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ORIGINAL PAPER

Deficiency of mismatch repair genes is less frequently observed in signet ring cell compared with non-signet ring cell gastric cancer

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Abstract

Signet ring cell (SRC) gastric cancer at advanced stage has poor prognosis. While a recent study reported nearly one-third of SRC cases contain tumors with deficient mismatch repair (MMR) genes, other studies in SRC have been inconclusive. To re-analyze the results, we performed immunohistochemical staining of MLH1, MSH2, MSH6 and PMS2 proteins in 38 SRC gastric tumors compared with 109 non-SRC (NSRC) tumors from 94 patients. In contrast to the previous study, all SRC gastric tumors normally expressed MMR proteins, whereas 22 of 109 of NSRC (20%) showed deficient MMR proteins. To reinforce our results, we referred to the Cancer Genome Atlas (TCGA) genomic database and found that only 6 (6%) of 99 samples with diffuse gastric tumors showed deficient MMR, whereas 64 (21%) of 304 in intestinal gastric tumors showed deficient MMR. Our results as well as the TCGA database indicated that MMR genes are infrequently inactivated in SRC gastric cancer. These findings indicate that SRC patients may not be the best candidates for immuno-oncology therapy.

Keywords Microsatellite instability · Signet ring cell · Non-signet ring cell · Gastric cancer · Immune checkpoint inhibitor

Abbreviations

SRC	Signet ring cell
MMR	Mismatch repair
NSRC	Non-signet ring cell
TCGA	The Cancer Genome Atlas
PD-1	Programmed death 1
PD-L1	Programmed death 1 ligand 1
dMMR	Deficient mismatch repair
MSI	Microsatellite instability
IHC	Immunohistochemistry
MSI-H	Microsatellite instability-high
MSS	Microsatellite stable

Introduction

Gastric cancer is one of the most common cancers and leading causes of cancer-related mortality in the world [1]. Signet ring cell (SRC) carcinoma of the stomach is frequently observed in young individuals and females in particular [2]. Once SRC of the stomach progresses to the advanced stage, the tumors show decreased chemosensitivity and therapeutic options become limited [3]. Molecular targeted drugs and immuno-oncology therapy have provided benefit in prolonging the overall survival of patients with cancer in advanced stages. Therefore, identification of the molecular and genetic changes in SRC will provide new therapeutic strategies for SRC gastric cancer patients.

Immune checkpoint inhibitors targeting programmed death 1 (PD-1) and PD-1 ligand 1 (PD-L1) have been

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developed and used for cancer therapy [4, 5]. Approximately, 10–20% of cancer patients treated with immune checkpoint inhibitors showed durable response. Several biomarkers, including PD-L1 protein expression and tumor heterogeneity, have been demonstrated to correlate with the efficiency of immunotherapy [6–8]. Mismatch repair (MRR) proteins such as MLH1, MSH2, MSH6 and PMS2 play critical roles in the DNA repair pathway [9]. Loss of function of these proteins led to the accumulation of somatic mutations in tumors, which exhibit the mutator phenotype. In deficient MMR (dMMR) tumors, repeat sequences (e.g. mono- or di-nucleotide repeats) are frequently shortened, a phenomenon known as a microsatellite instability (MSI) [10]. Among several types of cancers, colorectal, endometrial and gastric cancers frequently exhibit dMMR [11]. Previous studies have also shown that dMMR is associated with the response to immunotherapy [7]. Although several studies have reported the frequency of MSI or dMMR in gastric cancers, the studies in SRC have yielded inconsistent results [12–15]. Tamura et al. reported no MSI-high (MSI-H) tumors in SRC gastric cancers (0%, 0/14) [12]. However, other groups indicated that MSI-H tumors were observed in 4% [13], 14% [14] and 17% [15] of SRC gastric cancers. However, Jin et al. recently showed that 33% of SRC gastric cancers exhibited MMR gene deficiency [16], implying that immuno-oncology therapy may be beneficial for SRC patients. Based on these discordant results, we should re-analyze the frequency of MSI and dMMR gastric cancers and examine the possibility that patients with SRC gastric cancers may have benefit for immuno-oncology therapy.

Here, we performed immunohistochemistry for MMR protein expression in 147 tumor samples from 94 gastric cancer patients, including 37 SRC patients and 57 non-SRC (NSRC) patients. In addition, we also examined The Cancer Genome Atlas (TCGA) and genome projects to evaluate MSI status in gastric cancers.

Materials and methods

Patients

In 2006, the Japanese government initiated the Basic Law (The Cancer Control Act) to encourage in-hospital cancer registration. The Cancer Control Act was established by the Japanese government in December 2013 and the law was enforced in January 2016. Since 2006, we have accumulated information on all malignant neoplasms that have been consecutively seen at our hospital. Our registry enrolled 20,086 patients with various cancer types from 2007 to 2017. We surveyed these patient records and identified 2346 patients with gastric cancer. Of these, 205 patients (9%) were histologically diagnosed as SRC and 2141 patients (91%) as NSRC. From these patients, we selected 94 gastric cancer patients for this study (62 males and 32 females; median age 71 years, range 28–94), including 37 patients with SRC and 57 patients with NSRC. Written informed consent was obtained from all patients in this study. This study was approved by the Institutional Review Board at our hospital [17].

Histology

Histological data were characterized by Lauren classification. We considered diffuse cancer as SRC or poorly differentiated gastric cancer and intestinal or mixed types as NSRC gastric cancer.

Immunohistochemistry (IHC)

We performed IHC on a total of 147 samples from the 94 gastric cancer patients, including 38 samples from 37 SRC patients and 109 samples from 57 NSRC patients. Serial 3-µm-thick sections of formalin-fixed paraffin-embedded tissues were deparaffinized and antigen activation was performed by heat treatment. Expressions of four MMR proteins (MLH1, MSH2, MSH6 and PMS2) were evaluated. IHC was performed on tumor samples using the OptiView DAB IHC Detection kit on the Ventana BenchMark ULTRA system (Roche, Tucson, AZ). We used primary antibodies against anti-MLH1 (M1; Ventana), anti-MSH2 (G219-1129; Ventana), anti-MSH6 (44; Ventana) and anti-PMS2 (EPR3947; Ventana). For PMS2 staining, the OptiView Amplification kit was used for development with diaminobenzidine solution. Positive staining of nuclear expression in the tumor lesion was considered as a proficient MMR tumor, whereas negative staining for at least one protein expression was considered dMMR. Normal tissue adjacent to tumor tissue served as a positive control.

To validate the specificity of IHC staining of MMR proteins, we performed MSI analysis and IHC on 126 tumor samples, including gastric cancers ($n=62$), lung cancers ($n=26$), colorectal cancers ($n=18$), breast cancers ($n=8$), ovarian cancers ($n=4$), pancreatic cancers ($n=2$), malignant pleural mesotheliomas ($n=2$), esophagus cancer ($n=1$), cholangiocarcinoma ($n=1$), prostate cancer ($n=1$) and bone metastasis ($n=1$). We confirmed all data were concordant between MSI and IHC data [18, 19] (in submission).

Data analysis of the cancer genome atlas

We obtained mutational, histological and patient information of TCGA from cBioPortal [20]. Fourteen data sources related to esophagus and stomach cancers were deposited in cBioPortal that included genetic information of 3089 tumor samples. After curating these datasets, we selected

four studies that included mutation profiles, the number of mutations, MSI status and histological information (Lauren class: e.g., intestinal and diffuse) of 976 gastric cancers. The 573 overlapping samples across the four projects were omitted, and the remaining 403 samples were subjected to subsequent analysis [21–24]. We classified tumors into MSI-H tumors and microsatellite stable (MSS) tumors (the latter of which included the MSI low tumor group). Genetic alterations (e.g., truncating, in-frame and missense mutations) in *MLH1, MSH2, MSH6* and *PMS2* genes were depicted using OncoPlot. The functional relevance of each mutation was evaluated by OncoKB and cBioPortal databases [25, 26].

Results

Low frequency of dMMR in SRC gastric cancer

This study included 147 samples from 94 gastric cancer patients, including 37 SRC patients and 57 NSRC patients, with 38 and 109 samples, respectively. We performed IHC for MMR proteins including MLH1, MSH2, MSH6 and PMS2 on 38 SRC and 109 NSRC tumor samples. MMR proteins were expressed at normal levels in all 38 SRC tumors (Fig. 1; Table 1). In contrast, 20% of NSRC cases (22/109) were dMMR tumors. Among the NSRC tumors, 21 tumors showed negative expression of MLH1 and PMS2 and one tumor was negative for MLH1, PMS2 and MLH6 expression (Table 1). These data showed that dMMR is a very rare event in SRC of the stomach.

We next referred the database of cBioPortal and used TCGA datasets of gastric cancers. In total, fourteen datasets including 3089 esophagus and stomach cancers were deposited. After curation, we selected 976 gastric cancers from four datasets that included mutation profiles, the number of mutations, MSI status and histological information (Lauren class) [21–24]. The 573 overlapping samples were omitted and the remaining 403 samples were subjected to subsequent analyses. We were able to retrieve genetic alterations, MSI and histological data.

Among the 403 patients with intestinal and diffuse types of gastric cancers, 99 patients showed diffuse gastric cancer and 304 patients showed intestinal gastric cancer. Of the 99 diffuse gastric cancers, only 6 (6%) were MSI-H tumors while the remaining 93 (94%) were MSS tumors (Fig. 2; Table 2). In contrast, of the 304 intestinal gastric cancers, 64 (21%) were MSI-H while 240 (79%) were MSS tumors. The frequency of MSI-H tumors was low or absent in diffuse gastric cancer in the four studies. In fact, three of the four studies showed no MSI-H in the diffuse type of 36 gastric cancers (Table 2). These results showed MSI occurred less frequently or was completely absent in diffuse gastric cancer compared with intestinal gastric cancer.

Tumor mutational burdens in diffuse and intestinal gastric cancer

To examine whether immune therapy may show possible benefit in patients with MSI-H diffuse gastric tumors, we analyzed the tumor mutational burden. As expected, in both diffuse and intestinal cancers, the number of mutations was higher in MSI-H tumors than in MSS tumors (Table 3). Interestingly, the number of mutations was significantly smaller in MSS diffuse cancers (mean, 74 mutations) than MSS intestinal cancers (139 mutations) ($p < 0.01$), whereas the number of mutations was comparable between MSI-H diffuse (1152 mutations) and intestinal tumors (1273 mutations).

Discussion

The SRC neoplasm is a rare tumor. Of the 2346 gastric tumors in our Cancer Registry Database, only 205 patients (9%) were SRC gastric cancer cases. However, because of its poor prognosis, better treatment options for SRC gastric cancer patients at advanced stage are required. In September 2017, nivolumab was approved in Japan for the treatment of advanced gastric cancer as the third line therapy. In 2018, nivolumab treatment was launched in other Asian countries, including Taiwan and Korea [27]. During the same period, a very high prevalence of dMMR in the SRC type of gastric cancer among several types of gastric cancers was reported in Asia [16]. This report prompted us to conduct this study.

We searched other studies on the prevalence of dMMR in SRC and found that the frequency of MSI in SRC was different among studies [12–15] and varied from 0% (0/14) to 33% (29/89) (Table S1). The most recent study showed the highest occurrence of dMMR (33%) in SRC [16]. Our results clearly indicated that none of the 38 tumor samples from the 37 patients with SRC had MMR protein expression, indicating proficient MMR. We also performed analysis using TCGA and genomic databases on gastric cancer and examined 403 patients with gastric cancer. Of the 99 diffuse gastric cancers, only 6 patients (6%) showed MSI-H tumors. Our findings and the database results clearly indicated that SRC gastric cancers exhibit MSI-H at a low frequency. The reason for this discrepancy is unclear, but it might be related to the evaluation threshold of positive or negative staining by IHC. We classified a tumor as a dMMR tumor when one of the four MMR proteins (MLH1, MSH2, MSH6 and PMS2) showed loss of expression in tumor nuclei compared with adjacent non-neoplastic tissue.

IHC analysis is a simple and very informative method to effectively select patients with gastric cancer for immuno-oncology therapy. Our current results indicate that 20% of intestinal carcinoma patients may receive benefit from

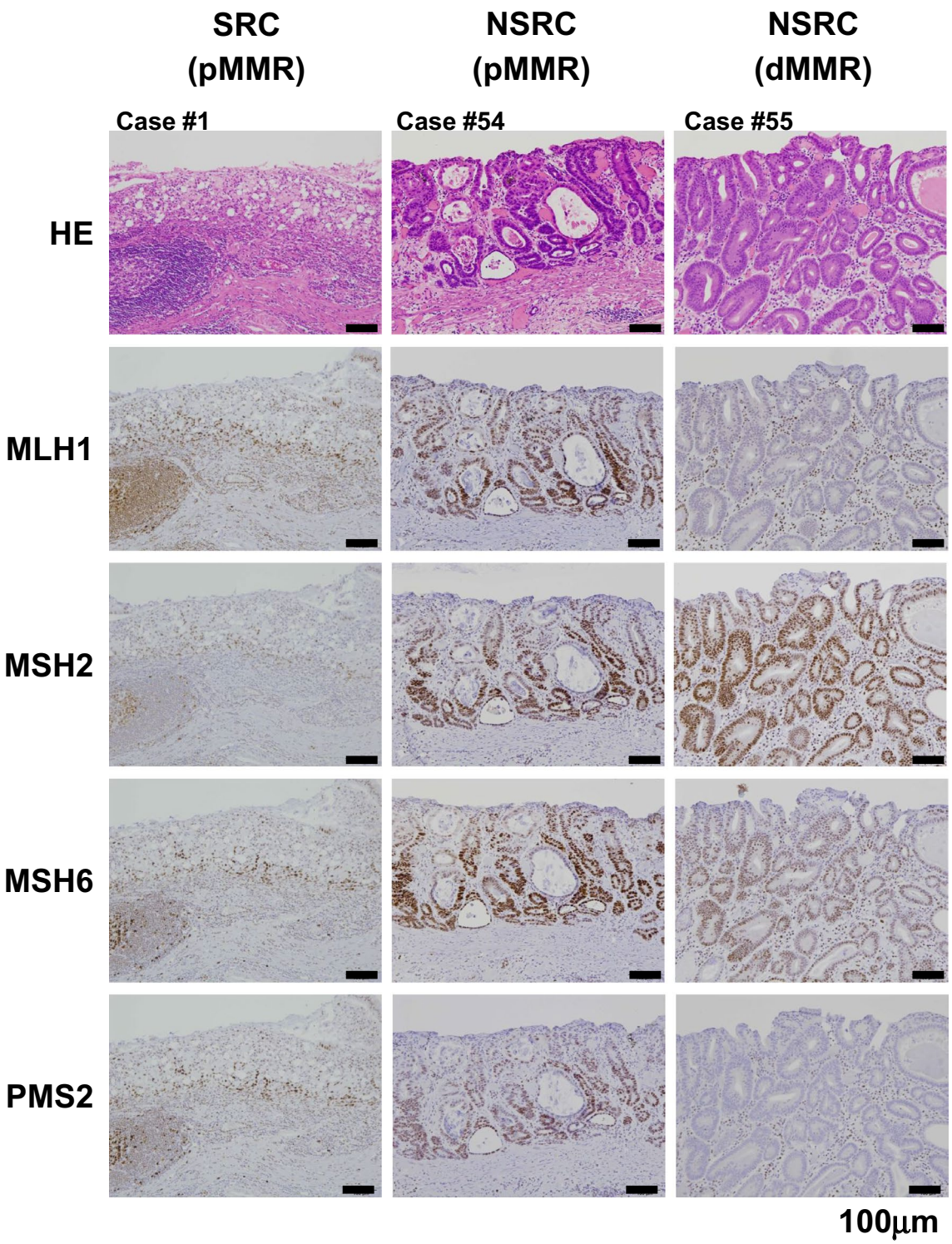

Fig. 1 Mismatch repair (MMR) protein expression in signet ring cell (SRC) and non-SRC (NSRC) gastric cancers. Representative images for hematoxylin-eosin staining and immunohistochemistry of MLH1, MSH2, MSH6 and PMS2. SRC (Case #1) and NSRC (Case #54) gastric tumors showed proficient MMR (pMMR). Loss of MLH1 and PMS2 proteins was detected in an NSRC case, indicating deficient MMR (dMMR) (Case #55)

Table 1 Frequency of loss of MMR protein expression in SRC and NSRC gastric tumors ($n = 147$)

	Loss of MLH1	Loss of MSH2	Loss of MSH6	Loss of PMS2	Total number of dMMR tumors
SRC ($n = 38$)	0/38 (0%)	0/38 (0%)	0/38 (0%)	0/38 (0%)	0/38 (0%)
NSRC ($n = 109$)	22/109 (20%)	0/109 (0%)	1/109 (1%)	22/109 (20%)	22/109 (20%)

MMR mismatch repair, *SRC* signet ring cell, *NSRC* non-signet ring cell, *dMMR* deficient mismatch repair

Fig. 2 OncoPlot showed microsatellite instability (MSI) status and genetic alterations in 403 patients with diffuse or intestinal gastric cancers from The Cancer Genome Atlas (TCGA) and genomic data. From cBioPortal, we used four datasets including tumor histology, MSI status and somatic mutations. Diffuse type (orange) and intestinal/mixed type (light green) gastric cancer were classified by Lauren criteria. MSI status was classified into MSI-high (MSI-H, blue) or MSI-low and microsatellite stable (MSS, light blue). Genetic alterations in mismatch repair genes (*MLH1, MSH2, MSH6* and *PMS2*) are presented. Genetic data were used from previous reports (Wang et al. *Nat. Genet*. [21]; Wang et al. *Nat. Genet*. [24]; TCGA, *Nature* [22]; TCGA, *Nature* [23])

Table 2 Frequency of MSI-H and MSS tumors in diffuse and intestinal gastric cancers

Lauren classification	MSI status	Nat. Genet. Wang et al. [21]	Nat. Genet. Wang et al. [24]	Nature TCGA [22]	Nature TCGA [23]	Total	MSI-H/ all (%)
Diffuse ($n = 99$)	MSI-H	0	0	0	6	6	6/99 (6%)
	MSS	2	28	6	57	93	
Intestinal ($n = 304$)	MSI-H	4	9	6	45	64	64/304 (21%)
	MSS	16	60	18	146	240	
	Total	22	97	30	254	403	

MSI-H microsatellite instability high, *MSS* microsatellite stable, *MSI* microsatellite instability, *TCGA* The Cancer Genome Atlas

Table 3 Relationship of tumor mutational burden and gastric histological characteristics

Histology	MSI status	Mean number of mutations	SD
Diffuse	Total ($n=99$)	140	279
	MSI-H ($n=6$)	1152	407
	MSS ($n=96$)	74	52
Intestinal	Total ($n=304$)	378	706
	MSI-H ($n=64$)	1273	945
	MSS ($n=240$)	139	354

MSI microsatellite instability, *SD* standard deviation, *MSI-H* microsatellite instability high, *MSS* microsatellite stable

immuno-oncology therapy. Despite the low frequency of MSI-H in SRC, the number of nonsynonymous mutations is almost the same in both MSI-H diffuse and intestinal tumors (Table 3). These results suggested that patients with MSI-H SRC gastric cancer may very well respond to immune-oncology therapy. However, unlike some previous reports [16], most SRC gastric cancers may not be MSI-H tumors.

To improve the prognosis in SRC gastric cancer patients, more effective therapeutic options need to be developed. Several reports have revealed somatic changes in SRC, and *TP53* (25%), *CDH1* (16%), *PIK3CA* (13%), *ERBB2* (6%), *LCE1F* (6%) and *OR8J1* (6%) were identified as significantly mutated genes in SRC [28]. *ALK* translocation was observed in 4% of SRC patients of the stomach (1/25) by fluorescence in situ hybridization [29]. *CLDN18-ARHGAP26/6* fusion genes were also identified in 25% of SRC patients (73/829) [28, 30]. *RHOA* activating mutations were observed in 25% of patients with diffuse gastric cancers (22/87) [30]. More studies to identify critical mutations with the aim of developing alternative therapeutic strategies are required for treatment of SRC gastric cancer patients.

Conclusions

Our results clearly revealed that dMMR was not frequently observed in patients with SRC or diffuse gastric cancers. Our findings also indicate that immuno-oncology therapy may have little benefit for patients with SRC gastric carcinoma, but may be useful in a significant number of NSRC gastric cancers.

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Compliance with ethical standards

Conflict of interest All authors declare no competing interests.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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