Original Article

KRAS gene status in gastric signet-ring cell carcinoma patients and acts as biomarker of MEK inhibitor

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Background: Signet-ring cell carcinoma (SRCC) is a specific subtype of stomach cancer with unique epidemiology. Here, we sought to explore the role of *KRAS* in SRCC.

Methods: *KRAS* status was studied both in The Cancer Genome Atlas (TCGA) and internal cohorts. Immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) were performed in formalin-fixed and paraffin-embedded (FFPE) samples. We explored patients' survival and clinicopathological characteristics in terms of *KRAS* mutation and expression. We also explored *KRAS* status and drug response curve of MEK/mTOR inhibitors in SRCC cell lines.

Results: Patients with *KRAS* mutations and copy number variation (CNV) showed higher mRNA level compared to non-mutant cases (P=0.003 and P<0.001). In internal cohort, 15 samples harbored *KRAS* mutations. Survival analysis showed that these patients had significantly lower overall survival (OS) (P=0.048). We further analyzed 75 patients with sufficient FFPE samples. Eight patients showed *KRAS* mutations and one patient showed *KRAS* amplification. The median OS was 12.5 months for patients with *KRAS* mutation, and 19.5 months for patients without *KRAS* mutation (P=0.005). Positive expression of *KRAS* as shown by IHC was detected in majority of SRCC samples, which was higher than our intestinal cohort (28% *vs.* 12.6%, P=0.033). We further explored the correlation between *KRAS* status and drug sensitivity in 4 SRCC cell lines. SNU601 and SNU668, which harbored *KRAS* mutation, were hypersensitive to MEK and mTOR inhibitors than *KRAS* wide type cell lines KATO-III and NUGC-4.

Conclusions: Our findings demonstrate that *KRAS* gene plays an important role in SRCC and reveals therapeutic potential of targeting tumors by inhibiting MEK and mTOR pathways.

Keywords: *KRAS*; point mutation; amplification; gastric signet-ring cell carcinoma (SRCC)

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Introduction

Gastric cancer (GC) is the fifth most common cancer in the world and currently accounts for 8.2% of all new cancer cases worldwide (1-3). Signet-ring cell carcinoma (SRCC) is defined as GC with at least 50% of signet-ring cell in the pathologic specimen according to World Health Organization (WHO) classification and is found in 8% to 30% of GC (4). Distinct epidemiology and oncogenesis of SRCC have been observed in previous studies. For example,

SRCC occurs more often in younger women groups and SRCC is associated with serosal invasion, lymph node metastasis and worse prognosis (4,5). Although The Cancer Genome Atlas (TCGA) has systematical analysis about four subtypes of GC through next generation sequencing. Few studies have been focused on SRCC molecular subtypes, and a significant proportion of clinical regimens are limited to chemotherapy.

KRAS is renowned as one of the most common driven genes in cancer. Research on RAS-driven cancers almost focused on RAS-coding mutations. Ninety-nine percent of cancer-associated *KRAS* genes mutations are characterized by single base missense mutations, which are found at residues G12, G13 or Q61 (6). In colorectal, pancreatic, and non-small cell lung cancers (NSCLCs), *KRAS* mutations have been found to have a prognostic impact (6-9). Other measures for *KRAS* alterations, although less studied than mutations, also plays an important role. Recently, studies suggest that amplification and overexpression of *KRAS* was associated with enhanced rates of metastasis or poor survival in ovarian and endometrial cancers (10,11). However, Ras proteins have been dismissed as "undruggable" for many years. Till now, only one therapeutic attack AMG510 had been yielded. AMG 510, which targets a *KRAS* mutation known as G12C was reported safety, tolerability and preliminary anti-tumour activity in a phase I trial (12).

More attention has been paid to downstream pathways of RAS, especially the RAS-RAF-MAPK pathway and the PI3k-Akt-mTor kinase pathway. The goal of inhibiting these pathways is to provide clinical benefit to patients with RAS driven cancers (13).

In this study, we systematically investigated *KRAS* gene alterations in TCGA database. We also investigated the association between *KRAS* gene status and clinical outcomes. Furthermore, sensitivity and response of 4 SRCC cell lines to MEK and mTOR inhibitors were also determined.

We present the study in accordance with the REMARK reporting checklist (available at http://dx.doi.org/10.21037/jgo-20-617).

Methods

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The present study was approved by the ethics committee of Drum Tower Hospital (No. 2016-196-01), and informed consent was obtained from all subjects.

Bioinformatics analysis

We used publicly available data from TCGA in this study. Clinical information, mRNA expression and gene mutation data from TCGA-STAD cohort contained 388 tumor samples were downloaded from the NCI's Genomic Data Commons (GDC) portal (https://portal.gdc.cancer.gov). Raw RNA-seq count matrix and clinical information were obtained using RTCGAToolbox (2.8.0) package in R (3.4.4). The count matrix was normalized and processed by log2 transformation using Deseq2 (1.18.1) package. Subsequently, statistical analyses were performed to evaluate the *KRAS* expression in STAD tissues and the correlation between *KRAS* expression and clinical outcomes. Additionally, to further analyze the overall survival of STAD patients, a Kaplan-Meier plotter was constructed according to the median *KRAS* expression value or *KRAS* alteration status.

Patients and samples

This study included 234 SRCC patients and 57 intestinal gastric adenocarcinoma patients who underwent gastrectomy at the General Surgery Department of Drum Tower Hospital between 2010 and 2016. All patients were pathologically confirmed by pathologist and the clinical data were collected from medical charts and pathology reports.

Immunohistochemistry

Seventy-five SRCC patients and 57 intestinal gastric adenocarcinoma patients in our internal cohort which had sufficient tumor tissues were fixed in formalin and embedded in paraffin. Sections of a thickness of 4 μm were obtained from the tissue paraffin blocks and used for IHC. Paraffin sections were deparaffinized in xylene and rehydrated in a graded alcohol series and distilled water. The slides were heated for antigen retrieval in 1 mmol/L EDTA (0.05% Tween 20, pH 8.0). Endogenous peroxidase was blocked by incubation with 3% H_2O_2 for 10 min. Then the sections were blocked with 10% goat serum at room temperature for 1 h. After washing with PBS, anti-KRAS (ab180772, Abcam, Cambridge, UK) antibody (1:100 dilution) was applied to the sections, and the sections were incubated at 4 ℃ overnight. The combination of primary antibody was detected by peroxidase staining with an avidin-

Table 1 Sanger sequencing primers of *KRAS* gene

Gene	Primer
KRAS exon 2	F: GGTACTGGTGGAGTATTTGATAGTGT
	R: TGAATTAGCTGTATCGTCAAGGCACT
	Sequence primer: forward primer
KRAS exon 3	F: CCAGACTGTGTTTCTCCCTTC
	R: TGCATGGCATTAGCAAAGAC
	Sequence primer: reverse primer

biotin complex system. *KRAS* expression was evaluated semi-quantitatively according to the degree and proportion of membrane staining at the same time: 0 manifested no staining is observed or membrane staining is observed in <10% of tumor cells; 1+ mean faint or partly membrane staining is found in >10% of tumor cells; 2+ represented weak to moderate complete membrane staining is detected in >10% of tumor cells; 3+ represented strong, complete membrane staining is observed in >10% of tumor cells

KRAS sequence

KRAS exon 2 and exon 3 mutations were identified by PCR on 50 ng DNA samples. The primers used were summarized in *Table 1*. The Sanger sequencing process was performed by STAB Vida (Caparica, Portugal). PCR products were then directly sequenced using the Applied Biosystems (ABI) PRISM 3730 XL. PCR amplification was repeated to confirm the results.

FISH for KRAS gene amplification

Four-micron-thick FFPE tissue sections were used for FISH testing. Hematoxylin and eosin (H&E)-stained score was determined by two independent observers who were blinded to the clinicopathological information of each sample. *KRAS* gene copy number was then investigated according to protocol from Abbot molecular (Abbot molecular, Wiesbaden, Germany). Briefly, slides were incubated at 56 ℃ overnight, deparaffinized, and hydrated. After incubation in 2× saline sodium citrate buffer (pH =7.0) at 75 ℃ for 30 min slides were digested with 20 μg/mL proteinase K in pre-warmed 50 mM Tris for 10–20 min at 37 ℃. Then rinse the slides 5 times in distilled water and then immerse slides in ice-cold 2× saline sodium citrate buffer for 20 s. After that, slides were dehydrated using ethanol in a series of increasing concentrations. Hybridization was performed in a humidified chamber at 37 ℃ for 14–18 h with a denatured DNA probe onto the selected area based on the presence of tumor foci on each slide. Finally, the slides were air dried in the dark and counterstained with 4,6-diamidino-2-phenylindole (DAPI). FISH signals were assessed with a Zeiss fluorescence microscope (Gottingen, Germany) by two independent investigators. The numbers of *KRAS* probe signals and *CHR12* CEP probe (CEP12) signals were counted for each nucleus, and an overall mean *KRAS*:*CEP12* ratio was calculated for each patient. Each nucleus was classified according to the number of copies of *KRAS* and *CEP12*. Gene amplification was defined as a ratio of *KRAS* to *CEP12* of ≥2, polysomy was defined as *CEP12* ≥3, disomy (normal) was defined by *CEP12* and *KRAS* signal =2, as previously described (14). Results were interpreted by a pathologist and a cytogeneticist and were reported using ASCO/CAP 2013 criteria.

Cell lines and culture conditions

KATO-III cell lines were obtained from the Chinese Collection of Research Bioresources. Other cell lines (SNU601, SNU668, and NUGC-4) were provided by the National Cancer Center Research Institute (Tokyo, Japan) in 2011. Cells were cultured in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Gibco, NY, USA) at 37 ℃ in a humidified atmosphere of 5% CO_2.

Drug treatment and cell viability assay

AZD6244 and AZD2014 were purchased from Selleck (Houston TX, USA). Cells were seeded in 96-well plates (3,000 cells per well) with antibiotic-free RPMI 1640 (Invitrogen) plus 10% fetal bovine serum at 37 ℃ with 5% CO_2 for 24 h. The cells were then treated with AZD6244 and AZD2014 for another 72 h to determine the 50% inhibition concentrations (IC50). The growth-inhibitory effects of AZD6244 and AZD2014 were tested by 3,4,5-dimethyl-2H-tetrazolium bromide assay (MTT; Sigma-Aldrich). Optical density was spectrophotometrically measured at 570 nM. Each experiment was carried out in triplicate and data are presented as geometric means.

Western blotting

Western blotting analysis was performed in four gastric

Table 2 Patients characteristics of TCGA cohort

Characteristic	All patients (n=388)
Age	
≥60 years	265
<60 years	117
No data	6
Gender	
Male	256
Female	131
No data	1
Histology	
Adenocarcinoma	202
Intestinal	175
Signet ring type	11
TNM T stage	
1–2	105
3	179
4	97
No data	7
TNM N stage	
0	114
1	105
2	76
3	79
X	11
No data	3
KRAS mutation	
WT	327
Mutation	30
Amplification	33
Both mutation & amplification	2

TCGA, The Cancer Genome Atlas.

cell lines as described previously (15). Cells were lysed in buffer containing 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, and 0.5% sodium-deoxycholate. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto to PVDF membranes. After blocked with 10% defatted milk, the membranes were incubated overnight at 4 ℃ with anti-c-K-Ras (1:1,000, Millipore) antibodies. After further washes, the membranes were incubated with the goat anti-rabbit/mouse peroxidase-conjugated secondary antibodies (Abcam), and the blots were developed using ECL (Millipore).

Statistical analysis

Data was analyzed using independent two-tailed *t*-test. Categorical data was analyzed using the two-side chi-square test. The survival distributions were obtained by the Kaplan-Meier survival analysis and compared using the log-rank test, considering death for cancer as the end point. All statistical calculations were performed with the Statistical Package for the Social Sciences for Windows version 22 (SPSS Inc., Chicago, IL, USA). Two-sided P values <0.05 were considered to indicate statistical significance.

Results

TCGA analysis

Genomic mutation, copy number variants and RNA expression data were extracted from TCGA. Patients with complete pathologic and genomic data were included for further analysis. Their characteristics were summarized in *Table 2*. There are 388 patients including 256 men and 132 women and the median age was 66. There are 8 specific histological types in the TCGA STAD dataset. Among the 388 patients, only 11 patients were pathologically diagnosed as SRCC. For tumor stage, 105 patients and 276 patients were diagnosed with T1–2 and T3–4 respectively. Two hundred and sixty patients had lymph nodes metastasis (N1–3) and 26 patients got distant metastasis (M1). Thirty patients (7.7%) harbored point mutations, largely [26/30] showed exon 12 or exon 13 mutations, which was consistent with previous studies. Thirty-three patients (8.5%) had more than one copy number amplification. Two of the patients had both *KRAS* point mutation and amplification. The majority of the patients [21/33] gained more than 12 copies amplification. We further investigated the relationship between *KRAS* alteration and its expression. Interestingly, patients harboring *KRAS* point mutations and copy number variation (CNV) showed higher mRNA level compared to non-mutant cases (P=0.003 and P<0.001) (*Figure 1*). Kaplan-Meier analyses of the entire patient cohort showed that *KRAS* alteration status had no

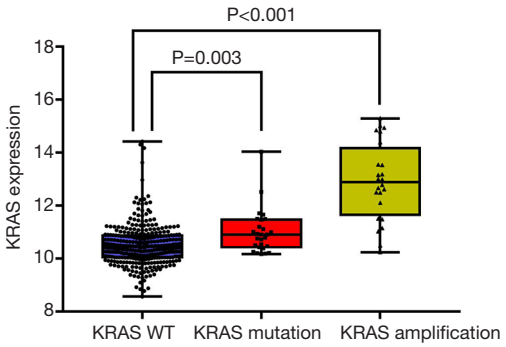

Figure 1 Comparison of *KRAS* mRNA expression among different *KRAS* status (WT, point mutations and CNV) in TCGA database. WT, wild type; CNV, copy number variation; TCGA, The Cancer Genome Atlas.

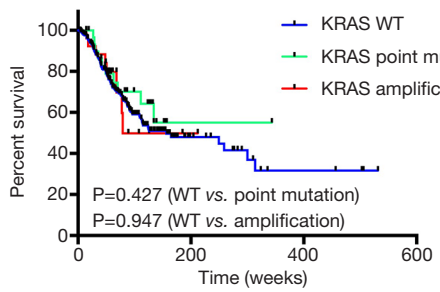

Figure 2 Survival analysis between patients with different *KRAS* status in TCGA cohort. WT, wild type; TCGA, The Cancer Genome Atlas.

significant prognostic value for patients' survival (*Figure 2*).

Internal cohort analysis

We further analyzed our internal cohort. Among them, 234 cases were histologically confirmed SRCC. The median age was 58.26 years old (range, 33–88 years old). Patients' characteristics were summarized in *Table 3*. Fifteen patients were detected with *KRAS* mutations. There were 8 G12V, 3 G12D, 1 G12S, 1 G12C, 1 G13D, and 1 G151A. Survival analysis showed that these patients had a significantly lower OS (*Figure 3*, P=0.048). We further did analysis on the 75 samples which had sufficient tumor tissues. In these 75 cases, 8 showed *KRAS* mutations with 7 G12V and 1 G151A. Two cases showed *KRAS* amplification. KRAS protein expression was assessed by IHC and the results showed that 54 patients were detected with negative (–) or weak positive (+) expression, whereas 21 patients were

Table 3 Patients characteristics of internal cohort

Characteristic	All patients (n=234)	Patients with IHC (n=75)	P
Age			0.406
≥60 years	78	29	
<60 years	156	46	
Gender			0.879
Male	174	57	
Female	60	18	
Tumor stage			0.319
IIIA	46	18	
IIIB	107	30	
IIIC	65	18	
IV	15	9	
TNM T stage			0.634
2	2	1	
3	142	52	
4	78	22	
TNM N stage			0.576
0	2	1	
1	7	3	
2	44	19	
3	181	52	
KRAS mutation			0.216
WT	219	67	
Mutation	15	8	
KRAS amplification	1	1	–
KRAS IHC			–
– ~ +	–	54	
++ ~ +++	–	21	

IHC, immunohistochemistry; WT, wild type.

KRAS median (++) or strong positive (+++). To investigate KRAS expression in GC, we next explored KRAS expression in another internal cohort which was consisted of 57 intestinal gastric adenocarcinoma patients. In intestinal cohort, 50 patients were detected with negative (–) or weak positive (+) expression, 7 patients were KRAS median (++) or strong positive (+++). Obviously, the majority of SRCC

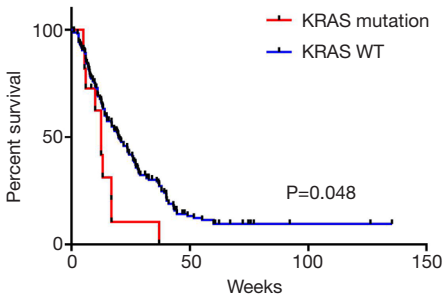

Figure 3 Survival analysis between *KRAS* wild type patients and *KRAS* mutation patients of internal cohort. WT, wild type.

patients were median or strong positive KRAS expression, which is higher than our intestinal cohort (28% *vs.* 12.6%, P=0.033, *Figure 4*). Survival analysis showed that the median OS was 12.5 months for patients with *KRAS* mutation, and 19.5 months for patients without *KRAS* mutation (P=0.005, *Figure 5A*). However, different *KRAS* expression levels had no effect on OS (P=0.095, *Figure 5B*).

Cell line investigations

To investigate the drug response of SRCC cell lines

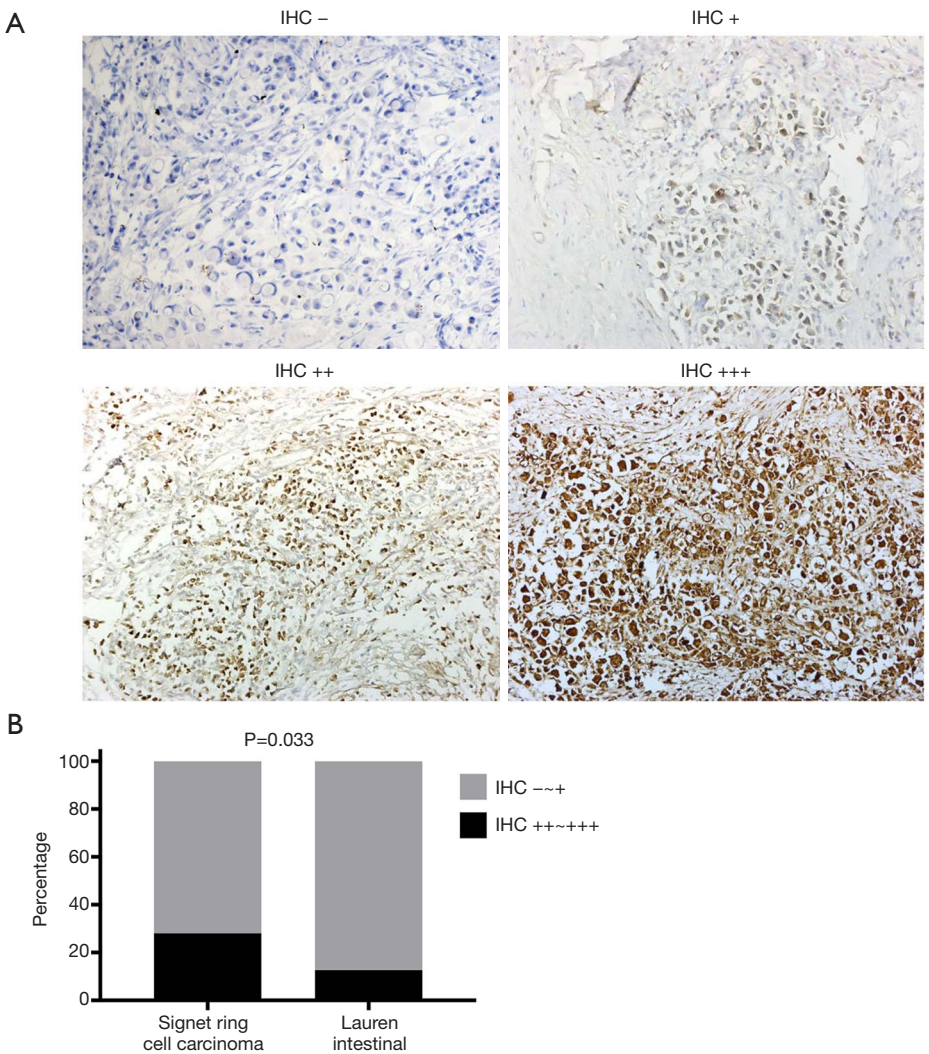

Figure 4 KRAS expression in gastric cancer tissues. (A) IHC staining of samples at different KRAS expression levels (magnification: ×200); (B) IHC analysis of KRAS expression between intestinal gastric adenocarcinoma patients and SRCC patients. IHC, immunohistochemistry; SRCC, signet-ring cell carcinoma.

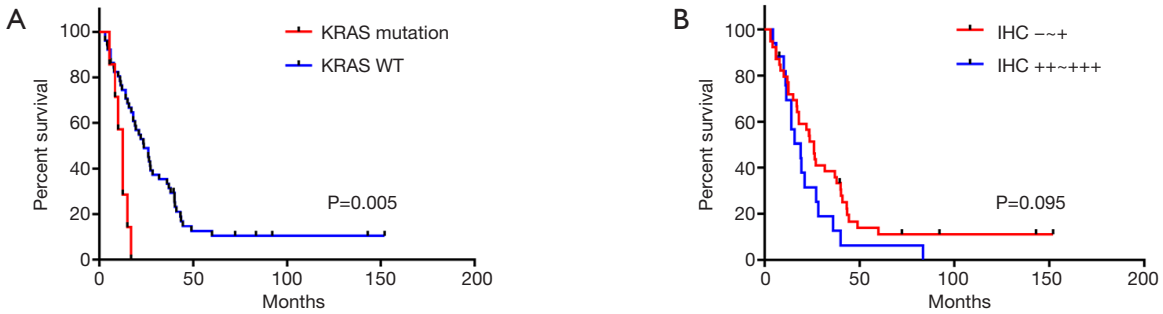

Figure 5 Survival analysis based on *KRAS* mutation status or expression level in 75 SRCC patients. (A) Survival analysis between *KRAS* wild type patients and KRAS mutation; (B) survival analysis between patients with low and high KRAS level based on IHC staining. IHC, immunohistochemistry; SRCC, signet-ring cell carcinoma.

Table 4 Drug sensitivity of MEK and mTOR inhibitors in different gastric cell lines

Cell lines	Cell type	*KRAS* status	Other alteration	AZD6244 IC50 (μmol/L)	AZD2014 IC50 (μmol/L)
SNU601	SRCC	G12D	–	0.663±0.041	1.560±0.021
SNU668	SRCC	Q61K	–	0.538±0.020	2.780±0.032
KATO-III	SRCC	WT	C-Met amplification	>15	7.637±0.085
NUGC-4	SRCC	WT	–	>20	>30

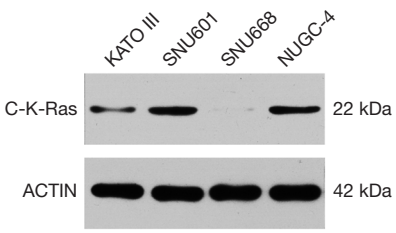

Figure 6 Western blot analysis of KRAS expression in different gastric cancer cell lines.

according to *KRAS* gene status, the cytotoxicity of MEK and mTOR inhibitors in four gastric SRCC cancer cell lines (SNU601, SNU668, KATO-III and NUGC-4) was assessed by MTT assay. *KRAS* alteration in cell lines according to sanger sequence was summarized in *Table 4*. Three cell lines, which were KATO-III, SNU-601 and NUGC-4, showed detectable KRAS expression (*Figure 6*). However, none of the cell lines showed *KRAS* amplification. All four cell lines were treated with MEK inhibitor AZD6244 and mTOR inhibitor AZD2014 for 72 h. SNU601 and SNU668, which harbored *KRAS* mutation, were more hypersensitive to AZD6244 and AZD2014 than other cell lines. They showed significantly lower IC50 than cells without *KRAS* mutations (*Table 4*).

Discussion

GC can be classified histologically into various types and SRCC is defined as cells with abundant intracytoplasmic mucin (16). SRCC is associated with more aggressive cancer and the disease is usually diagnosed at a distant stage (17). Although almost all SRCC belonged to diffused type, distinct epidemiology and oncogenesis of SRCC have been observed (5).

Gene alteration of *KRAS* has considered to be an important biological biomarker in several cancer types. Oncogenic Ras proteins promote multiple cancer related event, including excessive proliferation, loss of contact inhibition, increased motility, and so on (18). We firstly systematically investigated the *KRAS* gene alteration including point mutations and CNV in 389 patients in TCGA database. However, only 11 patients were pathologically diagnosed as SRCC. None of these 11 SRCC patients harbored *KRAS* point mutations or CNV, which made it unavailable for further subtype analysis. Regretfully, no survival difference was observed between different *KRAS* gene status in TCGA database. In consideration of the lower

prevalence of SRCC, our cohort included 234 SRCC patients was selected from thousands of GC samples. Previous studies have shown that SRCC tend to occur more often in younger women groups. However, 60/234 (25.6%) patients were women in our cohort. Our cohort was selected from GC surgery samples. Due to the advanced clinical stage, the majority of patients might not receive surgical treatment, resulting in a gender bias. In our internal SRCC cohort, 6.4% patients harbored *KRAS* point mutation in exon 2 and exon 3 which was consistent with previous report identifying 6.5% *KRAS* mutation rate in GC (19).

KRAS is the most frequently mutated oncogene compared with other RAS subtypes (NRAS, HRAS). *KRAS* mutations was previously mainly studied in endometrial cancer, however, the prognostic importance of *KRAS* mutation was inconsistent (11,20). Studies showed that *KRAS* mutations were associated with inferior survival in colon cancer patients (21,22) and in metastasis pancreatic cancer (23). Mutations in codons 12 and 13 are correlated with poor OS for colorectal cancer patients (24,25) and are predictors of resistance or sensitivity to EGFR-targeted therapy (26-28) by activated both the PI3K/AKT and MAPK signaling (29). Interestingly, we found that *KRAS* mutation were associated with unfavorable disease survival. We also found a higher KRAS expression level in SRCC cohort than our intestinal cohort (80% *vs.* 38.6%, P<0.001). To the best of our knowledge, this is the first study to demonstrate that KRAS expression was more common in SRCC and that *KRAS* mutation independently predicts worse OS in SRCC patients. Previous studies reported that *KRAS* mutations with MSS status presented a poor prognosis and a worse outcome (30). *KRAS* G12V mutation carriers have much shorter OS than other mutation carriers and wild-type groups (31). However, our study is a retrospective analysis, large prospective studies might be needed to validate our findings.

KRAS was long thought to be undruggable because the protein lacked traditional small molecule binding pockets. In 2019, AMG 510, which inhibits the oncogene *KRAS*, has achieved something which has eluded many other drug makers. The study of AMG 510 in 35 patients found no dose-limiting toxicities at the tested dose levels, and revealed a 50% partial response rate among a subgroup of 10 patients with KRAS-positive NSCLC (12). Despite more than three decades of effort, no effective Ras inhibitors have been approved. Thus, the question was raised about which pathway should be targeted. Though the MAPK pathway is preferentially activated by RAS, it has been also known to interact with p110α, the catalytic subunit of PI3K and PI3K signaling is firmly established under RAS (32). The direct downstream of the RAS signaling are the MAPK and PI3K signaling pathways, and MEK and mTOR inhibitors block these two pathways (33). More efforts have been put into MAPK, MEK and mTOR inhibitors. A pre-clinical study demonstrated that MAPK inhibitor showed superior efficacy in GC cell lines with *KRAS* mutation. In previous study, MEK inhibition showed significant treatment effects on KRAS-dependent CRC (34). Furthermore, MEK inhibitor could reduce peritoneal dissemination of KRAS addicted ovarian cancer. In addition, combined inhibition of the PI3K/mTOR/MEK pathway could induces apoptosis in pancreatic cancer cells (35). In our study, *KRAS* mutation was associated with increased sensitivity to MEK and mTOR inhibitors in gastric SRCC cell lines which was in line with the previous studies. Further studies also focused on tyrosine phosphatase SHP2, which was critical for mutant *KRAS*-driven cancers, and necessary for resistance mechanisms upon blockade of MEK (36). These results, together with our study, suggest that MEK and mTOR inhibitor might be a promising therapeutic options of signet ring cell GC with *KRAS* mutation.

Nowadays, new treatment methods for *KRAS* mutation are finally coming into view (37). Due to mutant KRAS proteins themselves are not strong antigens, efforts are now focus on increasing the capacity of the immune system to recognize *KRAS* mutants as neoantigens. Since this perspective has been aroused for many years, progress had been made in generating T-cell responses against Ras-derived epitopes (18). Immune checkpoint molecules play an important role in tumor immune escape, and the recent development of immune checkpoint inhibitors showed sufficient efficacy of anti-PD-1 antibody in specific subset of NSCLC patients. A recent pre-clinical study demonstrated that PD-L1 expression was positively associated with *KRAS* mutation in both *KRAS* mutated NSCLS cell lines and tissues. Further investigation revealed that PD-L1 was up-regulated by *KRAS* mutation through p-ERK signaling (38). The relationship of KRAS and PD-1 was also verified in clinical studies. In a retrospective study, advanced NSCLC patients treated with a single checkpoint inhibitor had at least one oncogenic driver change, patients with *KRAS* mutation showed more long-term response (12 months), higher partial or complete response rate and lower rapid progression rate (within 2 months) compared with other mutations (such as *EGFR*, *BRAF*, *MET*, *HER2*, *ALK*, *RET*, and *ROS1* mutations) (39). However, in colorectal

cancer, *KRAS* mutation reduced T cell infiltration and anti-PD-1 therapy resistance in mouse model (40). These data indicate potential role of immunotherapy in selected *KRAS* mutation.

In conclusion, our findings show for the first time that *KRAS* mutation is a promising prognostic marker in SRCC patients. Furthermore, *KRAS* mutation can be used as a predictive marker in patients treated with MEK and mTOR inhibitors. Given that SRCC was not sensitive to common chemotherapeutic agents, the results our study may facilitate further development of agents targeted agents in SRCC.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/jgo-20-617). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All experimental methods were carried out in accordance with the approved guidelines. This study was conducted with the approval of the Ethics Committee of Nanjing Drum Tower Hospital (No. 2016-196-01). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013), and informed consent was taken from all the patients

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