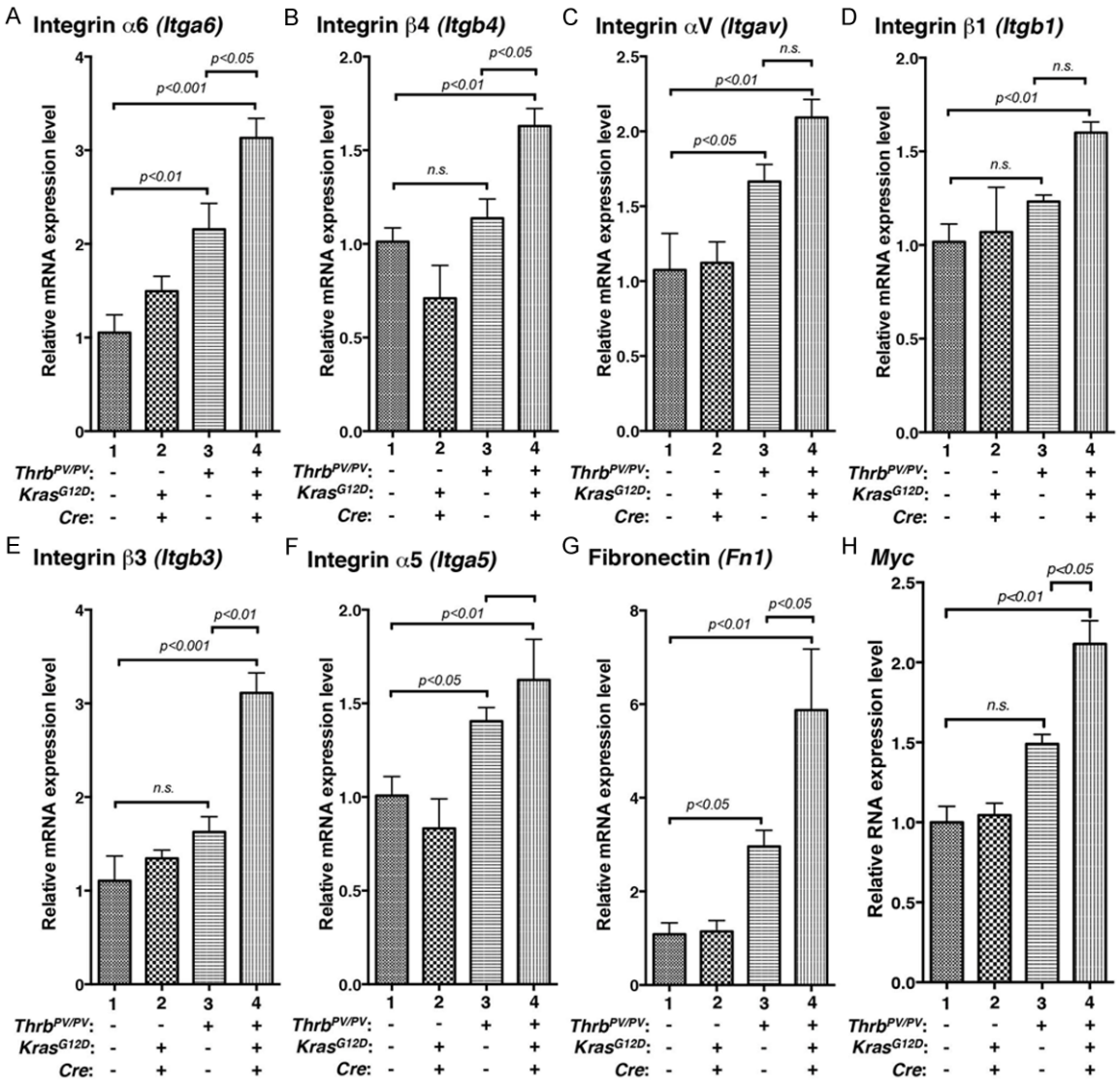

Figure 4. Validation of the extent in altered gene expression from arrays by quantitative real-time RT-PCR. Total RNA was extracted from thyroids of *wild-type*, *Kras*^{G12D}, *Thrb*$^{PV/PV}$, and *Thrb*$^{PV/PV}$*Kras*^{G12D} mice. Eight representative genes: (A) Integrin α6, (B) Integrin β4, (C) Integrin αV, (D) Integrin β1, (E) Integrin β3, (F) Integrin α5, (G) Fibronectin, and (H) *Myc*.

receptors that mediate cell binding to the extracellular matrix and link the extracellular environment to the intracellular cytoskeleton [22]. The different combination of 18 alpha subunits and 8 beta subunits gives rise to 24 distinct heterodimers [23]. Integrins belonging to the β1, αv, β7, and β4 subfamilies have been shown to potentiate signaling pathways in response to many growth factors [24, 25], cytokines [26], and TGFβ [22, 27]. Overexpression of integrin αvβ3 is associated with the progression and metastasis of several cancers including glioblastoma, carcinomas of the breast, prostate, pancreas, and lung [28]. Increased expression of αvβ6 is associated with greater invasive potential in oral squamous carcinoma, pancreatic, ovarian, breast, and lung cancer [28]. Integrin β4 forms a complex with ERBB2 and enhances activation of the transcription factors. In mice absent an integrin β4 signaling domain, both initiation and metastatic progression of mammary tumors induced by ERRB2 are significantly delayed, suggesting integrins act as cooperating oncogenes [29].

Our analyses of the gene expression profiles showed a global upregulation of integrins in *Thrb*$^{PV/PV}$*Kras*^{G12D} mice (**Figure 3B**). A hierarchi-

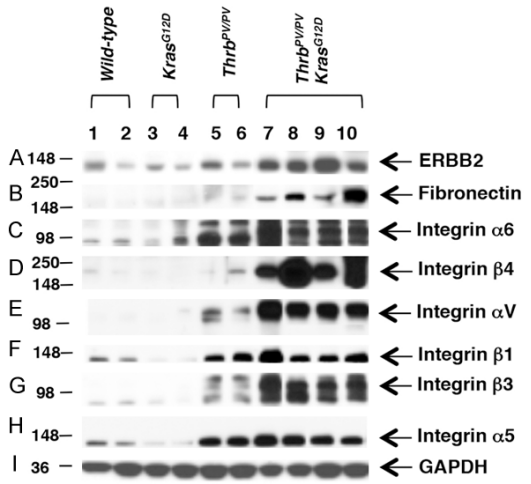

Figure 5. Protein abundance of integrin-ERBB2 signaling pathway in *wild-type*, *Kras*^{G12D}, *Thrb*$^{PV/PV}$, and *Thrb*$^{PV/PV}$*Kras*^{G12D} mice thyroids. Protein levels were measured using antibodies against (A) ERBB2, (B) Fibronectin, (C) Integrin α6, (D) Integrin β4, (E) Integrin αV, (F) Integrin β1, (G) Integrin β3, (H) Integrin α5, and (I) loading control GAPDH. Total protein extracts were prepared from thyroids of wild-type and *Kras*^{G12D} mice and thyroid tumors of *Thrb*$^{PV/PV}$ and *Thrb*$^{PV/PV}$*Kras*^{G12D} mice, and the Western blot analysis was carried out as described in Materials and Methods.

cal clustering heat map shows that the expression of integrins in *Thrb*$^{PV/PV}$*Kras*^{G12D} mice was closer to that of *Thrb*$^{PV/PV}$ mice than that of *Kras*^{G12D} mice (**Figure 3B**). However, it was also clear that the gene expression profiles in *Thrb*$^{PV/PV}$*Kras*^{G12D} mice were distinct from those in *Thrb*$^{PV/PV}$ mice.

We next used qRT–PCR to examine 3 integrin α genes and 3 integrin β genes for the validation of the gene expression profiles obtained by array analysis. Genes selected for validation were integrin α6, integrin β4, integrin αV, integrin β1, integrin β3, and integrin α5 as shown in **Figure 4A-F**. Consistent with array data, qRT–PCR showed that the expression of the 6 integrins (**Figure 4A-F**) was significantly increased in thyroid tumors of *Thrb*$^{PV/PV}$*Kras*^{G12D} mice. The expression patterns of integrins in *Thrb*$^{PV/PV}$ mice also had an upward trend to be in line with the findings in the array analysis. But the expression of integrins in *Thrb*$^{PV/PV}$ mice was lower than in *Thrb*$^{PV/PV}$*Kras*^{G12D} mice.

It is known that integrins bind to ligands such as fibronectin to transmit the signaling from the extracellular matrix into cytokeratin inside cells. Fibronectin has been implicated in the development of multiple types of human cancer [30, 31], and it has been associated with cell migration and invasion in several metastatic models [30, 32]. qRT–PCR determined that fibronectin (**Figure 4G**) was significantly increased with a pattern similar to integrins in *Thrb*$^{PV/PV}$*Kras*^{G12D} mice (**Figure 4A-F**).

In our previous studies, we found that upregulated MYC is crucial for the manifestation of aggressive tumor phenotypes [5]. We examined whether the *Myc* mRNA had an expression pattern similar to integrins. We found that the expression level of the *Myc* gene determined by qRT/PCR was significantly increased (**Figure 4H**). The expression changes of the *Myc* gene in 4 genotypes of mice were similar to that of integrins. Therefore, we identified increased Myc expression as one of the downstream effector of the integrin-ERBB2 signaling to promote tumor growth.

To confirm that ERBB2 had a crucial role in aggressive thyroid tumor growth, we further compared protein abundance of ERBB2 in thyroids of wild-type and *Kras*^{G12D} mice and in thyroid tumors of *Thrb*$^{PV/PV}$ and *Thrb*$^{PV/PV}$*Kras*^{G12D} mice. As shown in **Figure 5A**, there was no significant difference between ERBB2 protein levels in wild-type, *Kras*^{G12D}, and *Thrb*$^{PV/PV}$ mice. The level of ERBB2 protein was significantly increased only in *Thrb*$^{PV/P}$*Kras*^{G12D} mice. ERBB2 signaling has been shown to be regulated by integrins [28].

Because fibronectin is one of the ligands for integrins, we also examined its protein level. We found that the abundance of fibronectin was significantly higher in thyroid tumors of *Thrb*$^{PV/PV}$*Kras*^{G12D} mice than in WT, *Kras*^{G12D}, and *Thrb*$^{PV/PV}$ mice (compare lanes 7-10 to lanes 1-6, **Figure 5B**). Co-upregulation of ERBB2 and fibronectin supported the idea that interaction of 2 activated signaling pathways could be important for the tumor phenotypes observed in *Thrb*$^{PV/P}$*Kras*^{G12D} mice.

We also validated the protein abundance of integrins. Consistent with the highly elevated the mRNA expression (**Figure 4A-F**), Western blot analysis further showed that the protein abundance of integrins αV, β1, β3, and α5 was significantly elevated in thyroid tumors of *Thrb*$^{PV/PV}$*Kras*^{G12D} mice as compared with WT, *Kras*^{G12D}, and *Thrb*$^{PV/PV}$ mice (lanes compare

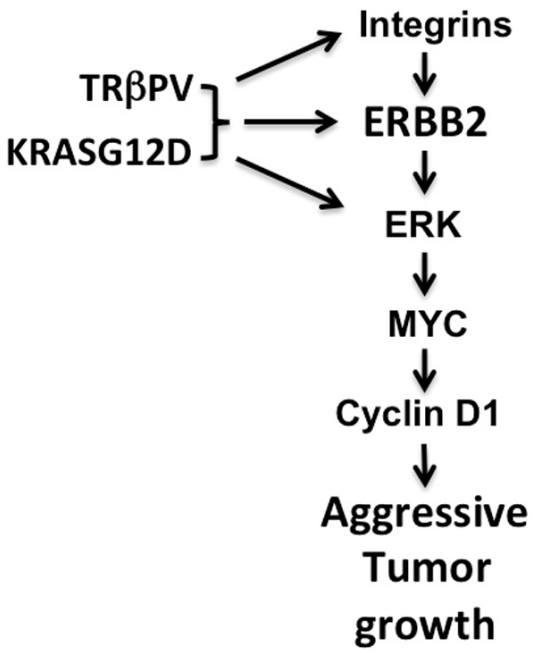

Figure 6. Proposed molecular pathways for integrin-ERBB2 signaling to stimulate proliferation of thyroid tumor cells in *Thrb*$^{PV/PV}$*Kras*^{G12D} mice. Synergistic oncogenic actions of TRβPV and Kras^{G12D} activate signaling mediated by integrins, ERBB2, and ERK, leading to increased expression of MYC. Elevated MYC stimulates downstream targets such as cyclin D1 to promote aggressive tumor growth in *Thrb*$^{PV/PV}$*Kras*^{G12D} mice.

lanes 7-10 to lanes 1-6, **Figure 5C-H**). ERBB2 has been shown to promote the formation of a multimeric complex that includes ERBB2 and α6β4 integrins. Cooperative signaling of ERBB2 and α6β4 integrins results in the activation of downstream factors that regulate carcinogenesis [29]. As shown in **Figure 5C** and **5D**, both integrin α6 and β4 were significantly increased in thyroid tumors of *Thrb*$^{PV/PV}$*Kras*^{G12D} mice. The enrichment of integrins/fibronectin and ERBB2 proteins indicated that their signaling is critical for the aggressive thyroid tumor growth.

Discussion

Recently, we genetically introduced the *Kras*^{G12D} mutation to express specifically in the thyroids of the *Thrb*$^{PV/PV}$ mice. Double mutant *Thrb*$^{PV/PV}$*Kras*^{G12D} mice have much worse survival than the mice with a single mutation in either the *Kras* or *Thrb* gene. The more aggressive thyroid tumor phenotypes result from a synergy of the oncogenic actions of 2 mutants in thyroids, leading to increased proliferation for rapid tumor expansion. However, the molecular events leading to the synergy of 2 mutants were not well characterized. In our current studies, through microarray gene profiling, the top upstream regulators identified are main growth factors or growth factor receptors, which promoted the aggressive phenotypes of undifferentiated thyroid cancer. We found that ERBB2 was increased only in the aggressive undifferentiated thyroid tumors of *Thrb*$^{PV/PV}$*Kras*^{G12D} mice, not in *Thrb*$^{PV/PV}$ mice with differentiated tumors. We further identified markedly increased integrins, such as β1, β3, β4, αV, α5 and α6, as activators to stimulate ERBB2-mediated downstream signaling in thyroid tumors of *Thrb*$^{PV/PV}$*Kras*^{G12D} mice. The present studies have uncovered the collaborative signaling by integrins and ERBB2 as one of the mechanisms in synergy mediated by the oncogenic actions of *Kras*^{G12D} and *Thrb*$^{PV/PV}$ mutants to promote the aggressive tumor growth.

The critical role of ERBB2 in cancer development and progression has been well documented. Overexpression of the *ERBB2* gene occurs in 15-30% of breast cancers [16]. Increased levels of ERBB2 are strongly associated with increased breast cancer recurrence and a poor prognosis [17, 18]. The expression of ERBB2 was detected in differentiated and undifferentiated thyroid cancer [19-21]. Binding of ligands, such as EGF or neuroregulins, to the extracellular domain of ERBB receptors results in the formation of receptor dimerization and activation of ERBB2. However, integrins such as β4 and α6 are known to amplify ERBB2 signaling [29, 33]. Importantly, it has been demonstrated that ERBB2 makes integrins collaborators in the initiation and progression of carcinogenesis. In mice without an integrin β4 signaling domain, the initiation and metastatic progression of mammary tumors induced by ERRB2 are significantly delayed [29]. In the presence of integrins, increased ERBB2 induces the intrinsic receptor kinase activity, ultimately leading to stimulation of multiple intracellular signaling cascades including the ERK pathway [14, 15].

In line with these reports, our present studies showed that there was a global upregulation of integrins, including α6 and β4, in thyroid tumors of *Thrb*$^{PV/PV}$*Kras*^{G12D} mice (**Figure 3B**). Abundance of ERBB2 was also markedly increased in the thyroid tumors of *Thrb*$^{PV/PV}$*Kras*^{G12D} mice

(**Figure 5A**). Moreover, we have previously shown activated ERK signaling and elevated MYC and cyclin D1 protein abundance in thyroid tumors $Thrb^{PV/PV}Kras^{G12D}$ mice [5]. Taken together, the present findings support the molecular pathways proposed in **Figure 6**. The multiple upstream regulators (i.e., integrins and ERBB2) are activated via synergistic oncogenic actions of TRβPV and KRASG12D, converging through ERK to increase MYC and cyclin D1 activation. These 2 critical growth signals downstream of ERK activation drive the aggressive thyroid tumor growth of $Thrb^{PV/PV}Kras^{G12D}$ mice.

The finding uncovered in the present studies that integrins-ERBB2 signaling contributes to aggressive tumor growth of undifferentiated thyroid cancer has important clinical implications. Currently, the modalities for the treatment of undifferentiated thyroid cancer are limited. ERBB2 has been shown to express in some differentiated and undifferentiated thyroid cancers [19-21]. Clinical trials are underway using anti-ERBB2 agents at various phases in several solid tumors [34]. Moreover, drugs against integrins are being developed to treat cancers [35]. Thus, integrins and ERBB2 could be tested as potential targets for the treatment of thyroid tumors with elevated integrins and ERBB2 expression and/or with resistance to therapy [36].

The prevalent genetic alterations identified in undifferentiated thyroid cancer are point mutations of the *RAS* genes together with the mutations of the *TP53* and *CTNNB1* genes. These observations suggest that mutations of the *RAS* genes alone might not be sufficient to bring out the aggressive dedifferentiated thyroid cancer. In our studies, we found mice harboring the single mutation of the $Kras^{G12D}$ gene did not develop thyroid tumors [5]. $Thrb^{PV/PV}$ mice with the mutation of the 2 alleles of the *Thrb* gene develop differentiated thyroid cancer. However, $Thrb^{PV/PV}Kras^{G12D}$ mice exhibit aggressive dedifferentiated thyroid cancer with large and rapid tumor growth [5]. The present studies show that synergistic oncogenic actions of TRβPV and KRASG12D brought out the aggressive tumor phenotypes. Thus findings from the studies using the mouse models support clinical observations that other oncogenic alterations in addition to a single mutation of the *RAS* gene are necessary to bring out the dedifferentiated thyroid cancer. At present, the homozygous mutations of the *THRB* gene have yet to be discovered in human dedifferentiated thyroid cancer. However, it is known that in $Thrb^{PV/PV}$ mice, altered signaling pathways such as the activation of the *CTNNB1* gene have been demonstrated. Thus, it is likely that synergistic oncogenic actions of TRβPV and KRASG12D could be, at least, the result of cross talk of KRASG12D- and TRβPV-mediated activated β-catenin signaling, which is consistent with observations in human patients [37]. Thus, the $Thrb^{PV/PV}Kras^{G12D}$ mouse is an excellent thyroid cancer model to further elucidate other altered signaling pathways during development of undifferentiated thyroid cancer.

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Disclosure of conflict of interest

The authors declare no conflicts of interest.

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