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 ThrbPV (ThrbPV/PV) spontaneously develop differentiated follicular thyroid carcinoma similar to human thyroid cancer. We recently demonstrated that targeting a RAS mutation (KrasG12D) to the thyroid of ThrbPV/PV mice (ThrbPV/PV/KrasG12D 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 KrasG12D mice and thyroid tumor lesions of ThrbPV/PV and ThrbPV/PV/KrasG12D mice. Analyses of microarray data identified 14 upstream regulators that were significantly altered in thyroid tumors of ThrbPV/PV and ThrbPV/PV/KrasG12D 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 ThrbPV/PV/KrasG12D 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, the 10-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 (ThrbPV/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 KrasG12D mutation to express specifically in the thyroids of the ThrbPV/PV mice. We found that double mutant ThrbPV/PV/KrasG12D 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 ThrbPV/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$^{PV/PV}$ and 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$^{PV/PV}$, Kras$^{SL-G12D}$, 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 littersmates 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’-cgtccctgtaga-caaatgtggt-3’; reverse, 5’-gaatttgccgtgagtgga-gt-3’. For Itga6, forward, 5’-CAGGTGTTGGA-ACAGCACAT, reverse, 5’-AAGAACAGGAGGA- GTGA-3’. For Itgb4, forward, 5’-GAGGGGCCCT-TATAGCTCAct-3’; reverse, 5’-GGTTCACAGAG- CCCCTTCT3’. For Itgb1, forward, 5’-TCTGTGCTA-TGTGTTGGAGACT-3’; reverse, 5’-CACAGTTGTG- ACGGCCACCT-3’. For Itgb3, forward, 5’-TGACATCGAGCAGGTGAAG-3’; reverse, 5’-GAGTACG- AAGGCCATGAC-3’. For Itgav, forward, 5’-TAAAGAGCAAGGAGCAATAT-3’; reverse, 5’-AATGCC- CCCAGGTGATGTTAG-3’. For Fibronectin, forward, 5’-GAGGGGCCCITCCAGCACAT, reverse, 5’-AAGAACAGGCAGGAGGATGA-3’. For Itga6, forward, 5’-CAGGTGTTGGA-ACAGCACAT, reverse, 5’-AAGAACAGGAGGA-GATGA-3’. For Itgb4, forward, 5’-GAGGGGCCCT-TATAGCTCAct-3’; reverse, 5’-GGTTCACAGAG- CCCCTTCT3’. For Itgb1, forward, 5’-TCTGTGCTA-TGTGTTGGAGACT-3’; reverse, 5’-CACAGTTGTG- ACGGCCACCT-3’. For Itgb3, forward, 5’-TGACATCGAGCAGGTGAAG-3’; reverse, 5’-GAGTACG- AAGGCCATGAC-3’. For Itgav, forward, 5’-TAAAGAGCAAGGAGCAATAT-3’; reverse, 5’-AATGCC- CCCAGGTGATGTTAG-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 Am J Cancer Res 2014;4(6):776-788
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 \(\text{Kras}^{G12D}\) mice and thyroid tumor cells of \(\text{Thrb}^{PV/PV}\) and \(\text{Thrb}^{PV/PV}\text{Kras}^{G12D}\) 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 \(\text{Kras}^{G12D}\) mice remain 1 cluster and the \(\text{Thrb}^{PV/PV}\) and \(\text{Thrb}^{PV/PV}\text{Kras}^{G12D}\) mice form another cluster, which indicates the \(\text{Thrb}^{PV/PV}\) mice might closer to \(\text{Thrb}^{PV/PV}\text{Kras}^{G12D}\) mice in the gene expression profiling.
Integrin-ERBB2 signaling in undifferentiated thyroid cancer

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 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-

![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.](image-url)
Integrin-ERBB2 signaling in undifferentiated thyroid cancer

Table 1. Upstream regulators of the common genes

<table>
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<tr>
<th>Upstream Regulator</th>
<th>Molecule type</th>
<th>p-value of overlap</th>
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<tbody>
<tr>
<td>EGF</td>
<td>Growth factor</td>
<td>7.48E-12</td>
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<tr>
<td>TGFβ1</td>
<td>Growth factor</td>
<td>1.90E-10</td>
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<td>ERBB2</td>
<td>Kinase receptor</td>
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<td>IL1B</td>
<td>Cytokine</td>
<td>1.73E-07</td>
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<td>VEGFA</td>
<td>Growth factor</td>
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<td>INSR</td>
<td>Kinase receptor</td>
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<td>TNF</td>
<td>Cytokine</td>
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<td>KRAS</td>
<td>Growth factor</td>
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<td>OSM</td>
<td>Cytokine</td>
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<td>ESR</td>
<td>Nuclear receptor</td>
<td>1.75E-05</td>
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</tbody>
</table>

Comparison between Thrb<sup>PV/PV</sup> and Kras<sup>G12D</sup> 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<sup>PV/PV</sup> vs Kras<sup>G12D</sup> mice

The distinct clusters of data derived from Kras<sup>G12D</sup>, Thrb<sup>PV/PV</sup>, and Thrb<sup>PV/PV</sup>/Kras<sup>G12D</sup> mice enabled us to compare the changes in gene expression due to either activated TRβPV signaling (compare Thrb<sup>PV/PV</sup>/Kras<sup>G12D</sup> mice with Kras<sup>G12D</sup> mice), activated Kas<sup>G12D</sup> signaling (compare Thrb<sup>PV/PV</sup>/Kras<sup>G12D</sup> mice with Thrb<sup>PV/PV</sup> mice) or synergistic signaling of both TRBPV and Kas<sup>G12D</sup> signaling in Thrb<sup>PV/PV</sup>/Kras<sup>G12D</sup> 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<sup>PV/PV</sup> vs Thrb<sup>PV/PV</sup>/Kras<sup>G12D</sup>. Furthermore, 204 genes were common between the comparison of Thrb<sup>PV/PV</sup> vs Thrb<sup>PV/PV</sup>/Kras<sup>G12D</sup> and Kras<sup>G12D</sup> vs Thrb<sup>PV/PV</sup>/Kras<sup>G12D</sup>. We found 906 genes were specific to the comparisons of Kras<sup>G12D</sup> vs Thrb<sup>PV/PV</sup>/Kras<sup>G12D</sup> and 458 were specific to the comparison of Kras<sup>G12D</sup> vs Thrb<sup>PV/PV</sup>. In addition, 1440 genes were common between comparisons of Thrb<sup>PV/PV</sup>/Kras<sup>G12D</sup> vs Thrb<sup>PV/PV</sup> and Thrb<sup>PV/PV</sup> vs Kras<sup>G12D</sup> 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<sup>PV/PV</sup>/Kras<sup>G12D</sup> 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<sup>PV/PV</sup> vs Thrb<sup>PV/PV</sup>/Kras<sup>G12D</sup> and Kras<sup>G12D</sup> vs Thrb<sup>PV/PV</sup>/Kras<sup>G12D</sup>—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<sup>PV/PV</sup>/Kras<sup>G12D</sup> and Thrb<sup>PV/PV</sup> 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-Kras2 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<sup>PV/PV</sup>/Kras<sup>G12D</sup> 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<sup>PV/PV</sup>/Kras<sup>G12D</sup> mice. Therefore, we further explored whether ERBB2 could play a major role in driving aggressive thyroid tumor growth in Thrb<sup>PV/PV</sup>/Kras<sup>G12D</sup> mice.
Integrin-ERBB2 signaling in thyroid tumors of Thrb<sup>PV/PV</sup> Kras<sup>G12D</sup> 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].

Table 2. Expression of Integrins

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Accession</th>
<th>Probeset_id</th>
<th>Total_probes</th>
<th>Mean</th>
<th>Percentile of Mean</th>
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To confirm the role of ERBB2 in thyroid carcinogenesis, we examined whether its expression was upregulated in thyroid tumors of Thrb<sup>PV</sup> Kras<sup>G12D</sup> 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<sup>G12D</sup> mice (bar 2) and Thrb<sup>PV</sup> 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<sup>PV</sup> Kras<sup>G12D</sup> mice (bar 4) was significantly higher than that in the thyroid of wild-type, Kras<sup>G12D</sup> mice, and thyroid tumors of Thrb<sup>PV</sup> mice. These increases in the Erbb2 mRNA levels indicated that the Erbb2 gene might be critical for the aggressive thyroid tumor growth in Thrb<sup>PV</sup> Kras<sup>G12D</sup> 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
Integrin-ERBB2 signaling in undifferentiated thyroid cancer

Figure 3. A. An increased mRNA expression of the Erbb2 gene in the thyroid of Thrb<sup>PV/PV</sup>Kras<sup>G12D</sup> mice. Total RNA was extracted from thyroids of wild-type, Kras<sup>G12D</sup>, Thrb<sup>PV/PV</sup>, and Thrb<sup>PV/PV</sup>Kras<sup>G12D</sup> 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-
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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<sup>PV/PV</sup>Kras<sup>G12D</sup> mice. The expression patterns of integrins in Thrb<sup>PV/PV</sup> mice also had an upward trend to be in line with the findings in the array analysis. But the expression of integrins in Thrb<sup>PV/PV</sup>Kras<sup>G12D</sup> mice was lower than in Thrb<sup>PV/PV</sup>Kras<sup>G12D</sup> 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<sup>PV/PV</sup>Kras<sup>G12D</sup> 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<sup>G12D</sup> mice and in thyroid tumors of Thrb<sup>PV/PV</sup> and Thrb<sup>PV/PV</sup>Kras<sup>G12D</sup> mice. As shown in Figure 5A, there was no significant difference between ERBB2 protein levels in wild-type, Kras<sup>G12D</sup>, and Thrb<sup>PV/PV</sup> mice. The level of ERBB2 protein was significantly increased only in Thrb<sup>PV/PV</sup>Kras<sup>G12D</sup> 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<sup>PV/PV</sup>Kras<sup>G12D</sup> mice than in WT, Kras<sup>G12D</sup>, and Thrb<sup>PV/PV</sup> 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<sup>PV/PV</sup>Kras<sup>G12D</sup> 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<sup>PV/PV</sup>Kras<sup>G12D</sup> mice as compared with WT, Kras<sup>G12D</sup>, and Thrb<sup>PV/PV</sup> mice (lanes compare
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Figure 6. Proposed molecular pathways for integrin-ERBB2 signaling to stimulate proliferation of thyroid tumor cells in \( Tbrb^{PV/PV}Kras^{G12D} \) mice. Synergistic oncogenic actions of TRPV 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 \( Tbrb^{PV/PV}Kras^{G12D} \) mice.

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 \( \alpha6\beta4 \) 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 \( \beta4 \) signaling domain, the initiation and metastatic progression of mammary tumors induced by ERR2 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 \( \alpha6 \) and \( \beta4 \), in thyroid tumors of \( Tbrb^{PV/PV}Kras^{G12D} \) mice (Figure 3B). Abundance of ERBB2 was also markedly increased in the thyroid tumors of \( Tbrb^{PV/PV}Kras^{G12D} \) mice.

Discussion

Recently, we genetically introduced the Kras\(^{G12D} \) mutation to express specifically in the thyroids of the \( Tbrb^{PV/PV} \) mice. Double mutant \( Tbrb^{PV/PV}Kras^{G12D} \) mice have much worse survival than the mice with a single mutation in either the Kras or \( Tbrb \) 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 \( Tbrb^{PV/PV}Kras^{G12D} \) mice, not in \( Tbrb^{PV/PV} \) mice with differentiated tumors. We further identified markedly increased integrins, such as \( \beta1, \beta3, \beta4, \alpha\nu, \alpha5 \) and \( \alpha\delta \), as activators to stimulate ERBB2-mediated downstream signaling in thyroid tumors of \( Tbrb^{PV/PV}Kras^{G12D} \) 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 \( Tbrb^{PV/PV} \) mutants to promote the aggressive tumor growth.

Integrins
\[ \downarrow \]
ERBB2
\[ \downarrow \]
ERK
\[ \downarrow \]
MYC
\[ \downarrow \]
Cyclin D1
\[ \downarrow \]
Aggressive Tumor growth

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 \( \alpha6\beta4 \) integrins. Cooperative signaling of ERBB2 and \( \alpha6\beta4 \) integrins results in the activation of downstream factors that regulate carcinogenesis [29]. As shown in Figure 5C and 5D, both integrin \( \alpha6 \) and \( \beta4 \) were significantly increased in thyroid tumors of \( Tbrb^{PV/PV}Kras^{G12D} \) mice. The enrichment of integrins/fibronectin and ERBB2 proteins indicated that their signaling is critical for the aggressive thyroid tumor growth.
(Figure 5A). Moreover, we have previously shown activated ERK signaling and elevated MYC and cyclin D1 protein abundance in thyroid tumors ThrbPV/PV/KrasG12D 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 ThrbPV/PV/KrasG12D 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 under way 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 KrasG12D gene did not develop thyroid tumors [5]. ThrbPV/PV mice with the mutation of the 2 alleles of the Thrb gene develop differentiated thyroid cancer. However, ThrbPV/PV/KrasG12D 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 ThrbPV/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 ThrbPV/PV/KrasG12D 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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