FULL PAPER

BJC

British Journal of Cancer (2013) 108, 1495–1501 | doi: 10.1038/bjc.2013.109

Keywords: *KRAS* mutation; *BRAF* mutation; DNA mismatch repair; gastric cancer; multicentre study

KRAS and *BRAF* mutations are rare and related to DNA mismatch repair deficiency in gastric cancer from the East and the West: Results from a large international multicentre study

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Background: Inhibitors of the epidermal growth factor (EGFR) signaling pathway have a major role in the treatment of *KRAS* wild-type colorectal cancer patients. The EGFR pathway has been shown to be activated in gastric cancer (GC). However, published data on *KRAS* and *BRAF* mutation status is limited in GC and has not been compared between GC from different geographic regions.

Methods: The prevalence of *KRAS* and *BRAF* mutations was established in 712 GC: 278 GC from the United Kingdom, 230 GC from Japan and 204 GC from Singapore. The relationship between *KRAS*/*BRAF* mutation status, DNA mismatch repair (MMR) status, clinicopathological variables and overall survival was analysed.

Results: Overall, 30 (4.2%) GC carried a *KRAS* mutation. In total, 5.8% of the UK GC, 4% of Japan GC and 1.5% of Singapore GC were *KRAS* mutant. *KRAS* mutant GC had fewer lymph node metastases in the UK cohort ($P=0.005$) and were more frequent in elderly patients in the Japan cohort ($P=0.034$). *KRAS* mutations were more frequent in MMR-deficient GC in the UK and the Japanese cohort ($P<0.05$). A *BRAF* mutation was only detected in a single Japanese GC.

Conclusions: This large multicentre study demonstrated that *KRAS* mutations and DNA MMR deficiency have a role in a small subgroup of GC irrespective of country of origin, suggesting that this subgroup of GC may have developed along a common pathway. Further studies need to establish whether concomitant mutations or amplifications of other EGFR signalling pathway genes may contribute to the activation of this pathway in GC.

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Received 29 October 2012; revised 12 February 2013; accepted 19 February 2013; published online 19 March 2013

Despite a steady decline in incidence over the last decades, gastric cancer (GC) is still the fourth most common cancer worldwide and the second most common cause of cancer-related death worldwide (Ferlay *et al*, 2010). Many GC patients present with locally advanced disease, which is treated with peri-operative cytotoxic combination chemotherapy and surgery in the West (Cunningham *et al*, 2006) and surgery followed by chemotherapy in the East (Sakuramoto *et al*, 2007). However, even with multimodality treatment, the 5-year overall survival (OS) is less than 40% in advanced disease (Cunningham *et al*, 2006). Recent advances in targeted therapy demonstrated a survival benefit of trastuzumab in patients with HER2-positive inoperable GC (Bang *et al*, 2010). GC is characterised by geographical and molecular heterogeneity, which may potentially impact on the development of targeted therapy for this disease. Mutations of *KRAS* and *BRAF*, two major players of the epidermal growth factor (EGFR) signalling pathway, are known to have predictive value for therapies with antibodies targeting EGFR, such as panitumumab and cetuximab in patients with metastatic colorectal cancer (Misale *et al*, 2012). Recent studies demonstrated sensitivity to cetuximab in *KRAS* wild-type, EGFR-expressing GC cell lines and xenografts (Heindl *et al*, 2012; Hotz *et al*, 2012; Kneissl *et al*, 2012).

The first study reporting a *KRAS* mutation in GC was a case report in 1986 (Bos *et al*, 1986). Since then, 50 studies have investigated the *KRAS* mutation status in GC. More than 80% of studies were conducted in Asian GC patients and only seven of these studies included tumour material from more than 100 patients (Lee *et al*, 1995; Hao *et al*, 1998; Yoo *et al*, 2002; Lee *et al*, 2003; Yashiro *et al*, 2005; Tajima *et al*, 2006; Deng *et al*, 2012). The largest Western study to date included 82 GC patients (Brennetot *et al*, 2003), whereas the largest Asian study from Korea included 319 GC patients (Lee *et al*, 2003). All studies focussed on the mutation status of *KRAS* codons 12 and 13 using a number of different methods. The median *KRAS* mutation frequency of all GC cohorts was 6.5% (range: 0%–36%) and was only slightly lower in the non-Asian GC (median 4%, range 0%–21%) compared with Asian GC (median 6%, range: 0%–36%).

Considering only studies with more than 100 GC patients, some of the authors reported a relationship between mutant *KRAS* and well-differentiated histology of GC (Yashiro *et al*, 2005), intestinal-type GC and higher pT stage (Yoo *et al*, 2002) and cancer location in the proximal third of the stomach (Lee *et al*, 1995).

Overall, the exact prevalence of *KRAS* mutations in locally advanced, resectable GC remains unknown and no definite conclusions can be drawn regarding the potential geographical heterogeneity or relationship of *KRAS* mutation status with clinicopathological data including survival. Furthermore, only a small number of studies investigated *BRAF* mutation status in small GC patient cohorts and reported a frequency ranging from 0% to 11% with no relationship to histopathological variables (Kim *et al*, 2003; Lee *et al*, 2003; Oliveira *et al*, 2003; Wu *et al*, 2004; Sasao *et al*, 2006; Stella *et al*, 2009; Corso *et al*, 2011).

Results from three published studies, all investigating less than 100 GC patients, suggest that there might be an association between *KRAS* mutation status and DNA mismatch repair (MMR) status (Brennetot *et al*, 2003; Zhao *et al*, 2004; Gylling *et al*, 2007), although no causal relationship between DNA MMR status and *KRAS* mutation status has been shown to date (for review see Castagnola and Giaretti (2005)).

The aim of the current study was to establish the frequency of *KRAS* and *BRAF* mutations in GC in a large multicentre study, investigate the relationship between *KRAS*/*BRAF* mutation status, DNA MMR status and clinicopathological variables including survival, and compare findings between GC from different geographic regions.

MATERIALS AND METHODS

Gastric cancer cohort from Leeds (UK). This study included 278 patients with sporadic gastric adenocarcinoma (GC) who underwent potentially curative surgery at the Department of Surgery, Leeds General Infirmary (Leeds, UK), between 1970 and 2004. None of the patients received any form of chemotherapy. Demographical, clinical and pathological data were retrieved from pathology reports, electronic patient hospital records and the Northern and Yorkshire Cancer Registry. Median follow-up time after surgery was 1.9 years, ranging from 0.11 to 20.48 years. Twenty-two patients died within 30 days after surgery and were excluded from survival analysis. Eight patients were lost from follow up. In total, 138 (49.6%) patients died from GC during the study period. The study was approved by the Local Research Ethics Committee (LREC No. CA01/122).

Gastric cancer cohort from Yokohama (Japan). This study included 230 patients with stage II/III sporadic GC who underwent potentially curative surgery at Kanagawa Cancer Center Hospital (Yokohama, Japan) between 2001 and 2010. In total, 125 (54.3%) patients received adjuvant chemotherapy (S-1 or Tegafur-uracil). Demographical, clinical and pathological data were retrieved from hospital records. Median follow-up time after surgery was 4.9 years, ranging from 0.5 to 10.4 years. None of the patients died within 30 days after surgery. Six patients were lost from follow up. Sixty-nine (30%) patients died from GC during the study period. The study was approved by the Local Research Ethics Committee.

Gastric cancer cohort from Singapore (Singapore). This study included 204 Chinese patients with sporadic GC who underwent potentially curative surgery in Singapore (Singapore General Hospital, National University Hospital and Tan Tock Seng Hospital) between 1994 and 2008. Twenty-six (12.7%) patients received adjuvant chemotherapy (5-Fluorouracil). Demographical, clinical and pathological data were retrieved from hospital records. Median follow-up time after surgery was 1.6 years, ranging from 0.2 to 13.1 years. None of the patients died within 30 days. Eight (3.9%) patients were lost from follow up. In total, 106 (52%) patients died from cancer and 11 patients died from other complications during the study period. This study was approved by the Local Research Ethics Committee and Institutional Review Board.

In all series, cases were staged according to TNM classification 7th edition (Sobin *et al*, 2009). Grade of differentiation was determined according to the WHO classification (WHO 2010) and morphological tumour type was classified according to Laurén's classification (Lauren, 1965).

DNA extraction. All haematoxylin/eosin-stained tissue sections from all resection specimens were reviewed by a histopathologist (HIG, NCTvG, YM, TArai, YK) and a representative formalin-fixed, paraffin-embedded tissue block containing the highest density of primary adenocarcinoma was selected. The area of interest contained more than 30% tumour cells in all cases and was marked on the slide by the histopathologist to facilitate macro-dissection. Depending on the size of the tumour up to five 10 μm sections were cut, deparaffinised using a standard protocol and the marked area of interest was dissected using a sterile scalpel blade. Genomic DNA from the Yokohama and Leeds cases was extracted using a protocol based on the QIAmp DNA Micro Kit (Qiagen, Hilden, Germany) as described previously (Buffart *et al*, 2011) and using the DNeasy blood and tissue kit (Qiagen) for the Singapore cohort as described previously (Deng *et al*, 2012).

***KRAS* and *BRAF* mutation detection.** In the Leeds GC cohort, mutation pre-screening using high-resolution melting technology followed by Sanger sequencing was used to detect *KRAS* codons 12,

13, 61 and *BRAF* codon 600 mutations as described in detail previously (Kramer *et al*, 2009; Heideman *et al*, 2012). In the Yokohama GC cohort, pyrosequencing was used to determine the mutations status of *KRAS* codons 12, 13 and 61 as well as *BRAF* codon 600 as described previously (Richman *et al*, 2009). In the Singapore GC cohort, Sanger sequencing and MassARRAY technology (Sequenom Inc., San Diego, CA, USA) were used to determine the mutation status of *KRAS* codons 12 and 13 as described previously (Deng *et al*, 2012). *KRAS* codon 61 and *BRAF* mutation status were not assessed in the Singapore GC cohort.

DNA extracted from normal tissues from the same patient was genotyped for *KRAS* and/or *BRAF* mutation status from all cases with *KRAS* and/or *BRAF* mutation to distinguish between somatic and germline mutation.

Assessment of the DNA MMR status

Immunohistochemistry for MLH1, MSH2, PMS2 and MSH6. For the Singapore GC cohort, immunohistochemistry (IHC) was performed using the Leica BOND-MAX autostainer (Leica Microsystems Ltd, Milton Keynes, UK). Tissue sections were treated with Leica Bond epitope retrieval solution (ER-2, Leica, cat. no: AR9640) for 20 minutes (min) at 100 °C and incubated with primary antibodies, MLH1 (1 : 50, Cell, Marque, Rocklin, CA, USA, cat. no: 285M-16), MSH2 (1 : 50, Biocare Medical, Concord, CA, USA, cat. no: CM219), MSH6 (1 : 150, Biocare Medical, cat.no: CM265) and PMS2 (1 : 150, Leica, cat. no: NCL-PMS2) for 20 min at room temperature. Leica Bond polymer refine DAB detection system was used according to the instructions of the manufacturer. Sections were counterstained with haematoxylin, dehydrated and mounted.

For the Yokohama GC cohort, IHC was performed manually as described previously (Grabsch *et al*, 2010) using 0.1 M citrate buffer pH 6.0 for antigen retrieval in a microwavable pressure cooker. Slides were incubated with primary antibodies, MLH1 (1 : 50, overnight at 4 °C, BD Pharmingen, Oxford, UK, cat. no: 550838), MSH2 (1 : 70, 60 min at 37 °C, Calbiochem, Watford, UK, cat. no: NA27), MSH6 (1 : 50, overnight at 4 °C, Invitrogen, Paisley, UK, cat. no: 18-0443) and PMS2 (1 : 25, overnight at 4 °C, BD Pharmingen cat. no: 556415). The Dako Real streptavidin-biotin detection kit (Dako, Ely, UK) or a tyramine-based amplification system and DAB were used as described previously (Grabsch *et al*, 2010). Sections were counterstained with haematoxylin, dehydrated and mounted.

The scoring system used was the same for both cohorts. GC with positive stained tumour cell nuclei were classified as MMR-proficient. GC were only classified as 'negative' (MMR-deficient) if the tissue section contained an internal positive control such as lymphocytes.

Microsatellite analysis. The MSI Multiplex System Version 1.2 (Promega, Southampton, UK, cat. no MD1641) was used for the detection of microsatellite instability according to the instructions of the manufacturer. This kit allows the co-amplification of BAT-25, BAT-26, NR-21, NR24 and MONO-27 from the same input DNA sample. The PCR products were separated by capillary electrophoresis using an ABI PRISM 3100 DNA sequencer and analysed with GeneMapper 3.5 software (Applied Biosystems, Paisley, UK). As the overall frequency of microsatellite instability was very low in the current cohorts, no distinction was made between low and high microsatellite instability. The kit includes a genomic DNA sample, which served as positive control, and nuclease-free water, which was used as negative control.

In 112 GC patients from Singapore, the MMR status was determined by IHC as well as by microsatellite analysis. All Singapore GC cases, which were negative for at least one of the MMR proteins by IHC, showed microsatellite instability and all cases positive for all four MMR proteins by IHC were microsatellite stable, a finding that is consistent with the published literature. A decision was therefore made to perform IHC on the Yokohama GC patients and microsatellite analysis on the Leeds GC patients, as available material was limited.

A case was classified as 'MMR-deficient' if either one of the MMR proteins was negative by IHC or the case showed microsatellite instability.

Statistical analysis. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS 15.0 for Windows, Chicago, USA).

Comparisons between the mutation status, the DNA MMR status and the clinicopathological variables were performed using the Mann–Whitney *U*-test (for two groups) or the Kruskal–Wallis test (for more than two groups). Analyses of overall survival (OS) were performed using the Kaplan–Meier method and differences between groups were tested by the log-rank test. Data from patients who died within 30 days after surgery were excluded from survival analysis. *P*-values less than 0.05 were considered significant.

RESULTS

***KRAS* and *BRAF* mutation status in the Leeds GC cohort.** *KRAS* exon 1 (codons 12 and 13) mutation data were available from 276 GC patients. Two (0.7%) GC failed to amplify. *KRAS* exon 2 (codon 61) data were available from 270 (97%) of the tested 278 GC. *BRAF* codon 600 data were available from 264 (95%) of the tested 278 GC.

A *KRAS* mutation was found in 16 (5.8%) GC. Twelve (75%) mutations occurred in *KRAS* codon 12, 2 (13%) in *KRAS* codon 13 and 1 (12%) in *KRAS* codon 61. The most common mutation was p.G12D, which was found in five (30%) GC, followed by p.G12V (4 GC) and p.G12A (2 GC). *KRAS* mutations p.G12C, p.G13C, p.G13D and p.Q61H were found in one GC each. No concurrent *KRAS* mutations were seen. None of the Leeds GC had a *BRAF* V600E mutation. With the exception of a *KRAS* p.V8V polymorphism (rs147406419), which was found in the tumour and normal DNA from one patient, all matched normal DNA showed *KRAS* and *BRAF* wild-type.

***KRAS* and *BRAF* mutation status in the Yokohama GC cohort.** *KRAS* exon 1 (codons 12 and 13) and exon 2 (codon 61) mutation data were available from all 230 GC patients. *BRAF* codon 600 data were available from 227 (99%) of the tested 230 GC.

A *KRAS* mutation was found in 10 (4%) GC. Six (60%) mutations were located in *KRAS* codon 12 and four (40%) in *KRAS* codon 13. No mutation was found in *KRAS* codon 61. The most common mutation was p.G12D, which was found in six (60%) GC, the remaining four GC had a p.G13D mutation. No concurrent *KRAS* mutations were seen. One (0.4%) GC had a *BRAF* V600E mutation and was *KRAS* wild-type at the same time. All matched normal DNA showed *KRAS* and *BRAF* wild-type.

***KRAS* and *BRAF* mutation status in the Singapore GC cohort.** *KRAS* exon 1 (codons 12 and 13) mutation data were available from 204 GC patients. Three (1.5%) GC showed a *KRAS* mutation. Two mutations were located in codon 12 (p.G12C and p.G12D) and one in codon 13 (p.G13D). All matched normal DNA showed *KRAS* wild-type.

Comparison of the *KRAS*/*BRAF* mutation status between the cohorts. There was no statistically significant difference in the overall frequency of *KRAS* mutations between the three GC cohorts with 5.8% (Leeds), 4% (Yokohama) and 1.5% (Singapore).

A total of 75% of *KRAS* mutations were located in codon 12 in the Leeds cohort compared with 60% in the Yokohama cohort and 67% in the Singapore cohort. A *KRAS* codon 61 mutation was only

found in the Leeds cohort, whereas a *BRAF* mutation was only found in the Yokohama cohort. No concurrent mutations were found in any of the patients.

***KRAS*/*BRAF* mutation status and clinicopathological characteristics.** Owing to the small number of mutations found, GC were categorised as '*KRAS* wild-type' or '*KRAS* mutant' for statistical analyses. When results from all three GC cohorts were combined for analyses, no significant relationship was found between *KRAS* mutation status and clinicopathological variables.

Because there were only three *KRAS* mutant GC in the Singapore cohort, no statistical analyses were performed within this cohort. One of the *KRAS* mutant Singapore GC was from a male patient and was staged as pT3N0M0. The two other *KRAS* mutant Singapore GCs were from female patients and both staged as pT4N1M0. All *KRAS* mutations in the Singapore GC cohort occurred in moderately differentiated intestinal-type GC and in patients younger than 70 years.

In the Leeds GC cohort, the only significant relationship found was that *KRAS* mutations were more common in Leeds GC with lower lymph node category (pN, $P = 0.005$, see Table 1). None of the patients with more than six lymph node metastases (pN3a/b) had *KRAS* mutations. A total of 81% of the *KRAS* mutant Leeds GC were of intestinal-type histology and 88% were locally advanced cancers with infiltration of the subserosa or beyond. However, due to the overall small number of cancers with *KRAS* mutations, these findings were not statistically significant. In contrast to the Singapore GC, 69% of *KRAS* mutant Leeds GC occurred in patients older than 70 years at the time of diagnosis.

In the Yokohama GC cohort, *KRAS* mutations were more frequent in the elderly patients aged $\geqslant 70$ years ($P = 0.034$). There was a trend for a higher *KRAS* mutation frequency in well-differentiated Yokohama GC ($P = 0.063$). The single *BRAF*-mutant Yokohama GC occurred in a 61-year-old male patient, was of poorly differentiated type histology and staged as pT3N2M0.

There was no relationship with any of the other clinicopathological variables tested (see Table 1).

***KRAS* mutation status and overall survival.** As expected, depth of tumour invasion (T category) and lymph node status (N category) were significant independent predictors of prognosis in all GC cohorts (data not shown).

Univariate overall survival (OS) analysis showed no significant difference when patients were stratified by *KRAS* mutation status irrespective of whether the results from all cohorts were combined for analysis or cohorts were analysed individually.

In the Leeds cohort, the OS rate at 3 and 5 years after surgery in patients with *KRAS* mutant GC was 42.9% and 35.7%, respectively, compared with 37.9% and 31.2% in patients with *KRAS* wild-type GC, $P = 0.5057$. In the Yokohama cohort, the OS rate at 3 and 5 years after surgery in patients with *KRAS* mutant GC was 81.8% and 71.6%, respectively, compared with 74.1% and 59.5% in patients with *KRAS* wild-type GC, $P = 0.5850$. There was also no significant difference in survival between patients with or without *KRAS* mutant GC in the Yokohama cohort when survival was analysed separately in patients treated with or without adjuvant chemotherapy. In the Singapore cohort, the OS rate at 3 and 5 years after surgery in patients with *KRAS* mutant GC was 66.7% for both time points compared with 51.7% and 47.3% in patients with *KRAS* wild-type GC.

***KRAS*/*BRAF* mutation status and DNA MMR status.** MMR status data were available from 264 Leeds GC of which 25 (9%) were classified as MMR-deficient. A higher incidence of *KRAS* mutations were noted in the MMR-deficient GC: 11 (5%) of the MMR-proficient and 5 (20%) of the MMR-deficient Leeds GC had a KRAS mutation ($P = 0.002$, Table 1). Four of the five *KRAS* mutant/MMR-deficient GC were intestinal-type GC, one showed a mixed histology.

MMR status data were available from 230 Yokohama GC of which 21 (9%) were classified as MMR-deficient. A higher incidence of *KRAS* mutations were noted in the MMR-deficient GC: 11 (3%) of the MMR-proficient and 3 (14%) of the MMR-deficient Yokohama GC had a *KRAS* mutation ($P = 0.019$, Table 1). One of the *KRAS* mutant/MMR-deficient GC was an intestinal-type GC, one a diffuse-type GC and one a mucinous GC. The Yokohama GC with *BRAF* mutation, which was the only case with BRAF mutation in the whole series, was classified as MMR-proficient as all four IHC markers were positive.

MMR status data were available from 122 Singapore GC of which 17 (14%) were classified as MMR-deficient. Of the three *KRAS* mutant GC, one showed MMR deficiency, one was classified as MMR-proficient and no data were available from the third case.

DISCUSSION

Five-year survival of patients with locally advanced GC is still poor in the East and the West even after modern multimodality treatment combining radical surgical resection with cytotoxic chemotherapy (Cunningham *et al*, 2006; Sakuramoto *et al*, 2007). Several clinical studies are underway to evaluate the potential efficacy of EGFR inhibitors in patients with metastatic oesophagogastric cancer, none of them is currently using a biomarker to select patients (Okines *et al*, 2011). In colorectal cancer, benefit from EGFR inhibitors has been restricted to patients with *KRAS* wild-type cancer (Misale *et al*, 2012). The determination of the prevalence of *KRAS*/BRAF mutation in a sufficiently large series of GC from different geographic regions appears to be an essential prerequisite for further worldwide clinical development of EGFR-directed therapy in GC.

The current study is the largest study to date investigating *KRAS* and *BRAF* mutation status and DNA MMR status in patients with locally advanced resectable GC originating from three different countries with different GC incidence, Caucasian patients from the UK, Japanese patients and Chinese patients from Singapore. A *BRAF* mutation was found in a single GC from Yokohama confirming the absence or very low frequency of *BRAF* mutations in GC reported previously (Lee *et al*, 2003; Oliveira *et al*, 2003; Zhao *et al*, 2004).

The prevalence of *KRAS* mutation in all primary resectable GC of this study was 4% and statistically not different between the different GC cohorts. From this result, which is in concordance with the published GC literature on *KRAS* mutation frequency (Hongyo *et al*, 1995; Lee *et al*, 1995; Zhao *et al*, 2004), there is no evidence to suggest that *KRAS* mutation frequency is related to GC incidence, aetiology or ethnicity, factors which are all significantly different in countries from the East and the West (Ferlay, 2010). Furthermore, in all investigated cohorts, *KRAS* mutation frequency was statistically not related to gender, tumour location, depth of invasion, grade of differentiation or tumour morphology. However, looking at the subgroup of all *KRAS* mutant GC investigated in the current study, almost two-third of *KRAS* mutant GC were intestinal-type GC, which is consistent with other studies (Miki *et al*, 1991; Yoo *et al*, 2002; Corso *et al*, 2011). It is difficult to compare our findings to the current GC literature as the studies published so far are contradictory. As such, *KRAS* mutations in GC were described as being exclusively seen in males (Liu *et al*, 2009) but also to be more common in females (Corso *et al*, 2011), more frequent in well-differentiated GC (Kihana *et al*, 1991; Hiyama *et al*, 2002; Yashiro *et al*, 2005), in distal cancers (Zhao *et al*, 2004), in proximal cancers (Lee *et al*, 1995), in early-stage cancers (Hongyo *et al*, 1995; Liu *et al*, 2009), whereas other studies found

Table 1. KRAS mutation status and relationship with clinicopathological variables and mismatch repair status in the Leeds and Yokohama gastric cancer cohort

	Leeds gastric cancer							Yokohama gastric cancer						
	Total		*KRAS* wild-type		*KRAS* mutated			Total		*KRAS* wild-type		*KRAS* mutated		
	n	**%**	***n***	**%**	***n***	**%**	***P*-value**	***n***	**%**	***n***	**%**	***n***	**%**	***P*-value**
Age group														
<70 years	112	41	107	96	5	4	0.434	161	70	157	97	4	3	0.034
⩾70 years	164	59	153	93	11	7		69	30	63	91	6	9	
Gender														
Male	164	59	155	95	9	5	0.791	162	70	155	96	7	4	0.975
Female	112	41	105	94	7	6		68	30	65	96	3	4	
Tumour location														
Proximal	66	24	64	97	2	3	0.421	69	30	67	97	2	3	0.693
Mid	72	26	66	92	6	8		93	40	89	96	4	4	
Distal	122	45	115	94	7	6		68	30	64	94	4	6	
Stump	8	3	7	88	1	12		0	0	0	0	0	0	
L. plastica	5	2	5	100	0	0		0	0	0	0	0	0	
Depth of invasion (pT)														
pT1a/b	20	7	18	90	2	10	0.735	8	4	8	100	0	0	0.214
pT2	23	8	23	100	0	0		42	18	38	91	4	9	
pT3	80	29	74	93	6	7		30	13	30	100	0	0	
pT4a/b	153	55	145	95	8	5		150	65	144	96	6	4	
Lymph node status (pN)														
pN0	85	31	76	89	9	11	0.005	40	17	40	100	0	0	0.160
pN1	51	19	48	94	3	6		55	24	51	93	4	7	
pN2	54	20	50	93	4	7		62	27	61	98	1	2	
pN3a/b	84	31	84	100	0	0		73	32	68	93	5	7	
Grade of differentiation														
G1	31	11	28	90	3	10	0.768	23	10	20	87	3	13	0.063
G2	88	32	84	96	4	4		53	23	50	94	3	6	
G3	156	57	147	94	9	6		154	67	150	97	4	3	
Laurén classification														
Intestinal	178	65	165	93	13	7	0.150	120	52	117	97	3	3	0.151
Diffuse	60	22	58	97	2	3		110	48	103	94	7	6	
Mixed	38	14	37	97	1	3		0	0	0	0	0	0	
Mismatch repair status														
Proficient	239	91	228	96	11	4	0.002	209	91	202	97	7	3	0.019
Deficient	25	9	20	80	5	20		21	9	18	86	3	14	

no such associations (Nanus *et al*, 1990; Arber *et al*, 2000; Lee *et al*, 2003). All previous studies suffer from investigating a relatively small number of GC patients making the interpretation of any statistical analysis difficult.

The higher frequency of *KRAS* mutations in patients with lower pN category in the Leeds GC cohort confirms a previous report from a small cohort of Chinese GC (Liu *et al*, 2009). It is currently unclear why no such relationship was seen in the Japanese GC cohort. *KRAS* mutation status was not related with survival in any of the three GC cohorts confirming a previous report in 140 Japanese GCs (Lee *et al*, 1995).

The current study showed that *KRAS* mutations are more frequent but not exclusive to MMR-deficient GC confirming results from a small previous study (Zhao *et al*, 2004). Other previous studies did not identify *KRAS* mutations in MMR-proficient GC, which could be related to the very small number of GC investigated (Brennetot *et al*, 2003; Gylling *et al*, 2007). Interestingly, these findings in GC are in contrast to results from studies in colorectal cancer where a lower incidence of KRAS mutations in MMR-deficient cancers has been described (Hutchins *et al*, 2011). Although this is currently the largest series of GC investigating more than 700 GC for MMR status and *KRAS* mutation status, the total number of GC showing *KRAS* mutation or MMR deficiency and *KRAS* mutation is still very small making interpretation difficult. However, the existence of a small subgroup of GC with distinct molecular characteristics may be related to the known heterogeneity of GC.

Further studies are required to characterise the *KRAS* mutant GC subgroup at a molecular level in order to better understand the biological effects of *KRAS* mutation in GC. It would be of particular interest to establish whether the RTK/RAS signalling pathway might be activated in GC due to multiple concomitant

mutations of genes related to RTK/RAS signalling or concomitant gene amplifications also present in only small subsets of GC (Deng *et al*, 2012). The presence of up to 40% concomitant EGFR pathway-related mutations has been reported in a small study ($n = 63$) of GC very recently (Corso *et al*, 2011). However, *BRAF* mutations do not seem to have any role in GC.

The current study has some limitations that are mainly related to the fact that this was a retrospective study. Although this is a very large series of GC with more than 700 patients, the interpretation of the results remains challenging, as the prevalence of *KRAS* mutation, DNA MMR deficiency and combined KRAS mutation/DNA MMR deficiency is relatively low. Hence, even this large multicentre study may still be underpowered to detect an association between *KRAS* mutation and overall survival. However, there was a trend in the current study that the presence of a *KRAS* mutation was associated with better overall survival in GC patients, which is in contrast to studies in colorectal cancer.

For the current study, we used DNA from a single tissue block found to be representative of the primary cancer based on morphology. Gastric cancer is known to be very heterogeneous and thus, by using only one block we may have underestimated the true mutation frequency. However, there is currently no evidence in the literature to support that *KRAS/BRAF* mutations are heterogeneous in GC or that the frequency differs between primary cancer and lymph node metastasis. For practical reasons, we have used different methods to evaluate the *KRAS* mutation status in the different patient cohorts. However, all methods have shown to be able to detect mutations in samples with less than 10% mutated tumour cells (Heideman *et al*, 2012) and all samples used for extraction had in effect more than 30% of tumour cells. Unfortunately, we do not have access to material from clinical studies investigating the efficacy of EGFR inhibitors. Hence, it remains to be shown whether *KRAS* mutation status predicts treatment response in GC patients.

In summary, this is the largest study to date investigating the *KRAS* and *BRAF* mutation status as well as DNA MMR status in locally advanced, resectable GC from the East and the West. The study confirms that *KRAS* mutations and DNA MMR deficiency have a role in a small subgroup of GC irrespective of country of origin of the patient.

These data suggest that neither *KRAS* mutations nor DNA MMR deficiency are related to the very different GC incidence in the East and the West. Similar *KRAS* mutation frequency and similar incidence of DNA MMR deficiency in GC patients from multiple cohorts may suggest that these particular subgroup of GC may have develop along a common yet to be identified pathway. Further molecular characterisation of these GC subgroups is needed to understand the biological effect of *KRAS* mutations and DNA MMR in GC.

ACKNOWLEDGEMENTS

We thank Marije Doeleman and Marinda van Moorsel for expert technical assistance. This work was supported by grants from the Sasakawa Foundation UK, Pathological Society of Great Britain and Ireland, Cancer Research UK grant C37059/A11941, Non-Profit Organizations Kanagawa Standard Anti-cancer Therapy Support System (Yokohama, Japan) and the Health Insurance Company (LPT grant, The Netherlands).

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