KSR1 is coordinately regulated with Notch signaling and oxidative phosphorylation in thyroid cancer

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Abstract

Kinase suppressor of RAS1 (KSR1) is a scaffold protein implicated in RAS-mediated RAF activation. However, the molecular function of KSR in papillary thyroid cancer (PTC) is unknown. Thus, this study aimed to characterize the role of KSR1 in patients with PTC. qRT-PCR and immunohistochemistry (IHC) revealed inter-tumor heterogeneities in the expression of KSR1 in PTC tissues. Interestingly, BRAFV600E-positive PTC showed higher KSR1 mRNA expression than BRAFV600E-negative PTC (P < 0.001). Gene Set Enrichment Analysis (GSEA) using public repositories showed that high KSR1 expression coordinately upregulated Notch signaling (nominal P = 0.019, false discovery rate (FDR) q-value = 0.165); this finding was supported by GeneNetwork analysis, indicating that KSR1 expression is positively correlated with NOTCH1 expression (r = 0.677, P = 6.15 × 10^-9). siRNA against KSR1 (siKSR1) significantly decreased ERK phosphorylation induced by BRAFV600E, resulting in reduced expression of NOTCH1 and HES1, targets of Notch signaling. GSEA revealed that high KSR1 expression was also associated with downregulation of genes related to oxidative phosphorylation (OxPhos). Consistent with this, electron microscopy showed that PTCs with high KSR1 expression exhibited structural defects of the mitochondrial cristae. Furthermore, siKSR1-transfected BCPAP and 8505C cells generated fewer colonies in colony-forming assays. In addition, GSEA showed that high expression of KSR2 and connector enhancer of KSR1 (CNKSR1) also coordinately upregulated Notch signaling (KSR2: nominal P = 0.0097, FDR q-value = 0.154 and CNKSR1: nominal P < 0.0001, FDR q-value = 0.00554), and high CNKSR2 was associated with downregulation of the OxPhos gene set (nominal P < 0.0001, FDR q-value < 0.0001). In conclusion, KSR1 is coordinately regulated with Notch signaling and OxPhos in PTC, because its scaffold function might be required to sustain the proliferative signaling and metabolic remodeling associated with this type of cancer.

Key Words
- kinase suppressor of RAS (KSR)
- connector enhancer of KSR (CNKSR)
- thyroid cancer
- notch signaling
- oxidative phosphorylation

Introduction

Papillary thyroid cancer (PTC) is the most common endocrine malignancy, and generally has favorable outcomes (DeGroot et al. 1990, Wada et al. 2003, Pacini et al. 2006, Kweon et al. 2013); however, a significant proportion of patients with PTC exhibit poor clinical outcomes such as lymph node (LN) metastasis, distant
metastasis, and even fatalty (Mirallie et al. 1999, Pereira et al. 2005, Bilimoria et al. 2007). Surgery followed by radioactive iodine therapy has been the gold-standard treatment regimen for differentiated thyroid cancer (DTC; Mazzaferr & Jhiang 1994, Pacini et al. 2006, American Thyroid Association Guidelines Taskforce on Thyroid N et al. 2009, Alzahrani & Xing 2013); however, recurrent or metastasized lesions sometimes cannot be cured by surgical resection or radioactive iodine therapy (Riesco-Eizaguirre et al. 2009, Xing et al. 2013). Recently, sorafenib (Nexavar) has been approved by the FDA to treat patients with locally advanced or metastatic DTC that is refractory to radioactive iodine; however, the Phase 3 DECISION trial did not demonstrate a significant improvement in overall survival (Brose et al. 2014).

The RAS–RAF–MAPK pathway is an intracellular chain of proteins that transduces a signal from a membrane receptor to the nucleus. In many cancers, including BRAFV600E-driven tumors, the sustained activation of this pathway causes uncontrolled growth and cell survival, promoting carcinogenesis (Hanahan & Weinberg 2011). The signaling pathway comprises many proteins, including various adaptor and scaffold proteins. Recent studies have shown that two of these scaffold proteins, kinase suppressor of RAS (KSR) and connector enhancer of KSR (CNKSR), regulate RAS-mediated RAF activation via novel mechanisms (Anselmo et al. 2002, Lanigan et al. 2003, Claperon & Therrien 2007, Brennan et al. 2011).

Several compounds, including sorafenib, dabrafenib, and vemurafenib, have inhibitory effects on the RAS–RAF–MAPK pathways (Bollag et al. 2010, 2012, Chapman et al. 2011, Hauschild et al. 2012); however, tumors acquire resistance to RAF inhibitors by several mechanisms (Lito et al. 2013). In BRAFV600E-driven tumors, ERK-dependent negative feedback suppresses rzeceptor tyrosine kinase (RTK)-mediated signaling, resulting in low levels of GTP-bound RAS (Villanueva et al. 2011, Lito et al. 2013); however, inhibition of ERK by RAF inhibitors substantially decreases ERK-dependent negative feedback, resulting in the restoration of RTK signaling and activation of RAS (Corcoran et al. 2012, Montero-Conde et al. 2013). Regarding the mechanism of drug resistance related to the loss of ERK-dependent negative feedback, we postulate that KSR and CNKSR play a role in the restoration of the ERK proliferative signal induced by RTK signaling (Therrien et al. 1998, Anselmo et al. 2002, Ohmachi et al. 2002, Lanigan et al. 2003, Claperon & Therrien 2007). In support of this idea, ERK activation induced by RTK signaling is mediated by RAF dimers that are insensitive to RAF inhibitors, indicating that formation of a signalosome complex tethered by scaffold proteins is a pivotal step in ERK signal propagation (Berman et al. 2003, Liu et al. 2009, Lito et al. 2013).

In this study, we investigated the expression status of KSR1 in patients with PTC before treatment with targeted agents, with the goal of gaining insights into the role of KSR1 in RAS–RAF–MAPK signal propagation in PTC and identifying potential targets for drugs that would be effective against PTC, regardless of the presence or absence of de novo or acquired drug resistance to novel targeted agents. To this end, we examined the expression levels of KSR1 by qRT-PCR and immunohistochemistry (IHC), which revealed KSR1 inter-tumor heterogeneity. Bioinformatic analysis showed that KSR and CNKSR are associated with Notch signaling and mitochondrial oxidative phosphorylation (OxPhos). Furthermore, the results of cell-based assays using siRNA against KSR1 (siKSR1) and electron microscopy (EM) of human PTC samples indicated that KSR1 participates in the propagation of the Notch signal, mitochondrial respiration, and colony formation in vitro under BRAFV600E-activated conditions.

Materials and methods

Subjects

We enrolled 165 patients who underwent thyroidec- tomy for the management of classical PTC between January 2013 and January 2014 at Yonsei Cancer Center, Severance Hospital, Seoul, South Korea. All samples were microscopically dissected at the time of surgery and validated by hematoxylin–eosin staining after the operation. The samples were taken from the central part of the cancer and from contralateral histologically normal tissue. All protocols were approved by the Institutional Review Board of Severance Hospital.

DNA isolation and sequencing

Genomic DNA from formalin-fixed paraffin-embedded specimens was prepared (QIAamp DNA FFPE Tissue Kit; Qiagen). To examine for the presence of the BRAF1799A mutation, the BRAF gene was amplified by PCR using the following primers: forward 5'-ATG CTT GCT CTG ATA GGA-3' and reverse 5'-ATT TTT GTG AAT ACT GGG GAA-3'. The PCR products were sequenced on an ABI PRISM 3100 automated capillary DNA Sequencer (Applied Biosystems).

RNA isolation and qRT-PCR

Total RNA was extracted using TRIzol (Invitrogen). cDNA was prepared from total RNA using the Super Script
First-Strand Synthesis System (Invitrogen). Real-time PCR measurement of individual cDNAs was performed using SYBR Green dye to measure duplex DNA formation on a Light Cycler System (Roche Diagnostics). cDNA were amplified using the following primers: KSR1, 5'-GGG GAG CAC AAG GAG GAC T-3' and 5'-GGG TTC AGG GGA ATA CAG G-3'; Notch 1 (NOTCH1), 5'-GAG GCG CTT GGA ACT ATG C-3' and 5'-GTT GTA CTC CGT CAG GTG GA-3'; Notch 4 (NOTCH4), 5'-GAT GGG CTA GAC ACC TAC AC-3' and 5'-CAT CGA CGA TTC GGA ACC AGT-3'; NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4 (NDUFA4), 5'-ATG CTC CGC CAG ATC ATC G-3' and 5'-TGC CAG AGC CAA GAG ATA CAG-3'; cytochrome c oxidase assembly protein (COX11), 5'-AGA GGG TAG AGC CTT TCT TTA G-3' and 5'-GGG TGT GAA AGG GTT CGA G-3'; KSR2, 5'-AGA GGG GCT TAG GAC CAA ATG-3' and 5'-CAT CGA CGA TTC GGA ACC AGT-3'; hairy and enhancer of split 1 (HES1), 5'-TCA ACA CGA CAC CGG ATA AAC-3' and 5'-GCC GCG AGC ATG TAT CTT TCT TCA-3'; CNKSR1, 5'-AAG CCT GAC AGA AGG ACT TCT-3' and 5'-AGG ACA TCA ATA GGG GTC TGT G-3'; CNKSR2, 5'-TCA GTT GTG GAT CTT GAT ATT GGA GC-3' and 5'-CGA CAG GAT GAT GTG GTC AC-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-GGG GCG AGA TCC CTC CAA AAT-3' and 5'-GGG TGT GGT CAT ACT TCT CAT GG-3'. QRT-PCR experiments were repeated three times, and each experiment was performed in triplicate. Relative mRNA expression levels were calculated using the equation 2ΔCt = 2^(-ACTarget − CtTarget) and then expressed as a ratio (level in PTC/level in matched normal thyroid tissue).

**IHC analysis**

Paraffin-embedded tissue sections (4 μm thick) were deparaffinized in xylene and stained with anti-KSR1 antibody (sc-25416, Santa Cruz Biotechnology). The avidin–biotin complex/HRP Kit (DAKO, Copenhagen, Denmark) was used according to a standard three-step technique. Negative controls were incubated with PBS instead of primary antibody, and normal adrenal gland tissues were used as positive controls.

**Public data and statistical analysis**

Data from the Gene Expression Omnibus (GEO) of NCBI (http://www.ncbi.nlm.nih.gov/projects/geo; accession no. GSE33630) were subjected to Gene Set Enrichment Analysis (GSEA) (Subramanian et al. 2005). Analysis of the correlation between KSR1 and NOTCH1 was performed using GeneNetwork (a free scientific web resource, http://www.genenetwork.org/). Statistical analysis was carried out using SPSS version 18.0 for Windows (IBM Corporation, Armonk, NY, USA) or GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). Comparisons of means were performed by paired t-test or Mann–Whitney U test. Data are expressed as means ± S.E.M., *P < 0.05, **P < 0.01, and ***P < 0.001. All reported P values are two sided.

**Cells, transfection, and immunoblot analysis**

BCPAP and 8505C cells were cultured in RPMI media (Sigma–Aldrich) and HeLa cells were grown in DMEM (Sigma–Aldrich). The cells were transiently transfected with pEF-mBRAFV600E or SiKSR1 (sc-35762, Santa Cruz Biotechnology) using Lipofectamine PLUS (Invitrogen). The cells were lysed in lysis buffer, and the lysates were separated by SDS–PAGE. After proteins were transferred to a nitrocellulose membrane (Amersham Biosciences), the membranes were blocked with 5% skimmed milk and incubated with the indicated primary antibodies overnight at 4 °C; after washing, the membranes were incubated with secondary antibodies for 1 h at room temperature. The immunoreactive bands were developed using peroxidase-conjugated secondary antibodies (WEST-ZOL, iNtRON Biotechnology, Kyungki-Do, South Korea). The primary antibodies used in this study were anti-BRAF (F-7) (sc-5284, Santa Cruz Biotechnology), anti-KSR1 (sc-25416, Santa Cruz Biotechnology), anti-Notch1 (#4380, Cell Signaling, Danvers, MA, USA), anti-phospho-p44/42 MAPK (Thr202/Tyr204) (#4376, Cell Signaling), and anti-actin (sc-1616, Santa Cruz Biotechnology).

**EM analysis**

Tissues were fixed in 2% paraformaldehyde (Sigma–Aldrich) and 2% glutaraldehyde (Sigma–Aldrich) overnight. After rinsing in 0.1 M cacodylate, samples were postfixed in 1% OsO4 in cacodylate buffer (Hampton Research, Aliso Viejo, CA, USA). The samples were dehydrated through graded ethanol (10–100%, Merck KgaA) for 10 min each, and then embedded in Epon (EMS, Hatfield, PA, USA). The prepared samples were analyzed by an EM (Tecnai G2 Spirit Twin; FEI company; Korea Basic Science Institute). The morphology of mitochondria cristae was quantified by measuring the lengths of cristae in a mitochondrion and normalizing the lengths by the area of the mitochondrion using ImageJ Software (NIH, Bethesda, MD, USA; Schneider et al. 2012). For comparison, the average ratio of cristae lengths to mitochondrial area in PTC samples with low KSR1 mRNA expression was set to 100% (Costa et al. 2010).
Colony formation assays

The cells (5.0×10^2 cells/well) were seeded into six-well cell culture plates and allowed to grow for 14 days. Every 2 days, the cells were transfected with siKSR1 (sc-35762, Santa Cruz Biotechnology) using JetPei (Polyplus-Transfection SA, Illkirch, France). The colonies were fixed in methanol and stained with crystal violet (Sigma–Aldrich). Visible colonies containing ~50 or more cells were counted. Three independent experiments were performed in triplicate (Franken et al. 2006).

Results

Expression of KSR1 in normal human thyroid tissues and PTCs

To evaluate the expression pattern of KSR1/2, we first performed qRT-PCR using cDNA from PTCs and matched contralateral normal thyroid tissues. The results revealed inter-tumor heterogeneity of KSR1 expression (Fig. 1A); however, 108 (65.5%) out of 165 cases expressed higher levels of KSR1 mRNA in tumors than that in control tissue, and the difference was statistically significant (paired t-test, P<0.001). Interestingly, the mean relative KSR1 mRNA level of BRAFV600E-positive PTC samples was higher than that of BRAFV600E-negative PTC samples (P<0.001, Fig. 1B). To validate our qRT-PCR data, we conducted IHC analysis using anti-KSR1 antibody (n=165). Tumor cells in PTCs with high levels of KSR1 mRNA consistently exhibited abundant protein expression relative to adjacent normal tissues (Fig. 1C and D). Furthermore, metastatic LNs from PTCs with high levels of KSR1 mRNA also exhibited markedly elevated KSR1 expression (Fig. 1E). Taken together, these data indicate that a subset of PTCs express higher levels of KSR1 mRNA than that of normal thyroid tissues, suggesting that PTC can be classified according to KSR1 expression status.

Figure 1

Expression of kinase suppressor of RAS1 (KSR1) in normal human thyroid tissues and papillary thyroid cancers (PTCs). (A) Result of qRT-PCR to compare KSR1 mRNA expression in PTCs vs matched normal thyroid tissues. (B) Comparison of KSR1 mRNA expression in PTCs according to BRAFV600E mutation status. Relative mRNA expression ratio in PTCs was calculated as the relative mRNA level in the PTC/relative mRNA level in matched normal thyroid tissue. Mean ratios were compared by paired t-test. ***P<0.001. (C, D and E) Representative immunohistochemical staining for KSR1 in tissue sections of PTC or metastatic lymph nodes (original magnification, ×200).
Analysis of enriched gene sets in PTCs expressing high levels of KSR1 mRNA

Next, we sought to identify enriched gene sets in PTCs expressing high levels of KSR1 mRNA. To this end, we used public repository GEO dataset GSE33630, which includes data from 49 PTCs obtained from Ukraine via the Chernobyl Tissue Bank. Among these, we selected the 16 PTCs with the highest KSR1 expression (high-KSR1 PTCs) and 16 PTCs with the lowest KSR1 expression (low-KSR1 PTCs) and subjected these samples to GSEA.

As summarized in Supplementary Table 1, see section on supplementary data given at the end of this article, the top 20 gene sets enriched in high-KSR1 PTCs included the wingless-type MMTV integration site family (WNT) signaling pathway (nominal $P=0.0014$, false discovery rate (FDR) $q$-value $=0.131$), colorectal cancer (nominal $P=0.0138$, FDR $q$-value $=0.1475$), and the Notch signaling pathway (nominal $P=0.019$, FDR $q$-value $=0.1647$) (Fig. 2A). Compatible with the results of GSEA, Gene-Network analysis using repository data (Record ID: Nominal $P$ value $=0.019$ Low KSR1 A BC High KSR1 DLL4 60 86 42 20 06 42 20 01 n = 53 $\rho=0.677$ P $=6.15 \times 10^{-9}$) Spearman’s rank correlation Relative mRNA expression Mean comparisons were performed by the Mann–Whitney U test. Data are mean ± s.e.m. and $P$ values are two-sided, **$P<0.01$.}

Figure 2
Bioinformatic analysis indicating the relationship between kinase suppressor of RAS1 (KSR1) and Notch signaling. (A) Gene Set Enrichment Analysis revealed enrichment of genes involved in Notch signaling in papillary thyroid cancers (PTCs) with high KSR1 expression. (B) Gene-Network Analysis revealed a strong positive correlation between KSR1 and NOTCH1 expression levels in repository data (Spearman’s rank correlation, $\rho=0.677$, $P=6.15 \times 10^{-9}$; INIA Adrenal Affy MoGene 1.0ST (Jun 12) RMA exon level: 10388917). (C) QRT-PCR data indicated mRNA expression of NOTCH1, NOTCH4, NDUF4, and COX11 in PTC samples with low KSR1 expression (white bars, $n=7$ per group) and in PTC samples with high KSR1 expression (blue bars, $n=7$ per group). Mean comparisons were performed by the Mann–Whitney U test. Data are mean ± s.e.m. and $P$ values are two-sided, **$P<0.01$.}
10388917) also revealed a strong positive correlation between KSR1 and NOTCH1 expression levels (Supplementary Fig. 1 and Fig. 2B). As shown in Supplementary Table 2, the top 20 gene sets enriched in low-KSR1 PTCs included OxPhos (nominal \( P < 0.001 \), FDR q-value = 0.0069) (Supplementary Fig. 2). In accord with the results of GSEA, qPCR data obtained using cDNA from the study subjects indicated that PTC samples with high KSR1 expression had higher NOTCH1 and NOTCH4 mRNA levels and lower NDUF44 and COX11 mRNA levels than PTC samples with low KSR1 expression (Fig. 2C). Taken together, the results of GSEA, GeneNetwork analyses, and qPCR suggest that high KSR1 expression is associated with upregulation of the Notch signaling pathway and downregulation of OxPhos.

**KSR1 is required for BRAFV600E-induced NOTCH1 activation**

Based on our observation of a relationship between KSR1 and Notch signaling, we asked whether KSR1 is required for BRAFV600E-induced Notch activation. As shown in Fig. 3A, NOTCH1 expression was markedly increased in HeLa cells by BRAFV600E, accompanied by robust ERK phosphorylation. Remarkably, when we treated HeLa cells with siKSR1, ERK phosphorylation by BRAFV600E was barely detectable, and NOTCH1 expression was also significantly affected (Fig. 3B). qRT-PCR analysis revealed that siKSR1 decreased expression of NOTCH1 and HES1, a representative of NOTCH1 target (Fig. 3C and D). Taken together, cell-based assays confirmed that KSR1 is required for Notch activation induced by BRAFV600E oncogenic kinase.

**Defects in cristae shape in PTCs with high KSR1**

Because our GSEA analysis indicated the reduction in OxPhos in high-KSR1 PTCs, as verified by qRT-PCR in our study subjects, we observed the mitochondrial morphology of PTC using EM. This analysis revealed that human PTCs expressing higher levels of KSR1 contained more fragmented mitochondria and exhibited more profound defects in cristae structure than PTCs expressing low levels of KSR1 (Fig. 4A and Supplementary Fig. 3, see section on supplementary data given at the end of this article). In addition, mitochondrial cristae length in PTC samples with high KSR1 expression was shorter than that in PTC samples with low KSR1 expression (\( P < 0.01 \), Fig. 4B).

**The effect of siKSR1 on cell proliferation**

Our GSEA, cell-based assays, and EM consistently indicated that KSR1 is related to NOTCH1 and OxPhos, all of which can affect cell proliferation during carcinogenesis. To confirm the impact of KSR1 on tumor behavior, we performed a colony-forming assay using BCPAP cells (BRAFV600E-positive PTC cells) and 8505C cells (BRAFV600E-positive anaplastic thyroid cancer cells) treated with siKSR1. After 14 days of growth with siKSR1 treatment for every 2 days, the number of colonies was significantly decreased (Fig. 4C and D), suggesting that KSR1 plays a pivotal role in cell proliferation in BRAFV600E-driven carcinogenesis.

**Figure 3**

Effects of kinase suppressor of RAS1 (KSR1) knockdown on activation of NOTCH1 signaling by oncogenic BRAFV600E kinase. (A) Forty-eight hours after transfection of HeLa cells with BRAFV600E, western blotting analyses were performed to detect the indicated proteins. NTM, transmembrane/intracellular region. (B) Forty-eight hours after co-transfection of HeLa cells with BRAFV600E and siKSR1, western blotting analyses were performed to detect the indicated proteins. NTM, transmembrane/intracellular region.

All data are means +/- S.E.M., *** \( P < 0.001 \).

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Supplementary Fig. 5A). In GSEA of CNKSR2, low pathway (nominal expression group included genes of the Notch signaling 20 gene sets enriched in the high CNKSR1 POxPhos (nominal expression was associated with genes related to CNKSR2 PCR revealed heterogeneous expression of CNKSR1/2 (Supplementary Table 3, see section on supplementary Table 5, Fig. 5F, and Supplementary Fig. 5B). Supplementary Fig. 4). As shown in Fig. 5B and C, qRT-data given at the end of this article, Fig. 5D, and n = 7 per group). See the detailed description in ‘Materials and methods’ section. (C) Representative figures of colony-forming assays. (D) Comparison of number of colonies between control and siKSR1-treated HeLa cells. Means were compared by Mann–Whitney U test. All data are means ± S.E.M., **P < 0.01 and ***P < 0.001.

Figure 4
Electron microscopy and colony-forming assays. (A) Mitochondrial morphology in human papillary thyroid cancers (PTCs) with low or high kinase suppressor of RAS1 (KSR1) expression (original magnification, ×30 000). (B) Morphometric analysis of mitochondrial cristae. Data represent mean ± S.E.M. (50 random selected mitochondrial/sample, Analysis of the enriched gene sets in PTCs expressing high levels of KSR2, CNKSR1, or CNKSR2
As our data suggested that KSR1 expression correlates with increased Notch1 signaling and decreased OxPhos, we decided to investigate whether KSR2 and CNKSR1/2, which are necessary for the regulation of RAF in the MAPK pathway, are also involved in these processes. In the case of KSR2, 85 (51.5%) PTC samples exhibited high mRNA levels (Fig. 5A), but the difference in expression between the tumor tissue and the control tissue was not statistically significant. The top 20 gene sets enriched in PTC samples expressing high levels of KSR2 included those of WNT (nominal P < 0.001, FDR q-value = 0.163) and Notch pathways (nominal P = 0.009, FDR q-value = 0.154) (Supplementary Table 3, see section on supplementary data given at the end of this article, Fig. 5D, and Supplementary Fig. 4). As shown in Fig. 5B and C, qRT-PCR revealed heterogeneous expression of CNKSR1/2 in PTC samples. As shown in Supplementary Table 4, the top 20 gene sets enriched in the high CNKSR1 mRNA expression group included genes of the Notch signaling pathway (nominal P < 0.001, FDR q-value = 0.006, Fig. 5E and Supplementary Fig. 5A). In GSEA of CNKSR2, low CNKSR2 expression was associated with genes related to OxPhos (nominal P < 0.001, FDR q-value < 0.001) (Supplementary Table 5, Fig. 5F, and Supplementary Fig. 5B). Taken together, these data indicate that, similar to the expression of KSR1, the expression of KSR2, CNKSR1, and CNKSR2 is also associated with the regulation of the Notch signaling pathway and OxPhos. Supporting this idea, KSR1 mRNA levels showed a strong positive correlation with KSR2 and CNKSR1 expression, and tended to correlate with CNKSR2 expression (Supplementary Fig. 6).

Discussion
The discovery of oncogenic BRAFV600E led to intensive investigation of the RAS–RAF–MAPK signaling pathway (Davies et al. 2002, Namba et al. 2003), but the exact mechanism of signal propagation through this pathway remains elusive. As the key roles played by protein–protein interactions (e.g. RAF homo- and heterodimers) in RAF activation have been revealed, the complexity of the RAS–RAF–MAPK pathway has taken central stage (Montagut et al. 2008, Bollag et al. 2010, Hatzivassiliou et al. 2010, Johannessen et al. 2010). In addition to RAF dimerization, additional regulatory interactions are important: for example, heat shock protein 90 stabilizes the RAF protein itself, whereas the adaptor protein 14-3-3 stabilizes the active conformation of RAF (Tzivion & Avruch 2002, Grbovic et al. 2006). KSR1/2, a pseudokinase scaffold protein, regulates the MAPK cascade (including MEK and ERK) by interacting with RAF proteins (Claperon & Therrien 2007). In addition, CNKSR1/2 are also necessary components in the regulation of the RAS–RAF–MAPK signaling pathway (Therrien et al. 1998).

In our study, expression levels of KSR1/2 and CNKSR1/2 were somewhat heterogeneous. Nonetheless, a significant proportion of PTCs exhibited elevated expression of these scaffold proteins relative to matched normal tissues, indicating that PTC can be classified by KSR1/2 and CNKSR1/2 expression status. Moreover, BRAFV600E positive PTC samples showed higher KSR1 mRNA expression, indicating that KSR1 scaffold function might be essential for BRAFV600E signaling. In GSEA, high expression of KSR1 and KSR2 was associated with coordinated upregulation of Notch signaling. Previous studies using pancreatic cancer cells suggested that the MAPK
pathway is able to promote Notch signaling activation by γ-secretase complex-dependent proteolysis (Tremblay et al. 2013). Although we could not demonstrate γ-secretase complex activity using the γ-secretase peptide cleavage assay (Farmery et al. 2003, McLeod et al. 2009), we observed an increase in the level of the transmembrane/intracellular region (NTM) of NOTCH1 upon BRAFV600E transfection, which is consistent with the results of the previous study. Remarkably, knockdown of KSR1 by siRNA abrogated NOTCH1 activation by BRAFV600E, suggesting that KSR1 is required for BRAFV600E-induced Notch activation. KSR1 expression was also associated with downregulation of gene sets related to OxPhos. This was verified by performing qRT-PCR expression analysis of NOTCH1, NOTCH4, NDUFA4, and COX11 mRNA using cDNA from PTC samples of our study subjects. In addition, EM clearly revealed defective mitochondrial cristae in PTC samples expressing high levels of KSR1. In light of these observations, we postulated that activation of Notch signaling combined with downregulated OxPhos strongly promotes cancer-cell proliferation. In support of this idea, colony-forming assays demonstrated that the proliferation of BCPAP and 8505C tumor cells, which harbor the BRAFV600E mutation, was significantly affected by KSR1 knockdown.

The transcription-mediated negative feedback provoked by BRAFV600E can repress MAPK activation induced by RTK signaling, resulting in the expression of dual-specificity phosphatases (DUSPs) and Sprouty (SPRY) proteins (Pratilas et al. 2009, Lee et al. 2012). However, selective BRAFV600E inhibitors restore RTK-induced MAPK signal propagation by inhibiting this negative feedback. In the resultant new steady state, KSR1/2 and CNKSR1/2 work coordinately to propagate the MAPK signal. Indeed, CNKSR1 is required for RAS signaling, linking RHO and RAS signal transduction (Jaffe et al. 2004). In addition, CNKSR1 acts as a scaffold protein for the AKT–FOXO signaling axis, and also promotes invasion of cancer cells via NFκB-dependent signaling (Fritz & Radziwill 2010, 2011, Fritz et al. 2010). In our GSEA, gene sets related to Notch signaling or OxPhos were enriched in high-CNKSR1 or low-CNKSR2 PTCs, respectively, suggesting that Notch signaling and OxPhos are regulatory targets of CNKSR1/2 through the RAS–RAF–MAPK signaling pathway.

In summary, our data indicate that KSR1/2 and CNKSR1/2, scaffold proteins of RAS–RAF–MAPK signaling, are consistently associated with upregulation of NOTCH1 signaling and downregulation of gene sets related to OxPhos. As PTCs exhibit a linear signal cascade during carcinogenesis regardless of the initiating oncogenic events, the enriched gene sets common to PTCs expressing high levels of KSR1/2 and CNKSR1/2 represent the most...
feasible druggable targets of PTC. Although we were not able to analyze the mutation status of PTCs derived from Ukraine via the Chernobyl Tissue Bank, the common enriched gene sets suggested by our GSEA also represent promising targets for the treatment of BRAFV600E PTCs exhibiting resistance to selective inhibitors.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-14-0270.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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