Am J Cancer Res 2020;10(3):897-907
www.ajcr.us /ISSN:2156-6976/ajcr0108211

Original Article

KRAS signaling enriched triple negative breast cancer is associated with favorable tumor immune microenvironment and better survival

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Received January 21, 2020; Accepted February 2, 2020; Epub March 1, 2020; Published March 15, 2020

Abstract: KRAS signaling is associated with cancer progression in several cancers. Upregulation of KRAS signaling is often seen in cancers that harbor high KRAS mutation rate, such as pancreatic cancer and non-small cell lung cancer (NSCLC). Less than 2% of breast cancers have KRAS mutation, however, the alteration of the effector signaling such as PI3K/AKT and MAPK pathways are well known. Mutated KRAS is known to function as immune suppressor in other cancers, but the role of KRAS signaling on tumor immune microenvironment (TIME) in breast cancer is not known. We hypothesize that the enrichment of KRAS signaling is associated with reduced patient survival as well as TIME in triple negative breast cancer (TNBC). Patient cohorts from Molecular Taxonomy of Breast Cancer International Consortium (METABRIC; n = 1903) and The Cancer Genome Atlas (TCGA; n = 982) were used. Higher expression of KRAS in breast cancer cell-lines (MCF7, BT474, and MDA-MB231) compared to MCF10A, which is a model of benign mammary cells was found. Both MEK and PI3K inhibitors suppressed MB231 cell proliferation in dose dependent manner. Gene Set Variant Analysis (GSVA) of the patient cohorts demonstrated two peaks by KRAS_SIGNALING_UP gene sets which were divided into KRAS-high and -low groups using median cutoff. There was no difference in KRAS mutation between KRAS-high and low. Despite its cell proliferation promoting role, KRAS-high patients demonstrated significantly better Disease-Free Survival and Overall Survival in triple negative breast cancer (TNBC). KRAS-high TNBC was associated with favorable tumor immune microenvironment with elevated B cells and CD8 T cells, monocytes, or M1 macrophage. It was associated with decreased CD4 central memory T-cells, but not Regulatory T-cells, or M2 macrophage detected by xCell. To elucidate the mechanism of this association, Gene Set Enrichment Analysis was performed. Inflammatory response, IL6/JAK-STAT3 signaling, and Interferon gamma response gene sets were enriched in KRAS-high TNBC patients in both METABRIC and TCGA cohorts. In agreement, cytolytic activity score, interferon gamma response score, and lymphocyte infiltrating signature score, were all significantly elevated in KRAS-high TNBC. In conclusion, we found that patients with enrichment of KRAS signaling gene sets were associated with inflammation and favorable tumor immune microenvironment as well as improved survival in TNBC.

Keywords: KRAS, triple negative, breast cancer, tumor immune microenvironment, KRAS signaling, TCGA, METABRIC, adaptive immune cells, innate immune cells

Introduction

KRAS signaling is associated with cancer progression in several cancers [1, 2]. Upregulation of KRAS signaling is often seen in cancers that harbor high KRAS mutation rate, such as pancreatic cancer and non-small cell lung cancer (NSCLC) [3-5]. In breast cancer, only less than 2% were reported to have KRAS mutation [6]; however, alteration of the effector signaling

such as PI3K/AKT and MAPK pathways are well known [7]. PI3K/AKT signaling pathway is the most aberrant pathway in breast cancer. Although the alteration of this pathway is mainly observed in hormone receptor positive cancer, recent studies reveal that the alteration is also found in approximately 30% of triple negative breast cancer (TNBC) patients [8-10]. In general, TNBC is the most aggressive and lethal breast cancer subtype. The role of tumor immune microenvironment (TIME) in TNBC is well recognized [11]. The efficacy of adjuvant and neoadjuvant chemotherapy and prognosis is associated with infiltration of immune cells in TNBC patients [11, 12]. KRAS mutant cells have been reported to create an immunosuppressive tumor microenvironment in colorectal cancer, making them less responsive to immune check point inhibition in mice model [13]. Also, other cancers which harbor high KRAS mutation rate such as pancreatic cancer and NSCLC are reported to have immunosuppressive tumor microenvironment [14]. Unlike those cancers the mutation rate of KRAS in breast cancer is low. Therefore, the clinical relevance of upregulation of KRAS signaling pathways remains elusive.

Gene set enrichment analysis (GSEA) is a computational algorithm that enables one to analyze the molecular profiles of the data set [15]. This analysis assesses the effect of certain gene to the biological activity of gene set of interest [16, 17]. Gene Set Variant Analysis (GSVA) is the analysis to further explore the biological activity of a signaling pathway [18]. Utilizing GSVA, we can line up the patients in the order of how much KRAS signaling is upregulated.

In the current study, we hypothesize that the upregulation of KRAS signaling is associated with TNBC poor survival and its tumor immune microenvironment.

Materials and methods

Cell culture

MCF10A, MCF7, SKBR3, BT474, and MB231 cells were obtained from the JCRB (Japanese Collection of Research Bioresources) Cell Bank. All cells were cultured in accordance to the manufacturer's guidelines.

Inhibitor experiment and cell viability

On the day before the administration of inhibitors, MB231 cells were seeded into 6-well plates to a concentration of 0.5×10^5/well. PI3K inhibitor LY294002 (CST, USA) and MEK inhibitor PD98059 (Calbiochem, USA) were used for the transfection of the cells as previously reported [19].

The trypan-blue dye exclusion test was performed to examine the percentage of viable cells.

Clinical data acquisition

The gene expression levels of Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) as well as The Cancer Genome Atlas (TCGA) were retrieved through cBioPortal as described previously [20-22]. Total of 1903 patients from METABRIC cohort and 982 patients from TCGA cohorts were included in this study. The Institutional Review Board at Roswell Park Comprehensive Cancer Center was waived for this study because we used the de-identified and publicly available data bases such as METABRIC and TCGA.

Gene set variation analysis (GSVA) and gene set enrichment analysis (GSEA)

Gene Set Variation Analysis (GSVA) is a method to estimate variation of gene set enrichment through the samples of expression data set [18]. The patients were divided by the median cutoff of GSVA scoring of KRAS_SIGNALING_UP gene set variation analysis as either KRAS-high or -low group.

Gene Set Enrichment Analysis (GSEA) was conducted in order to examine the relationship between KRAS_SIGNALING_UP and other Hallmark gene sets. This analysis was performed using the publicly available software provided by Broad Institute (http://software.broadinstitute.org/gsea/index.jsp) as previously reported [15-17, 22-26].

xCell, cytolytic activity score (CYT) and other immunological factors

As previously described, we used a computational algorithm, xCell to estimate the cell composition of immune cells within tumor [27]. CYT

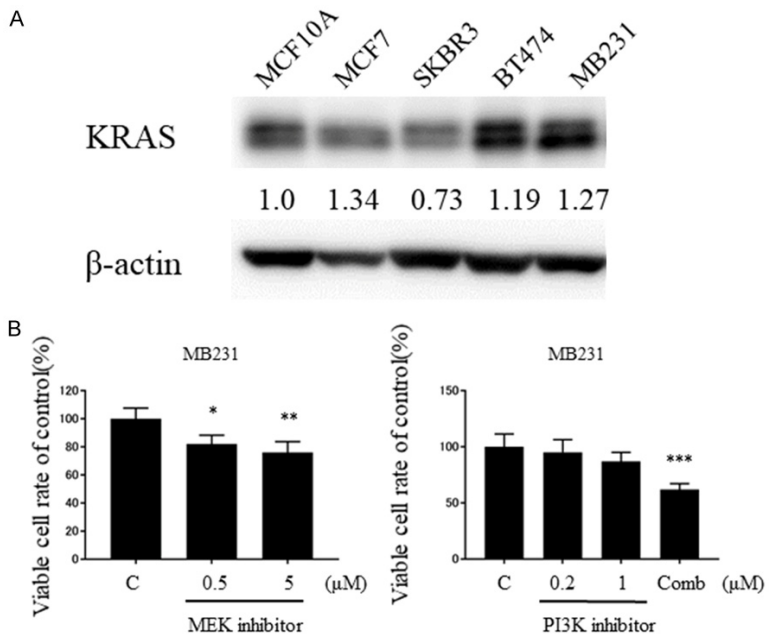

Figure 1. KRAS signaling was functioning in the breast cancer cell lines. A. Up-regulation of KRAS expression in breast cancer cell lines when compared with MCF10A. Densitometric values were calculated for KRAS. B. Cell growth suppression after transfection of MB231 cells with MEK inhibitor or PI3K inhibitor at 72 H. Comb: PI3Ki 1 + MEKi 5 (μM). **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

score was calculated using the expression values of granzyme A and perforin as previously reported [20, 21, 24, 28-31]. IFN-gamma response scoring and Lymphococyte Signature scoring were utilized as described previously [32-34].

Statistical analyses

All of the statistical analyses were conducted by using R software (http://www.r-project.org/), Bioconductor (https://www.r-project.org/). For the survival analysis, Kaplan-Meier method with the log-rank test was performed to compare the survival curves between KRAS_SIGNALING_UP high group and low groups as previously described [35]. In all analyses of this study, $P < 0.05$ was considered statistically significant.

Results

KRAS was expressed higher in breast cancer cell-lines compared from MCF10A, and both MEK and PI3K inhibitors suppressed MB231 cell proliferation in dose dependent manner

To assess whether KRAS signaling is functioning in breast cancer cell lines, we compared the KRAS expression levels of breast cancer cell lines-MCF7, SKBR3, BT474, MB231 with MCF10A, which models a normal mammary cell. We found that the expression levels of KRAS were upregulated in MCF7, BT474, and MB231 cells when compared with MCF10A (**Figure 1A**). To further explore the function of KRAS signaling in breast cancer cells, we inhibited the downstream of KRAS pathway by administering PI3K inhibitor (LY294002) and MEK inhibitor (PD98059) and the number of viable cells were counted. As a result, both PI3K inhibitor and MEK inhibitor significantly suppressed the number of MB231 cells in dose dependent manner. These results suggest that KRAS is expressed higher in breast cancer cell-lines compared from MCF10A, and that KRAS signaling promote MB231 cell proliferation (**Figure 1B**).

Patient distribution with the gene sets KRAS_SIGNALING_UP scoring demonstrated bimodality and higher scores in TNBC

To investigate the clinical relevance of the KRAS signaling in breast cancer patients, gene sets variation analysis (GSVA) was performed to assess the patient distribution of KRAS_SIGNALING_UP gene sets. Interestingly, the histogram of METABRIC cohort demonstrated bimodality (**Figure 2A**). Since the histogram demonstrated two peaks, we chose median as cutoff for dividing the group into KRAS-high and KRAS-low groups.

We also found that the score of KRAS_SIGNALING_UP was higher in TNBC compared with estrogen receptor (ER) positive subgroup (**Figure 2B**). This result implied that KRAS signaling is more clinically relevant in TNBC.

Since KRAS mutation is highly relevant in other cancers, the relationship between KRAS mutation and upregulation of KRAS signaling was assessed, but there was no difference in

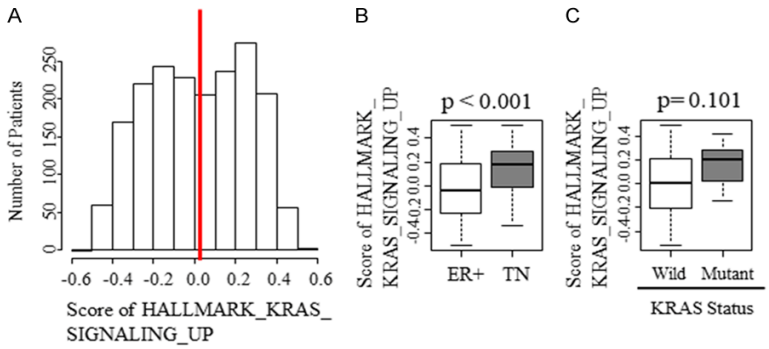

Figure 2. The patient distribution demonstrated bimodality with the scoring of gene set KRAS_SIGNALING_UP and higher scores in TNBC. A. Histogram of KRAS_SIGNALING_UP patients. Red line demonstrates median cutoff. B. The difference in scoring between estrogen receptor positive (ER+) and triple negative (TN) subgroups. C. The difference in scoring between KRAS wildtype and mutant.

KRAS_SCORING_UP score between KRAS wildtype and mutant (**Figure 2C**).

KRAS-high score was associated with improved disease-free survival (DFS) and overall survival (OS) in TNBC

Since KRAS signaling promotes cell proliferation in breast cancer, we expected that KRAS-high patients to have worse survival. To our surprise, KRAS-high group demonstrated significantly better disease-free survival when compared with low group in TNBC (**Figure 3**; $P < 0.045$). This was consistent with overall survival (OS) in TNBC. However, the whole cohort demonstrated significantly better survival with OS (**Figure 3**; $P = 0.002$) alone and not with DFS (**Figure 3**; $P = 0.904$).

KRAS-high TNBC enrich immune related gene sets in both METABRIC and TCGA cohorts

Given the unexpected better survival of KRAS-high patients, we investigated its mechanism by performing GSEA on TNBC, which has significantly high enrichment of KRAS signaling. Interestingly, KRAS-high group significantly enriched the immune related gene sets, INFLAMMATORY_RESPONSE ($P < 0.001$, NES = 1.78, FDR < 0.01), IL6_JAK_STAT3_SIGNALING ($P < 0.01$, NES = 1.71, FDR < 0.01), and INTERFERON_GAMMA_RESPONSE ($P < 0.05$, NES = 1.61, FDR < 0.05) compared with KRAS-low group in METABRIC cohort (**Figure 4A**). These results were validated with TCGA cohort, where INFLAMMATORY_RESPONSE ($P < 0.001$, NES = 2.57, FDR < 0.001), IL6_JAK_STAT3_SIGNALING ($P < 0.001$, NES = 2.33, FDR < 0.001), and INTERFERON_GAMMA_RESPONS ($P < 0.001$, NES = 2.15, FDR < 0.001) were significantly enriched in KRAS-high group (**Figure 4B**).

KRAS-high group demonstrated higher cytolytic activity score (CYT), interferon gamma response score and lymphocyte infiltrating signature score

To further elucidate the effect of KRAS signaling on cancer immunity, multiple scores that reflect immune activity were measured. Interferon (IFN) gamma response score, which also reflects immune activity, was significantly higher in KRAS-high group in both whole cohort and TNBC of TCGA cohort (**Figure 5A**; $P < 0.001$ and $P < 0.05$, respectively). The similar finding was observed in lymphocyte infiltrating signature score that reflect the amount of tumor infiltrating lymphocytes of METABRIC cohort (**Figure 5B**; $P < 0.001$ and $P < 0.001$, respectively). Furthermore, CYT score, which demonstrates immune cytolytic activity, strikingly echoed the results of GSEA as CYT scores of KRAS-high group were significantly higher in both whole cohort and TNBC compared with low group (**Figure 5C**; $P < 0.001$ and $P < 0.001$, respectively). This finding was validated with TCGA cohort (**Figure 5D**; $P < 0.001$ and $P < 0.001$, respectively). Taken together, cancer immunity is enhanced in KRAS-high TNBC.

KRAS-high TNBC is associated with anti-tumor immune microenvironment

Previous studies have demonstrated that tumor infiltrating lymphocyte are a positive predictive biomarker for breast cancer [36]. To this end, we hypothesize that KRAS-high TNBC attract adaptive and innate immune cells. We analyzed the intra-tumoral immune cell composition using a computational algorithm, xCell, on transcriptomic profiles of METABRIC and TCGA cohort. Interestingly, anti-tumor infiltrating lymphocytes, such as B cells and CD8 T cells were higher in KRAS-high TNBC with

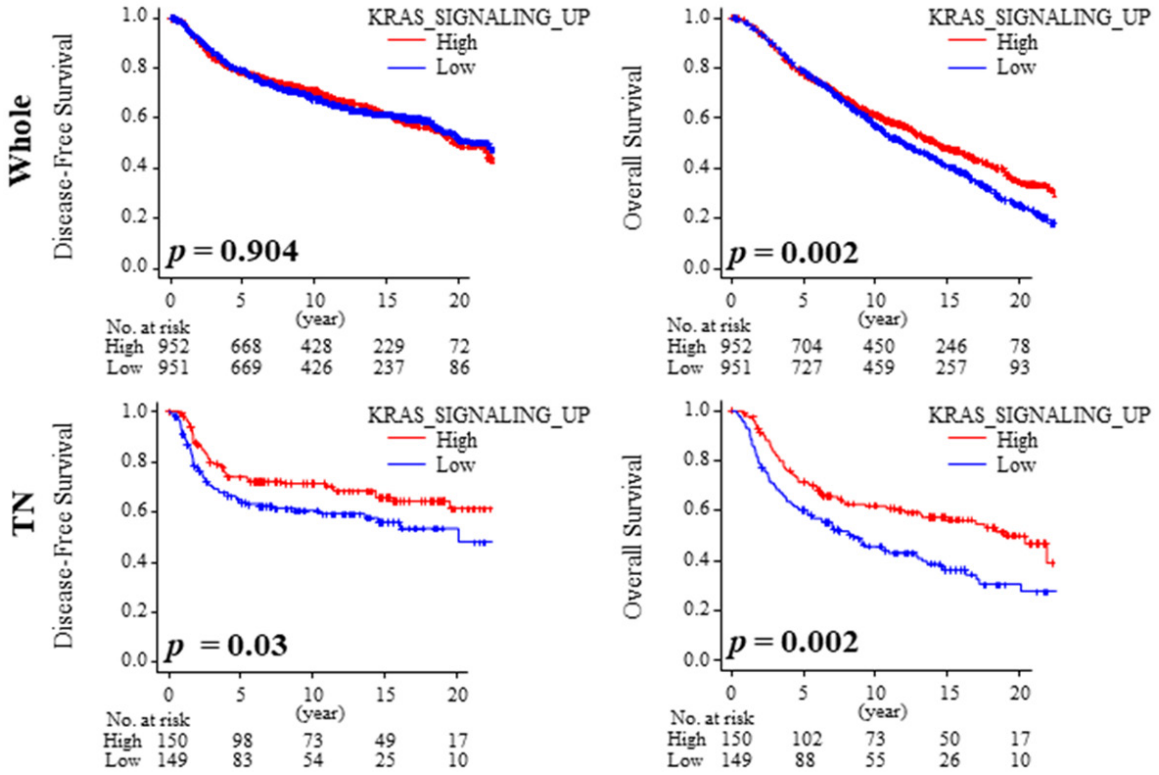

Figure 3. KRAS-high TNBC demonstrated favorable prognostic outcome. Kaplan-Meier analysis of Disease-Free Survival (DFS) and Overall Survival (OS) in whole and TNBC.

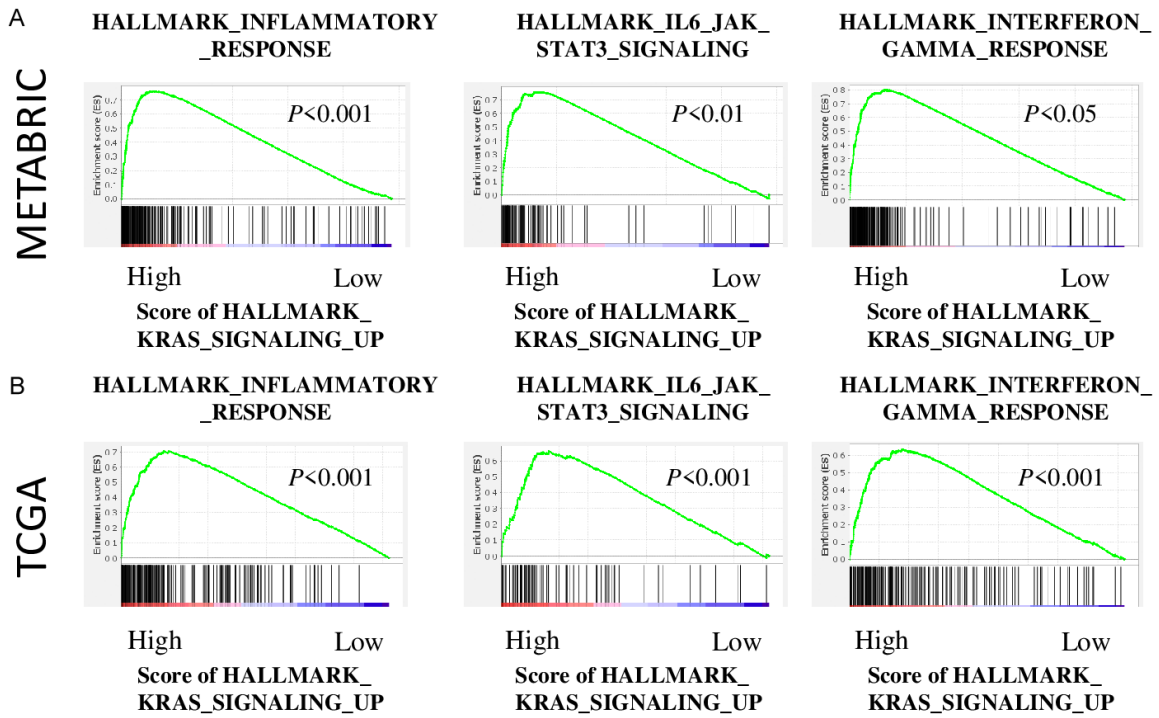

Figure 4. KRAS-high group enriched immune related gene sets in both METABRIC and TCGA cohorts. A. The enriched gene sets of METABRC cohort. B. The enriched gene sets of TCGA cohort.

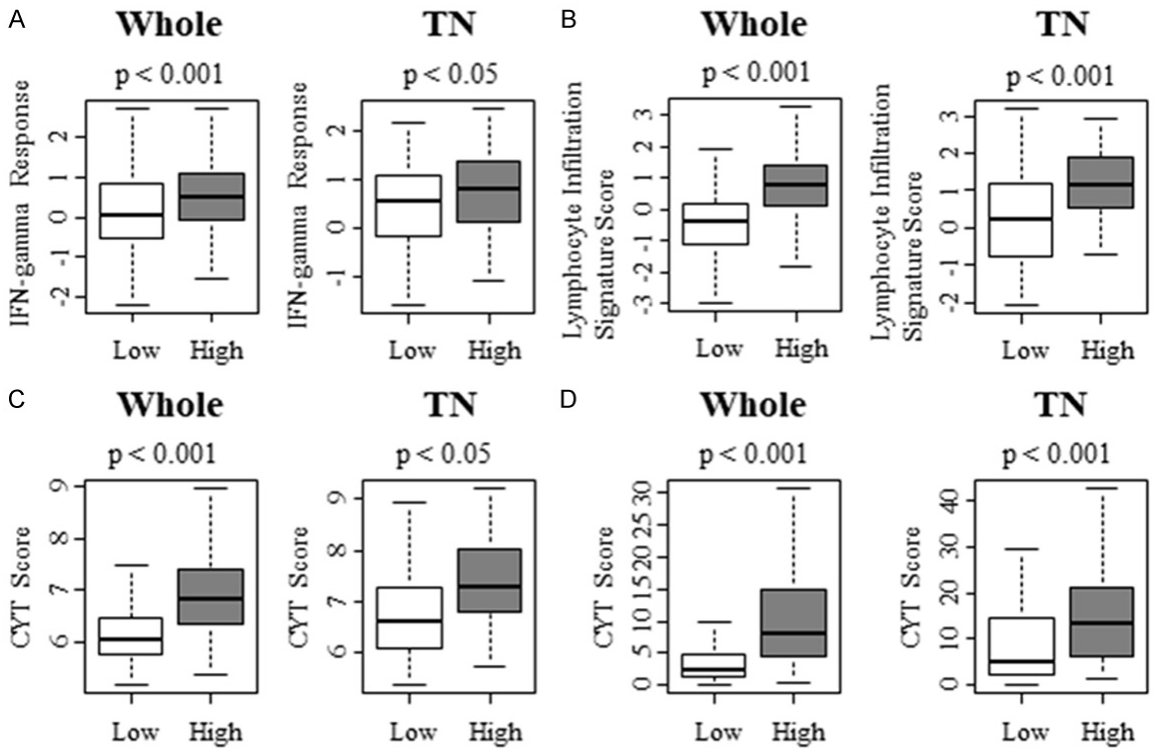

Figure 5. KRAS-high group demonstrated higher immune related scoring and cytolytic activity score (CYT) in whole and TN subgroup. A. IFN gamma Response Score of whole and TN subgroup with TCGA cohort. B. Lymphocyte Infiltrating Signature Score of whole and TN subgroup with TCGA cohort. C. Cytolytic activity score (CYT) of whole and TN subgroup with METABRIC cohort. D. CYT scoring of whole and TN subgroup with TCGA cohort.

METABRIC cohort (**Figure 6A**; $P < 0.001$ and $P < 0.001$, respectively). These results were echoed with TCGA cohort (**Figure 6B**; $P < 0.001$ and $P < 0.001$, respectively). On the contrary, pro-cancer immune cells, CD4 central memory T cells (Tcm) were significantly lower in KRAS-high TNBC in both cohorts (**Figure 6A**; $P < 0.001$ and **Figure 6B**; $P < 0.001$, respectively), whereas regulatory T cells (Treg) were not consistent with two cohorts. With METBARIC, Treg was significantly higher in KRAS-high TNBC (**Figure 6A**; $P < 0.001$), however, that did not reflect in TCGA (**Figure 6B**; n.s.).

Regarding the innate immune cells, monocytes and anti-tumor macrophage M1, pro-tumor macrophage M2 were significantly higher in KRAS-high TNBC with METABRIC cohort (**Figure 7A**; $P < 0.001$, $P < 0.001$ and $P < 0.001$, respectively). However, only monocytes and M1 were validated with TCGA cohort (**Figure 7B**; $P < 0.001$ and $P < 0.001$, respectively). These results implicate that KRAS-high TNBC possess higher overall anti-cancer immunity.

Discussion

We demonstrated that KRAS is expressed higher in breast cancer cell-lines compared to MCF10A, and both MEK and PI3K inhibitors suppressed MB231 cell proliferation in dose dependent manner. GSVA demonstrated two peaks by KRAS_SIGNALING_UP gene sets and was divided into KRAS-high and -low groups using median cutoff. TNBC had significantly higher KRAS signaling compared with ER positive BC. There was no difference in KRAS mutation between those groups. Unexpectedly, we found that KRAS-high TNBC was associated with better DFS and OS. Utilizing xCell, KRAS signaling associate with anti-tumor immune microenvironment in TNBC. KRAS-high TNBC enriched immune related gene sets, inflammatory response, IL6/JAK-STAT3 signaling, interferon gamma response in both METABRIC and TCGA cohorts. To further clarify the contribution of KRAS signaling to the tumor immune microenvironment, we assessed CYT score, IFN gamma response score and lymphocyte infiltrating signature score which were significantly

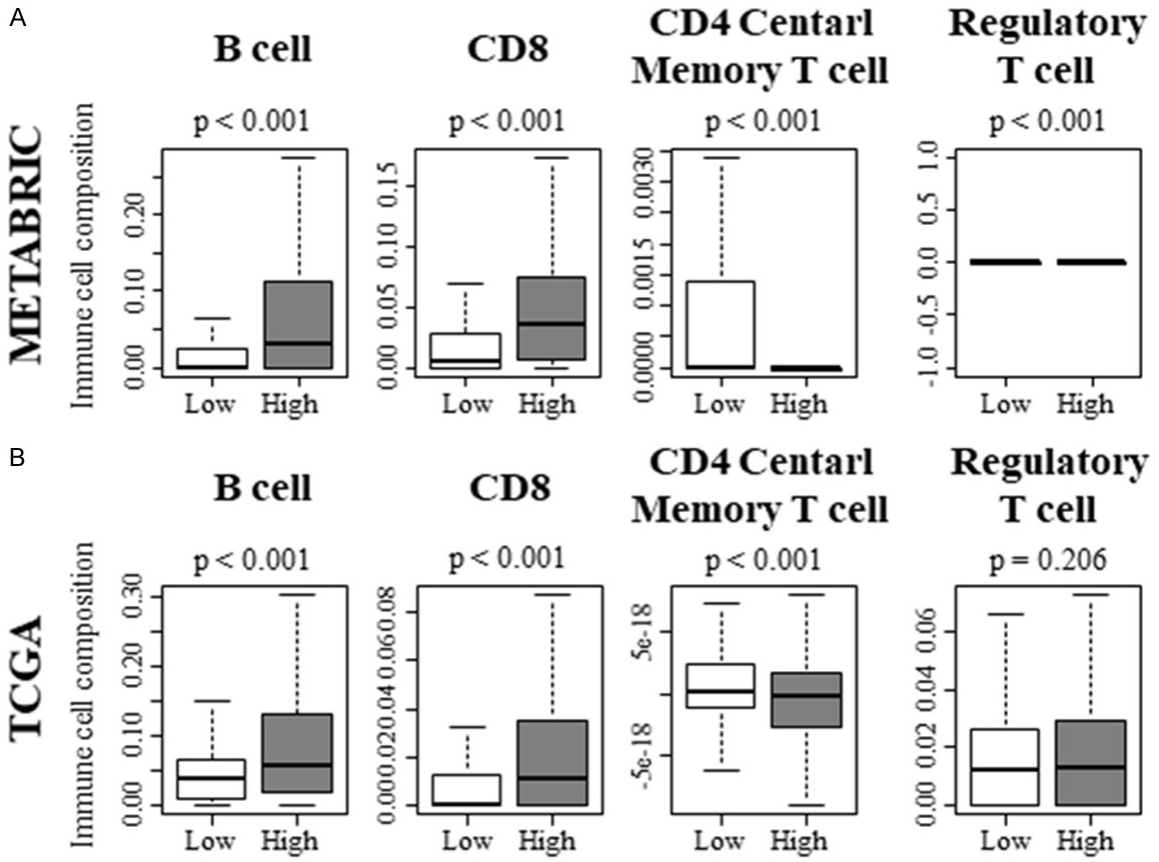

Figure 6. Adaptive immune cells contribute to the shift of tumor immune microenvironment toward anti-cancer environment in KRAS-high TNBC. A. Adaptive immune cell composition within a tumor of METABRIC cohort. B. Adaptive immune cell composition within a tumor of TCGA cohort.

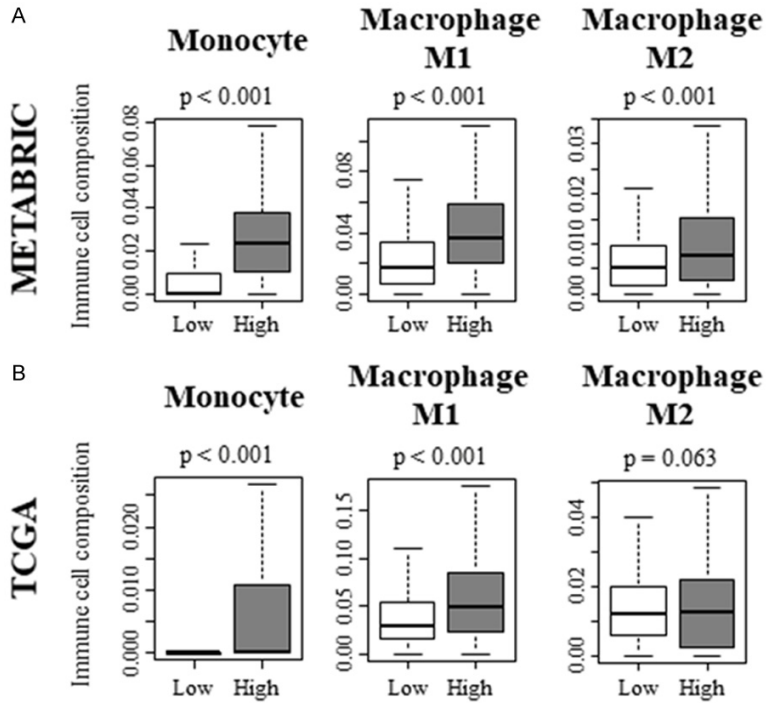

Figure 7. Innate immune cells contribute to the shift of tumor immune microenvironment toward anti-cancer environment in KRAS-high TNBC. A. Innate immune cell composition within a tumor of METABRIC cohort. B. Innate immune cell composition within a tumor of TCGA cohort.

higher in KRAS-high group versus KRAS-low group in the cohorts analyzed.

To date, we have published multiple reports utilizing GSEA [22-25, 30, 37, 38]. This analysis allows us to demonstrate how many biological pathways associate with the different gene expression levels of interest [15]. GSVA possesses the ability to detect

the underlying mechanism over a sample population [18]. To best of our knowledge, this is the first study to utilize GSVA for elucidating the clinical relevance of KRAS signaling pathway and its association with TIME.

It was quite unexpected to find that TNBC with high KRAS signaling to be associated with better survival. Since it is well recognized that infiltration of certain immune cells is associated with better outcome for breast cancer patients, we hypothesized that KRAS-high TNBC is associated with favorable TIME. It is well established that infiltration of CD8 cells to the tumor microenvironment is associated with favorable prognostic outcome. Also, according to previous study, high infiltration of B cells is associated with better survival [39]. Our results demonstrated that KRAS-high TNBC exhibits high number of infiltrating B cells and CD8 T cells in both METABRIC and TCGA. Furthermore, the infiltration of M1, innate immune cells which associate with anti-cancer TIME, was higher with KRAS-high TNBC.

On the contrary, some immune cells act as pro-cancer TIME. The higher percentage of CD4 central memory T cell (Tcm) and regulatory T cell contribute to form the immunosuppressive TIME and hence worse outcome for breast cancer patients [40, 41]. Also, high fraction of M2 macrophage has been reported to be associated with worse survival in breast cancer patients [42]. Of those pro-cancer immune cells, only Tcm was significantly upregulated in both METABRIC and TCGA cohorts. In agreement with previous reports, we found that lower percentage of Tcm in KRAS-high TNBC, which had better OS and DFS. KRAS-high TNBC demonstrated higher number of infiltrating Treg and M2 in METABRIC. However, these results were not supported in TCGA, where there was no significance between KRAS-high and KRAS-low TNBC. Overall, TIME shifted towards anti-tumor TIME and we speculate that this was reason behind those survival benefits in TNBC.

We found that KRAS signaling enriched cancer immunity related HALLMARK gene sets, such as, INFLAMMATORY_RESPONSE, IL6_JAK_STAT3_SIGNALING, and INTERFERON_GAMMA_RESPONSE. These results prompted us to further explore the association between KRAS signaling pathway and TIME by performing analysis of immune related scoring system. KRAS mutation is reported to be associated with the immunosuppressive TIME in other cancers [14]. However, in this current study upregulation of KRAS signaling was associated with better TIME in all and TNBC patients. The favorable TIME was demonstrated by the high scores of CYT scoring within KRAS-high group in both METABRIC and TCGA cohorts. This demonstrates that the ability to attack the cancer cells is increased in KRAS-high group. Also, this was consistent with IFN gamma response score and Lymphocyte Infiltrating Signature Score which suggest that the TIME shifts to anti-tumor immune microenvironment.

The current study has a few limitations. First, this study was retrospective study conducted using the public accessible databases, METABRIC and TCGA. Even though these databases offer significant amount of clinical information, these databases also lack some clinical data for certain number of patients. TNBC is less prevalent when compared to ER positive subtype, which may result in a decrease of reliability of the results.

In conclusion, we found that enrichment of genes related with KRAS signaling is associated with improved DFS and OS in TNBC patients. KRAS_SIGNALING_UP high TNBC was found to be associated with anti-tumor immune microenvironment, which was demonstrated by immune cell composition analysis, GSEA, CYT, interferon gamma response score as well as lymphocyte infiltration signature score.

Acknowledgements

This work was supported by NIH grant R01CA-160688 to K.T., and National Cancer Institute (NCI) grant P30CA016056 involving the use of Roswell Park Cancer Institute's Resources.

Disclosure of conflict of interest

None.

Abbreviations

CYT, Cytolitic activity score; METABRIC, Molecular Taxonomy of Breast Cancer International Consortium; GSEA, Gene Set Enrichment Analysis; GSVA, Gene Set Variant Analysis; TCGA, The Cancer Genome Atlas; Tcm, CD4 central

memory T cell; TN, Triple negative; TNBC, Triple negative breast cancer; Treg, Regulatory T cell.

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