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NTRK gene alterations were enriched in hepatoid or enteroblastic differentiation type of gastric cancer

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ABSTRACT

Aims

Currently, the clinicopathological characteristics of gastric cancer (GC) with oncogenic NTRK alterations are not well known. Although NTRK fusion has been identified as prevalent in DNA mismatch repair protein deficient (dMMR) colorectal cancer (CRC), the relationship between NTRK alterations and dMMR protein expression in GC has not been previously explored.

Methods Our study comprised 51 cases of EBV(Epstein-barr virus)-associated gastric carcinomas, 94 cases of dMMR GC, 90 cases of gastric adenocarcinoma with hepatoid or enteroblastic differentiation (GAHED) and 256 cases of conventional GC. Furthermore, to investigate the connection between NTRK fusion and dMMR proteins, we collected dMMR tumours of various types, including 21 cases of duodenal adenocarcinomas, 46 endometrioid carcinomas and 82 CRCs. NTRK fusion and amplification were screened in GC and various types of dMMR tumours using fluorescence in situ hybridisation (FISH), while cases positive for FISH translocation underwent next-generation sequencing testing.

Results Our findings revealed the existence of two cases each of NTRK fusions and NTRK amplifications, which were all enriched in case of GAHED. Additionally, following an analysis of several types of cancers, we discovered that NTRK gene alterations were only present in dMMR CRC.

Conclusions Our results indicate that NTRK gene alterations are not enriched in GC with dMMR but are specifically enriched in cases of GAHED.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ NTRK fusion has been found to be enriched in DNA mismatch repair protein deficiency (dMMR)-type colorectal cancer (CRC).

WHAT THIS STUDY ADDS

⇒ NTRK gene alterations were enriched in hepatoid or enteroblastic differentiation type of gastric cancer (GC) not dMMR-type GC. NTRK gene fusion enrichment in dMMR is a unique phenomenon only in CRC but not in other dMMR tumours.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ In clinical practice, targeted screening for NTRK gene alterations in hepatoid or enteroblastic differentiation type of GC cases can be performed, which is beneficial for reducing screening costs.

BACKGROUND

Gastric cancer (GC) ranks as the fifth most common cancer and the third-leading cause of cancer-related deaths worldwide.[1] The incidence of GC varies significantly across geographical locations, with a higher concentration in developing countries,[2] particularly in China, which accounts for nearly half of the global GC-related fatalities. Globally, the median survival rate for advanced-stage GC is less than 12 months.[3] GC is a highly aggressive and heterogeneous malignancy that can be divided into various classifications based on distinct patterns, signatures and molecular mechanisms. Improved understanding of each GC subtype is crucial for developing appropriate therapies and advancing personalised medicine.

Although GC incidence rates have declined in recent decades worldwide,[4] progress in targeted treatments has been limited. In November 2018, Larotrectinib, a Trk inhibitor, was granted accelerated approval for the treatment of solid cancers with NTRK fusions in advanced/metastatic settings or when alternative treatments are infeasible, regardless of histological classification.[5 6] Larotrectinib has also been approved by the National Medical Products Administration for treating NTRK fusion-positive cancers in China. As GC is highly prevalent in China, NTRK fusion represents an essential therapeutic target.

In colorectal carcinoma (CRC), NTRK gene rearrangements are highly enriched in MLH1/PMS2 deficiency or MSI-H (microsatellite instability-high) cases.[7–9] Although prior studies have reported that NTRK gene abnormalities are exceedingly rare in GC, these studies did not accurately classified GC's histological and molecular subtypes.[10 11] Certain tumour driver gene abnormalities, such as HER2, have been found, to occur frequently in gastric adenocarcinoma with hepatoid or enteroblastic differentiation (GAHED).[12] Therefore, it is imperative to conduct research to ascertain whether NTRK gene abnormalities are enriched in specific GC subtypes.

In this study, we evaluated NTRK gene status using immunohistochemistry (IHC), and fluorescence in situ hybridisation (FISH) in three distinct GC subtypes, including EBV(Epstein-barr virus)-associated gastric cancer (EBVaGC), DNA mismatch repair protein deficient (dMMR) GC and GAHED. We compared these subtypes with conventional GC to analyse the prevalence of NTRK gene alterations

in GC. Finally, we examined the clinicopathological features of cases with NTRK alterations.

MATERIALS AND METHODS

Patients

We established a selected cohort of GC patients who underwent surgical resection by retrieving all cases from January 2011 to December 2021 in the computerised database of the Department of Pathology, Affiliated Nanjing Drum Tower Hospital, Nanjing University Medical School, Nanjing, China. The inclusion criteria were as follows: (1) gastric adenocarcinoma confirmed by pathology, (2) available pathological tissue samples and (3) clear pathological subtypes. The exclusion criteria included the following: (1) primary tumour with extracolonic or appendiceal location, (2) presence of simultaneous cancer, (3) preoperative neoadjuvant chemotherapy, radiation therapy or immunotherapy and (4) insufficient clinicopathological data.

All cases were histopathologically diagnosed according to the fifth edition of the WHO classification of digestive system tumours and were staged following the rules specified in the eighth edition cancer staging manual of the American Joint Cancer Committee. The definition of GAHED included GC with morphological features of hepatoid and/or enteroblastic differentiation as well as harbouring a positive immunophenotype of more than one biomarker of AFP, SALL4 and GPC3 in this study. Morphologically, GC with hepatoid differentiation usually presents a trabecular or solid growth pattern surrounded by sinusoidal vascular channels. In contrast, GCs with enteroblastic differentiation often show a tubular and/or papillary growth pattern with cytoplasmically clear tumour cells. The diagnosis of gastric adenocarcinoma with enteroblastic differentiation (GAED) and hepatoid carcinoma (HC) in this study was based on the morphological features described above when the area of HC or GAED accounted for more than 30% of the total number of tumours. Sometimes a mixture of hepatoid or enteroblast differentiated GC was present; when the sum of the two components exceeded 30%, it was also included in this study. In total, 33 HC cases, 42 GAED cases and 15 cases with mixed growth of HC and GAED were analysed.

Patient consent for surgical resection and clinical research was obtained in all cases before surgical resection.

Immunohistochemistry

AFP (Clone: 07I5D2, dilution 1:200, ZSGB-BIO, China), GPC3 (Clone: SP86, dilution 1:100, MXB Biotechnologies, China), SALL4 (Clone: 6E3, dilution 1:100, ZSGB-BIO, China), MLH1 (Clone: ES05, dilution 1:100, Dako Denmark A/S, Denmark), PMS2 (Clone: EP51, dilution 1:100, Dako Denmark A/S, Denmark), MSH2 (Clone: RED2, dilution 1:100, ZSGB-BIO, China), MSH6 (Clone: EP49, dilution 1:150, ZSGB-BIO, China) and Pan-TRK (Clone: EPR17341, Abcam, USA) IHC staining was carried out on the automatic Ventana Bench Mark Ultra system (Roche Diagnostics, Basel, Switzerland) using an automated staining protocol.

IHC staining scores were calculated by multiplying the staining intensity (0=no staining, 1=mild staining, 2=moderate staining and 3=strong staining) by the percentage of immunoreactive tumour cells (0–100). The immunostaining result was considered to be 0 or negative when the score was <25; 1+ or weak when the score was 26–100; 2+ or moderate when the score was 101–200; or 3+ or strong when the score was 201–300. This scoring method was applied to all cases and all markers. The results of IHC were interpreted independently by two pathologists who were blinded to all clinical and pathological data. For Pan-TRK IHC, cases were scored as positive if there was unequivocal staining in any percentage of tumour cells in any pattern (nuclear, cytoplasmic and/or membrane) locations. The absence of any staining was scored as negative. For the interpretation of IHC staining results for MLH1, PMS2, MSH2 and MSH6, a case was considered negative if there was no staining in tumour cells but nuclear positivity in non-tumour cells, including lymphocytes. Cases in which one or more of these four proteins were negative were considered as dMMR GC.

EBV-encoded RNA in situ hybridisation

In situ hybridisation staining was also performed on the automatic Ventana BenchMark Ultra system (Roche Diagnostics, Basel, Switzerland) using EB RNA probes (ZSGB-BIO, China). Both negative (without the probes) and positive controls were conducted in each run. Nuclear staining intensity was considered positive. GC with positive EB RNA expression was defined as EBVaGC.

Fluorescence in situ hybridisation

Four-micrometer-thick, formalin-fixed and paraffin-embedded tissue sections were used for FISH. FISH testing for NTRK gene rearrangements was performed using the NTRK1/2/3 Dual Color Break Apart Probe (Anbiping, China). NTRK gene break-apart was performed and interpreted according to a previously described method.[9] FISH testing for NTRK gene amplification using the NTRK1/2/3 Dual Color Probe (Empire Genomics,

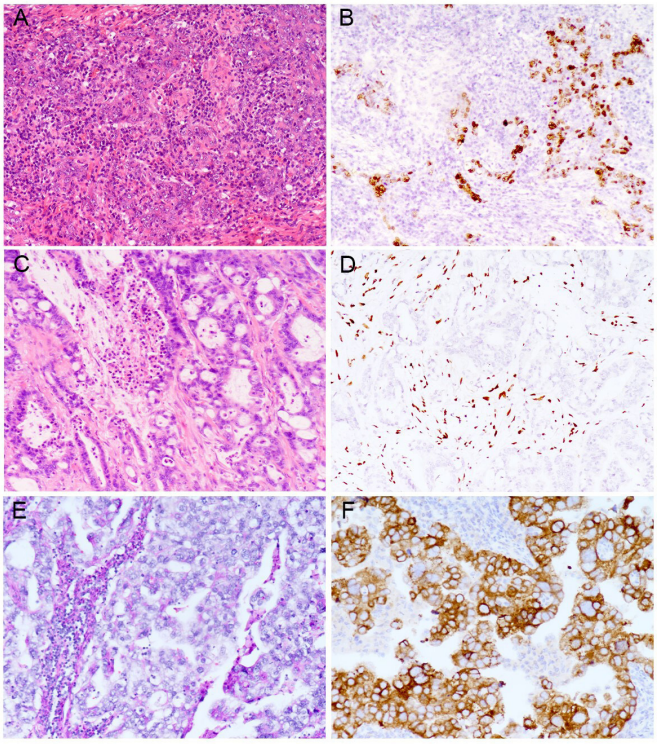

Figure 1 Microscopic morphology and immunohistochemistry (IHC) characteristics of different subtypes of gastric cancer (GC). H&E staining of EBVaGC (A); EBER staining show positive in EBVaGC (B); H&E staining of dMMR GC (C); immunohistochemical stain show negative for MLH1 (D); H&E staining of GAHED (E); immunohistochemical stain show positive for GPC3 (F). H&E, IHC×20. dMMR, mismatch repair protein deficient; EBVaGC, EBV-associated gastric cancer; EBER, Epstein-Barr Virus encoded RNA.

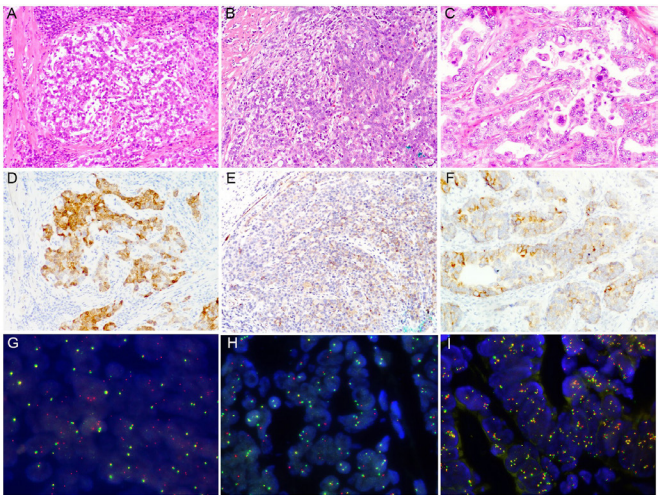

Figure 2 Microscopic morphology, immunohistochemistry (IHC) and molecular characteristics of NTRK gene alterations in the GAHED subtype. Microscopic morphology of NTRK gene alteration cases (A–C). Immunohistochemical staining for patients 1–3 showing positive staining for Pan-TRK (D–F). The amplified region of the NTRK gene appears red, while the signals related to the centromere of the chromosome appears green (control). NTRK3 amplification (G); NTRK1 break-apart (H); NTRK1 amplification (I). H&E, IHC×20, FISH×100. FISH, fluorescence in situ hybridisation; GAHED, gastric adenocarcinoma with hepatoid or enteroblastic differentiation.

USA) was also carried out on each case. NTRK gene amplification was considered when the ratio of signals of NTRK/signals of control genes was more than two or the average signals of NTRKs were more than ten.

ArcherDx assay (RNA-based next-generation sequencing)

Target-specific libraries for next-generation sequencing (NGS) were constructed using the Archer Universal RNA Reagent Kit v2 (ArcherDx, Boulder, CO). Library sequencing was performed using a MiSeqDx instrument (Illumina, San Diego, California, USA). NGS data were analysed using the Archer Analysis Pipeline Virtual Machine (https://archerdx.com). The ArcherDx assay was performed only for FISH NTRK break-apart positive cases.

RESULTS

Design cohort of GC

This study analysed a cohort of GC cases based on the morphological features and genotype. The cohort included 51 cases of EBVaGC, 94 cases of MMR protein deficient GC (dMMR GC), 90 cases of GAHED and 256 cases of conventional GC (online supplemental data 1). EBVaGC was characterised by the present of EBV infection (figure 1A,B), while dMMR GC showed the loss of expression of one or more than one MMR protein (figure 1C,D).[13 14] GAHED exhibited hepatoid and/or enteroblastic differentiation and positive immunophenotypes for than one biomarker of AFP, SALL4 and GPC3 (figure 1E,F).

Clinicopathological features of NTRK gene alterations in the GAHED subtype

Through FISH testing, four cases were identified as having NTRK gene translocation or amplification. The clinical features of the four cases with NTRK gene alterations are summarised in table 1. All four cases carrying NTRK genetic alterations displayed the morphological characteristics of GAHED and were male patients with a mean age of 63 years (aged between 57 and 67 years old). Tumour size ranged from 3.5 to 5.6 cm (mean: 4.8 cm), and all tumours were in the gastric antrum. Clinical follow-up was available for three of the four patients (patients 2, 3 and 4). Patient 2 underwent radical gastrectomy and died 63 months later due to cachexia. Patient 3 underwent radical gastrectomy and died 10 months later due to complications from liver metastases. Patient 4 was treated with camrelizumab plus oxaliplatin-gemcitabine for five cycles. After 24 months of follow-up, repeat CT and positron emission tomography(PET) scans did not identify recurrence.

Most notably, three GC cases with NTRK alterations showed the same morphological features of adenocarcinoma with enteroblastic differentiation. Cases 1–3 were composed of well-differentiated papillary or tubular-type adenocarcinoma with clear cytoplasm (figure 2A–C). Despite similarities, each of these four cases displayed variable features. In cases 1 and 4, the tumour showed glandular structures surrounded by chronic inflammatory cells (figure 2A); in case 2, part of the invasive area exhibited a hepatoid pattern mixed with a papillary pattern (figure 2B); in case 3, the tumour partly displayed a yolk-sac tumor-like carcinoma, representing reticular or papillary structures composed of cuboidal or columnar cells and focal necrosis (figure 2C). Pan-TRK IHC showed diffuse expression in all cases, with variable intensity in both cytoplasmic and membrane patterns (figure 2D–F). All four cases exhibited SALL4 and GPC3 expression, with coexpression of CD10 protein in the enteroblastic differentiation area.

All samples underwent FISH testing using NTRK1/2/3 break-apart probes and NTRK1/2/3 amplification probes. Of all the samples, only two cases with NTRK translocation and two cases with NTRK amplification (figure 2G–I) were found, and all the NTRK gene alteration cases were enriched in GC with enteroblastic differentiation areas. One case with NTRK1 gene translocation and one case with NTRK2 gene translocation were further validated by RNA-based NGS. RNA-based NGS confirmed the TPM3-NTRK1 fusion and NTRK2-SMCHD1 fusion (table 1).

Tumours with NTRK alterations showed heterogeneity, especially case 4 with NTRK2 translocation. This case displayed both glandular differentiation areas (figure 3A) and solid adenocarcinoma areas (figure 3B). Meanwhile, pan-TRK and CD10 IHC positivity were only observed around enteroblastic

Table 1 Clinical features of the four cases with NTRK gene alterations in gastric cancer

Case ID	Sex	Location	Size (cm)	Lauren	TNM	NTRK gene alteration	Follow-up
1	M	Antrum	5.0	Intestinal	T3, N3b, M0	NTRK1 Amplification	LTF
2	M	Antrum	3.5	Intestinal	T3, N1, M0	NTRK3 Amplification	DOD (63 months)
3	M	Antrum	5.6	Mixed	T3, N3b, M1	TPM3-NTRK1 fusion	DOD (10 months)
4	M	Antrum	5.2	Intestinal	T3, N2, M0	NTRK2-SMCHD1 fusion	NED (24 months)

DOD, dead of disease; LTF, lost to follow-up; M, male; NED, no evidence of disease.

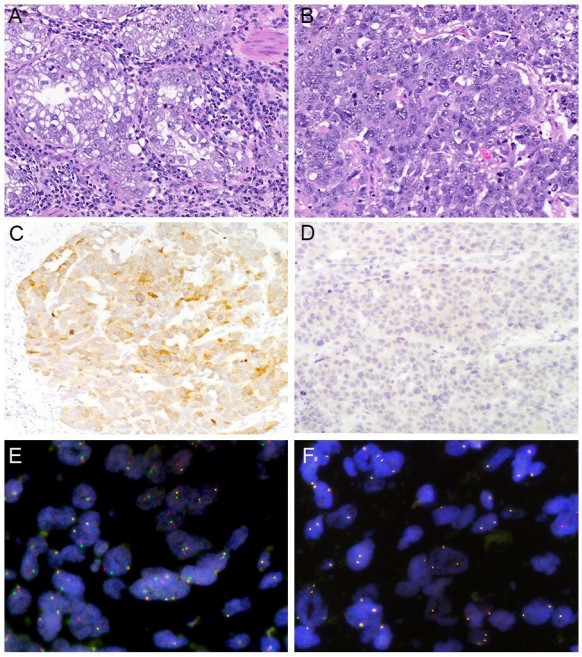

Figure 3 Tumours with NTRK alterations showed heterogeneity. Case 4 with NTRK2 translocation showed FISH positivity only in the area of glandule differentiation (A), whereas no NTRK fusion or amplification was found in the solid adenocarcinoma area (B). Pan-TRK IHC was positive only in the area of gland differentiation (C) and negative in the solid adenocarcinoma area (D). FISH showed positivity only in glandule differentiation (E) and negativity in the solid adenocarcinoma area(F). H&E, IHC×20, FISH×100. FISH, fluorescence in situ hybridisation; IHC, immunohistochemistry.

differentiation (figure 3C), and negative in the solid hepatoid adenocarcinoma area (figure 3D). FISH showed positivity only in glandular differentiation and negativity in the solid adenocarcinoma area (figure 3E,F).

NTRK gene fusion was only enriched in CRC but not in other tumours harbouring dMMR

The enrichment of NTRK gene fusion enrichment in the dMMR subtype of colorectal cancer (CRC) is a widely held belief in previous studies. However, our study found that NTRK gene fusion was not enriched in dMMR GC. To investigate this further, we collected various types of dMMR tumours, including 21 cases of duodenal adenocarcinomas, 46 endometrioid carcinomas and 82 CRCs (online supplemental data 2). All of these cases showed loss of expression of one or more than one biomarker of PMS2, MLH1, MSH2 and MSH6. All of these cases underwent FISH testing using the NTRK1/2/3 Dual Color Break Apart Probe. In addition to five (5/82, 6.10%) dMMR CRCs, none of the tumours with dMMR were confirmed to carry pan-TRK expression and NTRK translocation. Five dMMR CRCs with FISH NTRK translocations were also confirmed by RNA-based NGS (table 2).

DISCUSSION

GAHED is a comparatively rare subtype of GC, accounting for only 1.3%–5.4% of all cases.[15–18] However, GAHED presents special morphology with both adenocarcinomatous and hepatocellular differentiation and is characterised by several immunohistochemical markers, such as AFP, GPC-3, SALL4 and Hap-Par 1.[19 20] Previous studies have shown that GAHED is the most common AFP-producing GC, and GAHED is commonly believed to have more aggressive biological behaviour and poorer prognosis than cancers without hepatocellular carcinoma(HCC)-like morphology.[21] Because of the special histological and morphological features, the origin of GAHED may also have a unique underlying mechanism. Based on the molecular characteristics, the TCGA Research Network defined four molecular subtypes of GC: EBVaGC, MSI-H GC, genomically stable tumours and chromosomally unstable tumours,[22] whereas GAHED is a heterogeneous cancer with different clinical outcomes, biological behaviour and genetic alterations.[23] However, therapeutic targets specific to this unique subgroup have not been identified.

In this study, screening by NTRK FISH and confirmed by RNA-based NGS, we found NTRK gene alterations accounted for 0.8% (4/491) of all gastric adenocarcinomas with enrichment in GAHED (4.4%, 4/90), especially in cases with enteroblastic differentiation, which morphologically presented tubular and/or papillary characteristics. In total, four cases with NTRK alterations were found, including two cases with NTRK fusion and two cases with NTRK amplification. Subsequently, in our cohort, NTRK gene alteration tumours (GAHED and dMMR colorectal adenocarcinoma) were further investigated and found to have molecular biological differences.

Clinicopathologically, all GAHED patients who exhibited NTRK gene alterations in our study were found to be male, with tumours located in the gastric antrum. Microscopically, all four cases showed adenocarcinoma with enteroblastic differentiation, out of which three cases were classified as Lauren intestinal type. An intriguing observation from our study was that even in the case with heterogeneity (case 4), the NTRK alteration was observed only in tumour cells exhibiting a tubular differentiation pattern, whereas no NTRK fusion or translocation was found in the solid pattern. This observation highlights the distinctive histological and molecular features of GAHED exhibiting NTRK alterations.

In CRC, NTRK fusion has been reported to be enriched in tumours with dMMR immunophenotype.[7–9] However, in this study, we did not observe this phenomenon in GC. In all 94 dMMR GC cases, none of the cases were confirmed to display NTRK fusion. To further investigate the relationship

Table 2 Clinical features of the five cases with NTRK gene alterations in colorectal adenocarcinoma

Case ID	Sex	Size (cm)	Differentiation	TNM	NTRK gene alteration	Mismatch repair protein expression
1	M	6.0	Low	T3, N0, M0	TPM3-NTRK1 fusion	MLH1/PMS2 deficiency
2	M	8.0	Low	T3, N0, M0	TPR-NTRK1 fusion	MLH1/PMS2 deficiency
3	F	6.0	Low	T3, N1, M1	TPM3-NTRK1 fusion	MLH1/PMS2 deficiency
4	M	5.0	High	T3, N0, M0	NTRK3-hoxc13 fusion	PMS2 deficiency
5	F	2.6	High	T3, N0, M0	5′-telomere-NTRK1 fusion	MLH1/PMS2 deficiency

F, female; M, male.

between the expression of dMMR protein and NTRK fusion, we collected various types of tumours that were confirmed to be dMMR by IHC, including 21 duodenal adenocarcinomas, 46 endometrioid carcinomas and 82 CRC. However, the results were the same as those in GC; in addition to five (5/82, 6.10%) dMMR CRC, none of the tumours with dMMR were confirmed to carry NTRK fusion. Our results further demonstrated that NTRK gene fusion was only enriched in dMMR CRC but not in other tumours with dMMR, which is a unique phenomenon exclusive to CRC. This may be mostly because the MMR proteins we selected did not fully mirror the function of the dMMR system. In cancers other than CRC, multiple signalling pathways may participate in the 'crosstalk' between MMR-related pathways and NTRK-related pathways, so the relationship between MMR pathways and NTRK-related pathways is complex and not straightforward. In different tumours we included, we all used the expression of the same four MMR proteins (MLH1, PMS2, MSH2 and MSH6) to indicate the function of the MMR system. These four proteins have been shown to contribute to the development of some cancers, such as CRC, ovarian clear cell and endometrioid carcinoma,[24 25] whereas in some other cancers, the role of the MMR pathway in tumour development is unclear. In addition to MLH1, PMS2, MSH2 and MSH6, there are also other DNA MMR proteins, including MSH3, MLH3 and PMS1. However, accurate prediction of the status of the MMR pathway has not reached a consensus in other tumours. Therefore, even though there was a relationship between MMR protein expression and NTRK gene alterations in other tumours, the MMR protein may exclude MLH1, PMS2, MSH2 and MSH6.

In conclusion, our clinicopathological data and molecular analysis of the largest series of different types of GC combined with our new clustering analysis results based on gene expression profile data (1) is the first to report NTRK gene alterations that were enriched GAHEDs but not in dMMR GCs. Out of a 51 EBVaGCs, 94 dMMR GCs, 90 GAHEDs and 256 conventional GCs, only two cases with NTRK fusions and two cases with NTRK amplification were identified, and all four cases were observed in GAHEDs. (2) We clearly defined the biological nature of GAHED with NTRK gene alterations as adenocarcinoma with enteroblastic differentiation. Through further collection of duodenal adenocarcinomas with dMMR, endometrioid carcinoma with dMMR, and CRC with dMMR, we demonstrated that (3) NTRK gene fusion enrichment in dMMR is a unique phenomenon only present in CRC but not in other dMMR tumours.

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Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval The Medical Ethics Committee at Nanjing Drum Tower Hospital granted ethics approval for this study.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement The data that support the findings of this study are available from the corresponding author upon reasonable request.

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