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ORIGINAL ARTICLE

KRAS Mutation in Gastric Cancer and Prognostication Associated with Microsatellite Instability Status

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Abstract Microsatellite instability (MSI) is one of the subgroups based on the new molecular classification of gastric cancer (GC). In this study, we analyzed the role of KRAS status in MSI GC and the impact of MSI status on KRAS mutation. We performed analysis on 595 GC patients. Polymerase chain reaction (PCR) was used for the screening of KRAS mutation (exon 2) and 5 quasi-monomorphic mononucleotide repeats, namely, BAT-26, BAT-25, NR -24, NR-21, and NR-27 were used to determine the MSI status. The KRAS and MSI status were then compared with clinicopathologic data of the GC patients. MSI GC was found in 20.3% of all cases. KRAS mutation was seen in 24 patients; 18 were MSI (75%) and 6 were microsatellite stable (MSS) (25%). MSI GC patients with KRAS mutation were older and mostly female, but MSS presented more advanced T and N stage of the disease, more cardia tumors, and adjuvant treatment. Five-year survival was 72.2% for KRAS mutation patients with MSI and 0% for MSS ($p < 0.001$). Although KRAS mutations in GC are linked with MSI in the majority of cases, KRAS mutations with MSS status presented with a poor prognosis and a worse outcome. In multivariate analysis, MSI was associated with better survival ($p < 0.001$) but KRAS was with worse survival ($p = 0.304$). Our study suggests that KRAS mutations are based on MSI status rather than different codon subtypes of mutation, and such a division could be used to determine the GC patient outcome.

Keywords Stomach cancer · KRAS mutation · Mismatch repair deficiency · Molecular · Prognosis

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Introduction

Gastric cancer (GC) is still one of the most common malignancies around the world [1]. Even as we observe a decline in incidence during the past few decades, it is still the third most common cause of cancer death worldwide [1]. We have different treatment options and they vary according to the region and stage of the disease [2]. As in other cancers, a survival benefit was achieved from the development of targeted therapies such as transtuzumab in HER-2 positive GC patients or ramucirumab against VEGFR2 [3, 4]. In addition, improvements in our understanding of the molecular, genetic and immunological background of cancer and usage of modern diagnostic tools, tailored surgical techniques, and more accurate radiotherapy helped in forming the modern multidisciplinary approach to fighting cancer [3, 5–9]. GC has been shown to be a heterogeneous disease with molecular and geographical variants. Recently, two different groups proposed new and more versatile classifications of

GC, dividing them into four subtypes [5, 6]. These subtypes are based on molecular data together with genomic information. In both proposed classifications, microsatellite instability (MSI) is one of the new subtypes.

MSI is described as a genomic instability caused by inactivation of DNA mismatch repair genes [10]. MSI is commonly detected in colon and endometrial cancers. It is also observed in GC, and its presence in this malignancy is associated with older age of patients, female gender, intestinal histotype, non-cardia tumors, lower number of metastatic lymph nodes, and better survival [11–14].

Additionally, we also know that mutations in KRAS are crucial in determining the efficacy of antibodies targeting epidermal growth factor (EGFR) in metastatic colorectal cancer (CRC) [15]. Such mutations have an impact on cell proliferation and also inhibition of apoptosis due to deregulation of the MAPK signaling pathway. In GC, the studies on cell lines and xerographs showed sensitivity to Cetuximab in KRAS wild-type, EGFR-expressing cases, but also independent of KRAS status [16, 17]. Studies on the clinical role of KRAS in GC are limited because of the number of cases [18–20].

It is also worth mentioning that KRAS mutation in colorectal cancers is seen in MSI and also microsatellite stable (MSS) tumors, but in GC, KRAS mutations are observed generally in MSI subtype [20]. The strong association of KRAS with MSI status in GC leads us to questions regarding the relationship between these two molecular factors. The first question is, what is the influence of KRAS mutation on MSI subgroup of patients? The second question is how does the MSI status influence patients with or without KRAS mutation?

In this study, we tried to answer both of these questions by analyzing the role of KRAS status in MSI GC, as well as the impact of MSI status on KRAS mutation.

Material and Methods

General Information About Patients

We used a biobank of collected tissue from 472 patients after surgical treatment of primary gastric cancer. All of these patients were treated in the General Surgery and Surgical Oncology Department, University of Siena, Italy. We used material collected from patients operated between 1990 and 2011. No preoperative oncological treatment had been administered. The tissue material used for the study includes tumoral and normal frozen tissue collected directly after resection in the operating theater. From the University of Singapore, we analyzed 123 GC patients also without preoperative treatment. The tissue material used for the study includes tumoral and normal frozen tissue collected directly after resection in the operating theater.

KRAS Samples Preparation

Genomic DNA was extracted by tumoral and constitutional fresh frozen samples tissues using a standard protocol (Gentra Systems, Minneapolis, USA). The DNA concentration was calculated by spectrophotometry.

Polymerase Chain Reaction (PCR) Used for the Screening of KRAS (exon 2)

Mutation analysis of KRAS codons 12 and 13 mutation were performed by PCR amplification and direct sequencing using the protocol used by Oliveira et al. [21].

PCR reactions were carried out in a volume of 20 μl containing 100 ng/μl genomic DNA template, 1X Reaction Buffer, 0.5 μM of each PCR primer, $MgCl_2$ 1.25 mM, 0.15 mM of each dNTPs, Taq Polymerase 0.5 U/μl (Euroclone). The reactions were performed in programmable thermocyclers according to protocol standard.

A 5 μl aliquot of each PCR reaction was run on a 2% agarose gel to confirm the size, quantity, and purity of each PCR product. The remaining 15 μl of PCR amplified bands were extracted from the gel with the Invisorb® Spin DNA Extraction Kit (INVITEK). Samples were then purified and 2 μl aliquot of purified PCR product was cycle sequenced using Big Dye Terminator Kit (Applied Biosystems, Foster City, CA) in a total volume of 20 μl. Samples were then purified and sequenced using an automated DNA sequencer ABI PRISM 310 Genetic Analyser (Applied Biosystems, Milan, Italy) according to the protocol of the manufacturer. Sequencing was performed in both strands.

All sequence alterations in these genes were validated with a second independent PCR.

Pentaplex Polymerase Chain Reaction and Microsatellite Analysis

A detailed description of MSI analysis was described in our previous paper [13]. Shortly we used 5 quasi-monomorphic mononucleotide repeats, namely, BAT-26, BAT-25, NR -24, NR-21, and NR-27. Following the definition of National Cancer Institute workshop on MSI for cancer, we contemplated a tumor as MSI when 2 or more markers showed instability on 5 loci (MSI-H) [22].

A detailed description of pathological, clinical, surgical and follow up data were also given in our previous publication [13].

Statistical Analysis

Statistical analysis was performed with the X2 test or Fisher exact test to compare categorical variables. The Mann-Whitney U test was used to compare continuous variables

not normally distributed. Cumulative survival was calculated by the life table method of Kaplan and Meier, and the log-rank test was used to distinguish significant differences. Statistical significance was determined at p value <0.05.

Survival curves were estimated using the Kaplan-Meier method were compared using a log-rank test, considering death for cancer as the end-point (cancer-related survival).

Cox proportional hazards model was used to calculate the hazard ratio for each variable in the univariate and multivariate analyses. Statistical analysis was done using Stata IC 2012.

Results

General Results

Of 595 patients, we have 121 MSI GC patients and 24 KRAS mutations. Of these 24 patients, 18 were MSI (75%) and 6 were MSS (25%). For wild-type (wt) KRAS, MSI was observed in 103 patients (18%), and MSS in 468 patients (82%). The difference of MSI status between wt KRAS and mutant KRAS had statistical significance ($p < 0.001$).

Clinical and Pathological Factors Associated with KRAS According to MSI or MSS Status

The analysis of clinicopathological factors of MSI group for wt KRAS and mutant KRAS are presented in Table 1. Statistically significant factor for KRAS mutation positive values was the patients were older. The other factors were without statistical significance. The second analysis was done for KRAS group between MSI and MSS patients. Clinicopathological factors for KRAS MSI and MSS groups were presented in Table 2. Statistically significant KRAS MSS patients were younger, mostly men, with more advanced T and N stage of the disease, with adjuvant therapy and with cardia position (here $p = 0.059$).

This result clearly shows that MSI status is the most important factor associated with KRAS mutation. It is difficult to show the different subtypes of KRAS mutation in MSS group but both 12 V mutation (the only 2 patients with 12 V mutation found in the group of 595 patients), and for 1 patient from 12C,12D, 13D, and 39insTGG.

Survival Analysis

We also looked for differences in overall survival. For the group of MSI GC patients, median overall survival was 85 months (95% CI 62 to 129 months) in wt KRAS and 108 months (95% CI 45 to not reached months) in KRAS mutation ($p = 0.19$) **(**Fig. 1**)**. Additionally, for the group of MSI GC patients overall survival for wt KRAS patients was 59.2% and for KRAS mutation 72.2% ($p = 0.811$). The second analysis was done analyzing a group of patients presenting only mutations in KRAS. The overall survival for this group according to MSI status was performed, median overall survival was 10 months (95% CI 5 to 129 months) in MSS and 108 months (95% CI 45 to not reached months) in MSI ($p < 0.001$) **(**Fig. 2**)**, Five year survival for KRAS mutation patients with MSI was 72.2% and for MSS, 0% ($p < 0.001$). Next, we analyzed only patients with MSS status; in this analysis, the 5-year survival was 0% for patients presenting with KRAS mutation and for patients without that mutation, 35.6% ($p < 0.001$).

Cox regression analyses were performed, with OS as the end point, and included the variables reported in Table 3. In univariate analysis MSI-H, KRAS mutation, female gender were correlated with a reduction in the risk of death. However, KRAS status did not significantly influence survival. Other variables such as R+ surgery, Lauren/mixed diffuse histology, $T \geq 3$ and $N \geq 1$ were correlated with an increase in the risk of disease recurrence. Interestingly, when we analyzed separately the role of MSI status in the reduction of DFS in the cohort of KRAS mutated was 0.11, 0.02–0.47 95% CI $p = 0.003$ and in the cohort of KRAS wild type patients was 0.56, 0.42–0.76 95% CI $p = 0.0001$, highlighting the effect of MSI-H mainly in the KRASmut patients. In the multivariate analysis performed in the entire population, MSI status was associated with a lower risk of death (HR = 0.53, 95% CI 0.39–0.72, $P = 0.001$) and KRAS status with a higher risk of death (HR = 1.32, 95% CI 0.77–2.24, $P = 0.304$). However, this association did not reach statistical significance.

Discussion

KRAS Mutation and MSI in GC Patients

Currently, available clinical and histopathological knowledge of GC shows great variability, forcing us to look deeper into molecular and genetic factors. For GC, currently only Transtuzumab against Her2/neu and ramucirumab, a monoclonal antibody against VEGFR-2, are used as tailored treatments for small subgroups of patients [23, 24].

MSI subtype status seems to play an important role in the prognosis of GC. Current knowledge based on many studies shows that this subtype has a very good outcome. Also in our previous analysis, we found that it is associated with older age of patients, females, non-cardia intestinal or tubular/poorly differentiated histology [13]. The impact of MSI does not play a role in diffuse or signet ring/mucinous histotypes [13]. The role of KRAS mutation in GC based on presence or absence of MSI is unknown.

The KRAS occurrence described in MSI GC is about 20–26% [5, 25–27]. In the currently published The Cancer Genome Atlas (TCGA) based on close to 300 MSI GC

Table 1 Analysis of clinicopathological data in group of MSI GC patients according to KRAS mutations

MSI-H	Total	KRAS wt		KRAS mut		P value
Patient (n)	*121*	*103*		*18*		
Age, y (median)	*75 (67;80)*	*72 (65;79)*		*79 (76;84)*		*0.009*
Sex						*0.052*
Male	*51*	*47 (45.6%)*		*4 (22.2%)*		
Female	*70*	*56 (54.4%)*		*14 (77.8%)*		
pT						0.315
1	7	7	*6.8%*	0	*0%*	
2	22	19	*18.4%*	3	*16.7%*	
3	40	31	*30.1%*	9	*50%*	
4	52	46	*44.7%*	6	*33.3%*	
pN						*0.403*
0	51	44	*42.7%*	7	*38.9%*	
1	23	19	*18.4%*	4	*22.2%*	
2	25	19	*18.4%*	6	*33.35*	
3a	11	11	*10.7%*	0	*0%*	
3b	11	10	*9.7%*	1	*5.6%*	
M						*0.741*
M0	112	95	*92.2%*	17	*94.4%*	
M1	9	8	*7.8%*	1	*5.6%*	
Stage						*0.853*
I	21	19	*18.4%*	2	*11.1%*	
II	44	37	*35.9%*	7	*38.9%*	
III	47	39	*37.9%*	8	*44.4%*	
IV	9	8	*7.85*	1	*5.6%*	
Lauren						*0.166*
Diffuse/mixed	29	27	*26.2%*	2	*11.1%*	
Intestinal	92	76	*73.8%*	16	*88.9%*	
Tumor site						*0.302*
Non cardia	112	95	*94.1%*	17	*100%*	
Cardia	6	6	*5.9%*	0	*0%*	
*WHO**						*0.343*
Papillary	1	1	*1%*	0	*0%*	
Poor	46	37	*38.9%*	9	*60%*	
Signet & Mucinous	23	22	*23.2%*	1	*6.7%*	
Tubular	40	35	*36.8%*	5	*33.3%*	
Adjuvant						*0.095*
No	88	72	*69.9%*	16	*88.9%*	
Yes	33	31	*30.1%*	2	*11.1%*	

*11 cases with unclassified WHO cases histotype are excluded

patients, the KRAS mutations were observed in 23% of all cases [5]. In the same study, KRAS mutations were observed in 6% of the MSS group. In our paper, KRAS mutation is seen in a total of 4.03% of all GC patients, but in 14.9% of MSI GC, and 1.2% of MSS cases. The difference is most likely associated with whole exome sequencing in TCGA study, considering hot spot and non-hot spot regions. The other study by Cristescu et al. who also proposed a new molecular classification of GC based on 300 GC patients had the following results: KRAS mutation was seen in 23.3% of MSI group, and 4.4% in the MSS group ($p = 0.0006$) [6].

In the paper by van Grieken et al., based on multicenter analysis of GC the authors revealed that KRAS mutation is frequently associated with MSI GC [28]. The authors did not link this result with survival. Other studies also showed that KRAS mutation is strongly linked with MSI status. Zhao et al. found 7 of 8 KRAS mutation GC to have also MSI status [29]. A recent publication by Queirós et al. showed KRAS mutation

Table 2 Clinicopathological analysis of GC patients with KRAS mutation according to microsatellite instability status

KRAS mut	*Total*	*MSS*		*MSI-H*		*P value*
Patient (n)	*24*	*6*		*18*		
Age, y (median)	*77 (72;81)*	*70 (59;78)*		*79 (69;86)*		*0.018*
Sex						*0.046*
Male	*8*	*4 (66.7%)*		*4 (22.2%)*		
Female	*16*	*2 (33.3%)*		*14 (77.8%)*		
pT						0.018
1	0	0	*0%*	0	*0%*	
2	3	0	*0%*	3	*16.7%*	
3	9	0	*0%*	9	*505*	
4	12	6	*100%*	6	*33.3%*	
pN						*0.029*
0	7	0	*0%*	7	*38.9%*	
1	4	0	*0%*	4	*22.2%*	
2	10	4	*66.7%*	6	*33.35*	
3a	1	1	*16.7%*	0	*0%*	
3b	2	1	*16.7%*	1	*5.6%*	
M						*0.394*
M0	22	5	*83.3%*	17	*94.4%*	
M1	2	1	*16.7%*	1	*5.6%*	
Stage						*0.178*
I	2	0	*0%*	2	*11.1%*	
II	7	0	*0%*	7	*38.9%*	
III	13	5	*83.3%*	8	*44.4%*	
IV	2	1	*16.7%*	1	*5.6%*	
Lauren						*0.394*
Diffuse/mixed	2	0	*0%*	2	*11.1%*	
Intestinal	22	6	*100%*	16	*88.95*	
Tumor site						*0.059*
Non cardia	21	4	*66.7%*	17	*100%*	
Cardia	2	2	*33.3%*	0	*0%*	
*WHO**						*0.688*
Papillary	0	0	*0%*	0	*0%*	
Poor	11	2	*40%*	9	*60%*	
Signet & Mucinous	1	0	*0%*	1	*6.7%*	
Tubular	8	3	*60%*	5	*33.3%*	
Adjuvant						*0.006*
No	18	2	*33.3%*	16	*88.9%*	
Yes	6	4	*66.7%*	2	*11.1%*	

*4 cases with unclassified WHO cases histotype are excluded

in GC was found in 5 of 19 (26%) MSI GC patients [27]. The authors performed entire KRAS coding sequence but did not find mutations outside codon 12 and 13 hot spots. In our study, 14.9% of the MSI GC group had KRAS mutations. Additionally, we showed the difference of MSI status for wt KRAS and for KRAS mutation was statistically significant. Additionally, it is important to mention that also KRAS amplification is an alternative mechanism for activating KRAS.

KRAS Mutation and Colorectal Cancer

In CRC, KRAS mutations are observed in both MSI and MSS tumors; however, in GC — as is the case in endometrial cancer — the majority of KRAS mutations occur in the MSI group [19, 20, 30, 31]. Also, we confirm that in the case of GC, KRAS mutations are in more than ¾ of cases found in MSI group.

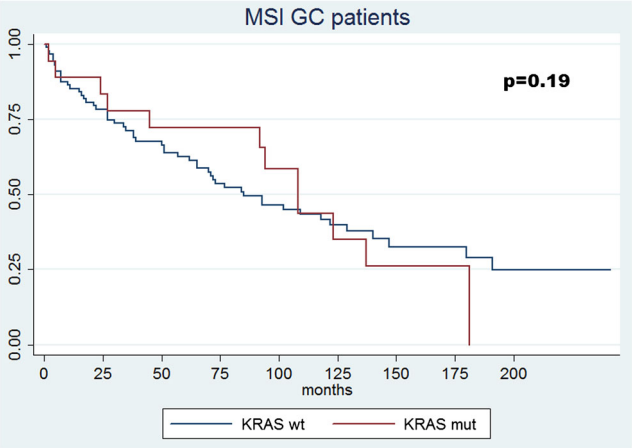

Fig. 1 Estimated OS for MSI GC patients with wild type KRAS (blue) or KRAS mutation (red)

In a study by de Cuba et al., colon cancer patients with BRAF or KRAS mutations had worse survival in the MSI group than wild-type cancer group [32]. Similar results were found in endometrial cancer, where the presence of MSI status showed to be a strong factor associated with decreased disease-free survival [33]. In our research, KRAS mutant patients in MSI group had better survival, however, this finding was not statistically significant.

In CRC, 25% of KRAS mutations are associated with codon number 12 and 10% of mutations with codon number 13 [34]. In our paper, 10 MSI patients showed KRAS mutation in 12D, 7 patients in 13D, and one in 12C. For the MSS group, there were 2 patients with KRAS mutation 12 V, one with mutation 39insTGG, one patient with mutation 13D, one with mutation 12C and one with 12D. No substantial link was found between KRAS codon mutation types and MSI status.

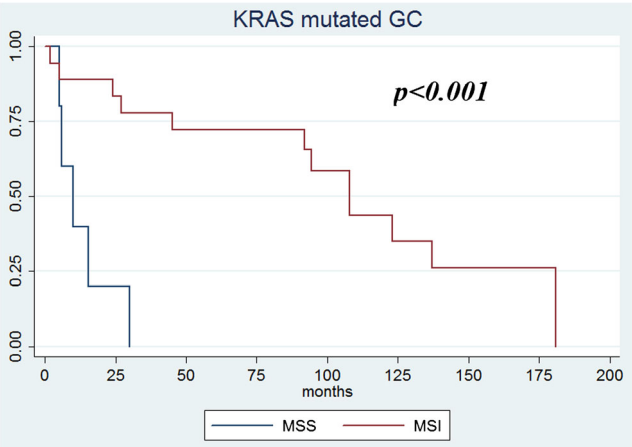

Fig. 2 Estimated OS for KRAS mutated GC patients with MSS (blue) or MSI (red)

Table 3 Univariate analysis of clinical-pathological variables including MSI and KRAS status in patients

Variables	Hazard ratio	95% CI	P value
MSI-H	0.56	0.42–0.73	0.0001
KRAS mut	0.84	0.51–1.37	0.50
Female	0.66	0.53–0.82	0.0001
Age > 70	1.30	1.03–1.64	0.02
R+ Surgery	3.61	2.80–4.66	0.0001
Lauren diffuse/mixed	1.26	1.02–1.55	0.03
T ≥ 3	3.16	2.81–3.58	0.0001
N ≥ 1	3.05	2.28–4-08	0.0001

KRAS Mutation in MSI GC and Tumor Location

Werneke et al. presented interesting results: KRAS mutations were associated with MSI status and were more common in proximal GCs [35]. Mutation in KRAS intestinal GC had a worse prognosis in comparison the with wild type of KRAS. Our results showed that proximal location is more common for KRAS MSS tumors. In our series, 2 patients out of 21 (9.5%) were cardia tumors. Both were MSS, KRAS 12 V and both had a very bad prognosis and aggressive clinicopathological factors.

Limitations

One of the limitations of this paper is the small number of patients presenting with KRAS mutation. However, this is a first study analyzing the strong relationship between KRAS mutation and MSI status in GC and its outcome. We hope that this analysis will encourage the search for this molecular relationship using a bigger group of patients.

Conclusions

The rising interest in molecular and genetic links to clinicopathological data will probably improve the treatment and outcome of GC patients. We presented our data about KRAS mutations in GC patients, which showed a significant link with MSI status.

From our paper, we can conclude a few key points. The first is that KRAS mutations in GC are linked with MSI in the majority of cases. Patients with KRAS mutation and MSI are older, usually female, and present better survival than wt KRAS MSI patients, so it is a positive factor for survival of GC patients. KRAS mutations with MSS status present with a very bad prognosis and here, KRAS mutation is linked to a worse outcome than other MSS GC patients. Based on our study, it is not recommended to characterize KRAS mutations

based on different codon subtypes of mutation, but rather on MSI status.

While KRAS mutation status is not a prognostic or predictive biomarker in GC per se, the subtype-specific analysis may indeed identify clinically relevant subgroups of patients that ultimately may influence a treatment decision. Further analysis of a larger group of patients is needed to evaluate the outcome of these findings.

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Compliance with Ethical Standards

Conflict of Interest The authors declare they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions. Informed consent or substitute for it was obtained from all patients for being included in the study.

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