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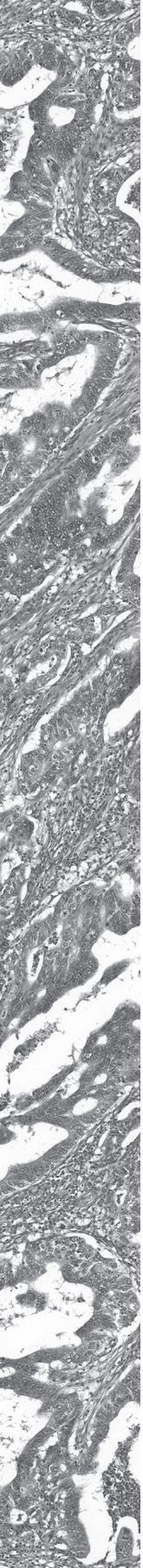
CONTENTS



Chapter 1

General Introduction

GASTRIC CANCER

Gastric cancer (GC) is the fifth most common cancer worldwide with more than 1 million new cases diagnosed annually and is accountable for more than 782,000 deaths each year (1). Globally, GC incidence rates are twofold higher in males than females (1). GC incidence is highest in Asia, whereas in Northern Europe, North America and Africa the rates are lower (1). The high GC incidence in Asian countries is the reason why a two-yearly population based GC screening programme has been implemented in countries such as Japan and South Korea with the aim of improving patient survival due to detection of GC at an early stage (2, 3). In other countries, screening programmes are not cost effective due to the lower incidence (4). In The Netherlands (NL), the estimated World Standardised Rate (WSR) [number of cancers per 100,000, adjusted for age distribution of the world population] for incidence and mortality in 2018 is 8.0 and 5.8, respectively (5). The estimated WSR for incidence and mortality in the UK for 2018 is 7.8 and 5.6, respectively (5).

On the basis of associated risk factors, GC can be classified as cardia and non-cardia GC (6). Cardia GC arises in the region directly distal from the gastro-oesophageal junction (6). Risk factors for cardia GC include alcohol consumption, tobacco smoking, increased body mass index and gastro-oesophageal reflux disease (7-10). The increasing incidence of cardia GC has been attributed to rising global obesity levels which itself is related to increased gastro-oesophageal reflux disease (7). Non-cardia GC arises in the distal stomach (6) and has been associated with *Helicobacter pylori (H. pylori)* infection and chronic atrophic gastritis (11, 12). The declining incidence of non-cardia GC in recent years has been attributed to decreasing *H.pylori* infection rates (13, 14). Other risk factors for GC include diet (high consumption of salty food and low consumption of fruit and vegetables) (15) and genetic predisposition (16).

In Western countries, the Lauren classification remains the most widely used histological classification system, which classifies GC into diffuse, intestinal and mixed-type (17), see figure 1. Other schemes proposed over the years include Nakamura (18), Ming (19), Goseki (20), Carneiro (21), Solcia (22), Japanese Gastric Cancer Association (23) and World Health Organisation [WHO] (24), which for adenocarcinoma alone has over ten different subcategories (see table 1).

In addition to numerous histological classification schemes, several molecular GC classifications have been proposed in recent years, including from our own research group (25-27). In 2013, a classification system proposed by the Singapore-Duke group divided GC into proliferative, metabolic and mesenchymal subtypes, based on genetic and epigenetic expression of drug-responsive clusters (28). Subsequently, in 2014 The Cancer Genome Atlas described four distinct molecular GC subtypes: Epstein-Barr virus (EBV) positive, microsatellite instable (MSI), genomically stable (GS) and chromosomally instable (CIN) (29). The EBV subgroup is further characterised by *PIK3CA* mutations, programmed death ligand 1/2 (PD-L1/2) overexpression, hypermethylation (CpG island methylator phenotype (CIMP)), *CDKN2A*

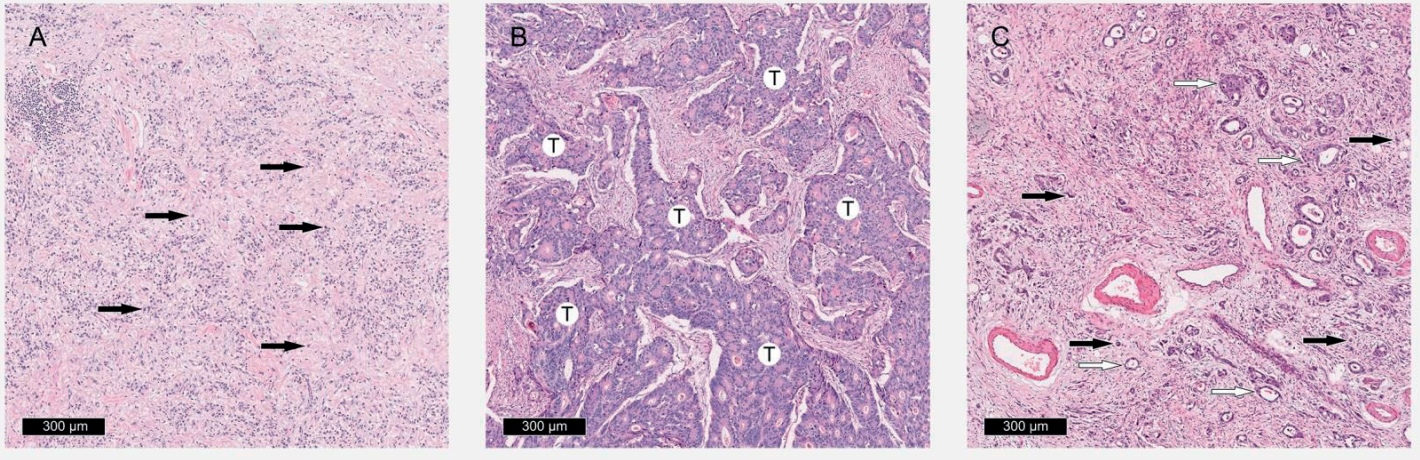


Figure 1 | Examples of histological phenotypes in gastric cancer according to the Lauren classification
(A) diffuse type GC with poorly cohesive individual tumour cells or small groups of tumour cells (black arrows)
(B) intestinal type GC with tumour cells showing glandular or tubular differentiation (marked with 'T')
(C) mixed type GC, with a mixture of diffuse type tumour cells (black arrows) and intestinal type tumour cells forming glandular structures (white arrows).

silencing and increased immune cell infiltration. The MSI phenotype is most frequently the result of a defective mismatch repair (MMR) mechanism due to somatic mutation of one of the MMR genes (such as MLH1, MLH2, MSH6 or PMS2) or hypermethylation of the

Table 1 | Histological classification of gastric cancer according to the World Health Organisation

Histological classification of gastric cancer
Adenocarcinoma
Tubular adenocarcinoma
Parietal call adenocarcinoma
Adenocarcinoma with mixed subtypes
Papillary adenocarcinoma
Micropapillary carcinoma
Mucoepidermoid carcinoma
Mucinous adenocarcinoma
Signet-ring cell carcinoma
Poorly cohesive carcinoma
Medullary carcinoma with lymphoid stroma
Hepatoid adenocarcinoma
Peneth cell carcinoma
Squamous cell carcinoma
Adenosquamous carcinoma
Carcinoma, undifferentiated
Large cell carcinoma with rhabdoid phenotype
Pleomorphic carcinoma
Sarcomatoid carcinoma
Carcinoma with osteoclast-like giant cells
Gastroblastoma
Neuroendocrine tumour
Neuroendocrine tumour, grade 1
Neuroendocrine tumour, grade 2
Neuroendocrine tumour, grade 3
Gastrinoma
Somatostatinoma
Enterochromaffin-cell carcinoid
Enterochromaffin like-cell carcinoid, malignant
Neuroendocrine carcinoma
Large cell neuroendocrinecarcinoma
Small cell neuroendocrine carcinoma
Mixed neuroendocrinenon–neuroendocrine neoplasm

Adapted from (24)

MLH1 promoter (30). The most common molecular alterations in GS GC are *CDH1* or *RHOA* mutations, *CLDN18-ARHGAP* gene fusion and increased expression levels of genes/proteins involved in cell adhesion pathways. The CIN subtype is characterised by TP53 mutations and activation of genes in the receptor tyrosine kinase pathway, such as *EGFR*, *HER2*, *FGFR2*, *MET* and *KRAS* (29). Sohn *et al*. suggested a prognostic value of the TGCA GC classifier and predicted chemotherapy survival benefit using this classification system (31). In 2015, The Asian Cancer Research Group proposed a molecular GC classification system based on MSI, microsatellite stable (MSS)/epithelial to mesenchymal transition, MSS/TP53 active and MSS/TP53 inactive GCs (32). This classification system was shown to have prognostic value and was validated in two additional Asian GC cohorts (32). Figure 2 shows a comparison of the main molecular classification systems proposed in GC. Subsequently, several research groups have proposed classification of GC using Epstein-Barr encoded RNA *in situ* hybridization and immunohistochemistry as a surrogate for molecular classification in GC (33-35). Despite all these classification efforts (molecular and histological), decisions regarding patient treatment are still currently based upon the clinical stage of the disease (36, 37) and patient's performance status and preferences.

Figure 2 | Comparison of molecular classifications systems in gastric cancer
Abbreviations: CIN, chromosomal instability; EBV, Epstein–Barr virus; EMT, epithelial–mesenchymal transition; FU, fluorouracil; GS, genomically stable; MSI, microsatellite instability; MSS, microsatellite stable.
Adapted from (35).

OESOPHAGEAL CANCER

Oesophageal cancer (OeC) is the seventh most common cancer worldwide, with an estimated 572,000 new cases and 509,000 deaths in 2018 (1). Globally, the incidence of OeC is two to threefold higher in males compared to females (1). The incidence of OeC is highest in Eastern Asia, and Eastern and Southern Africa (1). In NL, the WSR for incidence and mortality in 2018 is 3.5 and 2.8, respectively (5). The estimated WSR for incidence and mortality in the UK for 2018 is 3.7 and 3.0, respectively (5). With the exception of high-risk areas of China (38), population screening is not proven to be cost effective for OeC (39).

The two main histological subtypes of OeC are squamous cell carcinoma (SqC) and adenocarcinoma (AdC). More than 90% of OeC in the world are SqC with the highest incidence in Eastern countries, whereas UK and NL have the highest incidence of AdC in the World (1). Molecular characterisation of OeC by TCGA revealed that the molecular profile of oesophageal AdC more closely resembles GC than oesophageal SqC (40). Nevertheless, trials for patients with metastatic disease often include patients with GC, oesophageal adenocarcinoma and oesophageal squamous cell cancers in the same clinical trial. This is also true to a lesser extent for patients with resectable disease.

The risk factors for oesophageal SqC include tobacco smoking, alcohol consumption and diet, whereas obesity, gastro-oesophageal reflux disease and Barrett's oesophagus are associated with oesophageal adenocarcinoma (39). Population cancer screening for high-risk patients with Barrett's oesophagus is recommended (41).

OESOPHAGEAL AND GASTRIC CANCER: DIAGNOSIS, PROGNOSIS AND TREATMENT

GC and OeC are often grouped together under the term oesophagogastric cancer (OGCa) due to similarities in diagnosis and treatment strategies.

Patients with early stage OGCa are often asymptomatic. Due to the absence of an OGCa screening programme, patients in Western countries most commonly present with locally advanced disease at the time of diagnosis. Gold standard diagnosis of OGCa is by endoscopic biopsy and histopathological assessment. The Union for International Cancer Control (UICC) tumour-node-metastasis (TNM) staging system is used for clinical staging to decide patient treatment and pathological staging after resections in Europe (42). The TNM stage is a combination of depth of tumour invasion (T stage), number of tumour positive lymph nodes (N stage) and the presence of distant metastases (M stage). Figure 3 shows a schematic representation of T stage in GC and OeC. The TNM stage groupings are established and regularly updated on the basis of their prognostic relevance, with high TNM stage being associated with a worse prognosis in OGCa (43, 44). The definition whether a tumour is a gastric or

oesophageal cancer is dependent on the macroscopic location of the bulk/epicentre of the tumour with respect to the gastro-oesophageal junction. In accordance with TNM8, tumours are categorised as being either OeC (including the oesophagogastric junction) or GC (42).

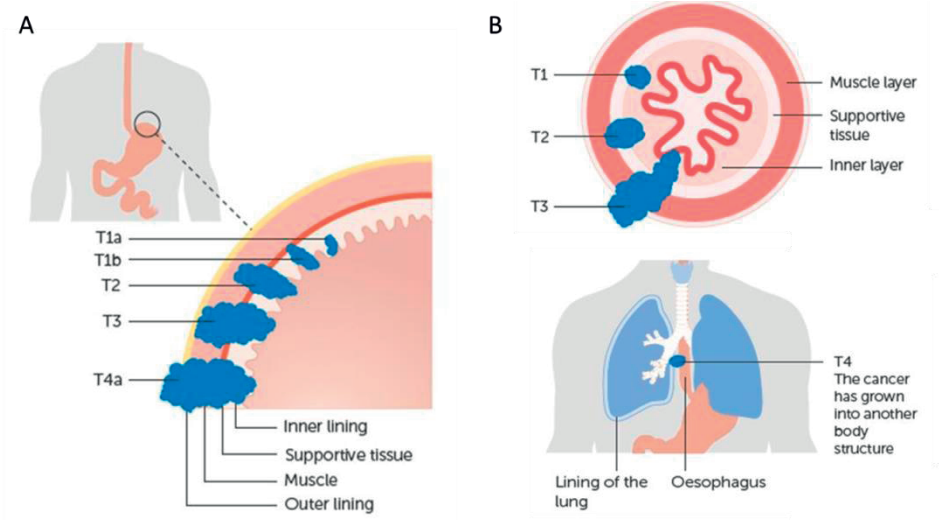


Figure 3 | Schematic representation of the depth of tumour invasion in gastric cancer (A) and oesophageal cancer (B). The T stage increases with increasing depth of the tumour.
Image reuse credit: Cancer research UK

Treatment of oesophagogastric cancer patients

Treatment decisions are currently based on TNM stage and patient related factors (36, 45-50). Thus, in early disease where the cancer is restricted to the mucosa and without clinical evidence of lymph node metastasis e.g. cT1aN0M0, endoscopic resection would be the preferred treatment option depending on size of the tumour, absence of ulceration and absence of poor differentiation (51). In locally advanced resectable OGCa (≥cT2N0), which is the focus of this thesis, clinical trials have demonstrated the benefit of neoadjuvant/perioperative combination chemo(radio)therapy followed by surgical resection as the gold standard treatment in the West (52-55) and surgical resection followed by adjuvant chemotherapy in the East (56, 57). OGCa patients presenting with unresectable or metastatic disease are treated with combination chemotherapy and have a median life expectancy of less than 12 months if treated with cytotoxic chemotherapy (58). For a summary of treatment options for patients with OGCa, see figure 4.

Treatment of locally advanced gastric and gastro-oesophageal junction cancer

In Europe, the results from the FLOT4 trial have been reported very recently (55) which resulted in a change of the standard perioperative chemotherapy backbone for the treatment

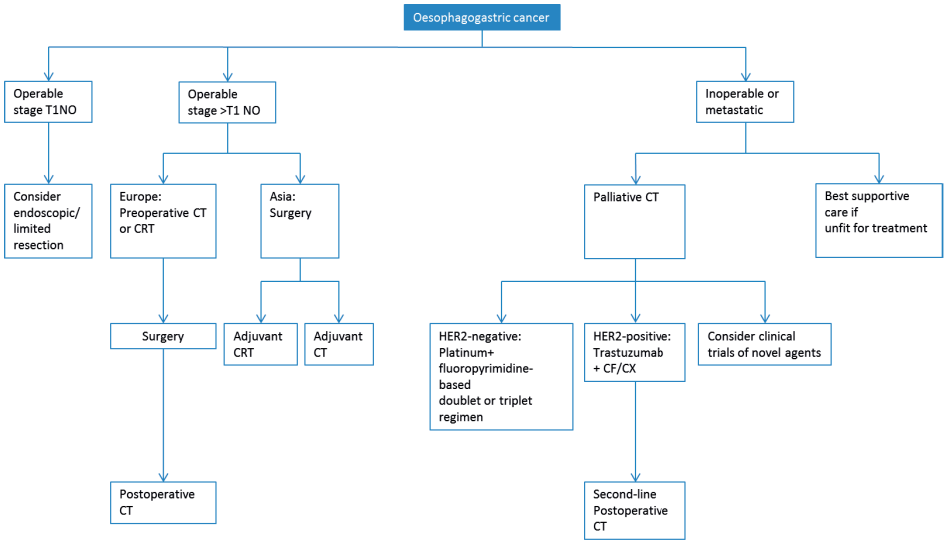


Figure 4 | Treatment algorithm for patients with oesophagogastric cancer in Europe and Asia
Abbreviations: CT, chemotherapy; CRT, chemoradiotherapy; HER2, human epidermal growth factor receptor 2; CF, cisplatin and 5-fluorouracil; CX, cisplatin and capecitabine
Adapted from (36).

of patients with locally advanced resectable gastric or gastro-oesophageal junction cancer from ECF/ECX (epirubicin, cisplatin, 5-fluorouracil/epirubicin, cisplatin, capecitabine (53)) to FLOT (fluorouracil plus leucovorin, oxaliplatin, and docetaxel). However, even with FLOT, 5-year overall survival reaches only 45%. Other attempts such as the use of preoperative chemotherapy combined with postoperative chemoradiotherapy in the CRITICS trial or the addition of targeted treatment such as bevacizumab in the ST03 trial did not improve overall survival compared to existing standard treatment regimens for patients with resectable GC (59, 60).

In Asia, the ACTS-GC and CLASSIC trials, randomising patients with pathological TNM stage II-III disease, established the benefit of adjuvant chemotherapy, with a 5-year survival of 72-78% (56, 57). The addition of radiotherapy into the adjuvant treatment in the ARTIST trial did not improve patient survival (61).

For an overview of survival outcomes in studies evaluating different treatment regimens in patients with locally advanced resectable gastric cancer, see table 2.

Treatment of locally advanced oesophageal cancer

OeC patients in the UK are usually treated with neoadjuvant chemotherapy (cisplatin/5-FU) followed by surgery based on the Oe02 trial (52). The Oe05 trial, which in comparison to Oe02 doubled the number of neoadjuvant chemotherapy cycles and increased the number of chemotherapy drugs to 3, was unable to demonstrate benefit from more intensified

Table 2 | Overall survival reported in randomised phase III clinical trials, evaluating treatment regimens in patients with localised resectable gastric cancer

Trial/ registry No./ authors	Regimen	No. of patients	Outcome
SWOG 9008/ INT-0116 (69)	Surgery vs. surgery + CRT	275 vs. 281	Median OS: 27 months vs. 36 months; 3-year OS rate, 41% vs. 50%; HR, 1.35; 95% CI: 1.09-1.66; P = 0.005
ARTIST (61)	Surgery (D2) + CT vs. surgery (D2) + CRT	228 vs. 230	5-year OS rate, 73% vs. 75%; HR, 1.130; 95% CI: 0.775-1.647; P = 0.5272
ACTS GC (57, 70)	Surgery (D2) vs. surgery (D2) + S1	530 vs. 529	3-year OS rate, 70.1% vs. 80.1%; HR, 0.68; 95% CI: 0.52-0.87; P = 0.003
CLASSIC (56)	Surgery (D2) vs. surgery (D2) + XELOX	520 vs. 515	5-year OS rate, 69% vs. 78%; HR, 0.66; 95% CI: 0.51-0.85; P = 0.0015
MAGIC (53)	Surgery vs. ECFx3 + surgery + ECFx3	253 vs. 250	5-year OS rate, 23.0% vs. 36.3%; HR, 0.75; 95% CI: 0.60-0.93; P = 0.009
FLOT4-AIO (55)	FLOTx4 + surgery + FLOTx4 vs. ECFx3 + surgery / ECFx3	356 vs. 360	Median OS: 50 months vs. 35 months; 5-year OS rate, 45% vs. 36%; HR, 0.77; 95% CI: 0.63-0.94; P = 0.012
ST03 (60)	(ECX + bevacizumab) x3 + surgery + (ECX + bevacizumab)x3 vs. ECXx3 + surgery / ECXx3	533 vs. 530	3-year OS rate, 48.1% vs. 50.3%; HR, 1.09; 95% CI: 0.91-1.29; P = 0.36

Abbreviations: SWOG 9008/INT-0116, Southwest Oncology Group 9008/Intergroup trial 0116; CRT, chemoradiation therapy; OS, overall survival; HR, hazard ratio; CI, confidence interval; ARTIST, Adjuvant Chemoradiation Therapy in Stomach Cancer; CT, chemotherapy; ACTS GC, Adjuvant Chemotherapy Trial of S-1 for Gastric Cancer; CLASSIC, Capecitabine and Oxaliplatin Adjuvant Study in Stomach Cancer; XELOX, capecitabine/oxaliplatin; MAGIC, Medical Research Council Adjuvant Gastric Infusional Chemotherapy; ECF, epirubicin, cisplatin, and 5-fluorouracil; FLOT4-AIO, perioperative chemotherapy with docetaxel, oxaliplatin, and fluorouracil/leucovorin (FLOT) versus epirubicin, cisplatin, and fluorouracil or capecitabine (ECF/ECX) for resectable gastric or gastroesophageal junction (GEJ) adenocarcinoma; FLOT, 5-fluorouracil, leucovorin, oxaliplatin, and docetaxel; ECX, epirubicin, cisplatin, and capecitabine; ST03, Chemotherapy With or Without Bevacizumab or Lapatinib to Treat Operable Oesophagogastric Cancer.
Adapted from (71).

therapy and 3-year overall survival remained poor at 39% with median overall survival at 26 months (62). Thus, although the Oe02 trial closed over 20 years ago, Oe02-style treatment remains one of the standard treatment options.

OeC patients in NL are treated with neoadjuvant chemoradiotherapy followed by surgery, based on the results from the CROSS trial, which reported a 5-year survival of 47%, with median overall survival of 49 months (54).

Several meta-analyses have failed to show improved survival with the addition of radiotherapy to neoadjuvant chemotherapy (63, 64). For a conclusive answer to this question, we need to wait for the results from the ongoing Neo-AEGIS trial, which compares survival in patients treated with perioperative chemotherapy versus neoadjuvant chemoradiotherapy followed by resection (65).

For an overview of survival outcomes in studies evaluating treatment regimens in localised resectable oesophageal cancer, see table 3.

Table 3 | Overall survival reported in randomised phase III clinical trials, evaluating treatment regimens in patients with localised resectable oesophageal cancer

Trial/ registry No./ authors	Regimen	No. of patients	Outcome
RTOG 8911 (72)	Surgery vs. CFx3 + surgery	227 vs. 213	Median OS, 16.1 vs. 14.9 months; HR, 1.07; 95% CI: 0.87-1.32; P = 0.53
OE02 (52, 73)	Surgery vs. CFx2 + surgery	402 vs. 400	5-year OS rate, 17.1% vs. 23%; Median OS, 13.3 vs. 16.8 months; HR, 0.79; 95% CI: 0.67-0.93; P = 0.004
OE05 (62)	CFx2 + surgery vs. ECX x4 + surgery	451 vs. 446	Median OS, 23.4 vs. 26.1 months; HR, 0.90; 95% CI: 0.77-1.05; P = 0.19
FNCLCC/FFCD (74)	Surgery vs. C x3 + surgery + CFx3	111 vs. 113	5-year OS rate, 24% vs. 38%; HR, 0.69; 95% CI: 0.50-0.95; P = 0.02
CROSS (54, 75)	Surgery vs. CRT (with carboplatin and paclitaxel) + surgery	188 vs. 178	5-year OS rate, 33% vs. 47% Median OS, 24 vs. 49.4 months; HR, 0.657; 95% CI: 0.495-0.871; P = 0.003
POET (76, 77)	Induction CT + surgery vs. induction CT + CRT + surgery	59 vs. 60	3-year OS rate, 27.7% vs. 47.7%; P = 0.07; study closed early due to poor accrual
Burmeister et al (78)	CT + surgery vs. CRT + surgery	36 vs. 39	Median OS, 29 vs. 32 months; P = 0.83
Ajani et al (79)	CRT + surgery vs. induction CT + CRT + surgery	63 vs. 63	Median OS, 45.62 vs. 43.68 months; P = 0.69
NeoRes (80)	CT + surgery vs. CRT + surgery	91 vs. 90	3-year OS rate, 49% vs. 47%; P = 0.77

Abbreviations: RTOG 8911, Radiation Therapy Oncology Group trial 8911; CF, cisplatin plus fluorouracil; OS, overall survival; HR, hazard ratio; CI, confidence interval; ECX, epirubicin, cisplatin, and capecitabine; FNCLCC, Federation Nationale des Centres de Lutte Contre le Cancer; FFCD, Federation Francophone de Cancerologie Digestive; CROSS, Chemoradiotherapy for Oesophageal Cancer Followed by Surgery Study; POET, PreOperative therapy in Esophagogastric adenocarcinoma Trial; CT, chemotherapy; CRT, chemoradiation therapy; NeoRes, Neoadjuvant Chemotherapy versus Chemoradiotherapy in Resectable Cancer of the Oesophagus and Gastric Cardia.
Adapted from (71).

CHALLENGES IN OESOPHAGOGASTRIC CANCER PATIENT MANAGEMENT

When a patient is confronted with a cancer diagnosis, the individual patient wants to know whether he/she will survive the cancer ('what is my prognosis?') and whether the proposed treatment will work ('will I benefit from the treatment and live longer, or will I only have side effects with a poor quality of life?'). As shown from the studies/summary above, there has been very little progress in improving the outcome of OGCa patients with locally advanced

resectable disease in the last decade. The patient's treatment is still determined based on the clinical stage of the disease (see figure with the treatment algorithm). However, OGCa patients with the same clinical or pathological TNM stage receiving the same chemotherapy and surgical treatment can have very different outcomes (66, 67) suggesting that only a subset of OGCa patients truly benefit from chemotherapy, with the remaining patients suffering unnecessary toxicities. The clinical team currently has no patient specific biomarkers to support the discussion with the patient and provide satisfactory answers to individual patient's questions.

For OGCa patients with locally advanced resectable disease, the clinical team needs to be able to distinguish between patients with (1) 'indolent' disease most likely curable by surgery alone, (2) 'aggressive' disease which can be influenced by chemotherapy and (3) 'aggressive' disease, resistant to standard chemotherapy and for which other therapy options might need to be considered, for example via participation in ongoing trials. Despite the recently proposed histological and molecular classifications (25-27, 29, 40, 68) and the continued use of multimodal treatment, the challenge and the clinical need remains to identify clinically relevant biomarkers in order to improve and individualise the management of OGCa patients with locally advanced resectable disease.

Tumour heterogeneity and the tumour microenvironment have been suggested as potential factors influencing OGCa patient outcome, and are discussed below.

Tumour heterogeneity

OGCa is known to be a very heterogeneous disease at the molecular and histological level, both between tumours (inter-) and within the same tumour (intra-). Figure 5 provides an example of histological intertumour heterogeneity with respect to the relative tumour content per area in GC. Tumour heterogeneity has been proposed as one of the reasons for the disappointing results of recent clinical trials in OGCa patients (81). The numerous proposed molecular and morphological classification systems in GC have focussed on the heterogeneity of the epithelial component of a tumour (82). Heterogeneity of other components within OGCa and its relationship with patient prognosis and/or response to chemotherapy has not been investigated in detail.

Tumour microenvironment

Tumours including OGCa are highly complex tissues composed of neoplastic epithelial cells and 'stroma', the material in between the tumour cells, which includes fibroblasts, extracellular matrix, vessels and immune cells (see figure 5). In GC, our previous research has shown that the expression of stroma-related gene sets and the morphometric quantification of the tumour-stroma proportion is related to patient prognosis (83). In OeC, we demonstrated that the quantity of the intratumoural stroma in the pretreatment biopsies predicts benefit from neoadjuvant chemotherapy in patients recruited into the Oe02 trial (84). In addition, there

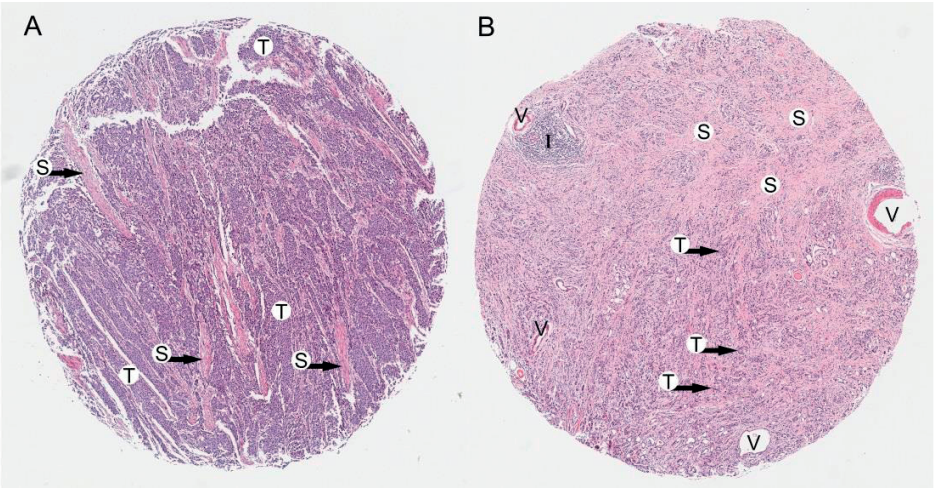


Figure 5 | Intertumour heterogeneity of the tumour microenvironment in gastric cancer. (A) High proportion of tumour epithelial cells [T] relative to the stroma [S]. No immune cell clusters in the stroma visible.
(B) Low proportion of tumour epithelial cells [T] relative to the stroma [S]. Within the stroma, there are vessels [V] and immune cell clusters [I] are visible.
Haematoxylin/Eosin stained tissue microarray cores from the CLASSIC trial. Cores taken from the area of highest tumour density in both cases. Core diameter = 3mm

is evidence to suggest that interactions between tumour cells and stroma resident immune cells may influence tumour progression. This could explain why patients with EBV-positive and MSI GCs, which are usually characterised by a relative high number of tumour-infiltrating lymphocytes, have a better prognosis compared to those with EBV-negative and MSS GCs (85, 86). As with proposed tumour-cell based biomarkers, clinical validation of stroma-based biomarkers is still lacking.

AIM AND OUTLINE OF THE THESIS

The aim of this thesis was to identify prognostic and predictive biomarkers in locally advanced resectable OGCa. We begin by focussing on the molecular characterisation of OGCa. We review the literature on *KRAS* and *BRAF* mutations in GC in **chapter 2**. In **chapter 3**, we follow on from this review and investigate the relationship between *KRAS* mutation and copy number status, histological phenotype, clinicopathological variables and survival in GC. In **chapter 4**, we determine the frequency of EBV and MMR in a large multicentre derived series of OeC and GC and relate the results to clinicopathological variables including patient survival. In **chapter 5,** the focus shifts to the tumour microenvironment where we investigate the intratumour heterogeneity of the relative tumour/stroma content in the diagnostic biopsy of OeC patients to predict survival benefit from neoadjuvant chemotherapy. In **chapter 6**,

we investigate the role of tumour infiltrating lymphocytes as a prognostic and predictive biomarker in GC. **Chapter 7** discusses the implications of our research in the context of the current literature and the future perspectives of the clinical management of OGCa patients.

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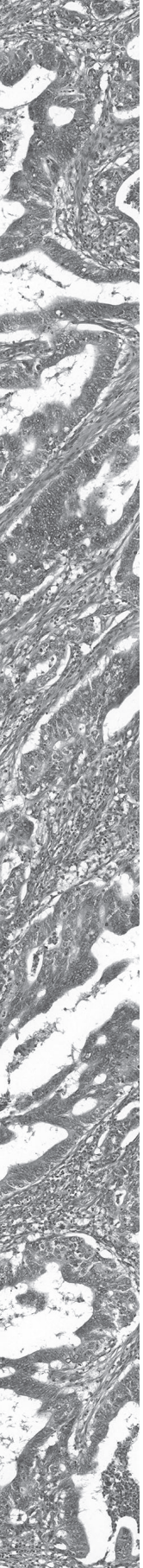

Chapter 2

KRAS, *BRAF* and Gastric Cancer

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Gastric Cancer Precision Medicine. Chen L, Kim SY and Sugimura H, eds. 1st ed. AME publishing Company, 2017: 7-25

ABSTRACT

Gastric cancer (GC) remains a major worldwide health problem and survival rates continue to be poor in patients with advanced stage disease despite multimodal treatment combining different chemo(radio)therapy regimens with surgery or best supportive care. Thus, there is an urgent clinical need to identify new potential drug targets in order to improve survival for GC patients.

KRAS encodes a small guanosine triphosphatase and point mutations in codons 12 and 13 of *KRAS* have been detected in many human cancers. *BRAF* is a member of the RAF family of protein kinases and has a hotspot for mutations in codon 600 (so called V600E mutation). KRAS and BRAF proteins are both components of the MAPK/ERK pathway. When mutated, *KRAS* becomes constitutively active resulting in enhanced BRAF activity. *KRAS* and *BRAF* mutations in colorectal cancers are known predictors of poor response to EGFR targeting agents. This PubMed and Web of Science based review aimed to analyze and summarize the current literature on mutations in *KRAS* and *BRAF* in GC and their relationship to clinicopathological and molecular variables including *KRAS* amplification. In total, 69 studies were included in this review. The median incidence of a *KRAS* mutation was 6.5% ranging from 0-29%. The median incidence of *KRAS* mutations was similar in studies from the East and the West (East: 6%, ranging from 0-20%; West 7.5%, ranging from 0-29%). *KRAS* amplifications were reported at an incidence of 1-9%. The median *BRAF* mutation incidence in GC was 0%, ranging from 0% to 12%. Due to the low incidence and often small study size, many of the published studies had insufficient statistical power to detect a potential relationship between *KRAS* mutation status and clinicopathological variables including patient survival.

In summary, the current literature on *KRAS* and *BRAF* in GC is still limited and very heterogeneous making any comparisons between different studies difficult. *BRAF* V600E mutations are very rare in GC. Interestingly, the incidence of *KRAS* mutations in GC is much lower than that in colorectal cancer and there appears to be no difference by ethnicity of the patients. *KRAS* mutations and *KRAS* amplifications seem to be mutually exclusive suggesting the need to screen GC patients for both genetic aberrations. So far, all clinical studies in unselected patients with metastatic GC have failed to show a significant benefit for EGFR targeting therapy. However, there has been a recent report indicating that the subgroup of signet ring cell GC, which is known to be resistant to standard cytotoxic chemotherapy, has a higher incidence of *KRAS* mutations (15%). Thus, EGFR targeted therapy in this particular histological subtype of GC could potentially be a promising treatment option in the future.

INTRODUCTION

Gastric cancer (GC) is a common cancer with a worldwide incidence of nearly one million cases per year (1). In 2012, there were an estimated 723,100 GC deaths worldwide, making GC the third most frequent cause of cancer related death. There is large geographic variation in GC incidence, with the highest incidence rates in Eastern Asia (particularly in Korea, Mongolia, Japan, and China), Central and Eastern Europe, and South America and lowest rates in Northern America and most parts of Africa. The incidence of GC in men is about twice as high as in women (2) and approximately 10% of GCs have a familial component (3). *Helicobacter pylori* (*H. pylori*) infection is an established risk factor for developing GC. 89% of cases of non-cardia GC worldwide are attributed to this bacterium (4). Survival of GC patients remains poor. The overall 5-year survival of patients with locally advanced unresectable, recurrent or metastatic GC is 5-20% if treated with cytotoxic chemotherapy (5), increasing to 36% in patients with locally advanced resectable GC treated with perioperative chemotherapy followed by surgery (6). Thus, there is an urgent clinical need to identify new potential drug targets in order to and improve survival for GC patients.

Macroscopically, GCs are categorized according to the Borrmann classification into type I (polypoid), type II (fungating), type III (ulcerating), and type IV (diffusely infiltrating) (7). Histologically, GCs are most commonly categorized using the Lauren classification into intestinal, diffuse and mixed/indeterminate type (8). The intestinal-type occurs more commonly in elderly patients, whereas the diffuse-type is seen in particular in young female patients and has a poorer prognosis (9). In the West, the relative proportion of intestinal-type GC is up to 74% intestinal-type (10) compared to 44% in the East (11). Staging of GC is performed using the UICC (12), AJCC (13) or JGCA (14) Tumor Node Metastasis (TNM) staging system which follow same principles but have some minor variations.

Molecular aberrations are known to play an important role in the development of GC. In addition to mutations in oncogenes, such as *TP53*, *APC*, *CDH1*, *p16* and *PTEN*, or tumor suppressor genes such as β-*catenin*, *BRAF, KRAS*, *PIK3CA* and *ERBB2* (15), microsatellite instability (MSI) caused by deficient DNA mismatch repair (MMR) has been identified in 15% to 30% GC (16). DNA aneuploidy, a surrogate marker for chromosomal instability, has been reported in 24-85% GC (17) and Epstein-Barr Virus (EBV) infection has been identified in approximately 9% GCs (18). Several different molecular classifications of GCs have been proposed recently (19). For a recent review on this subject see Tan *et al*. (20).

The focus of this review is on the existing literature on genetic alterations in *KRAS* and *BRAF* in GC. Reported incidence of mutations in *KRAS* and *BRAF* and their relation to clinicopathological and molecular variables including *KRAS* amplification are analyzed and summarized. Literature on *KRAS*/*BRAF* epigenetic changes has been excluded from this review. Results from GC are compared with studies investigating *KRAS* and *BRAF* mutations in colorectal cancer (CRC) and cancer of the small bowel. Furthermore, the clinical relevance

of determining the mutational status and DNA copy number of these genes in relation to patient treatment for GC will be discussed.

METHODS

The Web of Science (from 1988-14th May 2015) and PubMed (from 1946-14th May 2015) databases were searched for all known_gene aliases of *KRAS* and *BRAF* (gene aliases from www.genecards.org, accessed on 8th May 2015). These aliases were used as search terms in combination with ("gastric cancer" or "stomach cancer" or "gastric carcinoma" or "stomach carcinoma", see table 1).

Table 1 | Search terms used in PubMed and Web of Science

	Search Term
KRAS	("KRAS" OR "Kirsten Rat Sarcoma Viral Oncogene Homolog" OR "KRAS2" OR "RASK2" OR "V-Ki-Ras2 Kirsten Rat Sarcoma 2 Viral Oncogene Homolog" OR "V-Ki-Ras2 Kirsten Rat Sarcoma Viral Oncogene Homolog" OR "c-Ki-ras" OR "K-Ras 2" OR "CFC2" OR "NS" OR "C-K-RAS" OR "K-RAS2A" OR "K-RAS2B" OR "K-RAS4A" OR "K-RAS4B" OR "KI-RAS" OR "KRAS1" OR "NS3" OR "C-Kirsten-Ras Protein" OR "Cellular C-Ki-Ras2 Proto-Oncogene" OR "GTPase KRas" OR "K-Ras P21 Protein" OR "Oncogene KRAS2" OR "PR310 C-K-Ras Oncogene" OR "Transforming Protein P21" OR "Ki-Ras" OR "c-K-ras") AND ("gastric cancer" or "gastric carcinoma" or "stomach cancer" or "stomach carcinoma")
BRAF	("BRAF" OR "V-Raf Murine Sarcoma Viral Oncogene Homolog B" OR "V-Raf Murine Sarcoma Viral Oncogene Homolog B1" OR "Proto-Oncogene B-Raf" OR "BRAF1" OR "RAFB1" OR "NS7" OR "94 KDa B-Raf Protein" OR "B-RAF1" OR "B-Raf Proto-Oncogene Serine/Threonine-Protein Kinase (P94)" OR "Murine Sarcoma Viral (V-Raf) Oncogene Homolog B1" OR "Serine/Threonine-Protein Kinase B-Raf" OR "EC 2.7.11.1" OR "p94") AND ("gastric cancer" OR "gastric carcinoma" OR "stomach cancer" OR "stomach carcinoma")

Eligibility to be included in the current review was restricted to original articles reporting gastric cancer (GC) studies using human tissue, blood or plasma samples irrespective of sample size and stage of disease. Other tumors of the stomach such as lymphomas or gastrointestinal stromal tumors, and cell line studies were excluded. The reference lists of publications eligible to be included in this review were searched for further relevant articles. Each article was analyzed for information on study size, geographical origin of patient cohort (East versus West), age, gender, survival, and whether any chemo(radio)therapy was given. With regard to DNA isolation from tumor tissue, the reported tumor cell density, number of blocks used, and tissue processing (frozen versus formalin-fixed paraffin embedded (FFPE)) were analyzed. Furthermore, information on the mutation incidence, the mutation detection method and investigated codons was collected from each study. The relationship of mutation status with clinicopathological variables, DNA mismatch repair status and microsatellite instability, and DNA ploidy was noted.

RESULTS

The initial database searches found 1369 articles in total. After screening, applying exclusion criteria and including additional articles from references, the final number of articles used for this review was 69. For a Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram illustrating the manuscript selection process, see Figure 1.

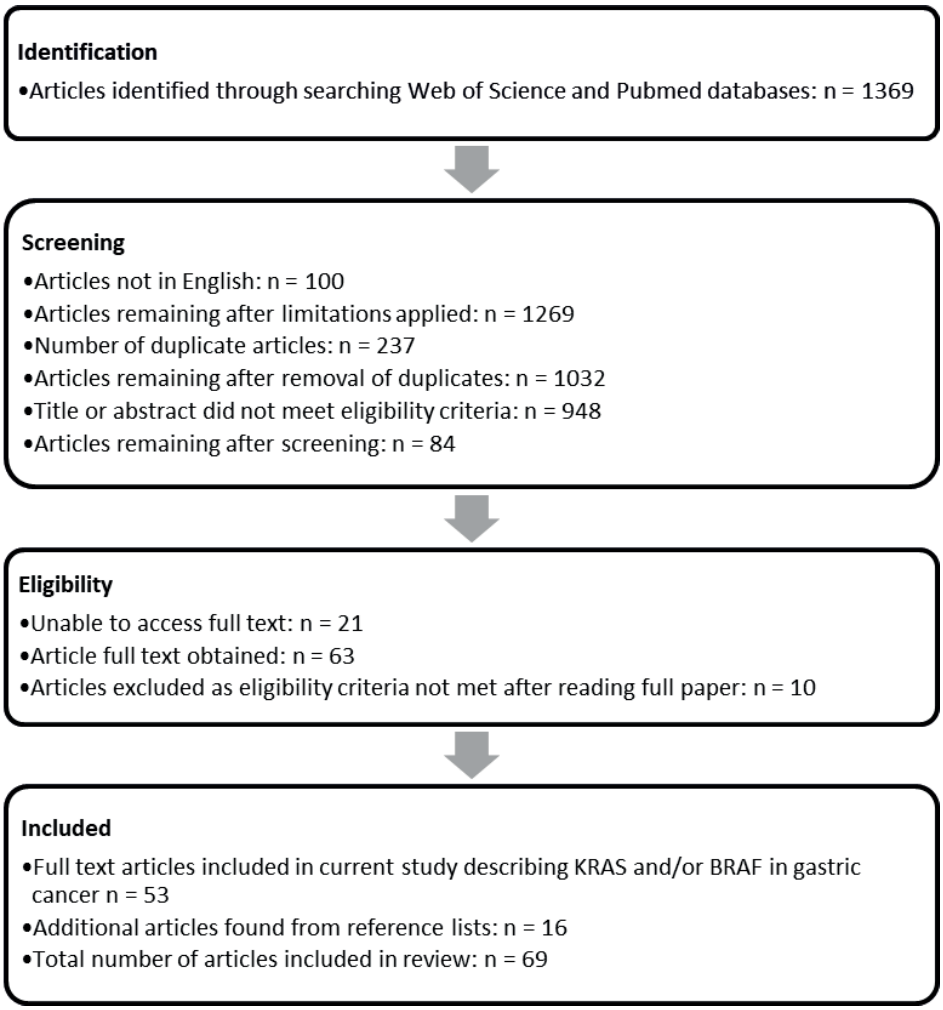

Figure 1 | PRISMA flow diagram showing the number of studies included at each stage of the review process

KRAS

Mammalian cells encode three functional RAS genes: *HRAS, KRAS and NRAS* (21, 22). Although these different isoforms share a similar structure, their expression and/or activation

differs by tissue and cancer types (23-25). This review will focus on *KRAS* as it is the most frequently mutated RAS gene in GC (26).

Kirsten Rat Sarcoma Viral Oncogene Homolog (*KRAS*) was discovered in 1982 by Chang EH *et al* (21). *KRAS* is a tumor suppressor gene which is located on chromosome 12p12 (www.genecards.org, accessed 8th May 2015). It has six exons and alternative splicing of exon 4 produces KRAS4A and KRAS4B which contains 188 and 189 amino acids, respectively (27). *KRAS* encodes a small guanosine triphosphatase (GTPase) protein with a molecular mass of 21.6 kD (28).

The KRAS protein contains four domains which determine the interaction with GTP (G-domain, amino acids 1-165), the anchoring of the protein in the plasma membrane (hypervariable region at the C-terminus, amino acids 165-188) as well as the binding of other regulators and effectors such as RAF and PI3K (28).

KRAS cycles between an inactive GDP-bound state and an active GTP-bound state (29). Activation of KRAS is triggered through a number of different types of receptors including tyrosine kinase receptors such as epidermal growth factor receptor (EGFR), as well as cytokine receptors, T cell receptors, and subunits of heterotrimeric G proteins (30). Active RAS-GTP undergoes a conformational change affecting its interaction with various downstream effector molecules such as RAF and Mitogen-Activated protein kinase kinase (MAPK) (31) or PI3K/AKT (32). This in turn activates nuclear transcription factors inducing a cascade of cellular processes such as proliferation, angiogenesis, apoptosis, or cell survival (26). Mutant *KRAS* functions as an oncogene inducing malignant transformation of cells due to permanent activation of downstream effectors (33).

KRAS mutations have been found in many human cancers. The most common mutations are located in codon 12 or 13 in exon 1, and less frequently in codon 61, 63, 117, 119 and 146 (28). Mutations in codons 12 and 13 are known to result in conformational changes and permanent expression ('activation') of the KRAS protein (34). Overexpression of *KRAS* as a result of loss of p16INK4 or loss of p53 has also been reported (35). For a more general review on *KRAS* mutations in human cancer, see Jancik *et al* (28).

KRAS in Gastric Cancer

KRAS mutations

The first report of a *KRAS* mutation in a single gastric cancer (GC) was published in 1986. Investigators described the presence of a single mutated *KRAS* allele (gly-12 to ser), together with a 30-50 fold amplification of the other *KRAS* allele (36). Since this first publication, 64 studies have reported on the incidence of *KRAS* mutations in GC, with the majority of studies (61%) originating from Asia (see table 2a and 2b). Two studies compared *KRAS* mutations between GC patients from the East and the West (37, 38). Forty-five (70%) studies investigated the *KRAS* mutation status in patient cohorts comprising less than 100 patients.

Table 2a | Published literature on *KRAS* mutation status in gastric cancer excluding studies testing chemotherapeutic agents

Reference	Year	Origin	Total n	mut *KRAS* n (%)	Comment
Nagata *et al*	1990	Japan	25	2 (8)	
Victor *et al*	1990	South Africa	11	0	
Kihana *et al*	1991	Japan	35	3 (9)	3 of 7 adenoma had mut *KRAS*; mut *KRAS* in well diff GC only
Miki *et al*	1991	Japan	31	4 (13)	mut *KRAS* only found in intestinal-type
Capella *et al*	1991	Europe	14	1 (7)	
Ranzani *et al*	1993	Europe	32	3 (9)	1 mut *KRAS* also had allelic losses
Koshiba *et al*	1993	Japan	37	1 (3)	no mut *KRAS* in 13 adenoma
Craanen *et al*	1995	Europe	45	0	only early GC tested
Sakurai *et al*	1995	Japan	19	0	only early GC tested
Hongyo *et al*	1995	Europe	34	7 (21)	only intestinal-type GC tested; no mut *KRAS* in stage III
Lee *et al*	1995	South Korea	140	11 (8)	mut *KRAS* more common in DNA aneuploid and in upper third GC
Hosoi *et al*	1995	Japan	31	0	biopsy samples tested
Hao *et al*	1998	China	206	0	
Iwaya *et al*	1998	Japan	5	1 (20)	synchronous primary cancers of the esophagus and other organs
Arber *et al*	2000	USA	32	1 (3)	
Russo *et al*	2001	Europe	63	5 (8)	mut *KRAS* not related to DNA ploidy
Lee *et al*	2002	South Korea	71	1 (1)	
Yoo *et al*	2002	South Korea /US	104	10 (10)	mut *KRAS* related to intestinal-type GC and higher pT
Hiyama *et al*	2002	Japan	48	4 (8)	mut *KRAS* related to well diff histology type, younger age and H. pylori infection
Lee *et al*	2003	South Korea	319	9 (3)	mut *KRAS* related to advanced GC
Brennetot *et al*	2003	Europe	82	10 (12)	mut *KRAS* only seen in MSI not in MSS GC
Kim *et al*	2003	South Korea	66	4 (6)	
Wu *et al*	2004	Japan	62	1 (2)	mut *KRAS* GC related to MSI; *KRAS* and *BRAF* mutations were exclusive
Zhao *et al*	2004	China	94	8 (9)	7 of 8 GC with mut *KRAS* were MSI. All mut *KRAS* in GC from antrum
Yashiro *et al*	2005	Japan	180	20 (11)	only advanced GC tested. mut *KRAS* more common in well diff GC and Bormann type I. No relationship with H-pylori infection
Oliveira *et al*	2005	Europe	25	6 (24)	only MSI GC tested
Tajima *et al*	2006	Japan	133	7 (5)	only early GC tested; no *KRAS* mutation in 63 gastric adenoma

Table 2a | (continued)

Reference	Year	Origin	Total n	mut *KRAS* n (%)	Comment
Sasao *et al*	2006	Japan	55	1 (2)	
Kusano *et al*	2006	Japan	78	4 (5)	
Gylling *et al*	2007	Europe	59	4 (7)	mut *KRAS* only seen in MSI not in MSS GC
Tajima *et al*	2007	Japan	134	8 (6)	only differentiated GC tested
Kimura *et al*	2007	Japan	66	3 (5)	
Liu *et al*	2009	China	52	5 (10)	mut *KRAS* only seen in males
Mita *et al*	2009	Japan	86	0	5% *KRAS* amp
Betge *et al*	2011	Austria	12	1 (8)	GC with concomitant renal cancer
Liu *et al*	2011	China	58	6 (10)	mut *KRAS* only seen in males
Corso *et al*	2011	Europe	63	11 (18)	only MSI GC tested; mut *KRAS* more common in elderly patients
Chen *et al*	2011	China	123	12 (10)	*KRAS* tested in blood
Saxena *et al*	2012	India	62	0	
Matsubara *et al*	2013	Japan	71	1 (1)	
Van Grieken *et al*	2013	Europe/Japan/ Singapore	712	29 (4)	mut *KRAS* associated with MMR-deficient GC. In Europe cohort mut *KRAS* associated with pN, in Japan cohort mut *KRAS* associated with elderly patients
Kim et al	2013	South Korea/Japan	30	2 (7)	mut *KRAS* associated with CIMP
Warneke *et al*	2013	Europe	475	17 (4)	mut *KRAS* associated with worse survival in proximal GC. Mut *KRAS* intestinal-type GC with worse prognosis than *KRAS* wild-type intestinal-type. 9% *KRAS* amp.
Kim *et al*	2014	South Korea	17	1 (6)	early and advanced GC tested. Missense mutation detected
Kim *et al*	2014	South Korea	89	3 (3)	only metastatic GC tested. *KRAS* amp in 2 cases; 1 case had increased copy number
Peng and Zhao	2014	China	126	9 (7)	tissue and plasma tested
Palacio-Rua *et al*	2014	Colombia	29	2 (7)	
Qian *et al*	2014	China	131	8 (6)	mut *KRAS* and *KRAS* amp (5%) mutually exclusive; associated with different outcomes
TGCA	2014	Multicenter	215	36 (17)	
Ali *et al*	2015	USA	116	12 (10)	6% *KRAS* amp. Includes 36 samples from metastatic sites
Lu *et al*	2015	China	156	7 (4)	mut *KRAS* associated with pN0 GC
Deng *et al*	2015	Singapore	139	1 (1)	9% *KRAS* amp
Cristescu *et al*	2015	South Korea	223	18 (8)	8% *KRAS* amp
Yoda *et al*	2015	Japan	50	4 (8)	8% *KRAS* amp

Abbreviations: mut *KRAS*, mutant *KRAS*; MSI, microsatellite instability; well diff, well differentiated; PFS, progression free survival; OS, overall survival; CIMP, CpG island methylator phenotype; *KRAS* amp, *KRAS* amplification.

Table 2b | Published literature on *KRAS* mutation status in gastric cancer studies investigating chemotherapeutic agents

Reference	Year	Origin	Total n	mut *KRAS* n (%)	Stage of disease	Treatment	Sample type used *for KRAS* testing	Mutant *KRAS* relationship to survival
Pinto *et al*	2009	Europe	32	3 (9)	advanced unresectable. Includes some junctional cancer	Cetuximab + cisplatin and docetaxel	not specified	not reported (no association with ORR)
Han *et al*	2009	South Korea	38	0	recurrent metastatic	Cetuximab + oxaliplatin/ leucovorin/5-fluorouracil	not specified	no mut *KRAS*
Park *et al*	2010	South Korea	30	4 (13)	metastatic	cetuximab + chemotherapy	primary tumor	no association with PFS and OS
Lordick *et al*	2010	Europe	52	1 (3)	metastatic or locally advanced unresectable	Cetuximab + oxaliplatin/ leucovorin/5-fluorouracil	not specified	not reported
Moehler *et al*	2011	Europe	29	0	advanced	Sunitinib monotherapy	not specified	no mut *KRAS*
Rohrberg *et al*	2011	Europe	7	2 (29)	advanced	Erlotinib + bevacizumab	not specified	no association with PFS, OS and DC
Woll *et al*	2011	Europe	13	0	metastatic or locally advanced unresectable	Oxaliplatin, irinotecan + cetuximab	biopsies/ resected primary tumor	no mut *KRAS*
Okines *et al*	2013	Europe	494	30 (6)	unresectable and/or metastatic GC. Includes some esophageal and junctional ca	REAL 3: Epirubicin, oxaliplatin and capecitabine ± panitumumab MAGIC: Epirubicin, cisplatin, 5-fluorouracil	REAL 3: pre-treatment biopsies MAGIC: resections	REAL 3: no association with RR MAGIC: no association with OS
Richards *et al*	2013	USA	40	5 (13)	metastatic	docetaxel plus oxaliplatin ± cetuximab	not specified	not reported (no association with response)
Takahashi *et al*	2014	Japan	164	8 (5)	Advanced	Cisplatin/S-1/5-fluorouracil/5-fluorouracil + methotrexate/ paclitaxel + capecitabine + cisplatin ± bevacizumab in metastatic disease	resection	No association with OS

Abbreviations: mut *KRAS*, mutant *KRAS;* PFS, progression free survival; OS, overall survival; DC, disease control; ORR, objective response rate; ca, cancer; RR; response rate.

Gastric cancer cohorts

The median number of patients per study was 61, ranging from 5 to 712 patients. Excluding three international multicenter studies and two studies that did not mention the geographical origin of their patients, there were 39 (66%) studies from the East and 22 (37%) studies from the West. Studies from the East had a higher median study size of 66 patients, ranging from 5 to 319 patients compared to studies from the West with a median study size of 33 patients, ranging from 7 to 494 patients. The largest GC study was an international multicenter study including 712 GCs: 278 GC from the United Kingdom, 230 GC from Japan and 204 CG from Singapore (38).

Twenty-five (39%) studies performed *KRAS* testing on samples from multiple centers (19, 37-60), 20 (31%) studies used samples from a single center (61-80), and the remaining did not report this information. Twenty-seven (42%) studies were performed using DNA extracted from formalin-fixed paraffin embedded tissue samples (37-39, 41, 42, 44, 45, 47, 48, 50-52, 56, 61, 63-66, 68, 69, 72-74, 81-84). With the exception of 11 studies which did not report at all which tissue was used (40, 54, 77, 80, 85-91), all other studies used DNA from 'paraffin embedded tissue' (fixation method not reported) (43, 92-94), frozen tissue (19, 46, 53, 59, 60, 67, 70, 71, 75, 76, 78, 79, 95-98), blood or plasma samples (99), or a combination of the above (49, 55, 57, 58, 62). Of the studies using tissue samples, 37 (59%) used DNA extracted from resection specimens (38, 39, 44, 46, 47, 50, 52-54, 60-64, 67, 68, 70, 71, 73-76, 78-82, 84, 88-91, 93, 95-98), 10 (16%) used a combination of biopsy and resection specimens (37, 40, 45, 51, 65, 69, 72, 87, 92, 94) and two (3%) used biopsy specimens (77, 86). The remaining 14 (22%) did not report on the type of specimen used (19, 41-43, 48, 49, 55-59, 66, 83, 85). No study reported extracting DNA from multiple blocks, thus we have assumed that all studies used a single block for DNA extraction. Thirty-seven (59%) studies considered the tumor cell density of the tissue prior to DNA extraction by either performing microdissection or preselecting areas of tumor with tumor cell density ranging from >20% to >80% (19, 37-40, 44, 46-54, 61, 62, 64-71, 73-76, 81, 82, 84, 89, 93, 94, 98). Twenty-two (34%) studies investigated only subgroups of GC patients, thus 8 (36%) studies investigated advanced disease (40-44, 61, 62, 82), 4 (18%) studies metastatic and advanced GC (48, 49, 81, 94), 3 (14%) studies early GC (45, 65, 84), 2 (9%) studies metastatic disease (66, 90), 2 (9%) studies compared early with advanced disease (46, 93), one (5%) study intestinal GC (47), one (5%) study MSI GC (85) and one study (5%) investigated GC with concomitant renal cancer (63).

KRAS mutation detection methods

A wide variety of methods was used to detect *KRAS* mutations. Twenty-six (41%) studies used polymerase chain reaction (PCR) (37, 43, 44, 49, 60, 61, 66, 70, 74, 75, 80, 88, 98) or single-strand conformation polymorphism (SSCP) (39, 45, 47, 52, 64, 65, 71, 72, 85, 93, 95, 97, 99) for mutation screening, followed by confirmatory direct Sanger sequencing. Other methods used to detect *KRAS* mutations included restriction fragment length polymorphism

(RFLP) (51, 76-78, 83, 86), next-generation sequencing (NGS) (19, 46, 48, 59, 67, 81, 87, 96), pyrosequencing (63, 68), Q-PCR (41, 94), nested and COLD-PCR (55), denaturing gradient gel electrophoresis (DGGE) (89, 91), dot blot hybridization assay (56-58, 69, 73, 82), high-resolution melting analysis (HRMA) (42, 50, 53, 54) and direct Sanger sequencing (62, 79). The largest international multicenter study used a combination of HRMA followed by Sanger sequencing, pyrosequencing, and MassARRAY (38). One study used RFLP and SSCP followed by direct sequencing (92), while other studies used a combination of RFLP and dot blot hybridization (84) or a combination of Q-PCR and Sanger sequencing (40). One study did not report which *KRAS* mutation detection method was used (90).

Investigated KRAS codons

Excluding eight studies that performed whole genome sequencing, 49 (88%) studies published information on investigated codons for mutation testing. The remaining seven (13%) studies did not provide any information which codons they investigated, however, they later report only mutations in specific codons. All studies investigated multiple codons, with 49 (100%) investigating codon 12, 45 (92%) codon 13, 18 (37%) codon 61, and 1 codon 146. Only a single study investigated all four codons (codons 12, 13, 61 and 146) (62) and one study investigated codon 59, in addition to codons 12, 13 and 61 (93).

Incidence of KRAS mutations

The overall median incidence of a *KRAS* mutation in GC was 6.5% ranging from 0-29%. The median *KRAS* incidence was similar in studies from the East and the West (East: 6%, ranging from 0-20%; West 7.5%, ranging from 0-29%). Likewise, the largest international multicenter study reported an overall incidence of *KRAS* mutations of 4.2% which did not differ between Eastern and Western countries (UK: 6%, Japan 4%, Singapore 2%) (38).

Of the 36 studies that reported the location of the mutations in *KRAS*, 154 mutations were found in codon 12, 66 mutations in codon 13, 6 mutations in codon 61. No mutation has been found so far in codon 146. The only study to report *KRAS* mutations in codon 11, was the result of SSCP and direct sequencing of exon 1. This revealed that 2 of the 7 mutations found in 34 GCs were located in codon 11, all other mutations were in codons 12 and 13 (47). Another study, in addition to identifying one *KRAS* mutation in codon 12 and two *KRAS* mutations in codon 13, also found one K5N mutation in exon 2 and five A59T mutations in exon 4 (93). There was only a single report of a single GC having multiple mutations in codon 12 and codon 13 (78).

KRAS mutation status and clinicopathological variables

Twenty-nine (45%) studies have investigated the relationship between *KRAS* mutation status and one or more clinicopathological variables (19, 37, 38, 40, 46, 47, 50-54, 56, 60, 62-64, 68, 69, 71-73, 75, 76, 82, 88, 91, 93, 96, 98). These included grade of tumor differentiation, Lauren classification, tumor location, tumor invasion depth (pT), lymph node status (pN),

Borrmann classification, age, gender, and infection with *H.Pylori* or EBV. The most frequent investigated association was between *KRAS* mutation status and pT, followed by gender and age reported in 33%, 30% and 30% of studies, respectively.

KRAS mutation and age

Nineteen (30%) studies investigated the relationship between patient age and *KRAS* mutation status mostly suggesting that *KRAS* mutations are more frequent in elderly GC patients. Seven (37%) studies reported individual ages or the median age of patients with a *KRAS* mutation (19, 46, 60, 62, 63, 69, 96), whereas the remaining studies stratified patient age into a range of subcategories (38, 50, 52-55, 68, 72, 76). Only Hiyama *et al* reported a significantly higher incidence of *KRAS* mutations in patients younger than 60 years (72). One study reported an equal number of *KRAS* mutations in patients ≤65 years old and >65 years old (54). All other studies found *KRAS* mutations more frequently in elderly patients although this association often did not reach statistical significance (38, 50, 52, 53, 55, 68, 76, 98).

KRAS mutation and gender

Nineteen (30%) studies investigated the relationship between gender and *KRAS* mutation status in GC. Although no statistically significant relationship between *KRAS* mutation status and gender was found, most studies seem to suggest that *KRAS* mutations are more frequent in males. Nine (47%) studies found a higher incidence in males (38, 46, 50, 55, 62, 68, 69, 72, 76), 3 (16%) studies reported that *KRAS* mutations were exclusively found in males (53, 54, 63) whereas 4 (21%) studies found an equal incidence of *KRAS* mutations in males and females (60, 75, 91, 96).

KRAS mutation and tumor location

Twelve (19%) studies investigated the relationship between *KRAS* mutation status and GC location within the stomach. Tumors in the upper third of the stomach had a significantly higher incidence of *KRAS* codon 12 mutations compared to GCs in the middle or lower (3%) third of the stomach (76). Summarizing and interpreting the results from the other studies is difficult as stomach area categorization varied substantially between studies. We therefore defined that GCs located in the cardia or upper third are 'proximal' and GCs located in all other regions are 'distal'. These studies found a higher incidence of *KRAS* mutations in distal GC (19, 37, 38, 60, 63, 64, 68, 72, 75, 91).

KRAS mutation and Borrmann classification

A single study investigated the relationship between *KRAS* mutation status and macroscopic classification according to Borrmann. This study investigated *KRAS* codons 12 and 13 in 108 GC patients with advanced disease and found a significant relationship between *KRAS* mutation status and Borrmann Type 1 (polypoid) GC (82). The incidence of *KRAS* mutation

was 6/14 (43%), 8/29 (28%), 2/11 (18%), and 4/54 (7%) in Borrmann type 1 to 4 GCs, respectively. Interestingly all *KRAS* mutations in polypoid GCs were located in codon 12. This is in contrast to a study investigating 48 GC which did not find any relationship between macroscopic appearance (classified according to the Japanese Research Society for Gastric Cancer) and *KRAS* mutation status (72).

KRAS mutation and primary tumor invasion depth (pT category)

Twenty-one (33%) studies investigated the relationship between *KRAS* mutation status and pT in GC. Unfortunately, different staging systems were used in different publications and some studies compared groups of pT categories against each other making the results interpretation difficult. None of the studies reported a significant association between pT category/stage and *KRAS* mutation status. Overall, there was a higher incidence of *KRAS* mutations in higher pT (pT 2-4) GC compared to lower pT (pT1) GC (19, 37, 38, 47, 50, 53, 54, 60, 63, 68, 75, 76, 82, 88, 91, 93, 96).

KRAS mutation and lymph node status (pN category)

Eleven (17%) studies investigated the relationship between *KRAS* mutation status and presence of lymph node metastases with conflicting results. Five (45%) studies found that *KRAS* mutant GCs tended to have either no lymph node metastases (46, 50, 53, 54) or significantly fewer lymph node metastases (38). Whereas other studies report that *KRAS* mutations are more frequent in GCs with lymph node metastases (19, 63, 68, 91, 96).

KRAS mutation and histological subtype according to Lauren classification

Seventeen (27%) studies including a total of 2583 patients investigated the association between *KRAS* mutation status and histological subtype according to the Lauren classification (19, 37, 38, 40, 46, 47, 56, 60, 62, 63, 68, 72, 75, 76, 88, 91, 93). Although 11 (65%) of studies reported a higher incidence of *KRAS* mutations in intestinal-type GC (see figure 2), this association did not reach statistical significance in any of the studies (19, 37, 38, 40, 56, 60, 62, 68, 72, 75, 91).

KRAS mutation and grade of tumor differentiation

Fifteen (23%) studies investigated the relationship between *KRAS* mutation and grade of tumor differentiation reporting discordant results. One (7%) study investigating advanced disease found that *KRAS* mutations were significantly more frequent in histologically differentiated GC (82), three (20%) studies found a higher incidence of *KRAS* mutations in well-differentiated GCs (47, 69, 72) whereas nine (60%) studies reported a higher incidence of *KRAS* mutations in poorly-differentiated GCs (38, 46, 50, 53, 54, 63, 73, 75, 76). Two studies (13%) found the same incidence of *KRAS* mutations in well- and poorly- differentiated GC (40, 96).

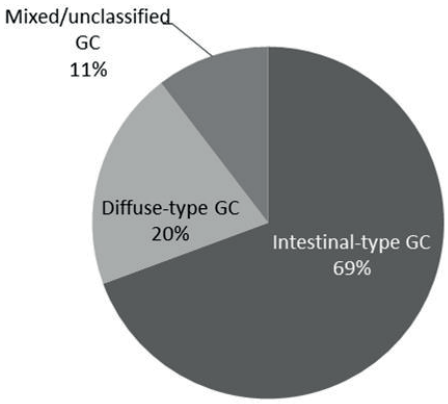

Figure 2 | Distribution of *KRAS* mutation incidence in gastric cancer (GC) by Lauren classification.

KRAS mutation and survival

Seven (11%) studies investigated the relationship between *KRAS* mutation status and survival (38, 41, 62, 66, 68, 76, 79), The largest international multicenter study reported a trend towards better survival in patients with a *KRAS* mutant GC (38) . In contrast, subgroup analysis in a different study showed that the median survival of patients with *KRAS* mutant proximal GCs was significantly shorter (3.5±3.1 months) compared with *KRAS* wild-type GCs (12.7±0.7 months, p = 0.021) (68). The same study found that *KRAS* mutant intestinal-type GCs had a worse prognosis compared to *KRAS* wild-type intestinal-type GC, however this difference was not significant on univariate analysis (p=0.098). Similarly, patients with a *KRAS* mutant GC in the upper third of the stomach may have improved survival over patients with *KRAS* mutant GC in the middle or distal stomach (76).

KRAS mutation and chemotherapeutic agents

Ten (16%) studies investigated the relationship between *KRAS* mutations and the use of chemotherapeutic agents (see table 2b). Four studies (40%) did not find any association between *KRAS* mutation status and progression free survival or overall survival (40, 41, 62, 66), three (30%) studies did not detect any *KRAS* mutations (42, 44, 94) and two (20%) studies did not find an association between *KRAS* mutations and response to chemotherapy (43, 90).

KRAS mutation and H.pylori infection

Six (9%) studies have investigated the relationship between *H. pylori* infection and *KRAS* mutation status. Three studies reported a higher incidence of *KRAS* mutations in *H.pylori* infected GCs, but the difference was not significant or statistical analysis was not performed (47, 82, 97). In contrast, thirteen (87%) *KRAS* mutant GCs were found to be *H.pylori* negative, compared to two *H.pylori KRAS* mutant GCs (68). One study reported an equal

incidence of *KRAS* mutations in *H.pylori* positive and negative GCs (75). The study by Hiyama *et al* found that *KRAS* mutations in *H. pylori*-chronic gastritis were significantly more frequent in patients with GC than those without and in patients with *KRAS* mutated GC than in *KRAS* wild-type GC (72).

KRAS mutation and EBV infection

Four (6%) studies investigating a total of 848 GC for *KRAS* mutation status and EBV infection found no relationship between EBV and *KRAS* mutation (19, 63, 68, 97).

***KRAS* mutation status and molecular variables**

KRAS mutation and DNA mismatch repair deficiency/microsatellite instability (MMR/MSI)

Thirteen (20%) studies investigated the relationship between *KRAS* mutation status and MMR/MSI with controversial results. One study which included only MSI GC reported that 18% harbored a *KRAS* mutation (98). Eight (62%) studies reported a higher incidence of MSI in *KRAS* mutant GCs (39, 63, 67, 70, 74), which was significant in three studies (19, 75, 91). This finding was supported by one study which found that *KRAS* mutations were more frequent in MMR-deficient GC (38). In contrast, two studies reported that *KRAS* mutant GC were more frequently microsatellite stable (MSS) (46, 68).

KRAS mutation and DNA ploidy

Three (5%) studies investigated the relationship between DNA ploidy and *KRAS* mutation status. Two investigated DNA ploidy by DNA flow cytometry. One study investigated *KRAS* mutations in codons 12 and 13 (71), whereas the other study focused on codon 12 (76). Another study investigated DNA ploidy by NGS (19). No associations were reported in any study.

KRAS amplification

Eight (13%) studies investigated *KRAS* amplification in addition to *KRAS* mutations with contradictory results. Three studies found that the incidence of *KRAS* amplification varied between 5% and 9% but was higher than that of *KRAS* mutation in GC (between 0% and 4%) (59, 68, 80). In contrast, four studies found that *KRAS* mutations are more frequent than *KRAS* amplifications in GC (48, 67, 87). One study, reported similar frequencies of *KRAS* amplification (6%) and *KRAS* mutation (6%) (79). Interestingly, the 5-year survival of patients with a *KRAS* amplification was worse than that of the patients *KRAS* mutant GC (HR 3.0, 95% CI: 1.3-7.0). Furthermore, *KRAS* amplification and *KRAS* mutation were exclusive. Deng *et al* reported that patients with GC with a *KRAS* amplification had a significantly poorer prognosis, however, as only one *KRAS* mutation was detected, the relationship between *KRAS* mutation and prognosis could not be analyzed (59).

BRAF

BRAF is a member of the RAF family of protein kinases which has three members: *ARAF, BRAF* and *CRAF* (100). All RAF proteins share a common structure (101), but *BRAF* is the only one known to be activated by mutation in human cancer, and therefore the focus of this review (102).

BRAF is also known as v-raf murine sarcoma viral homolog B1 (100) and was discovered in 1988 by Ikawa *et al* (103). *BRAF* is a proto-oncogene and is located on chromosome 7 (7q34) (www.genecards.org, accessed 8th May 2015). *BRAF* exists in multiple spliced forms, which seem to exhibit tissue specific expression patterns (104).

The BRAF protein is 75 to 100 kDa and has three conserved regions (CR): CR1, CR2 and CR3 (100). CR1 and CR2 are located at the N-terminus and are both regulatory domains, whereas CR3 is a kinase domain and is located at the C-terminus. CR1 is composed of the RAS-binding domain and a cysteine-rich domain binding RAS and membrane phospholipids. CR2 is a serine/threonine rich domain which when phosphorylated can bind regulatory proteins. CR3 is the protein kinase domain which is regulated through phosphorylation (101).

After RAS is activated via extracellular stimuli, it activates BRAF by phosphorylation of two residues in the kinase domain. Activated BRAF phosphorylates and activates MEK1 and MEK2 which then activate MAP kinases ERK1 and ERK2. ERK1/2 activates numerous cytoplasmic and nuclear targets including transcription factors (100).

More than 65 different mutations have been identified in *BRAF* in human cancer. Most of these mutations are in exon 11 or exon 15 in the catalytic kinase domain (100). The most frequently detected *BRAF* mutation is a single amino acid substitution (V600E) in exon 15 (105). *BRAF* is most commonly mutated in melanomas (67%) and CRC (10%) (105, 106). Mutant *BRAF* displays an elevated kinase activity (105) and becomes insensitive to negative feedback mechanisms (107). For a review on *BRAF* mutations in benign and malignant human tumors, see Michaloglou *et al* (108).

BRAF in gastric cancer

In total, 22 studies have investigated the incidence of *BRAF* mutations in GC. Seven (32%) studies screened for *BRAF* mutations by PCR, followed by direct sequencing (43, 61, 62, 68, 70, 75, 98, 109). Other detection methods included denaturing high pressure liquid chromatography, SSCP (39, 40, 52, 93, 110), HRMA (42), NGS (46, 48, 81), amplification-refractory mutation system-PCR, PCR-high resolution melting (50), real-time PCR, immunohistochemistry using a mutation-specific probe (111) or a combination of the above (38, 88, 112).

Fourteen (64%) studies used FFPE samples (38, 39, 42, 43, 48, 50, 52, 61, 68, 81, 88, 93, 109, 111), five (23%) used frozen tissue samples (46, 70, 75, 98, 110) and one study used a combination of FFPE and frozen samples (62). Two studies did not report this information (40, 112). Excluding the study that performed IHC, ten studies selected areas of tumor with a median tumor cell density of >55%, ranging from >20% to >80% (38, 46, 48, 50, 68, 70,

81, 98, 109, 110). Six studies performed microdissection of the selected area (39, 40, 52, 62, 75, 93). The remaining five studies did not provide this information (42, 43, 61, 88, 112).

All studies investigated the *BRAF* exon 15 'mutation hotspot' (V600E mutation). Some studies extended their mutation search to exon 11 and other regions of exon 15, or whole genome sequencing. The median *BRAF* mutation incidence in GC is 0%, ranging from 0% to 12% (38, 39, 42, 43, 46, 48, 50, 52, 61, 62, 68, 70, 75, 81, 88, 93, 98, 109-112). Only six of the BRAF mutations identified were in V600E of exon 15 (38, 40, 70, 110, 112). Six mutations were found in codon 396 and four mutations in codon 608 of exon 15 identified by Sasao *et al* (52). Lee *et al* found two mutations in codon 593 and the remaining five mutations were in codon 599 (V599 M) (93) and Okines *et al* identified a mutation in V600M and G596D of exon 15 (40).

The highest *BRAF* mutation incidence (12%) was reported in a Korean study of 17 early and advanced GC using whole-genome sequencing by NGS. The two mutations identified were missense mutations; one was detected in a mixed-type early cancer, the other one in an intestinal-type advanced cancer (46). There has been a single publication that used immunohistochemistry and a mutation specific antibody to detect the mutated BRAF protein as a surrogate for a *BRAF* mutation. All cases were negative (no evidence suggesting a *BRAF* mutation) (111).

Due to the low incidence of *BRAF* mutations no studies have reported a relationship between *BRAF* mutation status and DNA ploidy or clinicopathological variables. There are three studies that have investigated the relationship between microsatellite instability and *BRAF* mutation. *BRAF* mutations were not found in any of 37 MSI GC (110) which was confirmed in a study by Wu *et al* where the *BRAF* mutant GC was MSS (70). However, in another study the two *BRAF* mutant GC were found to be MSI (46).

EGFR pathway in gastric cancer

The EGFR pathway is known to be activated in GC (113). When EGFR is bound to its ligand, it triggers homodimerisation and heterodimerisation of the EGFR receptor. This activates a signaling cascade, including MAPK, through effector molecules RAS and RAF (113). Anti-EGFR monoclonal antibodies block ligand-induced binding EGFR tyrosine kinase activation by binding to the extracellular domain of EGFR (114).

DISCUSSION

KRAS and *BRAF* mutations in GC

Current literature investigating *KRAS* and *BRAF* mutations in GC is very heterogeneous in terms of sample size, patient ethnicity, patient treatment, mutation detection methods, tumor stage and grade of differentiation, as well as other clinicopathological variables.

The majority of studies (70%) investigated the *KRAS* mutation status in less than 100 patients. Such small studies may not be representative of the GC patient population and thus the patient selection bias may significantly influence any results. Thus, two of the smallest studies with five and seven patients reported some of the highest incidence of *KRAS* mutations, of 20% and 29%, respectively (41, 95). Similarly, for *BRAF*, the smallest study of 17 patients reported the highest *BRAF* mutation incidence of 12% (46). Furthermore, twenty-two (34%) studies investigating *KRAS* mutations deliberately selected subgroups of GC patients to study the *KRAS*/*BRAF* mutation status, such as advanced and/or metastatic disease and early disease.

Despite the much higher incidence in the East, the number of studies investigating the relationship between *KRAS* and *BRAF* in GC from the East and the West is almost equal. Nevertheless, potential bias due to differences in the histological subtypes (diffuse-type GC is more prevalent in the East), disease stage (GC is diagnosed at an earlier stage in the East) and patient survival (better overall survival in the East) (115) needs to be considered when comparing study results, particularly in the twenty studies that performed *KRAS* mutation testing on series from a single center. However, the incidence of *KRAS* mutations between East and West were comparable and do not seem to be related to the differences in GC incidence between the East and the West (38). Thus, bias due to the patient's country of origin appears to have no or minimal influence on the incidence of *KRAS*/*BRAF* mutations in GC.

An issue that was not addressed in any of the studies included in this review was the potential influence of tumor heterogeneity on the results. Tumor heterogeneity of *KRAS* and *BRAF* mutations has been described in CRC suggesting that more than one tumor block should be investigated if possible (116). None of the studies investigating *KRAS* and/or *BRAF* mutations in GC seem to have investigated multiple blocks. Studies either did not provide any information or investigated single blocks. Thus, it is impossible to assess whether the incidence of *KRAS* and/or *BRAF* mutations in GC is underestimated based on the current literature.

Over 10 different methods were used to detect *KRAS* and/or *BRAF* mutations in GC. It is known that the sensitivity (ratio of mutant to wild-type) of different methodologies varies between techniques (117), with COLD-PCR having the highest sensitivity (1%) and direct Sanger sequencing having the lowest (10-30%). Despite this low sensitivity, Sanger sequencing is considered the 'gold standard' technique due to its ability to detect substitutions, insertion and deletions. The median *KRAS* mutation incidence in GC appears to be similar irrespective of the detection method and thus, the detection methodology does not appear to effect the incidence of mutations detected in GC.

Several of the studies investigating the use of chemotherapeutic agents in the treatment of GC that also performed KRAS mutation testing, did not provide sufficient information on the type of tissue used for *KRAS* testing (biopsy/primary resection/recurrent resection/pre- or post-treatment), detection methods used, or codons investigated. Thus it is not possible to

accurately interpret the results and make comparisons between such studies. Future studies need to report detailed methodologies in order for conclusions to be drawn from the results.

A recent study suggested that *KRAS* amplifications contribute to the activation of *KRAS* in GC (80) and that activation by *KRAS* amplification may account for the low incidence of *KRAS* mutations in GC compared to other types of cancer (59). However, the results from studies comparing the incidence and relationship of *KRAS* mutations (0-10%) and *KRAS* amplifications (1-9%) in GC remain contradictory (48, 59, 67, 68, 79, 80, 87). However, three studies seem to indicate that *KRAS* amplifications and mutations are mutually exclusive (48, 79, 80) suggesting a need to screen GC patients for both *KRAS* mutations and amplifications.

Incidence of *KRAS* and *BRAF* mutations – comparison between gastric cancer, small bowel and colorectal cancer

According to the RASCAL collaborative, the incidence of *KRAS* mutation in CRC is 38% (118), and a similar incidence has been reported in other studies. Thus, the incidence of *KRAS* mutations in GC is much lower than in CRC. The incidence of *KRAS* mutations in small bowel adenocarcinomas seems to vary dramatically from 9-43% (51, 119-121). Based on data from four studies investigating each less than 100 patients and therefore partly comparable to that of GCs and partly similar to CRCs.

In contrast to GC, in CRC many studies have reported a significant association between *BRAF* mutation and either deficient mismatch repair status or microsatellite instability (106, 110, 122-126). This could be related to the fact that *BRAF* mutations are much more frequent in CRC (5-22% (127)) than in GC (0-12%). In adenocarcinomas of the small bowel, the incidence of *BRAF* mutations is comparable to those reported in GC (119-121). Whereas in CRC *KRAS* and *BRAF* mutations appear to be mutually exclusive (128), there are two reports indicating that GC can harbor a *KRAS* and *BRAF* mutations simultaneously (48, 93). In summary, *KRAS* mutations in GC are a rare event compared to other cancers of the gastrointestinal (GI) tract. Such differences in the incidence of these mutations between cancers of the GI tract may reflect differences in carcinogenesis.

Although no significant relationship between gender and incidence of *KRAS* mutations has been reported in GC, *KRAS* mutations are more frequently reported in males. In addition, the incidence of *KRAS* mutations is higher in intestinal-type than diffuse-type GC. Both observations may be explained by the fact that the incidence of GC in men is twice as high as in women (2) and that intestinal-type GC is found more frequently in males (129). In CRC, the worldwide incidence is also higher in males but the relative difference is not as prominent as in GC (746,000 new CRC cases per year in males versus 614,000 in females (2)). The relationship between *KRAS* mutations in CRC and gender is not consistent. One study found a higher incidence of *KRAS* mutations in females(130), whereas the QUASAR study did not find a difference (122) .

Twelve studies investigated the relationship between *KRAS* mutations and MMR/MSI in GC mostly suggesting a higher incidence of MSI in *KRAS* mutant GC compared to *KRAS* wild-type GC. This is in contrast to CRC, where *KRAS* mutant tumors are found to be less frequent MMR-deficient (118).

In CRC, patients with *KRAS* wild-type cancer seem to have a better survival (131). Few studies (9%) investigating the relationship between *KRAS* mutation status and survival in GC and the results do not concur with those from CRC.

KRAS and *BRAF* mutations and response to anti-EGFR therapy

In CRC, *KRAS* mutation and *BRAF* mutation are known predictors of poor response to EGFR targeted agents, such as cetuximab and panitumumab (132) and *RAS/BRAF* mutation screening is now part of routine clinical diagnosis. In contrast, the predictive value of *KRAS* and *BRAF* mutations in GC is far less clear. In vitro, several studies in *KRAS* wild-type GC cell lines reported sensitivity to EGFR targeting drugs (133-135). Other investigators report that, both *KRAS* mutant and wild-type GC cell lines were resistant to cetuximab (136). In GC xenografts, apoptosis was only induced in *KRAS* wild-type tumor cells treated with Cetuximab (136). Cetuximab was shown to reduce tumor volume, dissemination and vascularisation in EGFR-expressing, *KRAS* wild-type xenografts (133).

To date, the use of anti-EGFR agents (Cetuximab and Panitumumab) in phase III metastatic GC trials in patients has either showed no difference (137) or poorer survival than the control group (138). In the REAL3 trial, *KRAS* mutation status did not predict resistance to Panitumumab in GC (40).

Due to the low incidence of *BRAF* mutations in GC, a clinical trial which stratifies GC patients according to their *BRAF* status is probably not feasible due to the high number of patients that would need to be screened. Although all studies investigated the V600E mutation, three of the studies that also investigated exon 11 and 15 found *BRAF* mutations other than the hotspot V600E mutation (40, 52, 93). Thus, there could be an argument for investigating the whole length of the *BRAF* gene for mutations in GC.

CONCLUSIONS

In conclusion, despite the decrease in the incidence, GC remains a major worldwide health problem. *KRAS* was one of the first oncogenes discovered in GC in 1986. Nevertheless, the current literature on *KRAS* and *BRAF* in GC is still limited and very heterogeneous making any comparisons between different studies difficult. However, it appears that the incidence of *KRAS* mutations in GC is much lower than in CRC, does not differ significantly by ethnicity and that *BRAF* V600E mutations are very rare in GC. Due to the low incidence and often small studies, many of the published studies did not have enough power to detect a poten-

tial relationship between *KRAS* mutation status and clinicopathological variables including patient survival. Even fewer studies have assessed *KRAS* amplifications as a mechanism for *KRAS* activation. So far all clinical studies in unselected metastatic GC have failed to show a significant benefit for EGFR inhibitors. A recent meeting abstract reported the incidence of *KRAS* mutations in signet ring cell GC is higher (15%) than in other types of GC (139). As the incidence of this histological subtype of GC is increasing, particularly in the West (10) and as this subgroup of GC appears to be highly resistant to standard chemotherapy (140). EGFR targeted therapy in signet ring gastric cancer could potentially be a promising treatment option in the future.

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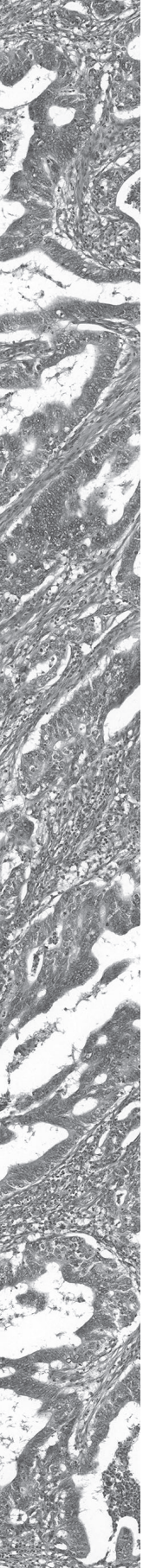

Chapter 3

KRAS status is related to histological phenotype in gastric cancer – Results from a large multicentre study

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ABSTRACT

Background | Gastric cancer (GC) is histologically a very heterogeneous disease, and the temporal development of different histological phenotypes remains unclear. Recent studies in lung and ovarian cancer suggest that *KRAS* activation (*KRAS*act) can influence histological phenotype. *KRAS*act likely results from *KRAS* mutation (*KRAS*mut) or *KRAS* amplification (*KRAS*amp). The aim of the study was to investigate whether *KRAS*mut and/or *KRAS*amp are related to the histological phenotype in GC.

Methods | Digitized Haematoxylin/Eosin stained slides from 1282 GC resection specimens were classified according to Japanese Gastric Cancer Association (JGCA) and the Lauren classification by at least two observers. The relationship between *KRAS* status, predominant histological phenotype and clinicopathological variables was assessed.

Results | *KRAS*mut and *KRAS*amp were found in 68 (5%) and 47 (7%) GCs, respectively. Within the *KRAS*mut and *KRAS*amp cases, the most frequent GC histological phenotype was moderately differentiated tubular 2 (tub2) type (*KRAS*mut: n=27, 40%; *KRAS*amp: n=21, 46%) or intestinal type (*KRAS*mut: n=41, 61%; *KRAS*amp: n=23, 50%). Comparing individual histological subtypes, mucinous carcinoma displayed the highest frequency of *KRAS*mut (JGCA: n=6, 12%, p=0.012; Lauren: n=6, 12%, p=0.013), and *KRAS*amp was more frequently found in poorly differentiated solid type (n=12, 10%, p=0.267) or indeterminate type (n=12, 10%, p=0.480) GC. 724 GCs (57%) had intratumour morphological heterogeneity.

Conclusions | This is the largest GC study investigating *KRAS* status and histological phenotype. We identified a relationship between *KRAS*mut and mucinous phenotype. The high level of intratumour morphological heterogeneity could reflect *KRAS*mut heterogeneity, which may explain the failure of anti-EGFR therapy in GC.

INTRODUCTION

Gastric cancer (GC) is histologically a very a heterogeneous disease, and this is reflected in the numerous proposed histological classification schemes (1). The temporal development of different histological phenotypes in GC remains unclear. Recent studies suggest that Kirsten Rat Sarcoma Viral Oncogene Homolog *(KRAS)* activation and downstream signalling can impact on the properties and functions of the tumour microenvironment (2), and thus may influence histological phenotype. Likely mechanisms of *KRAS* activation include *KRAS* mutation (*KRAS*mut) and *KRAS* amplification (*KRAS*amp) (3).

Mutations in *KRAS* have been identified in many human cancers and result in the constitutive activation of *KRAS* and the receptor tyrosine kinase (RTK) pathway (4). The frequency of *KRAS*mut is variable across different cancer types, with the highest frequency in pancreatic cancer (90%) followed by colon (34.6%), lung (16.5%) and ovarian (11%) cancer and the lowest frequencies in cervical (6.6%), prostate (5%) and oesophageal cancer (2%) (5). In a review of the literature we identified, on average, only 6.5% of GC have a *KRAS*mut (6). In colorectal cancer, routine testing for *KRAS*mut is now implemented as a predictor of response to anti-epidermal growth factor receptor (EGFR) therapy (7).

Several studies have demonstrated a relationship between *KRAS*mut status and histological phenotype in lung and ovarian cancer. In the subgroup of invasive mucinous adenocarcinoma of the lung, *KRAS* is mutated in up to 86% of cases (8). In ovarian cancer, *KRAS*mut has been identified in almost all cases with a mucinous histological phenotype (9). The relationship between *KRAS*mut status and histological phenotype in GC remains to be clarified (6).

The reported frequency of *KRAS*amp is 1-9% in GC (10-16). There are no reports of a relationship between *KRAS* DNA copy number and histological phenotype in other cancer types and in GC it has not been investigated in a large study. There is increasing recognition of the clinical importance of *KRAS*amp in GC. *KRAS*amp also associated with a worse survival (3, 10, 12), whereas *KRAS*mut do not appear to influence survival of GC patients (17).

Recently, image analysis on lung cancer HE stained sections using deep learning was predictive of mutation status (18), thus suggesting that morphological phenotype is reflective of molecular phenotype. Investigating the relationship between *KRAS* activation by *KRAS*mut and/or *KRAS*amp and histological phenotype may provide some insight into gastric adenoma-carcinoma sequence progression and the origin of histological heterogeneity. Based on the studies in lung and ovarian cancer, we hypothesise that *KRAS* activation influences histological phenotype and is associated with a mucinous phenotype in GC. This would suggest that *KRAS* activation is an early event in GC, occurring before the phenotype is determined.

The aim of this multi-centre GC study was to investigate the relationship of *KRAS* activation status (*KRAS*mut and/or *KRAS*amp) with the histological phenotype in a large series of GCs from UK, Japan and The Cancer Genome Atlas (TCGA). In addition, the relationship between *KRAS* status, clinicopathological variables, survival and microsatellite instability status was assessed.

MATERIAL AND METHODS

Patients

Kanagawa Cancer Center Hospital (KCCH), Yokohama, Japan

This cohort included 250 patients with TNM stage II/III GC who underwent potentially curative surgery at Kanagawa Cancer Center Hospital (Yokohama, Japan) between 2001 and 2010. One hundred and six (43%) patients were treated with surgery alone, 108 (43%), 22 (9%), 14 (6%) patients received S-1, Tegafur-uracil or S1 combined with other cytotoxic drug therapy, respectively. Demographical, clinical and pathological data were retrieved from hospital records. The study was approved by the Local Research Ethics Committee.

Leeds Teaching Hospitals NHS Trust (LTHT), Leeds, UK

This cohort included 277 patients with GC who underwent potentially curative surgery at the Department of Surgery, Leeds General Infirmary (Leeds, UK), between 1970 and 2004. Seven (3%) patients were treated by chemotherapy followed by surgery and the remaining 270 (98%) by surgery alone. Clinical and pathological data were retrieved from histopathology reports, electronic patient hospital records and the Northern and Yorkshire Cancer Registry. The study was approved by the Local Research Ethics Committee (LREC No. CA01/122).

The Cancer Genome Atlas

The TCGA stomach adenocarcinoma (STAD) clinicopathological and molecular dataset of 295 patients was obtained from the publically available TCGA database portal (19).

Tokyo Metropolitan Geriatric Hospital and Institute of Gerontology (TMGH), Tokyo, Japan

This cohort included 420 patients with 460 GC who were treated by surgery in the Tokyo Metropolitan Geriatric Hospital between 2000 and 2008. Three hundred and eighty patients had single carcinoma, and 36 had two or more carcinomas. Patients with Lynch syndrome were excluded from the current study. None of the patients underwent neoadjuvant chemotherapy. Histopathological examination and medical research were performed with informed written consent by the patients, and this work was approved by the ethics committee of the Tokyo Metropolitan Geriatric Hospital (#230225, R16-23).

Histopathological classification

pT and pN stage were reported according to 7th edition of the UICC TNM classification for GC (20).

In all cohorts, haematoxylin and eosin (H&E) stained formalin fixed paraffin embedded (FFPE) tissue sections from the resection specimens were reviewed. In the KCCH and LTHT cohorts, H&E stained slides were scanned at 40x magnification using an Aperio AT2 scanner

for review. In the TCGA cohort, H&E stained slides were viewed online using the cancer digital slide archive (http://cancer.digitalslidearchive.net/). In the TMGH cohort, classification was performed using the glass slide.

Histological classification according to JGCA scheme was performed (21). Mucinous carcinoma were defined by tumour cells in extra mucinous pools comprising an area greater than 50% of the total tumour. GC were classified as signet ring cell carcinoma when signet ring cells were present in more than 50% of the tumour volume. In cases where more than one histological phenotype was identified, the most predominant phenotype was recorded, and these GCs were categorised as heterogeneous. JGCA classification was converted to Lauren classification (22) according to table 1. As there is no Lauren classification for mucinous GC, we retained mucinous cancers as a separate category in order to distinguish them from other histological types.

Table 1 | Japanese Gastric Cancer Association histological classification of common types of gastric cancers in relation to Lauren classification.

	Histological classification
Lauren	**Japanese Gastric Cancer Association (JGCA)**
Intestinal	**Differentiated:** Papillary adenocarcinoma (pap) Tubular adenocarcinoma (tub) Well-differentiated (tub1) Moderately differentiated (tub2)
Diffuse	**Undifferentiated:** Poorly differentiated adenocarcinoma Non-solid type (por2) Signet-ring carcinoma (sig)
Mucinous	**Differentiated/undifferentiated:** Mucinous adenocarcinoma (muc)
Indeterminate	**Undifferentiated:** Poorly differentiated adenocarcinoma (por) Solid type (por1)

Table created after personal communication with H.Grabsch, March 12, 2019

DNA extraction

The area with the highest tumour cell density was identified on H&E stained sections and the whole tumour area, irrespective of subregions with different histological phenotypes was microdissected after staining with Shandon instant haematoxylin (Thermo Scientific, Cheshire, UK) using a sterile surgical blade. Tumour DNA from FFPE material was extracted from KCCH and LTHT GCs using QIAmp DNA Micro Kit (Qiagen,

Hilden, Germany) as previously described (23). DNA concentration was measured by ND-100 Spectrophotometer (Labtech International) and samples were diluted using Tris-EDTA buffer. In the TMGH cohort, DNA was extracted using a phenol-chloroform procedure as described previously (24).

KRAS gene copy number and data analyses

KRAS copy number status was investigated in KCCH, LTHT and TCGA cohorts. In the KCCH and LTHT cohort, *KRAS* gene copy number was determined by multiplex ligation-dependent probe amplification (MLPA) using the Salsa-FAM labelled MLPA reagent kit and probemix P458-A1 or the updated version -B1 (MRC Holland, Amsterdam, The Netherlands) as previously described (25). For further details on the *KRAS* probes included in this probemix see supplementary table 1. Fragment analysis of the MLPA reaction product was performed using capillary electrophoresis ABI-3130 XL (Applied Biosystems, California, USA) as previously described (25). Failed experiments were repeated at least twice before a case was finally excluded from the analyses.

KRAS DNA copy number data from 237 KCCH GC has been previously published (25), but was re-analysed using a different methodology in the current study. The output files (FSA files) from the sequencer were initially imported into Coffalyser.net for fragment analysis and results were exported as csv files. Subsequent analyses were performed using the MLPAInter method, as previously described (26), implemented in R. Samples were normalised per batch using reference samples processed in each batch. Quality control was performed to exclude samples with low overall intensity, with a large difference in intensity between short probes and long probes, with low intensity of denaturation controls, or high within gene variation, defined as the average of the standard deviation of log transformed values. Final values were calculated by averaging the peak height of each probe and then averaging the results of replicates. Copy number thresholds were set based on previously published studies (25, 27, 28), with a DNA copy number >1.31 categorised as amplification. This analysis was performed separately for KCCH and LTHT cohorts.

In TCGA, *KRAS*amp were determined by array-based somatic copy number analysis (29). Level 3 copy number segmentation data was downloaded from the TCGA data portal (19) and used to estimate copy number for *KRAS*. Based on previous studies, a LogRatio > 0.4 was categorised as amplification (30).

KRAS mutation status

*KRAS*mut data from a previous study were available for 230 KCCH and 275 LTHT GC patients (17). *KRAS*mut testing was performed on an additional 12 KCCH GCs as previously described (17). In TCGA, *KRAS*mut status was determined by whole exome sequencing (29) and results were downloaded from the TCGA database portal (19) for 289 patients. In the TMGH cohort, *KRAS* (codon 12 and 13) was examined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), using primers and methods previously described (31, 32).

Microsatellite instability (MSI) status

Immunohistochemistry of DNA mismatch repair proteins were used as a surrogate marker of MSI status. Results for MLH1, MSH2, MSH6 and PMS2 were available from 230 KCCH GCs,

and MLH1 and MSH2 from 253 LTHT GCs from a previous study (17). MLH1, MSH2, MSH6 and PMS2 immunohistochemistry was performed on additional 13 GCs from the KCCH cohort for this study, as previously described (17).

In TCGA, MSI was determined by a DNA based MSI-Mono-Derived-Dinucleotide Assay using four mononucleotide repeat loci and three dinucleotide repeat loci using a multiplex fluorescent-labeled PCR and capillary electrophoresis (29). Results were obtained from the TCGA database portal (19) for 295 GC patients. MSI-low GCs were grouped with microsatellite stable (MSS) GCs for further analyses following current guidelines (33).

In the TMGH cohort, mononucleotide repeats *BAT25* and *BAT26* were investigated, as previously described (34-36) and GC were classified as MSS or MSI.

Statistical analyses

All statistical analyses were performed using SPSS software version 23 (SPSS Inc., Chicago, Ill). The relationship between *KRAS*mut or *KRAS*amp and clinicopathological variables (age, gender, depth of invasion (pT), lymph node status (pN), TNM stage, Lauren classification (22), JGCA classification (21), MSI status and morphological heterogeneity status) were assessed using chi-squared or Fisher's exact test. The relationship between *KRAS*mut and survival in LTHT and KCCH cohorts has been published previously (17). Combining all cohorts, the relationship between *KRAS*mut or *KRAS*amp and 5 year overall survival was analysed using the Kaplan-Meier method and differences were assessed using the log rank test. A p-value of <0.05 was considered significant.

RESULTS

Patient characteristics

The median (range) age of GC patients was as follows; KCCH: 65 years (35-85 years), LTHT: 72 years (14-96 years), TCGA: 68 years (35-90 years), TMGH: 78 (51-96). For a summary of other patient clinicopathological variables in each cohort see table 2.

Histological classification of gastric cancer

Histological classification was available for 1271 GCs. Using the JGCA classification, the most predominant phenotype was tubular moderately differentiated [tub2] (n=408, 32%), followed by poorly differentiated solid type [por1] (n=229, 18%), poorly differentiated non-solid type [por2] (n=227, 18%), tubular well-differentiated [tub1] (n=219, 17%), papillary [pap] (n=71, 6%), signet-ring cell [sig] (n=66, 5%) and mucinous [muc] (n=51, 4%). According to Lauren classification, 293 (23%) GCs were classified as diffuse type, 698 (55%) as intestinal type, 51 (4%) as mucinous and 229 (18%) as indeterminate. Seven hundred and twenty-four GCs (57%) had intratumour morphological heterogeneity (see table 2).

KRAS mutation status and relationship with clinicopathological variables

*KRAS*mut status was available from 1266 GCs (KCCH n=242; LTHT n= 275; TCGA n=289, TMGH n=460). In total, 68 (5%) GCs were *KRAS* mutant, with the highest frequency of *KRAS*mut in the TCGA cohort (10%) and lowest frequency in the TMGH cohort (3%), see table 2. Within the *KRAS*mut GC, the most frequent histological phenotype was intestinal type (n=41, 61%) or tub2 (n=27, 40%) by Lauren and JGCA classification, respectively (see figure 1a). Comparing individual histological subtypes, mucinous phenotype displayed the highest frequency of *KRAS*mut by Lauren (p=0.013) and JGCA (p=0.012) classification, respectively (see figure 1b). *KRAS*mut was more frequent in MSI GC (p<0.001). For the comparison of *KRAS*mut status and other clinicopathological variables, see table 3. The 5 year overall survival rate in patients with *KRAS*mut or *KRAS* wildtype GC was 63.6% and 54.8%, respectively, p=0.541, see figure 2a.

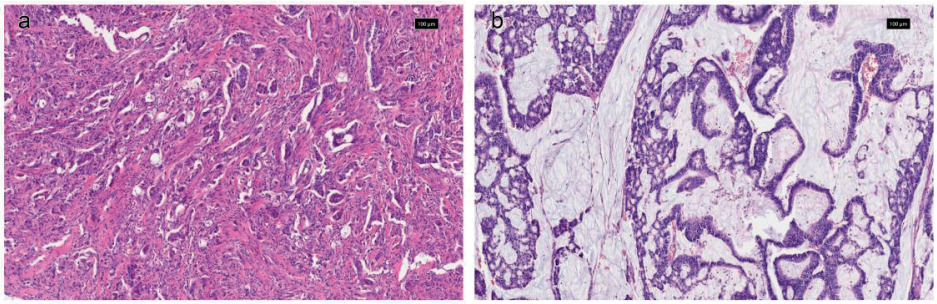

Figure 1 | Example of *KRAS* mutated GC with (A) moderately differentiated tubular (tub2) phenotype and (B) mucinous phenotype.

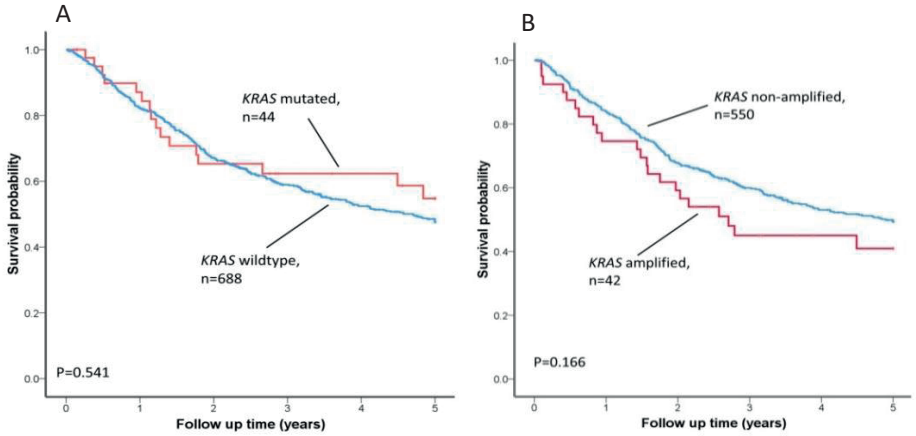

Figure 2 | Kaplan-Meier plots showing probability of overall survival in GC patients stratified by *KRAS* gene activation status. (A) Kaplan-Meier survival analysis showed no difference in survival when patients were stratified by *KRAS* mutation status. (B) Kaplan-Meier survival analysis showed no difference in survival when patients were stratified by *KRAS* amplification status.

Table 2 | Comparison of clinicopathological variables in each gastric cancer cohort

		Total		KCCH		LTHT		TCGA		TMGH	
		n	%	n	%	n	%	n	%	n	%
		1282		250	20	277	22	295	23	460	36
Age (years)	<65	343	27	122	49	78	28	123	42	20	4
	≥65	936	73	128	51	199	72	169	58	440	96
Gender	Male	769	60	175	70	164	59	182	62	248	54
	Female	513	40	75	30	113	41	113	38	212	46
T stage	pT1	272	21	6	2	20	7	11	4	235	51
	pT2	138	11	43	17	24	9	44	15	27	6
	pT3	350	28	34	14	79	29	155	54	82	18
	pT4	512	40	167	67	154	56	75	26	116	25
N stage	pN0	489	39	42	17	87	31	97	34	263	57
	pN1	247	19	58	23	52	19	64	23	73	16
	pN2	229	18	67	27	54	20	58	20	50	11
	pN3	306	24	83	33	84	30	65	23	74	16
TNM stage	I	307	24	0		34	12	32	12	241	53
	II	384	30	97	39	81	29	116	42	90	20
	III	507	40	153	61	151	55	111	40	92	20
	IV	67	5	0		11	4	20	7	36	8
Lauren classification	Diffuse	293	23	83	34	60	22	73	25	77	17
	Intestinal	698	55	103	42	145	54	156	53	294	64
	Mucinous	51	4	10	4	10	4	20	7	11	2
	Indeterminate	229	18	51	21	56	21	44	15	78	17
JGCA classification	Pap	71	6	5	2	9	3	17	6	40	9
	Tub1	219	17	18	7	55	20	23	8	123	27
	Tub2	408	32	80	32	81	30	116	40	131	29
	Por1	229	18	51	21	56	21	44	15	78	17
	Por2	227	18	63	26	52	19	71	24	41	9
	Sig	66	5	20	8	8	3	2	1	36	8
	Muc	51	4	10	4	10	4	20	7	11	2
Morphological heterogeneity	Homogenous	542	43	102	42	82	30	185	63	173	38
	Heterogeneous	724	57	140	58	189	70	108	37	287	62
***KRAS* mutation status**	Mutant	68	5	10	4	16	6	28	10	14	3
	Wildtype	1198	95	232	96	259	94	261	90	446	97
***KRAS* gene copy number**	Amplified	47	7	12	6	17	8	18	8		
	Other	602	93	196	94	199	92	207	92		
Microsatellite instability status	MSI	199	16	23	9	31	12	64	22	81	18
	MSS	1057	84	223	91	224	88	231	78	379	82

Note. Some variables do not add up to 1282 due to missing data.

Abbreviations: JGCA, Japanese Gastric Cancer Association; Pap, papillary adenocarcinoma; Tub1, well differentiated tubular adenocarcinoma; Tub2, moderately differentiated tubular adenocarcinoma; Por1, poorly differentiated adenocarcinoma solid type; Por2, poorly differentiated adenocarcinoma non-solid type; Sig, signet-ring cell carcinoma; Muc, mucinous adenocarcinoma; MSI, microsatellite instable; MSS, microsatellite stable; KCCH, Kanagawa Cancer Center Hospital; LTHT, Leeds Teaching Hospital Trust; TCGA, The Cancer Genome Atlas; TMGH, Tokyo Metropolitan Geriatric Hospital and Institute of Gerontology.

Table 3 | Comparison of clinicopathological variables and *KRAS* mutation status in all gastric cancer cohorts combined

		***KRAS* mutation status**				
		M n	**M %**	**WT n**	**WT %**	**P-value**
Age (years)	<65	13	4	319	96	0.167
	≥65	55	6	876	94	
Gender	Male	36	5	723	95	0.225
	Female	32	6	475	94	
T stage	pT1/pT2	20	5	388	95	0.639
	pT3/pT4	47	6	802	95	
N stage	pN0	31	6	455	94	0.158
	pN1-pN3	35	5	734	95	
TNM stage	I-II	35	5	651	95	0.756
	III-IV	31	6	533	95	
Lauren classification	Diffuse	7	2	283	98	0.013
	Intestinal	41	6	652	94	
	Mucinous	6	12	43	88	
	Indeterminate	13	6	215	94	
JGCA classification	Pap	7	10	64	90	0.012
	Tub1	7	3	212	97	
	Tub2	27	7	376	93	
	Por1	13	6	215	94	
	Por2	6	3	219	97	
	Sig	1	2	64	99	
	Muc	6	12	43	88	
Morphological heterogeneity	Homogeneous	31	6	506	94	0.550
	Heterogeneous	36	5	683	95	
Microsatellite instability status	MSI	33	17	165	83	<0.001
	MSS	32	3	1010	97	

Note. Some variables do not add up to 1282 due to missing data.
Abbreviations: JGCA, Japanese Gastric Cancer Association; Pap, papillary adenocarcinoma; Tub1, well differentiated tubular adenocarcinoma; Tub2, moderately differentiated tubular adenocarcinoma; Por1 , poorly differentiated adenocarcinoma solid type; Por2, poorly differentiated adenocarcinoma non-solid type; Sig, signet-ring cell carcinoma; Muc, mucinous adenocarcinoma; MSI, microsatellite instable; MSS, microsatellite stable.

KRAS amplification and relationship with clinicopathological variables

KRAS gene copy number status was available from 649 GCs (KCCH n=208, LTHT n=216, TCGA n=225). In total, 47 (7%) GCs had a *KRAS*amp (TCGA (8%), LTHT (8%) and KCCH (6%)), see table 2. Within *KRAS*amp GC, intestinal type (n=23, (50%) or tub2 (n=21, 46%) was the most frequent histological phenotype by Lauren and JGCA classification, respectively (see figure 3a). Comparing individual histological subtypes, *KRAS*amp was more frequently

found in indeterminate type (n=12, 10%) or por1 (n=12, 10%) phenotype by Lauren and JGCA classification, respectively (see figure 3b). There was no relationship between *KRAS*amp and histological phenotype or any other clinicopathological variables, see table 4. The 5 year overall survival rate in GC patients with and without *KRAS*amp was 47.6% versus 55.6%, respectively, p=0.166, see figure 2b.

Only two GCs from the TCGA cohort had a concurrent *KRAS*amp and *KRAS*mut; one was a mucinous GC, the other was a por2 GC according to JGCA classification.

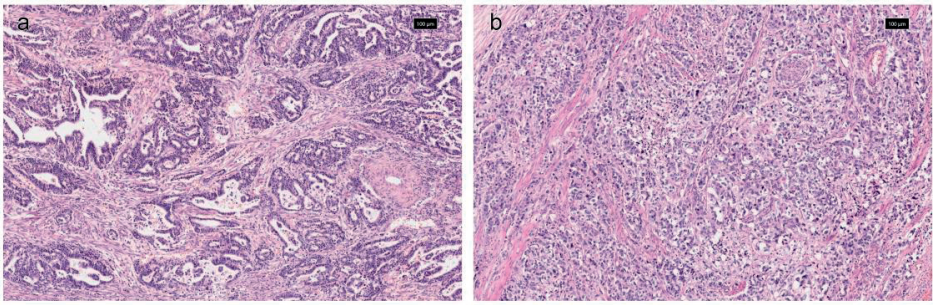

Figure 3 | Example of *KRAS* amplified GC with (A) moderately differentiated tubular (tub2) phenotype and (B) solid-type poorly differentiated adenocarcinoma (por1) phenotype.

Table 4 | Comparison of clinicopathological variables and *KRAS* copy number status in KCCH, LTHT and TCGA gastric cancer cohorts combined

		***KRAS* amplified n**	***KRAS* amplified %**	***KRAS* other n**	***KRAS* other %**	**P-value**
Age (years)	<65	21	8	235	92	0.462
	≥65	26	7	364	93	
Gender	Male	29	7	383	93	0.792
	Female	18	8	219	92	
T stage	pT1/pT2	8	7	109	93	0.867
	pT3/pT4	38	7	484	93	
N stage	pN0	7	4	163	96	0.058
	pN1-pN3	40	9	428	92	
TNM stage	I-II	14	5	262	95	0.061
	III-IV	32	9	325	91	
Lauren classification	Diffuse	10	6	168	94	0.480
	Intestinal	23	7	298	93	
	Mucinous	1	3	29	97	
	Indeterminate	12	10	107	90	

Table 4 | Comparison of clinicopathological variables and *KRAS* copy number status in KCCH, LTHT and TCGA gastric cancer cohorts combined (continued)

		***KRAS* amplified n**	***KRAS* amplified %**	***KRAS* other n**	***KRAS* other %**	**P-value**
JGCA classification	Pap	0	0	24	100	0.267
	Tub1	2	3	70	97	
	Tub2	21	9	204	91	
	Por1	12	10	107	90	
	Por2	9	6	144	94	
	Sig	1	4	24	96	
	Muc	1	3	29	97	
Morphological heterogeneity	Homogeneous	19	6	282	94	0.437
	Heterogeneous	27	8	315	92	
Microsatellite instability status	MSI	3	3	90	97	0.093
	MSS	44	8	494	92	

Note. Some variables do not add up to 822 due to missing data.
Abbreviations: JGCA, Japanese Gastric Cancer Association; Pap, papillary adenocarcinoma; Tub1, well differentiated tubular adenocarcinoma; Tub2, moderately differentiated tubular adenocarcinoma; Por1, poorly differentiated adenocarcinoma solid type; Por2, poorly differentiated adenocarcinoma non-solid type; Sig, signet-ring cell carcinoma; Muc, mucinous adenocarcinoma; MSI, microsatellite instable; MSS, microsatellite stable; KCCH, Kanagawa Cancer Center Hospital; LTHT, Leeds Teaching Hospital Trust; TCGA, The Cancer Genome Atlas.

DISCUSSION

This is the largest multicentre study to date to investigate the relationship between *KRAS* activation by mutation and/or amplification and histological phenotype in GC. The frequency of *KRAS*amp (7%) was slightly higher than that of *KRAS*mut (5%) which is consistent with other GC studies (10, 11, 37). The higher frequency of *KRAS*mut in the TCGA GC cohort compared to the other cohorts could be related to the methodology as TCGA used whole exome sequencing to test non-hotspot regions whereas other studies used less sensitive Sanger sequencing/PCR-RFLP. We found *KRAS*amp and *KRAS*mut were exclusive in >99% of GC, which is consistent with previous reports (11-13, 38).

The relationship between *KRAS*mut and histological phenotype had not been investigated in great detail and previous studies were limited by small sample sizes and hence lack of statistical power (6). In our study, we identified a relationship between *KRAS*mut and mucinous histological phenotype, which is concordant with higher frequencies of *KRAS*mut being reported in mucinous lung (8), ovarian (9) and colorectal cancer (39, 40). However, due to the relatively low frequency of GC with mucinous phenotype and *KRAS*mut (12%), it would not be feasible to use the presence of a mucinous phenotype as a predictor for the presence of a *KRAS*mut in GC. The main components of mucinous GCs are extracellular mucins, which are high molecular weight glycoproteins regulated by expression of the MUC2, MUC5AC

and MUC6 genes in humans (41). In mouse models with constitutively activated *KRAS* in the stomach, irregular MUC4+ cells were found with abnormal mucins confirmed by Alcian-blue staining (42). Interestingly, our study suggests a relationship between *KRAS*mut and mucinous phenotype, which is characterised by extracellular mucin, but is not related to signet-ring cell type GC, which is characterised by intracellular mucins. Our study confirmed the relationship between *KRAS*mut and presence of MSI, which our group and others have described previously in a smaller GC cohort (43, 44).

The prognostic significance of *KRAS*mut in GC remains controversial (6). In our study, there was no association with presence of *KRAS*mut and survival. Interestingly, in lung and colorectal cancer, *KRAS*mut are associated with a poor prognosis (45, 46), whereas in ovarian cancer, *KRAS*mut are associated with an improved prognosis (47).

The relationship between *KRAS*amp and clinicopathological variables, including histological phenotype in cancer is not well studied. In GC, we found no statistically significant relationship between *KRAS*amp and histological phenotype, or any other clinicopathological variables. In contrast, others found that the presence of *KRAS*amp is associated with a poor prognosis in GC (3, 10, 12). This difference might be due to case selection and methodology used.

In our study we used the JGCA scheme for the histological classification of GC and performed a conversion to the Lauren scheme, which is the most widely used histological classification system in Western countries (22). Previous studies investigating the relationship between *KRAS*mut and histological phenotype performed classification according to the Lauren scheme (6), for which there is no separate category for mucinous GC. The relatively large number of GCs classified as indeterminate according to the Lauren scheme comes from conversion from the JGCA por1 histological phenotype. Direct classification according to the Lauren scheme, would likely result in a higher proportion of GCs classified as either intestinal or diffuse.

In colorectal cancer *KRAS*mut is known to be an early event in the progression from normal colonic epithelial cell to adenoma, and finally to carcinoma (48). The evidence of sequential development by accumulation of genetic alterations, including *KRAS*mut, is still controversial in GC (49-51). We were unable to make any comments regarding the role of *KRAS*act in gastric carcinogenesis in our cohort as we did not investigate precancerous lesions in the current study. However, evidence from mouse models suggest that *KRAS*mut is one of the key molecular alterations involved in the development of stomach dysplasia (52) and GC (53). Based on the evidence from other cancer types that *KRAS*mut influence the progression of a mucinous histological phenotype, we therefore speculate based on our results that *KRAS*mut in GC is an early event in GC development whereas *KRAS*amp is likely to be a late event occurring after the histological phenotype has been established. This would correspond with experiments in mice expressing oncogenic *KRAS* in combination with E-cadherin and p53 loss, which resulted in a rapid progression of GC compared to wildtype mice (53).

Our study has some limitations. This is a retrospective study. Histological phenotyping was performed on a single slide. Given the high frequency of intra-tumoural morphological heterogeneity in this study and the previously reported intra-tumoural heterogeneity in *KRAS*mut status in GC (54), the sensitivity of some of the techniques used in the current study may not be sufficient to detect *KRAS* activation in subclones of tumour cells. As we did not perform microdissection of tumour subregions, we cannot comment on *KRAS* status heterogeneity within the same tumour. Furthermore, we used different techniques for DNA extraction, *KRAS*mut status analysis and MSI analysis in different cohorts included in the current study, each with differing sensitivities (55, 56).

In summary, we identified a relationship between *KRAS*mut and mucinous histological phenotype in GC. The high level of intratumour morphological heterogeneity could reflect *KRAS*mut heterogeneity, which may explain the failure of anti-EGFR therapy in GC.

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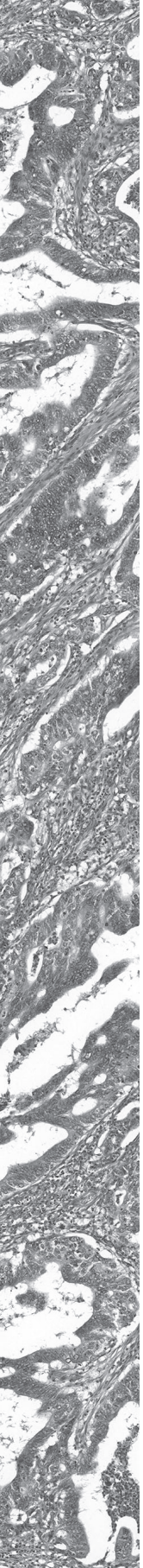
Supplementary table 1: Information on KRAS probes incorporated into MRC-Holland gastric cancer probemix

Probemix	Length (nucleotides)	Probe	Chromosome band	Exon	HG16 location	HG18 location
P458-A1	180	17596-L22078	12p12.1	2	12-025.289376	
P458-A1	392	09507-L22081	12p12.1	3	12-025.271583	
P458-A1	382	17605-SP0543-L21602	12p12.1	4	12-025.269833	
P458-A1	202	17597-SP0529-L22061	12p12.1	6	12-025.252102	
P458-B1	124	20117-L27312	12p12.1	6		12-025.252
P458-B1	197	20095-L27280	12p12.1	4		12-025.270
P458-B1	399	19323-L27531	12p12.1	3		12-025.272



Chapter 4

Epstein-Barr Virus and Mismatch Repair Deficiency status differ between Oesophageal and Gastric Cancer: a large Multicentre Study

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ABSTRACT

Background | Oesophageal (OeC) and gastric (GC) cancer patients are treated with similar multimodal therapy and have poor survival. There remains an urgent clinical need to identify biomarkers to individualise patient management and improve outcomes. Therapy with immune checkpoint inhibitors has shown promising results in other cancers. Proposed biomarkers to predict potential response to immune checkpoint inhibitors include DNA mismatch repair (MMR) and/or Epstein-Barr virus (EBV) status. The aim of this study was to establish and compare EBV status and MMR status in large multicentre series of OeC and GC.

Methods | EBV was assessed by EBV-encoded RNA (EBER) *in situ* hybridisation and MMR protein expression by immunohistochemistry (IHC) in 988 OeC and 1213 GC from multiple centres. In a subset of OeC, microsatellite instability (MSI) was tested in parallel with MMR IHC.

Results | Frequency of MMR deficiency (MMRdef) and MSI was low in OeC (0.8% and 0.6%, respectively) compared with GC (10.3%). None of the OeCs were EBER positive in contrast to 4.8% EBER positive GC. EBV positive GC patients were younger (p=0.01), more often male (p=0.001) and had a better overall survival (p=0.012). MMRdef GC patients were older (p=0.001) and showed more often intestinal-type histology (p=0.022).

Conclusions | This is the largest study to date indicating that EBV and MMRdef do not play a role in OeC carcinogenesis in contrast to GC. The potential clinical usefulness of determining MMRdef/EBV status to screen patients for eligibility for immune-targeting therapy differs between OeC and GC patients.

INTRODUCTION

Oesophageal cancer (OeC) and gastric cancer (GC) are the eighth and fifth most common cancer worldwide, respectively, with an estimated total of 1,407,000 new cases and 1,123,000 deaths in 2012 (1). The two main histological OeC subtypes are squamous cell carcinoma (SqC) and adenocarcinoma (AdC). The vast majority of GC are adenocarcinomas.

In Europe, the standard of care for OeC and GC patients with locally advanced resectable disease is chemotherapy or chemoradiotherapy, followed by surgery (2, 3). GC patients receive perioperative platinum/fluorouracil based chemotherapy. For OeC, patients with SqC are treated with preoperative chemoradiotherapy with carboplatin/paclitaxel. Patients with AdC receive perioperative platinum/fluorouracil or preoperative chemoradiotherapy. Nevertheless, survival remains poor, with 5-year overall survival between 36-47% (4, 5).

To date few targeted therapy options are available to OeC/GC patients with metastatic disease: trastuzumab for HER2 positive disease (6) and ramucirumab, a VEGFR-2 antagonist without biomarker based patient selection (7, 8). All other trials evaluating receptor tyrosine kinase or downstream signalling inhibitors in OeC/GC were unable to show a survival benefit (9). There remains an urgent clinical need to identify biomarkers to individualise and improve OeC/GC patient management.

DNA mismatch repair (MMR) has been used as a predictive biomarker for PD1 inhibitor therapy response in multiple different cancer types, including colorectal cancer (10). Evidence of Epstein-Barr virus (EBV) infection has been proposed as a potential marker for response to PD1/PDL1 inhibitors in GC (11). Pembrolizumab, an antibody against PD1, was approved by the FDA for the treatment of unresectable or metastatic solid tumours, including OeC and GC, with mismatch repair deficiency (MMRdef) or microsatellite instability (MSI)-High (12).

The potential of immunotherapy in OeC was shown recently in phase 2 trials in non-selected oesophageal SqC and GC patients treated with nivolumab, a monocolonal antibody inhibiting PD1, in second line treatment (13, 14) and in a phase 3 trial in heavily pretreated non-selected Asian GC patients (15). Furthermore, recent results from the phase 1b trials in patients with PD-L1 expressing OeC (KEYNOTE-028) and GC (KEYNOTE-012), showed promising activity of pembrolizumab in the metastatic setting (16, 17). In metastatic colorectal cancer, a phase 2 study demonstrated the clinical benefit of pembrolizumab in patients with MMRdef (18).

In addition to the potential role of MMR proteins in selecting patients for immunotherapy, MMRdef has shown prognostic value (19) and seems to predict a poor response to fluorouracil based chemotherapy in colorectal cancer (20, 21). It has been shown recently in MAGIC trial patients, that gastro-oesophageal cancer patients with MMRdef/MSI tumours treated with surgery alone survived longer compared to those treated with perioperative cytotoxic chemotherapy (22). In OeC, MLH1 and MSH2 deficiency has been shown to be associated with poor prognosis in small series of SqC (23).

To date, the frequency of MMRdef/MSI in OeC cancer remains unclear because of the small sample size of studies. The reported frequency of MSI-High (MSI-H) ranges from 0-27% but a number of previous studies did not distinguish between MSI-H and MSI-Low (MSI-L) (for an overview of all published studies on MMR and MSI in OeC, see table 1). The recent study by The Cancer Genome Atlas (TCGA) did not find MSI in any of the 162 OeC (24). With respect to the frequency of EBV infection in OeC, the majority of previous studies investigated SqC using different methodology, included relatively small number of patients and reported a frequency of EBV positivity from 0 to 36% (for an overview of all published studies on EBV

Table 1 | Summary of published literature relating to the frequency of mismatch repair deficiency and microsatellite instability in oesophageal cancer

Authors	Year	Oesophageal cancer type	Total n	MMRdef n (%)	MSI-High n (%)	Method
TCGA (24)	2017	SqC	90	NI	0	PCR
		AdC	70		0	
		undiff	2		0	
Pandilla *et al.* (26)	2013	SqC	60	NI	6 (10)	PCR
		AdC	30		2 (7)	
Farris *et al.* (27)	2011	SqC	76	5 (7)	5 (7)	IHC, PCR
Vasavi *et al.* (28)	2010	SqC	45	NI	12 (27)	PCR
		AdC	5		1 (20)	
Matsumoto *et al.* (29)	2007	SqC	62	NI	5 (8)	PCR
Falkenback *et al.* (30)	2005	AdC	59	2 (3)	2 (3)	IHC, PCR
Naidoo *et al.* (31)	2005	SqC	100	NI	5 (5)*	PCR
Uehara *et al.* (23)	2005	SqC	122	49 (40)	6 (5)*	IHC
Evans *et al.* (32)	2004	AdC	27	6 (22)	0	IHC, PCR
Araki *et al.* (33)	2004	SqC	100	NI	0	PCR
Hayashi *et al.* (34)	2003	SqC	30	NI	1 (3)	PCR
Ikeguchi *et al.* (35)	1999	SqC	20	NI	1 (5)*	PCR
Wu *et al.* (36)	1998	SqC	92	NI	5 (5)*	PCR
Muzeau *et al.* (37)	1997	SqC	20	NI	0	PCR
		AdC	26		0	
Gleeson *et al.* (38)	1996	AdC	17	NI	1 (17)	PCR
Keller *et al.* (39)	1995	AdC	15	NI	2 (13)*	PCR
Ogasawara *et al.* (40)	1995	SqC	35	NI	21 (60)*	PCR
Meltzer *et al.* (41)	1994	SqC	42	NI	1 (2)*	PCR
		AdC	36		2 (6)*	

Abbreviations: AdC, adenocarcinoma; SqC, squamous cell carcinoma; MMRdef, mismatch repair deficiency; MSI, microsatellite instability; PCR, polymerase chain reaction; IHC, immunohistochemistry; NI, not investigated; undiff, undifferentiated

*no distinction made between MSI-High and MSI-Low

in OeC see table 2). Thus neither MSI/MMRdef nor EBV status has been investigated in large series of OeC using the same methodology and relating results to clinicopathological variables and patient survival.

The aim of this multi-centre study was to establish the EBV and MMR/MSI status in 988 OeC, including patients from the Medical Research Council (MRC) Oe02 trial (25), from Leeds (UK) and from Cologne (Germany), and relate the results to clinicopathological variables, survival and treatment interaction (preoperative chemo(radio)therapy). As patients with resectable OeC and GC are often treated using similar neoadjuvant therapy regimens and recruited into the same clinical trials across different countries or continents, we compared the frequency of EBV positivity and MMRdef in OeC with that of 1213 GC from Leeds (UK) and Yokohama (Japan).

Table 2 | Summary of published literature relating to the frequency of Epstein-Barr virus in oesophageal cancer

Reference	Year	Oesophageal cancer type	Total n	EBV positive n (%)	Method
TCGA (24)	2017	SqC	90	0	Whole-exome sequencing
		AdC	70	0	
		undiff	2	0	
Genitsch *et al.* (42)	2015	AdC	118	0	EBER ISH
Farris *et al.* (27)	2011	AdC	76	1 (1)	EBER ISH
Sunpaweravong *et al.* (43)	2005	SqC	104	0	EBER ISH
Wu *et al.* (44)	2005	SqC	151	6 (4)	EBER ISH
		undiff	13	4 (31)	
Awerkiew *et al.* (45)	2003	SqC	23	8 (35)	PCR
		AdC	14	5 (36)	
Yanai *et al.* (46)	2003	SqC	34	0	EBER ISH, PCR
Mizobuchi *et al.* (47)	1997	SqC	41	0	PCR
Wang *et al.* (48)	1999	SqC	51	0	EBER ISH, PCR
Wang *et al.* (49)	1999	SqC	31	11 (36)	EBER ISH, PCR

Abbreviations: AdC, adenocarcinoma; SqC, squamous cell carcinoma; EBER ISH, EBV-encoded RNA in situ hybridisation; PCR, polymerase chain reaction; undiff, undifferentiated

MATERIAL AND METHODS

Oesophageal and gastric cancer

The definition whether a tumour is a gastric or oesophageal cancer is dependent on the macroscopic location of the bulk/epicentre of the tumour with respect to the gastro-oesophageal junction. Macroscopic images were not available to us for review as part of this study with the exception of the Japanese gastric cancer cases. In contrast to our Japanese colleagues who classify tumours as oesophageal, junctional or gastric, all other pathologists using the

TNM classification categorise tumours as being either oesophageal or gastric. We therefore reviewed the macroscopic images from the Japanese junctional cancers to classify them as either oesophageal or gastric according to TNM rules. For all other cases we have used the classification of the originally reporting pathologist.

Oesophageal cancer

Leeds Teaching Hospitals NHS Trust (LTHT), UK

The LTHT cohort included 223 OeC patients who underwent potentially curative surgery at the Department of Surgery, Leeds General Infirmary (Leeds, UK), between 1986 and 2006. 83 patients had preoperative chemotherapy. Clinical and pathological data were retrieved from pathology reports, electronic patient hospital records and the Northern and Yorkshire Cancer Registry. The study was approved by the Leeds Research Ethics Committee (LREC No. CA01/122).

University Hospital Cologne (UHC), Germany

The UHC cohort included 322 OeC patients who underwent potentially curative surgery at the Department of Visceral Surgery, University of Cologne (Cologne, Germany), between 1999 and 2013. 197 patients had pre-operative chemotherapy. Clinical and pathological data were retrieved from pathology reports and electronic patient hospital records. The study was approved by the Ethics Committee at the University Hospital, Cologne (reference number: 09-232).

Gastric cancer

Leeds Teaching Hospitals NHS Trust, UK

The GC LTHT cohort included 799 patients who underwent potentially curative surgery at the Department of Surgery, Leeds General Infirmary (Leeds, UK) between 1970 and 2004. 11 patients had preoperative chemotherapy. Demographical, clinical and pathological data were retrieved from pathological reports, electronic patient hospital records and the Northern and Yorkshire Cancer Registry. The study was approved by the Leeds Research Ethics Committee (LREC No. CA01/122).

Kanagawa Cancer Center Hospital (KCCH), Yokohama, Japan

The KCCH cohort included 414 patients with stage II-IV GC who underwent potentially curative surgery at the Kanagawa Cancer Center Hospital (Yokohama, Japan) between 2001 and 2010. None of the patients had preoperative chemotherapy, 202 patients were treated with chemotherapy after surgery. Demographical, clinical and pathological data were retrieved from pathological reports and patient hospital records. The study was approved by the Local Research Ethics Committee.

Cancer Staging

pT and pN stage was reported according to the Union for International Cancer control 6th and 7th edition of the TNM classification for OeC and GC, respectively.

The histological subtype of adenocarcinomas was established based on Lauren's classification (50). According to Lauren's classification signet-ring cell GCs were classified as diffuse-type cancer. As there is no category for mucinous cancers in the Lauren classification, such cancers were classified together with the mixed-type cancers which we used as a category for truly mixed-type cancers and cancers with indeterminate phenotype like the mucinous cancers. The histology type of the case, as stated in the pathology report, was used for statistical analyses.

Tissue microarray construction

Slides from all resection specimens were reviewed and a block with the highest tumour cell density was selected for tissue microarray (TMA) construction and/or marked for microdissection for DNA extraction (see below). The areas selected were representative of the overall histology of the case. The LTHT, KCCH and Oe02 trial cases were reviewed by HG, LH and GH, together with local pathologists. The UHC cases were reviewed by AQ. A total of 962 OeCs (417, 223 and 322 patients from the Oe02, LTHT, and UHC cohorts, respectively) and 1213 GCs (799 and 414 patients from LTHT and KCCH cohorts, respectively) were included in TMAs. TMA construction from the LTHT (OeC and GC) and Oe02 patient cohorts was performed using 0.6 mm tissue cores. 1.2 mm and 1mm tissue cores were used for the UHC and KCCH cohorts, respectively.

Immunohistochemistry for mismatch repair proteins

MMR immunohistochemistry (IHC) data from previous studies were available for 230 KCCH (51) and 175 LTHT (52) GCs. Additional 184 KCCH and 624 LTHT GCs were stained as part of the present study.

TMA sections from the Oe02 trial cohort were stained for MLH1, MSH2, MSH6, PMS2, from the UHC cohort for MLH1, MSH2 and MSH6 and from the KCCH and LTHT cohort (OeC and GC) for MLH1 and MSH2. For details on antigen retrieval, primary antibodies, detection system, staining protocols see table 1 in the supplementary material. For all cohorts, 3,3'-Diaminobenzidine (DAB) was used as a chromogen and haematoxylin as a counterstain.

A case was classified as MMR deficient (MMRdef) if tumour cell nuclei were negative for one or more MMR proteins in the presence of positively stained lymphocytes or fibroblasts as internal control. In the Oe02 trial cohort, 12 cases were negative for at least one MMR protein without positive internal controls on the TMA. For these cases IHC was repeated on full sections. A case was classified as MMR proficient (MMRprof) if tumour cell nuclei, irrespective of the number or intensity, were positive for all MMR proteins tested.

EBV RNA *in situ* hybridisation

EBV data from a previous study were available for 437 LTHT and 216 KCCH GC (52). Additional 362 LTHT and 198 KCCH GCs were stained as part of the present study. EBV status was determined on TMAs in the LTHT (OeC and GC), Oe02 and KCCH cohorts by EBV-encoded RNA (EBER) *in situ* hybridisation as previously described (53). In the UHC cohort, a fluorescein-conjugated oligonucleotide probe in conjunction with a monoclonal anti-fluorescein antibody and DAB as chromogen (Leica Biosystems, Wetzlar, Germany) was used according to the instructions of the manufacturer. EBV positivity was defined as presence of staining in tumour cell nuclei, irrespective of the number of nuclei or intensity.

DNA extraction

DNA was extracted using a protocol based on the QIAmp DNA Micro Kit (Qiagen, Hilden, Germany) as previously described (54). DNA concentration was measured by ND-100 Spectrophotometer (Labtech International) and adjusted to a final concentration of 1ng/µl.

Assessment of microsatellite instability

The MSI Analysis System, version 1.2 (Promega, Southampton, UK), was used for the detection of MSI in 419 Oe02 patients. This kit allows the simultaneous evaluation of 5 fluorescently labelled MSI markers: BAT-25, BAT-26, NR-21, NR-24 and MONO-27. PCR products were analysed using a 3100-Avant genetic analyser (Applied Biosystems, California, USA) as previously described (51). Instability in two or more microsatellite loci was categorised as MSI-high (MSI-H) and in a single loci as MSI-low (MSI-L). Absence of MSI in all 5 markers and MSI-L were grouped as microsatellite stable (MSS) for further analyses following current guidelines (55).

Statistical analyses

All statistical analyses were performed using SPSS version 23 software (SPSS Inc., Chicago, Ill). The relationship between EBV or MMR status and clinicopathological variables (age, gender, depth of invasion (pT), lymph node status (pN), Lauren classification and neoadjuvant treatment) were assessed using chi-squared for categorical variables and Mann-Whitney U for continuous variables. LTHT and KCCH GC data were combined for the analysis of the relationship between EBV or MMR status and overall 5-year survival and differences were assessed using the log rank test. P values less than 0.05 were considered significant.

RESULTS

EBV status

EBV data were available from 928 OeC patients (LTHT n=223; Oe02 n=383; UHC n=322) and 1178 GC patients (LTHT n=768; KCCH n=410). All OeC were EBV negative. A total of 56 (4.8%) GC were EBV positive (LTHT: n=30 (3.9%), KCCH: n=26 (6.3%)). Supplementary figure 1 illustrates EBV staining in GC.

Microsatellite status and mismatch repair protein expression

MSI data were available from 362 OeC from the Oe02 cohort. A total of 57 (13.6%) cases had to be excluded due to repeated technical failures. A total of 356 (98.3%) OeC patients were classified as MSS, 4 (1.1%) OeC as MSI-L (3 AdC and 1 SqC) and 2 (0.6%) OeC as MSI-H (both AdC). Supplementary figure 2 shows a typical capillary electrophoresis output for a MSI-H OeC and a MSS OeC. For 306 patients, MMR IHC (MLH1, MSH2, MSH6 and PMS2) data and MSI testing results were available and showed 99.0% concordant results. We therefore decided to only use IHC for the remaining cohorts.

MMR expression data were available from a total of 916 OeC (LTHT n=220; Oe02 n=374; UHC n=322). A sum of 43 (10.3%) and 3 (1.3%) OeC from the Oe02 and LTHT cohorts, respectively, were excluded due to technical failures. Seven (0.8%) OeC (5 AdC and 2 SqC) were classified as MMRdef (LTHT: 3 (1.4%) MLH1 deficient, Oe02: 1 (0.3%) MSH2 deficient, UHC: 3 (0.9%) MLH1 deficient). Patient clinicopathological variables and MMR status for OeC are summarised in table 3. Owing to the very small number of MMRdef in OeC, it was not feasible to perform any statistical analysis with clinicopathological data or survival.

MMR protein expression data were available from 1098 GC (LTHT n=702; KCCH n=396). A total of 113 (10.3%) cases were classified as MMRdef (LTHT: 70 (10.0%), KCCH: 43 (10.9%)). Supplementary figure 3 illustrates MMR protein expression in a MMRdef GC.

For 1063 GCs, both EBV and MMR data were available. A single GC from the LTHT cohort was MMRdef and EBV positive. This patient was male, 67 years old at the time of diagnosis, and survived 17 years despite having an advanced intestinal-type GC (pT4, pN3) in the resected specimen.

Relationship of EBV status and MMR status with clinicopathological variables in patients with gastric cancer

Patients with EBV positive GC were younger (median (range) age EBV positive GC: 63 years (32-89 years) versus 68 years (14-96 years) in EBV negative GC, p=0.01). A total of 48 (85.7%) patients with EBV positive GC were male compared with 8 (14.3%) of female patients (p=0.001). EBV positive GC patients had a better overall 5-year survival compared with EBV negative GC patients (60.7% versus 41.7%; hazard ratio 1.72, 95% confidence interval 1.12-2.63 [p=0.012]).

Table 3 | Mismatch repair status and clinicopathological variables in patients with oesophageal cancer

Clinicopathological variables		Mismatch repair proficient						Mismatch repair deficient					
		LTHT		Oe02		UHC		LTHT		Oe02		UHC	
		n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Sex	Male	137	63.1	294	78.8	287	89.9	2	66.7			3	100
	Female	80	36.9	79	21.2	32	10.1	1	33.3	1	100		
(y)pT(6)	T0	2	0.9			3	0.9						
	T1	32	14.7	27	7.2	63	19.7					1	33.3
	T2	38	17.5	36	9.7	63	19.7	1	33.3				
	T3	136	62.7	301	80.7	185	58	2	66.7	1	100	2	66.7
	T4	9	4.1	9	2.4	5	1.6						
(y)pN(6)	N0	83	38.2	123	33	122	38.2			1	100	3	100
	N1	133	61.3	250	67	197	61.8	3	100				
	unknown	1	0.5										
Histological type	Adenocarcinoma	165	76	275	73.7	319	100	2	66.7			3	100
	Squamous cell carcinoma	49	22.6	87	23.3			1	33.3	1	100		
	Other	3	1.4	11	2.9								
Neoadjuvant treatment	Yes	80	36.9	177	47.5	194	60.8	2	66.7	1	100	2	66.7
	No	133	61.3	196	52.5	125	39.2	1	33.3			1	33.3
	unknown	4	1.8										

Abbreviations: LTHT, Leeds Teaching Hospital Trust; Oe02, oesophageal cancer trial 02 (25); UHC, University Hospital Cologne

Patients with MMRdef GC were older (median [range] age MMRdef GC: 71 years [51-90 years] versus 68 years [24-96 years] in MMRprof GC, p=0.001). A total of 77 (69.4%) MMRdef GC had intestinal-type histology compared with 20 (18.0%) with diffuse-type histology (p=0.022). There was no difference in overall survival between MMRdef and MMRprof GCs (p=0.383). There was no relationship with any other clinicopathological variables (table 4).

A summary of the EBV, MMR and MSI status in each cohort is provided in table 5.

DISCUSSION

This is the largest gastro-oesophageal cancer study to date investigating MMR and EBV status in 988 OeC and 1213 GC. The extremely low frequency of MMR/MSI and lack of EBV infection in OeC relative to GC in our study confirms the recent TCGA results which investigated MSI and EBV in smaller series of 164 OeC (24) and 295 GC (11) using different methodologies.

All OeC were EBV negative which is consistent with the majority of previously published studies (42, 43, 46-48, 56). Therefore, we can conclude now that EBV does not play a role in OeC carcinogenesis neither in SqC nor in AdC. A small number of previous studies reported an EBV positivity rate between 1-36% in OeC (27, 44, 45, 49). This discrepancy is most likely related to different potentially less reliable methodology, such as PCR, which would also detect EBV in tumour-infiltrating lymphocytes (46) leading to false positive results. The present study used the generally accepted 'gold standard' EBER methodology. In our study EBV positive GC patients had a significantly better overall survival compared to EBV negative patients which is consistent with results from other studies (57).

In the Oe02 cohort, we detected a very low frequency of MSI-H (0.6%) using the Bethesda microsatellite panel (55). This result is consistent with the recent smaller TCGA study which found no MSI-H cases in 72 oesophageal AdC (24). However, our result is in contrast to the literature reporting a frequency of MSI-H in OeC between 0-27% in SqC (28, 33, 35, 37) and 0-20% in AdC (22, 27, 28, 30, 32, 37). Discrepancies in the frequency of MSI-H amongst studies could be related to different definitions of MSI-H (32), as well as differences in location (28) and number of microsatellite loci tested (35). Recent studies in GC suggest that a mononucleotide and dinucleotide markers different to those included in the so-called Bethesda panel might improve accuracy and sensitivity of MSI testing in GC (58, 59).

There are few small studies reporting a MMRdef frequency of 3-40% in OeC mostly based on IHC of MLH1 and MSH2 (23, 27, 30, 32). Some of the previous studies scores were based on staining intensity and cell proportions and classifying cases with weak staining and/or low percentages of positively stained tumour cells as MMRdef. Thus, when using our MMR scoring system where a case was classified as MMRprof, irrespective of the number of positive nuclei or staining intensity, the frequency of MMRdef in our study is comparable to previously published studies. Another potential reason for discrepant results in the literature could be the misclassification of AdC with a tumour bulk located in the stomach which extends into the GOJ as OeC. In contrast to the results from the MAGIC trial patients (22), there was no overall survival difference between MMRdef GC and MMRprof GC in our study. This is likely due to differences in disease stage, histological subtypes and age of GC patients in our study.

The frequency of MMRdef and EBV positivity in our GC cohort is consistent with the current literature (60-62). As the same methodology was used to stain GC and OeC, our GC results also indirectly support the reliability of the low frequency of MMRdef and EBV in OeC in the present study. Furthermore, our results are comparable with results from a smaller study in the MAGIC trial patients comparing the frequency of MSI and MMRdef in GC and OeC (22).

Our study has some limitations. First, this is a retrospective study. Secondly, due to limited tissue availability, we were unable to perform IHC for all four MMR proteins in all cases and we did not test all cases for MSI. However, evidence in the literature from GC found MMRdef was due to loss of MLH1 in 95.8% of cases, and deficiency in MSH6 and PMS2 was rare (60).

Table 4 | Comparison of mismatch repair and EBV status with clinicopathological variables in patients with gastric cancer

Clinicopathological variables		**Mismatch repair proficient**					
		LTHT		**KCCH**		**Total**	
		n	%	n	%	n	%
Gender	Male	415	59	250	63	665	61
	Female	214	30	102	26	316	29
	Unknown	3	0	1	0	4	0
(y)pT(7)	T1	83	12	34	9	117	11
	T2	69	10	52	13	121	11
	T3	179	25	52	13	231	21
	T4	301	43	214	54	515	47
	Unknown			1	0	1	0
(y)pN(7)	N0	206	29	70	18	276	25
	N1	123	18	80	20	203	18
	N2	146	21	91	23	237	22
	N3	156	22	111	28	267	24
	Unknown	1	0	1	0	2	0
Lauren classification	Intestinal	403	57	181	46	584	53
	Diffuse	145	21	154	39	299	27
	Mucinous/ mixed	82	12	15	4	97	9
	Unknown	2	0	3	1	5	0
Neoadjuvant treatment	Yes	8	1	177	45	185	17
	No	624	89	164	41	788	72
	Unknown			12	3	12	1

Abbreviations: KCCH, Kanagawa Cancer Center Hospital; LTHT, Leeds Teaching Hospital Trust

Similarly, a colorectal cancer study reported a positive predictive value and specificity of IHC for MMR proteins of 99.1% and 99.6%, respectively, compared with MSI (63). Our own study showed that MSI status is in 99.0% of cases concordant with the MMR IHC status. Another potential limitation is our inability to determine the proportion of junctional (GOJ) AdC versus true oesophageal or true gastric AdC which might potentially be clinically relevant. This is related to the fact that detailed pre-chemotherapy endoscopic information regarding the location was not available for most cases. There are very few studies investigating EBV and MMRdef in GOJ cancer with inconsistent results most likely related to low sample sizes (22, 42, 64) or differences in defining the GOJ (65).

Our OeC findings suggest that OeC carcinogenesis is not associated with EBV infection and MMRdef/MSI does not appear to be an important underlying mechanism in OeC, neither SqC nor AdC. The use of EBV and/or MMR/MSI status to determine OeC patient eligibility for immunotherapy or adjuvant cytotoxic therapy cannot be recommended and there remains the need to find alternative biomarkers for such therapy approaches in this patient population.

Mismatch repair deficient							EBV negative						EBV positive					
LTHT		KCCH		Total			LTHT		KCCH		Total		LTHT		KCCH		Total	
n	%	n	%	n	%	p value	n	%	n	%	n	%	n	%	n	%	n	%	p value
42	6	33	8	75	7	0.761	456	59	273	67	729	62	26	3	22	5	48	4	**0.001**
28	4	10	3	38	3		281	37	110	27	391	33	4	1	4	1	8	1	
							1	0	1	0	2	0							
5	1	3	1	8	1	0.074	105	14	37	9	142	12	4	1	2	0	6	1	0.794
2	0	5	1	7	1		75	10	58	14	133	11	5	1	4	1	9	1	
26	4	3	1	29	3		210	27	52	13	262	22	9	1	3	1	12	1	
37	5	32	8	69	6		348	45	236	58	584	50	12	2	17	4	29	2	
									1	0	1	0							
22	3	13	3	35	3	0.722	242	32	82	20	324	28	13	2	4	1	17	1	0.931
19	3	6	2	25	2		155	20	83	20	238	20	6	1	5	1	11	1	
14	2	8	2	22	2		152	20	96	23	248	21	7	1	7	2	14	1	
15	2	16	4	31	3		189	25	122	30	311	26	4	1	10	2	14	1	
									1	0	1	0							
49	7	28	7	77	7	**0.022**	461	60	204	50	665	56	20	3	15	4	35	3	0.919
10	1	10	3	20	2		185	24	156	38	341	29	6	1	10	2	16	1	
11	2	3	1	14	1		90	12	17	4	107	9	4	1	1	0	5	0	
		2	1				2	0	7	2	9	1							
1	0	16	4	17	2	0.305	11	1	185	45	196	17			13	3	13	1	0.293
69	10	27	7	96	9		727	95	185	45	912	77	30	4	13	3	43	4	
									14	3	14	1							

Table 5 | Summary of EBV, mismatch repair and microsatellite instability status in oesophageal and gastric cancer

		OeC						GC			
		Oe02		LTHT		UHC		LTHT		KCCH	
		n=443	%	n=223	%	n=322	%	n=768	%	n=410	%
EBV	Negative	383	100	223	100	322	100	738	96	384	94
	Positive	0	0	0	0	0	0	30	4	26	6
MMR	Proficient	373	100	217	99	319	99	632	90	353	89
	Deficient	1	0	3	1	3	1	70	10	43	11
Microsatellite	Stable	356	98	NI		NI		NI		NI	
	Instable-Low	4	1	NI		NI		NI		NI	
	Instable-High	2	1	NI		NI		NI		NI	

Abbreviations: EBV, Epstein-Barr Virus; GC, gastric cancer; KCCH, Kanagawa Cancer Center Hospital; LTHT, Leeds Teaching Hospitals NHS Trust; MMR, mismatch repair; MSI, microsatellite instability; MSS, microsatellite stable; OeC, oesophageal cancer; UHC, University Hospital Cologne; NI, not investigated

The difference in the frequency of MMRdef and EBV infection between OeC and GC indicate not only pathophysiological differences in oesophageal and gastric carcinomas but might also have important implications for patient selection for future treatment and study planning. In contrast to the current practice of recruiting patients with GC or OeC into the same trials, trials involving immunotherapy require most likely disease specific different designs and selection criteria for patients with OeC.

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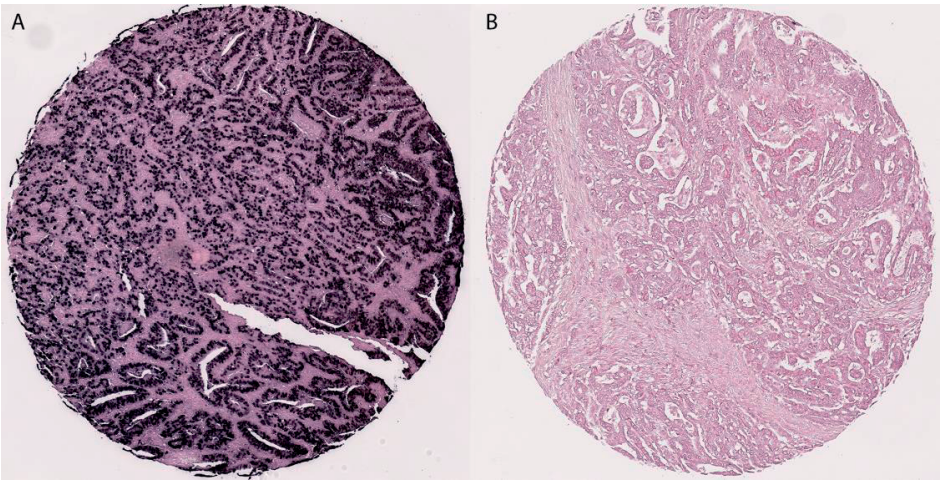
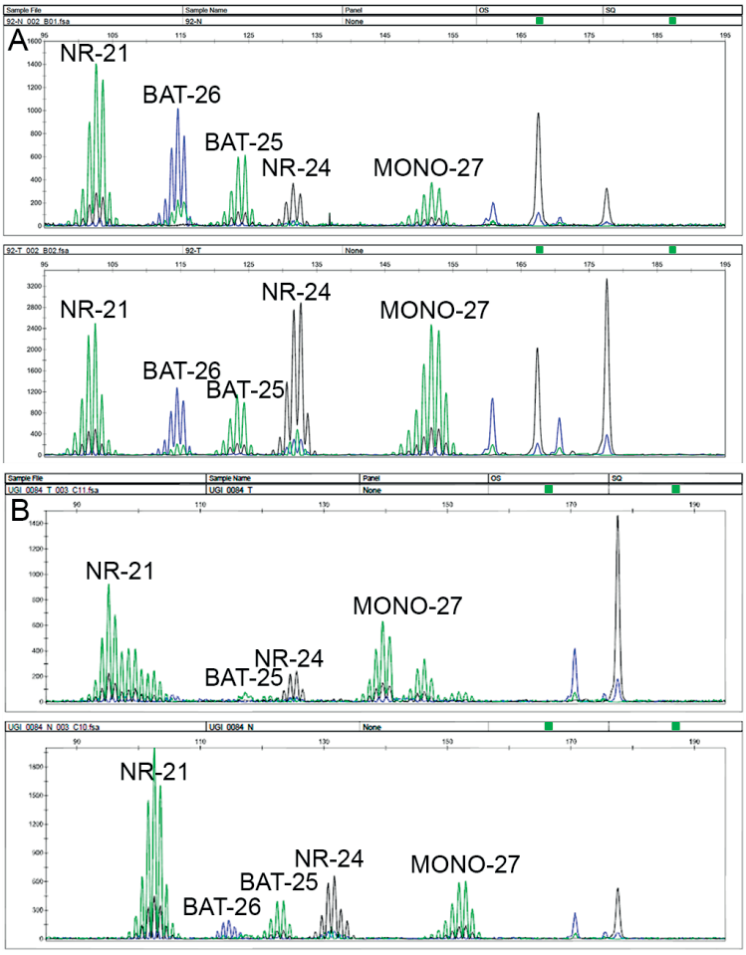
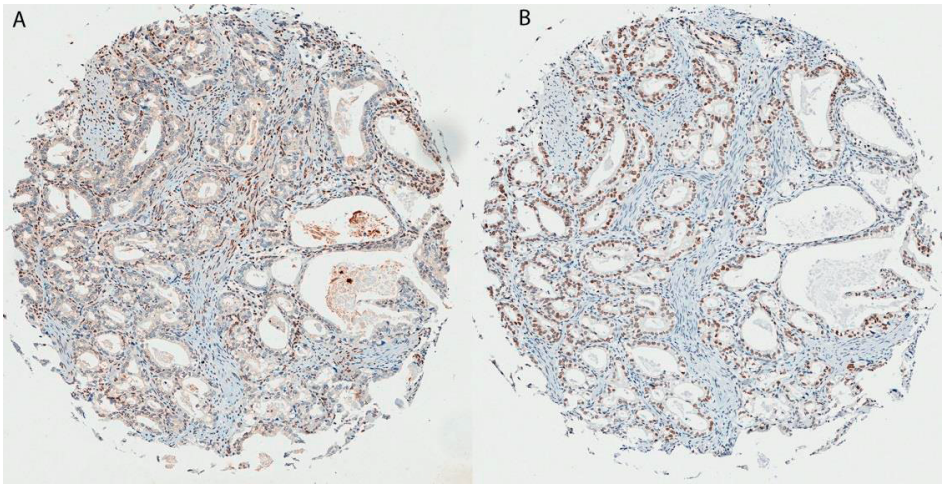


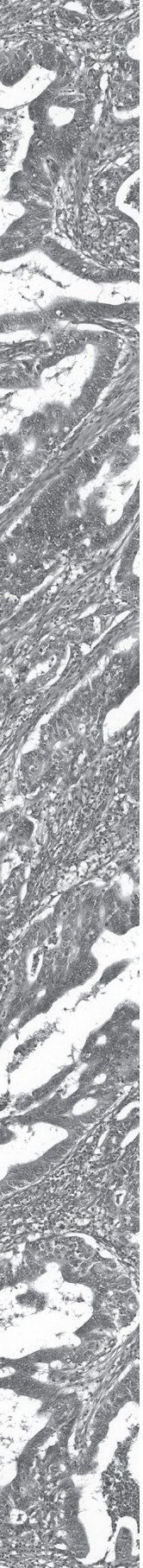
Supplementary figure 1 | Epstein Barr Virus-encoded RNA *in situ* hybridization staining. A: EBV positive gastric cancer (black = 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium, red = counterstain with nuclear fast red). B: EBV negative gastric cancer.



Supplementary figure 2 | MSI analysis output. A: Microsatellite stable oesophageal cancer (upper panel) and matched normal sample (lower panel). B: Microsatellite allele length changes in a microsatellite instable-high oesophageal cancer (upper panel) compared to the matched normal sample (lower panel).



Supplementary figure 3 | Expression of mismatch repair proteins by immunohistochemistry in a mismatch repair deficient gastric cancer. A: Tumour cell nuclei are negative for MLH1, adjacent lymphocytes are positive (brown = DAB, blue = haematoxylin counterstain). B: Tumour cell nuclei are positive staining for MSH2 (brown = DAB, blue = haematoxylin counterstain).



Chapter 5

Intratumour heterogeneity of the tumour content in the diagnostic biopsy predicts survival benefit from neoadjuvant chemotherapy in patients with oesophageal cancer – results from the OE02 trial

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Submitted

ABSTRACT

Objective | To establish a statistical model to objectively measure intratumour heterogeneity of the proportion of tumour (IHPoT) and to use this newly developed method to measure IHPoT in the pre-treatment biopsies from oesophageal cancer (OeC) OE02 trial patients.

Background | Despite the use of multimodal treatment, survival of OeC patients remains poor. One proposed explanation for the relatively poor response to cytotoxic chemotherapy is intratumour heterogeneity.

Methods | A statistical mixed effect model (MEM) was established for estimating IHPoT based on variation in haematoxylin/eosin stained pre-treatment biopsy pieces from the same individual in 218 OE02 trial patients (103 treated by chemotherapy followed by surgery (CS); 115 patients treated by surgery alone (S)). The relationship between IHPoT, prognosis, chemotherapy survival benefit and clinicopathological variables was assessed.

Results | 97 (44.5 %) and 121 (55.5%) OeCs showed high and low IHPoT, respectively. There was no significant difference in IHPoT between S (median (range): 0.1637 (0-3.17)) and CS (median (range): 0.1692: 0-2.69) patients (*P*=0.43). CS patients with low IHPoT had a significantly longer survival than S patients (HR=1.81, 95% CI: 1.20-2.75, *P*=0.005). There was no survival difference between CS and S patients with high IHPoT (HR=1.15, 95%CI: 0.72-1.81, *P*=0.566).

Conclusions | This is the first study suggesting that IHPoT measured in the pre-treatment biopsy can predict chemotherapy survival benefit in OeC patients. IHPoT may represent a clinically useful biomarker for patient treatment stratification. As implementation of MEM for IHPoT reporting in routine pathology is not feasible, future studies should determine if pathologists can reliably estimate IHPoT.

INTRODUCTION

Oesophageal cancer (OeC) is the eighth most common cancer worldwide with more than 572,000 new cases and 508,500 deaths in 2018 (1). The standard of care for OeC patients with locally advanced resectable disease is chemotherapy or chemoradiotherapy followed by surgery (2-5). Despite multimodal treatment, survival remains poor, with a 3-year overall survival rate of 39% (6). The recent OE05 trial demonstrated that intensifying treatment by using 3 drugs instead of 2 and increasing the number of chemotherapy cycles given pre-operatively did not improve OeC patient survival (6).

Decisions about OeC patient treatment are made at the time of diagnosis after confirming the presence of cancer in the endoscopic biopsy and clinical staging of the disease. We showed recently that the proportion of tumour (PoT) measured in the diagnostic biopsy is able to predict survival benefit from cytotoxic chemotherapy in a subgroup of OeC patients (7). During this previous study, we noticed that the PoT value can vary considerably between biopsy pieces from the same patient.

Considering that not only the absolute PoT value (7) but also intratumour heterogeneity of the proportion of tumour (IHPoT) might influence chemotherapy survival benefit, we hypothesized that OeC patients with relatively low IHPoT (e.g. similar PoT values measured in different biopsies from the same patient) will have greater survival benefit from neoadjuvant 5-fluoruracil/cisplatin chemotherapy compared to those with high IHPoT.

The current study had two aims: (1) to establish a statistical method to objectively measure IHPoT and (2) to use this newly developed method to measure IHPoT in the pre-treatment biopsies from OE02 trial patients. The relationship of IHPoT with clinicopathological variables, 5 year overall survival and chemotherapy survival benefit was analyzed.

METHODS

Study population

The UK Medical Research Council (MRC) OE02 trial randomized 802 patients with locally advanced resectable oesophageal cancer to surgery alone or 2 cycles of 5-fluoruacil + cisplatin chemotherapy followed by surgery (3, 8). Pre-treatment biopsy PoT values were available for 281 OE02 trial patients (140 patients treated with chemotherapy followed by surgery (CS) and 141 patients treated with surgery alone (S)) from our previous study (7).

The study was approved by the South East Research Ethics Committee, London, UK, REC reference: 07/H1102/111.

Measuring intratumour heterogeneity of the proportion of tumour

Of the 281 patients with a pretreatment biopsy PoT value from our previous study (7), 218 patients (S patients n=115, CS patients n=103) had PoT values from two or more tumour containing biopsies. Although a large number of studies in the literature use the term 'tumour heterogeneity', it is not clear under what conditions samples/values from the same tumour should be classified as 'heterogeneous'. We set out to establish a method to calculate an IHPoT index and to explore its predictive and prognostic value in patients with oesophageal cancer recruited into the OE02 trial.

In the field of multilevel data analysis, the mixed effects model (MEM) has been proposed as an appropriate model to analyse different quantities measured from the same individual(9-11), e.g., in our case the PoT values from different biopsy pieces of the same patient. We applied the R package "lme4" (12, 13) to build the MEM, which provides a value describing the level of variation between PoT values (IHPoT index). Theoretically, the obtained IHPoT index can range from zero (no heterogeneity) to infinity (maximal heterogeneity). Details of the statistical methodology including data structure can be found in the supplementary data file 1 and supplementary table 1.

Q statistic (14) was used to optimize the cut off point for the IHPoT index using all patients, with respect to overall survival calculated from the time of randomization to the date of death within the 5-year follow-up period. Patients were stratified by their IHPoT index into two groups: high and low IHPoT index. Low IHPoT index was defined as heterogeneity less than or equal to the cutoff point.

All other statistical analyses we performed using R (version 3.5.1). The relationship between IHPoT index and clinicopathological variables (depth of invasion ((y)pT), lymph node status ((y)pN) and (y)pTNM stage (UICC TNM classification 6th edition (15)), Mandard tumour regression grade(16), histological tumour type, resection margin status and tumour location) were assessed using chi-square and Fisher's exact tests.

The relationship between IHPoT index and 5 year overall survival (OS) was analyzed using the Kaplan-Meier method and log-rank statistics. Survival analyses were performed stratifying patients by IHPoT index and treatment arm to establish the predictive and prognostic value of IHPoT index. A p-value of <0.05 was considered significant.

As we previously found that patients with a total biopsy PoT value between 40% and 70% had a survival benefit from pre-operative chemotherapy, we additionally explored whether the improved OS in this particular patient subgroup might be related to the degree of intratumour heterogeneity.

It was unfeasible to perform multivariate analyses, including known prognostic factors such as depth of invasion and lymph node status, for two reasons. Firstly, detailed pre-treatment staging data were not collected in this trial (8). Secondly, using the pathological stage derived after surgery may not be representative of the stage in the biopsies from patients treated with neoadjuvant chemotherapy due to chemotherapy induced pathological changes.

RESULTS

The median number of biopsy pieces per patient was 3 (range: 2 to 12 pieces). In total, PoT values from 775 individual biopsy pieces from 218 patients were available for analysis.

The median IHPoT index was 0.1638 (range: 0 to 3.17). Tumours from 97 (44.5 %) OeC patients (48 (41.7%) S patients 49 (47.6%) CS patients) were classified as showing high IHPoT (IHPoT index > 0.2030). Tumours from 121 (55.5%) OeC patients (67 (58.3%) S patients, 54 (52.4%) CS patients) were classified as showing low IHPoT (IHPoT index ≤ 0.2030).

As expected, there was no significant difference in IHPoT in the pre-treatment biopsy pieces between S (median (range) 0.1637 (0 to 3.17) and CS (median (range) 0.1692 (0 to 2.69) patients (P=0.43). There was no significant difference in clinicopathological characteristics comparing patients with low or high IHPoT in each treatment group, with the exception of tumour location in the CS patients (Table 1). In particular, there was no difference by histological OeC subtype.

Intratumour heterogeneity of the proportion of tumour and survival

CS patients with low IHPoT in the pre-treatment biopsy had a significantly longer survival compared to S patients with low IHPoT (hazard ratio (HR) =1.81, 95% confidence interval (CI): 1.20-2.75, *P*=0.005, figure 1).

There was no significant difference in survival when comparing CS patients with high IHPoT in the pre-treatment biopsy to S patients with high IHPoT (HR=1.15, 95%CI: 0.72-1.81, *P*=0.566, figure 1).

In CS and S patients, 84 (55.6%) patients in the 40%≤PoT≤70% group had a low IHPoT index compared to 67 (44.4%) patients with PoT values < 40% or > 70%, p=0.956. The survival benefit from pre-operative chemotherapy seems to be even higher in the subgroup of CS patients with 40%≤PoT≤70% and low IHPoT (*n*=36, HR=2.71, 95%CI: 1.60-4.61, *P*<0.001, figure 1). In contrast, patients with 40%≤PoT≤70% and high IHPoT do not a survival benefit from chemotherapy (figure 1). In exploratory analysis, patients with PoT <40 % or > 70%, irrespective of the IHPoT index, do not seem to have a survival benefit from chemotherapy (supplementary figure 1).

There was neither a significant difference in survival of S patients comparing high versus low IHPoT (HR=0.76, 95%CI: 0.50-1.15, P=0.19) nor within the CS patients (HR=1.19, 95%CI: 0.75-1.90, *P*=0.45), figure 2.

Table 1 | Patient characteristics according to intratumour heterogeneity of the proportion of tumour index in each treatment arm

	Chemotherapy + surgery			Surgery alone		
	Low IHPoT *n* (%)	High IHPoT *n* (%)	p-value	Low IHPoT *n* (%)	High IHPoT *n* (%)	p-value
Age (years)						
≤ 65	32 (57)	24 (43)	0.477	39 (57)	29 (43)	0.883
> 65	22 (50)	22 (50)		28 (56)	22 (44)	
Gender						
Female	10 (46)	12 (56)	0.363	17 (50.0)	17 (50.0)	0.344
Male	4 4(56)	34 (44)		50 (59.5)	34 (40.5)	
Depth of invasion ((y)pT)*						
T0/Tis	2 (67)	1 (33)	0.055	0	0	0.353
T1	3 (33)	6 (67)		6 (50)	6 (50)	
T2	9 (82)	2 (18)		5 (83)	1 (17)	
T3	33 (57)	25 (43)		42 (58)	30 (42)	
T4	0	3 (100)		0	1 (100)	
Lymph node status ((y)pN)*						
N0	20 (51)	19 (49)	0.422	20 (59)	14 (41)	0.985
N1	27 (60)	18 (40)		34 (59)	24 (41)	
(y)pTNM stage*						
0	2 (67)	1 (33)	0.706	0	0	0.361
I	2 (33)	4 (67)		4 (44)	5 (56)	
II	19 (56)	15 (44)		21 (68)	10 (32)	
III	24 (59)	17 (42)		28 (55)	23 (45)	
Mandard tumour regression grade						
1	2 (67)	1 (33)	0.788	Not applicable		
2	1 (50)	1 (50)				
3	7 (70)	3 (30)				
4	13 (48)	14 (52)				
5	24 (59)	17 (42)				
Histological tumour type						
Squamous cell carcinoma	11 (50)	11 (50)	0.791	10 (46)	12 (55)	0.346
Adenocarcinoma	33 (57)	25 (43)		41 (62)	25 (38)	
others	1 (100)	0		2 (67)	1 (33)	
Resection margin status						
Positive	14 (50)	14 (50)	0.661	20 (61)	13 (39)	0.629
Negative	33 (55)	27 (45)		31 (55)	25 (45)	
Tumour location						
Lower	31 (46)	36 (54)	**0.010**	50 (62)	31 (38)	0.256
Middle	12 (57)	9 (43)		12 (46)	14 (53)	
Upper	11 (92)	1 (8)		5 (46)	6 (43)	

*No data is available for patients who did not proceed to surgery, *n*=43.

Abbreviations: IHPoT, intratumour heterogeneity of the proportion of tumour

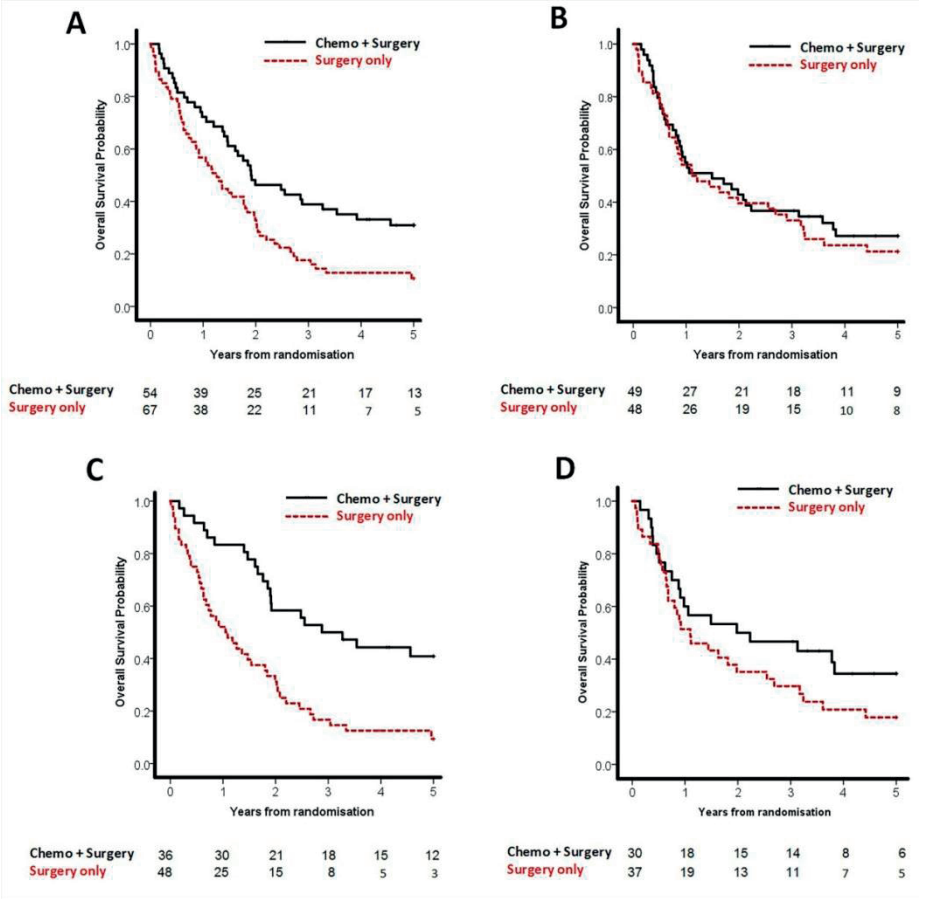

Figure 1 | Five years overall survival of patients treated with chemotherapy plus surgery (CS) versus surgery (S) alone stratified by intratumour heterogeneity of the proportion of tumour (IHPoT) index and absolute PoT value.
A | Patients with low IHPoT index: CS patients survived significantly longer than S patients (HR=1.81, 95%CI: 1.20-2.75, *P*=0.005).
B | Patients with high IHPoT index: There is no significant difference in survival between CS patients and S patients (HR=1.15, 95%CI: 0.72-1.81, *P*=0.566).
C | Patients with low IHPoT index and 40% ≤PoT≤ 70%: CS patients survived significantly longer than S patients (HR=2.71, 95%CI: 1.60-4.61, *P*<0.001),
D | Patients with high IHPoT index and 40% ≤PoT≤ 70%: There is no significant difference in survival between CS patients and S patients (HR=1.52, 95%CI: 0.85-2.70, *P*=0.153)

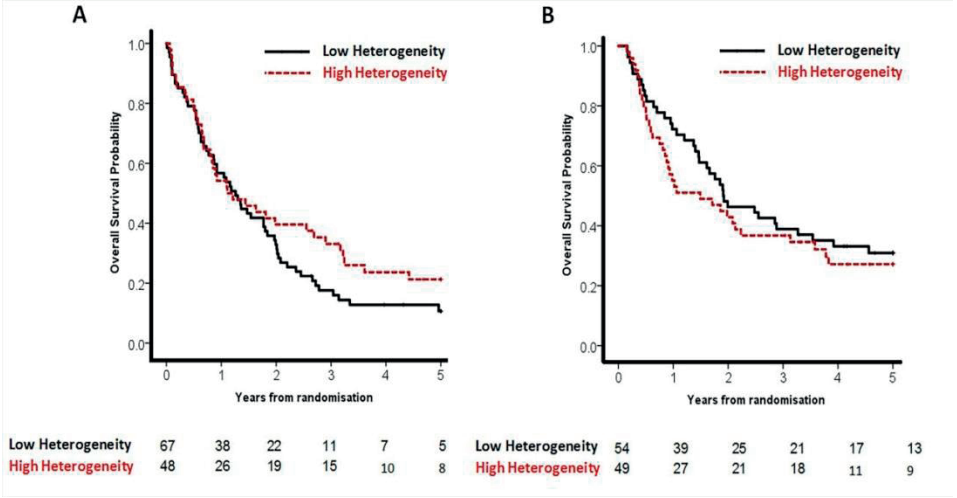

Figure 2 | Five years overall survival of patients with high versus low intratumour heterogeneity of the proportion of tumour (IHPoT) index within each treatment group.
A | There is no significant difference between the survival of S patients with high IHPoT index versus low IHPoT index (HR=0.76, 95%CI: 0.50-1.15, *P*=0.19)
B |There is no significant difference between the survival of CS patients with high IHPoT index versus low IHPoT index (HR=1.19, 95%CI: 0.75-1.90, *P*=0.45).

DISCUSSION

This is the first study to measure intratumour heterogeneity of the proportion of tumour (IHPoT) in routine Haematoxylin/Eosin stained pre-treatment endoscopic biopsies from oesophageal cancer (OeC) patients from the randomized UK MRC OE02 trial. We used a mixed effect model (MEM) to estimate the IHPoT level by modeling the probability of being tumour for each measurement point in the biopsy pieces.

Using a MEM, we found that patients with a low IHPoT index in the pre-treatment biopsy had a survival benefit from cytotoxic chemotherapy. We have previously shown that patients with an absolute PoT of 40%≤PoT≤70% calculated from all biopsy pieces had a survival benefit from pre-operative chemotherapy (7). We can now demonstrate that patients with tumours with a low IHPoT index and an absolute PoT value between 40% and 70% had the most survival benefit from pre-operative chemotherapy. In contrast, patients with a high IHPoT index derived little or no survival benefit from chemotherapy.

Recently, image analysis of HE stained sections from lung cancer was found to be predictive of mutation status (17), providing evidence that the morphological phenotype of the tumour is reflective of its molecular phenotype. Studies in oesophageal, head and neck and colon cancer have investigated 'intratumour heterogeneity' at the molecular level without providing a definition for intratumour heterogeneity as such. Existing data relating to 'intratumour

heterogeneity' are therefore difficult to interpret or compare with each other or our current study results (18-27) .

'Genetic heterogeneity' in cancer at the mutational or copy number level has been suggested to influence response to cytotoxic chemotherapy (28). In a study of 8 OeC patients, multi-region exome sequencing showed that 'intratumour genetic heterogeneity' is associated with a poor response to neoadjuvant chemotherapy (29). These results appear to be consistent with our morphology based study on a larger series of randomised clinical trial patients, including a control group of patients treated by surgery alone.

The predictive value of morphological intratumour heterogeneity of the tumour content in the pre-treatment biopsy identified in our study highlights the clinical need for multi-site sampling at the time of diagnostic endoscopy to enable the calculation of IHPoT and include this information in patient treatment decisions.

To the best of our knowledge this is the first study that has used a statistical method to objectively measure and clearly define intratumour heterogeneity. Results of our study suggest that intratumour heterogeneity of the tumour content is a potential useful biomarker for clinical decision making in patients with OeC. As implementation of MEM for IHPoT reporting in routine pathology is not feasible, future studies should determine if IHPoT in OeC biopsies can be reliably estimated by pathologists.

Limitations of our study include that this is a retrospective ad hoc analyse of a subset of available pre-treatment biopsies from OE02 trial patients containing multiple tumour containing biopsy pieces. In our study, we measured intratumour heterogeneity between biopsy pieces from the same patient, intratumour heterogeneity within individual biopsy pieces was not considered but may have an influence on our results.

In the era of whole genome sequencing and NGS, the increasing complexity of intratumour heterogeneity in cancer is becoming evident. However, the predictive value of molecular heterogeneity in response to therapy remains to be clarified and has not been implemented into clinical routine. We have shown that estimating IHPoT using a mixed effect model on digitized haematoxylin/eosin stained pre-treatment biopsy slides is predictive of survival benefit to cytotoxic chemotherapy in OeC patients from the Oe02 trial and may represent a clinically useful biomarker for patient treatment stratification.

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Supplementary data file 1 | Statistical methodology

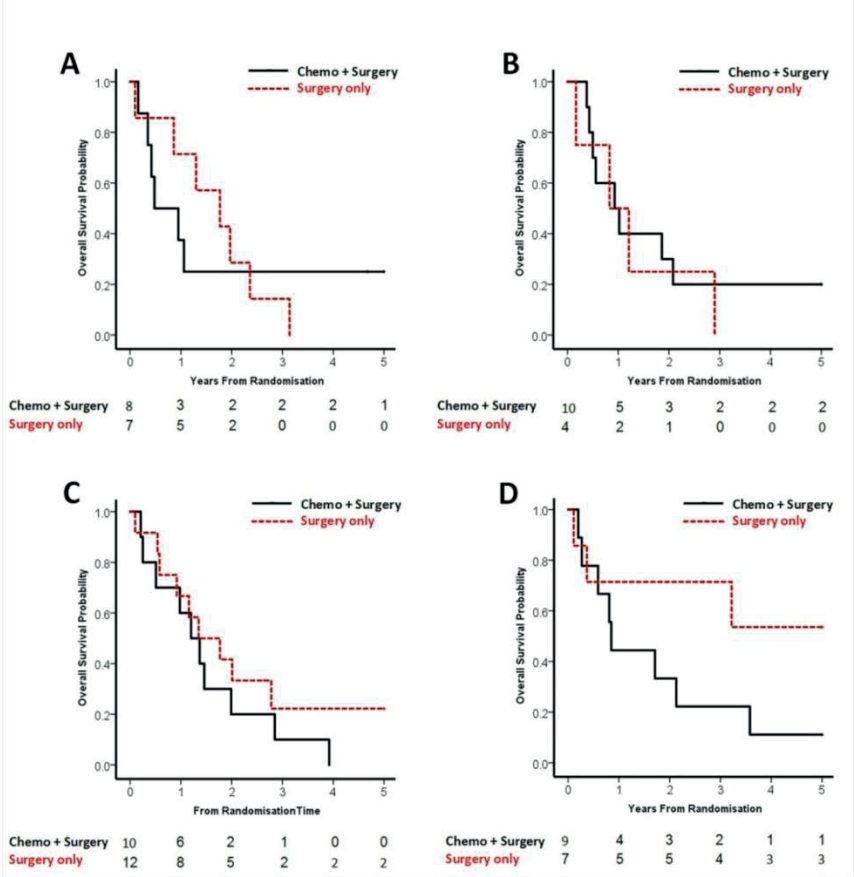
For the i^{th} biopsy piece we define Y_{ij} as a binary outcome variable with a value of 1 if the j^{th} point is tumour, otherwise the value is zero. Define P_{ij} as the probability that the j^{th} point is tumour, that is $Y_{ij} = 1$. Then the mixed effect model (MEM) is defined as follow:

$$\log\left(\frac{P_{ij}}{1-P_{ij}}\right) = \mu+\tau_i,$$

where, μ is the model intercept and τ_i is called the random effect which is a function of heterogeneity between the biopsy pieces. For the i^{th} biopsy piece, τ_i is assumed to have a normal distribution with mean zero and variance of σ^2 where σ^2 is defined as the intratumour heterogeneity between biopsy pieces of the given patient. Implementing MEM, using R package "lme4", we can estimate the σ^2 as the intratumour heterogeneity between the *K* biopsy pieces of the given patient.

Supplementary table 1 | Layout of dataset for applying mixed effect models

Patient ID	Biopsy piece ID	Point	Y_{ij}
1	1	1	1
1	1	2	0
1	1	3	0
.	.	.	.
.	.	.	.
.	.	.	.
1	1	n_1-1	1
1	1	n_1	0
1	2	1	0
1	2	2	1
1	2	3	0
.	.	.	.
.	.	.	.
.	.	.	.
1	2	n_2-1	1
1	2	n_2	0
.	.	.	.
.	.	.	.
.	.	.	.
1	K	1	0
1	K	2	1
1	K	3	1
.	.	.	
.	.	.	
.	.	.	
1	K	n_k-1	0
1	K	n_k	0



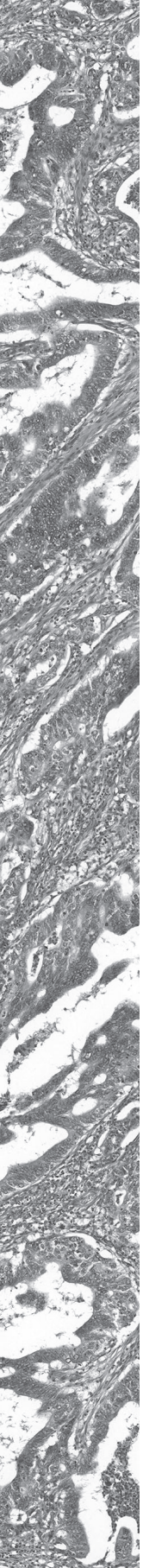
Supplementary figure 1 | Five years overall survival of patients treated with chemotherapy plus surgery (CS) versus surgery (S) alone group with low and high IHPoT and PoT<40% or PoT>70%.

A | Patients with low IHPoT index and PoT<40%: There was no significant difference in survival between CS patients and S patients (HR=1.001, 95%CI: 0.329-3.047, *P*=0.999).

B | Patients with high IHPoT index and PoT<40%: There was no significant difference in survival between CS patients and S patients. (HR=1.448, 95%CI: 0.454-4.615, *P*=0.532).

C | Patients with low IHPoT index and PoT>70%: There was no significant difference in survival between CS patients and S patients (HR=0.636, 95%CI: 0.257-1.575, *P*=0.328).

D | Patients with high IHPoT index and PoT>70%: There was no significant difference in survival between CS patients and S patients (HR=0.357, 95%CI: 0. 093-1.372, *P*=0.134).



Chapter 6

Density of tumour infiltrating lymphocytes predicts survival benefit from adjuvant chemotherapy in patients with stage II/III gastric cancer – results from the CLASSIC trial

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In preparation

ABSTRACT

Background | In Asia, patients with stage II-III gastric cancer (GC) are treated with adjuvant chemotherapy after potentially curative surgery. Despite being regarded as standard of care, adjuvant chemotherapy improves 5-year overall survival by only 9-11% suggesting that only a subset of GC patients benefit from treatment with adjuvant chemotherapy. We investigated tumour infiltrating lymphocytes (TILs) per mm^2 tissue area (TIL density) as a prognostic and/or predictive biomarker in patients with resectable, stage II-III GC from the CLASSIC trial.

Methods | HeteroGenius Medical Image Manager Cell Analysis Add-on was used to train a lymphocyte detection model and calculate TIL density using digital haematoxylin and eosin (HE) stained tissue microarrays constructed from resection specimens from 629 CLASSIC trial patients (325 treated by surgery plus adjuvant capecitabine and oxaliplatin chemotherapy; 303 treated by surgery alone). The relationship between TIL density, prognosis, survival benefit from chemotherapy and clinicopathological variables was analysed. For survival analyses, TIL density cut offs were established using Q statistics.

Findings | Results were available from 547 patients. Prognostic TIL density cut off was 470 TILs/mm^2, Predictive TIL density cut off was 870 TILs/mm^2. Patients with high TIL density GC had a significantly improved overall survival (OS) and disease free survival (DFS) compared with patients with low TIL density GC (OS: HR 0.56 [95% CI 0.41-0.77], p=0.0003; DFS: HR 0.54 [95% CI 0.41-0.71], p=0.00001). TIL density remained significant when known prognostic factors were included in the multivariate analysis (OS (HR 0.61 [95% CI 0.44-0.83], p=0.0022; DFS (HR 0.56 [95% CI 0.43-0.74], p=0.00006). Patients with low TIL density GC treated with surgery followed by adjuvant chemotherapy had an improved OS and DFS compared with patients treated by surgery alone (OS: HR 0.603 [95% CI 0.40-0.89], p=0.012, DFS: (HR 0.59 [95% CI 0.42-0.82], p=0.0021). In patients with high TIL density GC, there was no difference in OS or DFS between patients treated by surgery alone and patients treated by surgery followed by adjuvant chemotherapy (OS: HR 0.87 [95% CI 0.52-1.47], p=0.628), DFS: (HR 0.68 [95% CI 0.42-1.09], p=0.116). In multivariate analyses, TIL density was an independent predictive factor for survival benefit from adjuvant chemotherapy (OS: HR 0.56 [95% CI 0.38-0.84], p=0.005); DFS: (HR 0.57 [95% CI 0.41-0.80], p=0.0012).

Interpretation | Patients with low TIL density GC had a significant survival benefit from adjuvant chemotherapy. In contrast, patients with high TIL density GC had little or no survival benefit from adjuvant chemotherapy. TIL density measured on routine HE stained tissue sections may represent a new clinically useful biomarker identifying GC patients who may not require adjuvant chemotherapy and for whom treatment could be de-escalated.

INTRODUCTION

Despite a decline in gastric cancer (GC) incidence in recent years, GC remains the fifth most common cancer worldwide, with one million new cases and over 780,000 deaths in 2018 (1). Disease stage, patient performance status and patient preferences are currently used to determine patients treatment (2, 3). In Asia, the standard of care for patients with TNM stage II/III GC is D2 gastrectomy followed by adjuvant fluoropyrimidine based chemotherapy, based on results from the Japanese ACTS-GC trial (4) and the Korean CLASSIC trial (5). However, benefit from adjuvant chemotherapy is modest at 9-11% improved 5-year overall survival (4, 5). This suggests that only a subset of GC patients benefits from adjuvant chemotherapy whereas others might not need it or suffer from unnecessary side effects. Recent clinical trials increasing the number of drugs or chemotherapy cycles or adding radiotherapy failed to further improve survival in GC patients with resectable disease (6-16). Despite a number of recently published molecular classifications of GC (17-20), molecular classifiers are not yet used in the routine clinical settings to determine patients treatment with the exception of HER2 status in patients with metastatic GC (2, 21, 22). Thus, there remains an urgent clinical need to identify biomarkers that can predict (i) which individual GC patient requires and is likely to benefit from adjuvant chemotherapy to improve his/her prognosis, and (ii) which individual GC patient has a relatively good prognosis because of the individual's GC characteristics, would therefore not require adjuvant chemotherapy and might benefit from a de-escalating treatment strategy.

There has been a growing interest in the role of the tumour microenvironment in cancer progression and response to therapy (23). The clinical value of the morphological evaluation of tumour infiltrating lymphocytes (TILs) in haematoxylin and eosin (HE) stained sections has been demonstrated in a large number of studies in breast cancer (24-26), as well as in lung cancer (27), and urothelial carcinoma (28), which showed a relationship between TILs and prognosis and/or response to neoadjuvant or adjuvant chemotherapy. Furthermore, a semi-quantitative TIL score is already included in the routine histopathology report in melanoma (29-31) and will be recommended for routine pathology reporting in breast cancer in the new WHO classification.

Two recently published studies in GC suggest an interaction between the immune system and adjuvant chemotherapy. In the first RNA expression based study in CLASSIC trial patients, four gene classifiers were identified with predictive value, including granzyme B, an immune cell gene (32). Duan *et al.* identified a six immune-related gene signature in publically available data sets (33). Both studies were able to stratify GC patients into groups with different survival benefit from adjuvant chemotherapy based on the gene expression profile. The published evidence of the prognostic value of TILs in GC is inconclusive as studies have used different methods as well as different clinical endpoints investigating relatively

small single centre series (34, 35). A limited number of studies to date have investigated the prognostic value of TIL density based on the HE, using different methodologies (36-39).

HE based TIL density has not been measured in a large phase 3 GC trial in which patients were randomised to treatment by surgery alone (control group) or surgery followed by adjuvant chemotherapy (experimental group). Currently available evidence on TIL density based on the HE in other cancer types (24-26, 33) suggests that patients with high TIL density have a survival benefit from chemotherapy, but based on the results of the RNA based expression study in the CLASSIC series (32) we hypothesised that GC patients with low TIL density will have a survival benefit from adjuvant chemotherapy, whereas patients with high TIL density GC have little or no benefit.

The aim of this study was to quantify TIL density in GC resections specimens from patients recruited into the Korean CLASSIC trial and analyse the relationship between TIL density, prognosis, survival benefit from chemotherapy and clinicopathological variables.

METHODS

Patients

The CLASSIC trial (NCT00411229) was a randomised, open-label, multicentre, phase 3 study comparing D2 gastrectomy followed by adjuvant capecitabine and oxaliplatin chemotherapy with surgery alone in 1035 GC patients between 2006 and 2009 (5). Formalin-fixed, paraffin-embedded tissue blocks of the resected primary tumour from 629 patients were collected retrospectively. This study was approved by the local institutional review board of each participating institution.

Tissue microarrays (TMAs) were constructed sampling two 3mm diameter cores from blocks with the highest tumour density, as previously described (40). Four µm sections were cut, deparaffinised and stained with Haematoxylin/Eosin using a standard laboratory protocol. Stained tissue sections were dehydrated and coverslipped using glass coverslips and DPX (Sigma-Aldrich, St. Louis, USA). Slides were scanned at 40x magnification (Leica Aperio AT scanner).

Image analysis

Images were uploaded to MIM image analysis software (Medical Image Manager version 0.97, HeteroGenius Ltd., Leeds, UK). Obtaining the number of TILs per tumour area was a multistep process. For an overview of the workflow, see supplementary figure S1. Briefly, individual TMA cores were outlined and linked to a core identifier (supplementary figure S2). A colour model was built to identify area/pixels relating to all nuclei present in nine representative images from five GC cases. Subsequently, 4047 nuclei were manually annotated as being a lymphocyte versus non-lymphocyte and formed the basis of the cell model. The

resulting image analysis pipeline was applied to all TMA cores. For an example of image analysis based lymphocyte detection, see supplementary figure S3. Manual quality control of the lymphocyte detection was performed by a GI pathologist (HG) on 10% randomly selected cores, all cores with TIL density values greater or smaller than 2 standard deviations of the mean, and cases where the TIL/mm^2 measurement varied substantially between cores of the same patient (<50% or >200% TIL density of the other core). Based on this quality control we were satisfied with the detection of lymphocytes using the image analysis model.

Furthermore, all HE stained TMA cores were reviewed by an independent GI pathologist (MK) to (1) identify cores which contained only tumour epithelium e.g. no normal gastric epithelium to exclude from analyses and (2) to determine the histological tumour type according to Lauren (41) and World Health Organisation classifications (42).

TIL counts and surface area per core were added up and divided by the surface area of both cores (mm^2) to calculate TIL density (i.e. the number of TILs per mm^2 tissue area) per case. Cases with a total surface area of less than 1mm^2 were excluded from the analyses.

Epstein-Barr virus and microsatellite instability

Epstein-Barr virus (EBV)-encoded small RNA *in situ* hybridization data and microsatellite instability (MSI) status determined by PCR were available from previous studies (40, 43). MSI-Low classified GC were included in the microsatellite stable (MSS) GC groups for further analyses following current guidelines (44).

Statistical analyses

The relationship between TIL density and clinicopathological variables (age, sex, T stage, N stage, core Lauren classification, core WHO classification, EBV and MSI) was analysed using Chi-square or Fisher's exact test. In the CLASSIC trial, the stage of disease was originally reported according to the UICC TNM classification 6th edition (45). In our study, we converted the T and N category to UICC TNM 7th edition (46).

TIL density cut offs were established using Q statistics with the aim to identify the cut off which would identify groups of GC patients with large differences in prognosis and chemotherapy survival benefit (47). We assessed overall survival (OS), defined as time from randomisation to date of last follow up or death from any cause, and disease free survival (DFS), defined as time from randomisation to date of recurrence, death from any cause or date of last follow up, using the Kaplan-Meier method, log-rank tests, Cox proportional-hazards models and treatment interaction tests. The multivariate Cox proportional-hazards model was adjusted for age, sex, pT (depth of invasion) and pN (lymph node status). Interaction analysis was performed using Cox proportional-hazards model. P-values <0.05 were considered significant. Statistical analyses were performed using SPSS, version 23 (IBM Corporation, Somers, NY, USA) and R, version 3.3.2.

RESULTS

As previously reported, there was no significant difference in clinicopathological characteristics or survival when comparing patients included in this present TMA based study to the original CLASSIC trial population (40). The median (range) follow-up time for OS was 59 months (5 to 84 months) and 62 months (1 to 84 months) for patients treated with surgery alone and those treated with surgery followed by adjuvant chemotherapy, respectively. Median (range) follow-up time for DFS was 51 months (1 to 84 months) and 60 months (1 to 84 months) for patients treated with surgery alone and those treated with adjuvant chemotherapy after surgery, respectively.

After quality control (see material and methods), TIL values per mm^2 (TIL density) were available from 547 GC patients (265 in the surgery alone group, 282 in the surgery followed by adjuvant chemotherapy group, see consort diagram, figure 1). Results from two cores/patient were available for 399 (73%) patients, and one core/patients for 148 (27%) patients.

The median (range) TIL density of all patients measured in TMAs constructed from the resection specimen was 729 TILs/mm^2 (14 to 8161). There was no significant difference in TIL density between the two treatment groups (median (range) surgery alone group: 729 TILs/mm^2 (52 to 6164) versus surgery followed by adjuvant chemotherapy group: 726 TILs/mm^2 (14 to 8161), p=0.680).

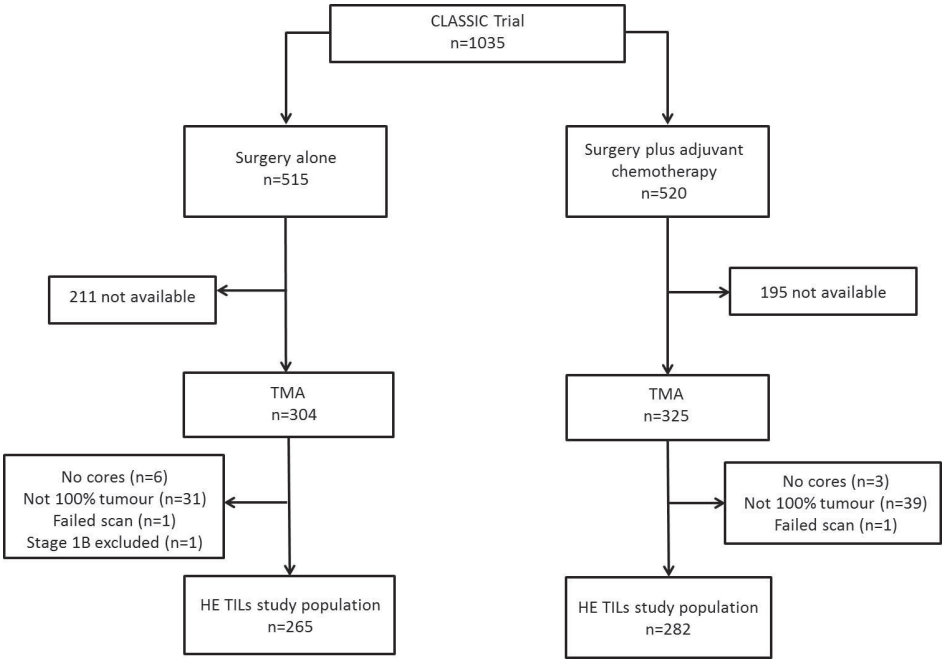


Figure 1 | consort diagram detailing how the final number of gastric cancers included in the study was reached

Prognostic value of TIL density

As there was no TIL density treatment interaction ($P_{interaction} > 0.05$), the prognostic effect of TIL density was estimated using all patients. Q statistics determined a cut off of 470 TILs/mm^2 being most appropriate to classify GC as having high versus low TIL density for this analysis. Patients with high TIL density (n=386, 70.6%) GC had a significantly improved OS and DFS compared to patients with low TIL density (n=161, 29.4%) GC (OS: HR 0.56 [95% CI 0.41-0.77], p=0.0003; DFS: HR 0.54 [95% CI 0.41-0.71], p=0.00001; figure 2). TIL density remained a significant prognostic factor when known prognostic factors age (<= 65 years vs > 65 years), sex, pT (T1/2 vs T3/4) and pN (N0 vs N1/2/3) are included in the multivariate analysis (OS (HR 0.61 [95% CI 0.44-0.83], p=0.0022; DFS (HR 0.56 [95% CI 0.43-0.74], p=0.00006); table 1 and 2).

Table 1 | Overall survival by prognostic 470 TILs/mm^2 cut off

	Overall Survival			
			HR (95%CI)	
	n(%)	5-year overall survival	Univariate	Multivariate
TILs ≤470/mm^2	161 (29.4)	61.5% (54.2-69.8)	1 (ref)	1 (ref)
TILs >470/mm^2	386 (70.6)	76.3% (72.1-80.8)	0.56 (0.41-0.77) p = 0.0003	0.61 (0.44-0.83) p =0.0022

HR, hazard ratio. Model was adjusted for age (<= 65 years vs > 65 years), sex, pT (T1/2 vs T3/4) and pN (N0 vs N1/2/3)

Table 2 | Disease free survival by prognostic 470 TILs/mm^2 cut off

	Disease Free Survival			
			HR (95%CI)	
	n(%)	5-year disease free survival	Univariate	Multivariate
TILs 470/mm^2	161 (29.4)	47.6 (40.4-56.0)	1 (ref)	1 (ref)
TILs >470/mm^2	386 (70.6)	67.2 (62.7-72.2)	0.54 (0.41-0.71) p = 0.00001	0.56 (0.43-0.74) p = 0.00006

HR, hazard ratio. Model was adjusted for age (<= 65 years vs > 65 years), sex, pT (T1/2 vs T3/4) and pN (N0 vs N1/2/3)

Predictive value of TIL density

Q statistics determined a cut off of 870 TILs/mm^2 being most appropriate to classify GC as having high versus low TIL density for this analysis. Patients with low TIL density (n=318, 51.8%) GC treated by surgery followed by adjuvant chemotherapy (n=165, 51.9%) had an improved OS and DFS compared to patients with low TIL density treated by surgery alone (n=153, 48.1%) (OS: HR 0.603 [95% CI 0.40-0.89], p=0.012), figure 3 and table 3; DFS: (HR 0.59 [95% CI 0.42-0.82], p=0.0021, figure 4 and table 4).

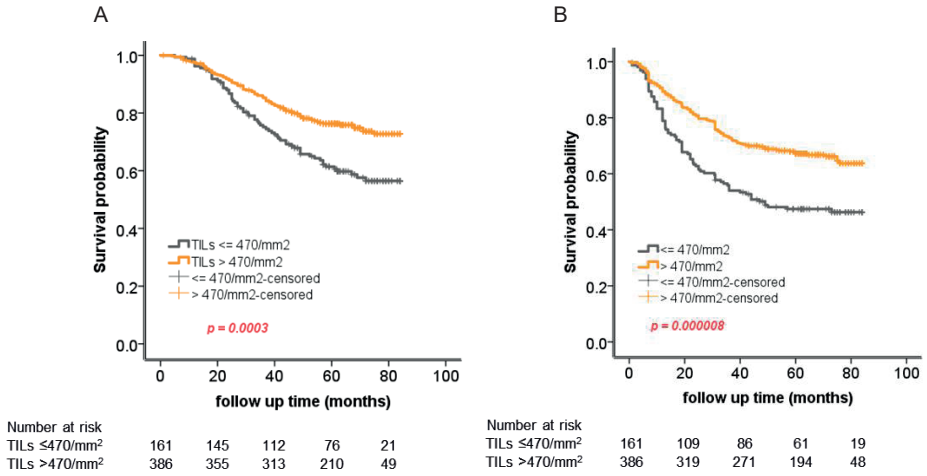


Figure 2 | Kaplan-Meier plots showing the prognostic value of TIL density for all patients by 470 TILs/mm^2 cut off for overall survival (A) and disease free survival (B).Patients with tumours with a TIL density >470 have a significantly better disease free survival and overall survival compared to patients with a TIL density of ≤ 470.

Patients with high TIL density (n= 229, 41.9%) GC treated by surgery followed by adjuvant chemotherapy (n=117, 51.1%) had the same OS and DFS as patients with high TIL density treated by surgery alone (n=112, 48.9%, OS: HR 0.87 [95% CI 0.52-1.47], p=0.628), figure 3 and table 3; DFS: (HR 0.68 [95% CI 0.42-1.09], p=0.116; figure 4 and table 4). In multivariate analyses, TIL density was an independent predictive factor for survival benefit from adjuvant chemotherapy (OS: HR 0.56 [95% CI 0.38-0.84], p=0.005); DFS: (HR 0.57 [95% CI 0.41-0.80], p=0.0012); table 3 and 4).

In summary, using two different TIL density cut-off points, patients can be stratified by prognosis and adjuvant chemotherapy survival benefit (figure 5). For examples of TMA cores containing low (≤870/mm^2), intermediate (>470/mm^2, ≤870/mm^2) and high (>870/mm^2) TIL density GCs, see supplementary figure S4.

Relationship between TIL density and clinicopathological variables

The relationship between TIL density (870 TILs/mm^2 cut off) and clinicopathological variables is summarised in table 5. There was no relationship between TIL density and age, sex or pN stage. Low TIL density was associated with higher pT stage and higher pTNM stage (pT stage: P=0.030, pTNM stage; P=0.014). High TIL density was more frequent in mixed/indeterminate type GC (P<0.001). Low TIL density was more frequent in mucinous type GC (P<0.001).

Relationship between TIL density, MSI status and EBV status

High TIL density (>870 TILs/mm^2) was more frequent in EBV positive GC (n=32 vs 10 in low TIL density, p=<0.001) and GC with MSI (n=21 vs 13 in low TIL density, p=0.017), see table 5. Importantly, 10 (24%) EBV positive GC and 13 (38%) GC with MSI showed low TIL density.

197 (39%) EBV negative GC and 197 (41%) GC with MSI low/MSS showed high TIL density. Due to very small number of GC in each subgroup, see table 5, it was not feasible to perform further statistical analyses.

Table 3 | Overall survival by predictive 870 TILs/mm^2 cut off

		Overall Survival			
	n (%)	5-year overall survival		HR (95%CI)	
		Surgery plus adjuvant chemotherapy	Surgery alone	Univariate	Multivariate
TILs ≤870/mm^2	318 (58.1)	74.2% (67.7- 81.4)	63.3% (55.9- 71.7)	0.603 (0.40-0.89) p = 0.012	0.56 (0.38-0.84) p = 0.005
TILs >870/mm^2	229 (41.9)	76.8% (69.3-85.1)	75.9% (68.2-84.5)	0.87 (0.52-1.47) p = 0.628	0.80 (0.47-1.35) p = 0.403

HR, hazard ratio. Model was adjusted for age (<= 65 years vs > 65 years), sex, pT (T1/2 vs T3/4) and pN (N0 vs N1/2/3)

Table 4 | Disease free survival by predictive 870 TILs/mm^2 cut off

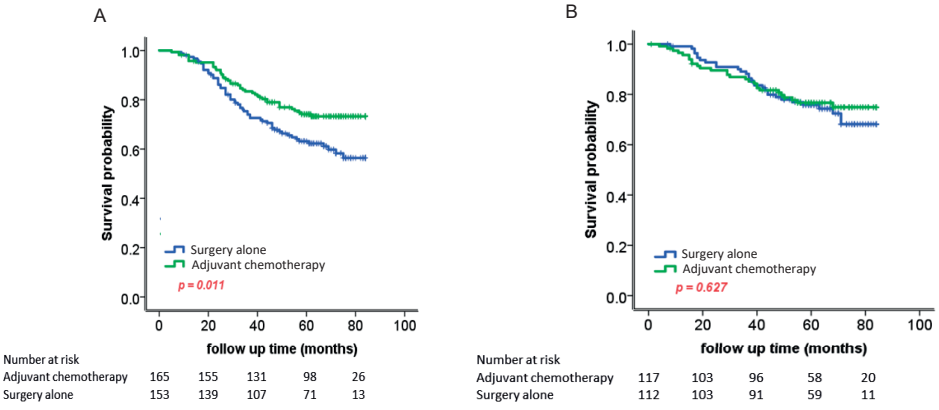
		Disease Free Survival			
	n (%)	5-year disease free survival		HR (95%CI)	
		Surgery plus adjuvant chemotherapy	Surgery alone	Univariate	Multivariate
TILs ≤870/mm^2	318 (58.1)	63.7% (56.7-716)	48.3% (41.0-56.9)	0.59 (0.42-0.82) p = 0.0021	0.57 (0.41-0.80) p = 0.0012
TILs >870/mm^2	229 (41.9)	73.2% (65.6-81.8)	64.3% (55.8-74.0)	0.68 (0.42-1.09) p = 0.116	0.68 (0.42-1.09) p = 0.114

HR, hazard ratio. Model was adjusted for age (<= 65 years vs > 65 years), sex, pT (T1/2 vs T3/4) and pN (N0 vs N1/2/3)

DISCUSSION

This is the first study to investigate the utility of TIL density measurement on routine Haematoxylin/Eosin stained sections for predicting survival benefit from adjuvant chemotherapy in GC patients in a randomised clinical trial which included a surgery alone control group.

Patients with low TIL density GC derived significant survival benefit from adjuvant chemotherapy, compared with patients with low TIL density GC who were treated by surgery alone. By contrast, the prognosis of patients with high TIL density GC could not be improved by adjuvant chemotherapy.

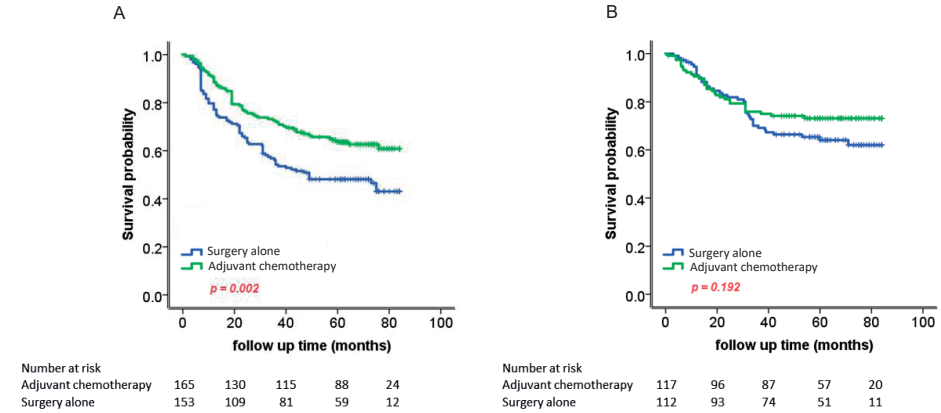


Number at risk					
Adjuvant chemotherapy	165	155	131	98	26
Surgery alone	153	139	107	71	13

Number at risk					
Adjuvant chemotherapy	117	103	96	58	20
Surgery alone	112	103	91	59	11

Figure 3 | Kaplan-Meier plots showing the predictive value of TIL density for overall survival by 870 TILs/mm^2 cut off.

Patients with low TIL density tumours (A) treated by surgery plus adjuvant chemotherapy have a better overall survival compared to patients treated by surgery alone. For high TIL density tumours (B), there is no difference in overall survival between patients treated by surgery alone and patients treated by surgery plus adjuvant chemotherapy.



Number at risk					
Adjuvant chemotherapy	165	130	115	88	24
Surgery alone	153	109	81	59	12

Number at risk					
Adjuvant chemotherapy	117	96	87	57	20
Surgery alone	112	93	74	51	11

Figure 4 | Kaplan-Meier plots showing the predictive value of TIL density for disease free survival by 870 TILs/mm^2 cut off.

Patients with low TIL density tumours (A) treated by surgery plus adjuvant chemotherapy have a better disease free survival compared to patients treated by surgery alone. For high TIL density tumours (B), there is no difference in disease free survival between patients treated by surgery alone and patients treated by surgery plus adjuvant chemotherapy.

Table 5 |Relationship of TIL density and clinicopathological variables HE TILs study population

		TIL density				
	Patients	**≤870/mm²**		**>870/mm²**		
	n	**n**	**%**	**n**	**%**	**P value**
Age						
<65 years	386	228	59	158	41	0.494
≥65 years	161	90	56	71	44	
Sex						
Male	393	226	58	167	43	0.634
Female	154	92	60	62	40	
T stage (TNM7)						
pT1/pT2	101	49	49	52	52	**0.030**
pT3/pT4	446	269	60	177	40	
N stage (TNM7)						
pN0	43	24	56	19	44	0.748
pN1-pN2	504	294	58	210	42	
TNM stage						
Stage II	183	93	51	90	49	**0.014**
Stage III	364	225	62	139	38	
Core Lauren classification						
Intestinal	184	115	63	69	38	**<0.001**
Diffuse	286	180	63	106	37	
Mixed/indeterminate	77	23	30	54	70	
Core WHO classification (2010)						
Tubular well differentiated	33	21	64	12	36	**<0.001**
Tubular moderately differentiated	123	71	58	52	42	
Tubular poorly differentiated	200	98	49	102	51	
Poorly cohesive	131	86	66	45	34	
Papillary	15	11	73	4	27	
Mucinous	24	22	92	2	8	
Mixed	21	9	43	12	57	
Epstein –Barr virus status						
Positive	42	10	24	32	76	**<0.001**
Negative	504	307	61	197	39	
Microsatellite instability (MSI) status						
MSI high	34	13	38	21	62	**0.017**
MSI low/ Microsatellite stable	482	285	59	197	41	

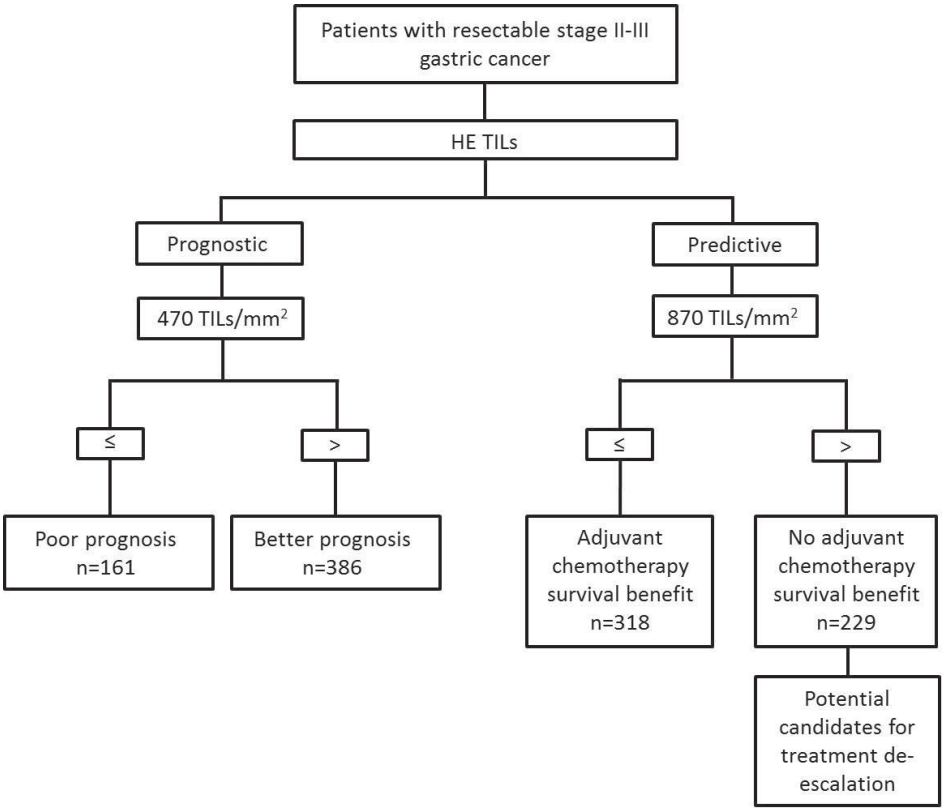


Figure 5 | Clinical subsets of patients with resectable gastric cancer by prognostic and predictive TIL cut-off points

The current study expands the recent results from an RNA based study in the same CLASSIC trial patient population which investigated the expression of immune cell related genes such as granzyme B and was able to stratify patients into groups with different risks of recurrence and different survival benefit from adjuvant chemotherapy (32). In the RNA-based study, patients classified as 'immune high' did not have a survival benefit from adjuvant chemotherapy, which is concordant with the results of our TIL density study performed on HE stained TMA cores. TIL density can be measured on the same section as used for diagnosis and as the size of the TMA cores used in this study are approximately equivalent to the amount of tissue of an endoscopic biopsy, the HE TIL based method could also be applied to biopsy specimens.

In contrast to the results from the current study, studies measuring stromal TILs on HE stained breast cancer (24-26) and a recent study using an immune related gene signature in publically available GC data (33), suggested that patients with high TIL density tumours might benefit most from adjuvant chemotherapy. These conflicting results could be due to different chemotherapy regimens or a different case mix in the GC study with respect

to stage of disease. In breast cancer and in our study TIL density was measured on the HE. Without immunohistochemistry we do not know the relative proportions of immune cell subtypes, which could be cancer specific and explain the discrepant results. Compared with our study, studies in breast cancer used different methods, including distinguishing between intraepithelial TILs and those in the stroma. More importantly, the above mentioned studies do not use samples from randomised clinical trials and also do not have a surgery alone control group or use an unmatched surgery alone group, and therefore are not able to distinguish between prognostic and predictive biomarkers. Without a control arm in our study we would also have concluded that patients with high TIL density have a survival benefit from adjuvant chemotherapy. By comparing with the control surgery group with the adjuvant chemotherapy group, we show that high TIL density GC patients also do well without chemotherapy.

Although we used a different methodology as most investigators of TILs in GC in the past, our study confirmed the prognostic value of TIL density in GC as described in recent meta-analyses (34, 35) and demonstrated that HE based TIL density can provide additional prognostic information, independent of the TNM stage.

Published studies suggest that EBV and MSI GC are often immunogenic (48-50). This was only partly confirmed in our study as up to 24% and 38% of EBV or MSI GC, respectively, showed low TIL density. Kim *et al*. found that TIL density is influenced by histological phenotype, however, this study investigated immune cell subtypes and therefore results are not directly comparable with our study (51). Our results suggest that TIL density might be a potentially better biomarker than EBV and MSI status for assessing tumour immunogenicity to guide conventional chemotherapy or immunotherapy based treatment decisions.

Our study has some limitations. This is a retrospective *post hoc* study from a subset of patients from the CLASSIC trial. However, this subset is representative of the whole trial population with respect to clinical characteristics and estimated 5-year DFS (40). TMA cores were taken from the area with the highest tumour content, which could have introduced some bias. In contrast to breast cancer studies, we cannot currently distinguish between stromal and intraepithelial TILs; however we obtain a continuous value (number per area) and not just a manual estimate, so our data should be more accurate and we will have higher statistical power for analysis. By using HE TIL density we get no information about TIL subtypes. However, we sought to establish a relatively easy to implement test that can be done from routine HE stained slides.

Ideally, the results of this study require validation in a second independent dataset before considering introducing it into the clinical routine. However, the only other GC trial comparing GC patients treated with surgery followed by adjuvant chemotherapy with GC patients treated by surgery alone is the ACTS-GC trial (NCT00152217), from which we were unable to obtain any material. By investigating TILs in the CLASSIC trial, the patient selection is restricted to Asian patients with stage II-III resectable GC. We have previously shown that TILs

vary between GCs from Asian and non-Asian patients (52), therefore our results also require validation in non-Asian patients to establish the generalizability of our findings.

In summary, this is the first study in GC to measure the density of TILs in HE stained TMA sections constructed from resection specimens from patients recruited in the CLASSIC trial with the aim of identify novel predictive biomarkers for this group of patients. TIL density was an independent prognostic and predictive biomarker in response to adjuvant chemotherapy in GC patients from the CLASSIC trial. TIL density may therefore represent a clinically useful biomarker for identifying GC patients for treatment de-escalation. For implementation of TIL density reporting in routine pathology, future studies should determine if TIL density in GC can be reliably estimated by pathologists or if TIL density needs to be quantified by a centralised image analysis service to provide accurate results with short turnaround time.

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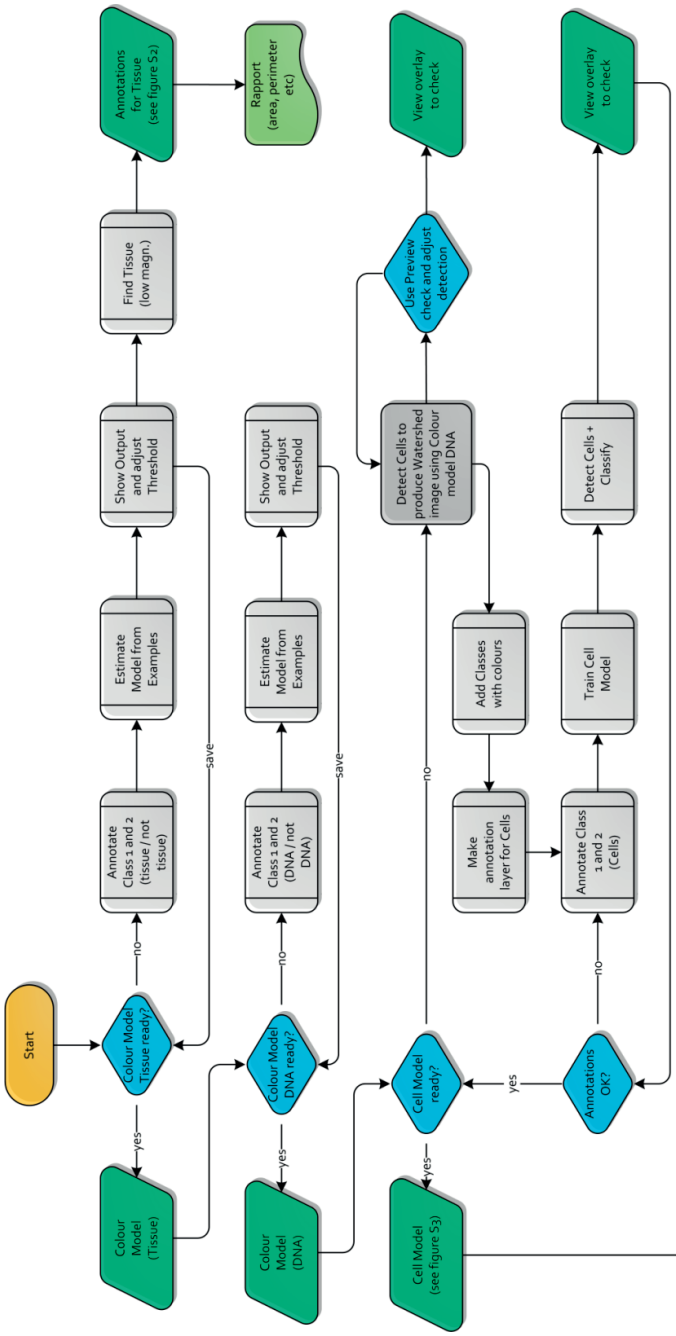


Figure S1 | Flowchart showing the workflow for the development of the cell model.

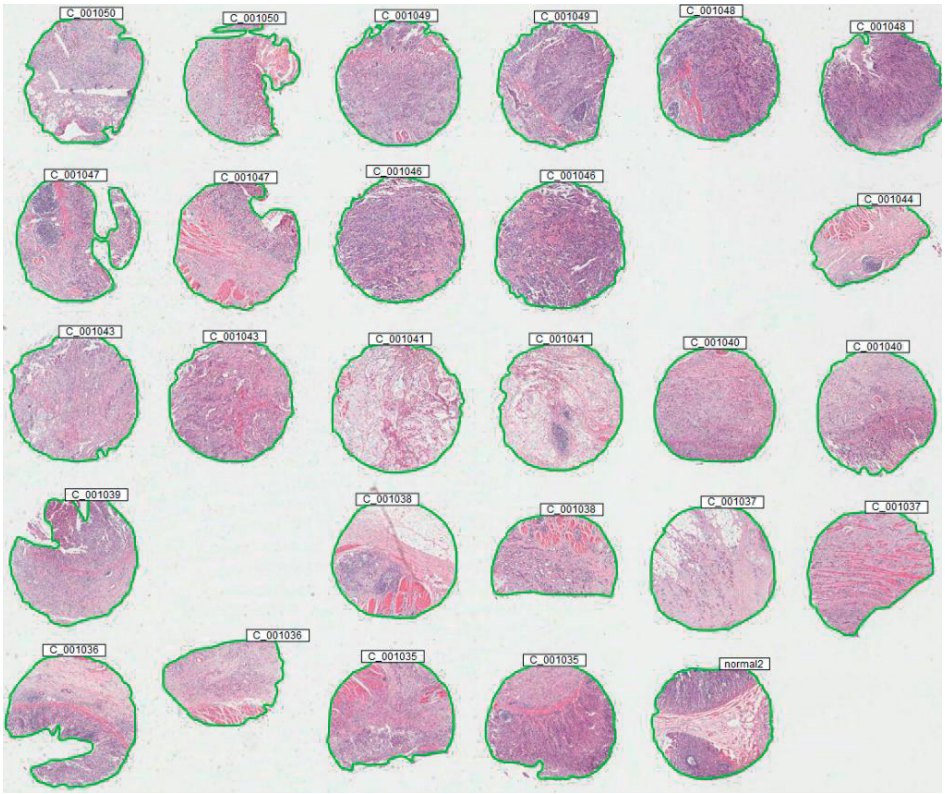


Figure S2 | Outlined (green) tissue microarray cores with a link to the core identifier.

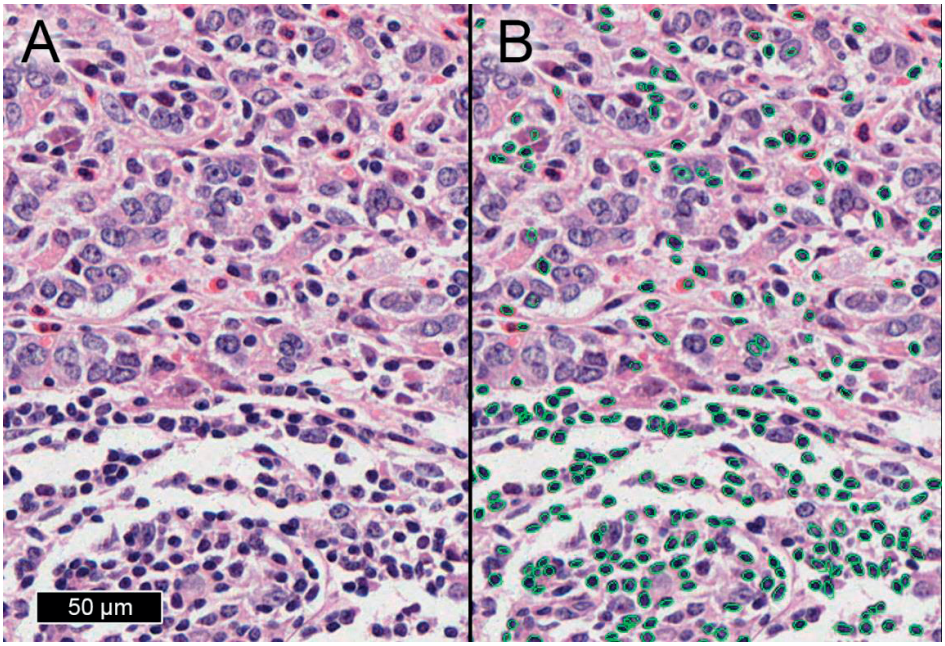


Figure S3 | Haematoxylin and eosin stained tissue microarray (a) no lymphocyte detection and b) with lymphocyte detection (green outline) by image analysis.

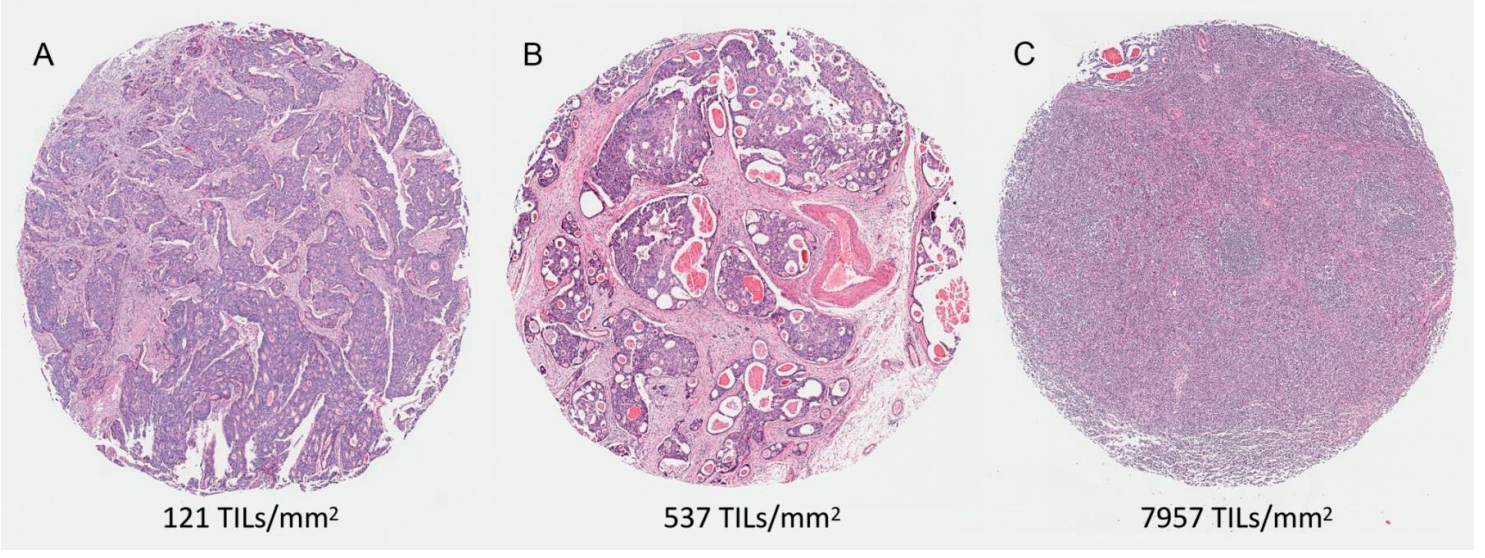
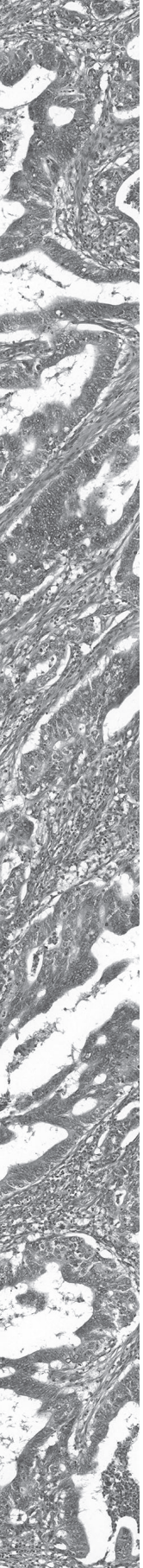


Figure S4 | Examples of TMA cores containing low (≤470/mm^2) TIL density tumours (A), intermediate (>470/mm^2, ≤870/mm^2) TIL density tumours (B) and high (>870/mm^2) TIL density tumours (C). Core diameter = 3mm



Chapter 7

General discussion

Emerging technologies partly adapted from: Hypothesis-free deep survival learning applied to the tumor microenvironment in gastric cancer

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Submitted

OESOPHAGOGASTRIC CANCER – THE CURRENT STATUS

Currently patient prognosis and treatment decisions in oesophagogastric cancer (OGCa) are based on TNM stage, patient performance status and patient's preferences (1). However, OGCa patients with the same TNM stage can have very different outcomes (2, 3). OGCa patients have a poor prognosis with a 5-year survival in Europe of 45-47% when diagnosed at a disease stage where the tumour is resectable and is treated with neoadjuvant/peri-operative chemo(radio)therapy followed by surgery (4, 5). The survival benefit of neoadjuvant/peri-operative chemotherapy is modest at 6-14% improved 5-year survival compared to treatment by surgery alone (4, 6), suggesting that only a subset of patients benefits from chemotherapy. Thus, there remains an urgent clinical need to identify biomarkers to individualise and improve OGCa patient management.

PROGNOSTIC AND PREDICTIVE BIOMARKERS IN OESOPHAGOGASTRIC CANCER: LOST IN TRANSLATION?

A prognostic biomarker is defined as a clinical or biological characteristic that provides information on clinical outcome independent of treatment. A predictive biomarker indicates the likely benefit of a treatment and is used to guide therapeutic decisions (7). The process of biomarker implementation to routine clinical practice is set out in roadmaps agreed by the scientific community starting with the identification of a clinical need for a biomarker, initial biomarker discovery, biomarker assay development, initial correlation to clinical outcome, validation and finally clinical qualification by prospective testing (8).

Despite comprehensive molecular characterisation of gastric cancer (GC) , none of the tumour-based biomarkers are currently used in clinical practice. The single centre Asian Cancer Research Group (ACRG) classification system was shown to have prognostic value and was validated retrospectively in two additional Asian GC cohorts (9), but has not been validated in a Western cohort. It was subsequently shown that The Cancer Genome Atlas (TCGA) GC classifiers may have prognostic value and may predict chemotherapy survival benefit (10). The results of this TCGA data based study have not been validated. Differences in clinicopathological variables between the TCGA and ACRG based studies are shown in table 1. Despite the higher incidence of GC in the East, many of the biomarker studies and clinical studies are performed in or at least include a substantial number of Western patients. The LOGiC and AVAGAST phase III clinical trials and other studies have consistently shown that there are geographical-based survival differences in GC patients (11, 12). Whilst this survival difference can in part be explained by earlier diagnosis in Eastern countries, and differences in treatment regimens (13), our research group has shown that Japanese and UK populations are different with respect to all clinicopathological data but also that a survival dif-

ference remains between the population after adjusting for all clinicopathological differences (14). Furthermore, we were also able to show that there are biological differences between Asian and non-Asian GC patients, such as the tumour immune response (15). These results highlight the need for geographical differences to be considered in validation studies before implementing a biomarker. Even within Asia there is a lack of validation studies hindering region-specific biomarker implementation into the clinic. Validation of the molecular studies conducted in our own research group identifying prognostic value or proposed relationship with response to chemotherapy is ongoing (16-18).

Table 1 | Comparison of clinicopathological variables in the TCGA and ACRG studies

	TCGA	**ACRG**
Ethnicity	25% East Asian	100% Korean
Histology	23% diffuse	45% diffuse
Stage	31% III/IV	57% III/IV
Location	19% GOJ	11% GOJ
Type of sample	Primary tumour	Primary tumour
Study type	Multi-centre	Single centre

Abbreviations: TCGA, The Cancer Genome Atlas; ACRG, Asian Cancer Research Group; GOJ, gastroesophageal junction.
Adapted from (19).

Biomarker research usually starts with single-centre, retrospective studies using relatively small local series and non-validated assays. Following the roadmap agreed by the scientific community, potential biomarkers then require validation in a second independent cohort. However, this is often not happening because there is no other series available, or if a validation study has been performed the results differ from the initial study due to factors such as sample size, sample mix, use of different methodologies, choice of cut offs and tumour heterogeneity (see below). After validation in a second independent series, potential biomarkers require validation in a prospective clinical trial setting where patients are randomised based on biomarkers status before implementation into the clinical routine can be considered. Such studies are very costly and time consuming particularly in OGCa patients in the West due to the relative low disease incidence resulting in a duration of over 10 years in order to recruit a sufficient number of patients and perform a 5-year patient follow up.

An untreated (no chemotherapy, only surgery) control group of patients, preferably from a randomised controlled clinical trial setting, is essential in predictive biomarker research in order to be able to distinguish between the prognostic and predictive value of a biomarker. In this thesis, we utilised tissue samples from two randomised clinical trials which still had a surgery alone (control) group; The UK Medical Research Council (MRC) OE02 trial which was a phase 3 study randomizing patients with locally advanced resectable OeC to surgery alone or 5-fluorouracil (5-FU) plus cisplatin chemotherapy followed by surgery **(chapter 5)**

(6, 20) and the randomised, open-label, multicentre, phase 3 CLASSIC trial comparing D2 gastrectomy followed by adjuvant capecitabine and oxaliplatin chemotherapy with surgery alone in GC patients **(chapter 6)** (21). In the CLASSIC trial study, we found that patients with low tumour infiltrating lymphocyte (TIL) density GC derived significant survival benefit from adjuvant chemotherapy, compared with patients with low TIL density GC who were treated by surgery alone. By contrast, the prognosis of patients with high TIL density GC could not be improved by adjuvant chemotherapy. Our results are in contrast to several breast cancer studies measuring stromal TILs on HE stained slides (22-24). However, none of the breast cancer studies included a surgery alone control group. Without a control arm in our study, we would also have concluded that patients with high TIL density have a survival benefit from adjuvant chemotherapy. By comparing the results between the surgery alone control group with the adjuvant chemotherapy group, we were able to demonstrate that high TIL density GC patients also do well without chemotherapy. Thus, being able to distinguish between prognostic and predictive biomarkers is crucial for accurate interpretation of study results. However, the inclusion of a surgery alone control group in biomarker research is becoming increasingly problematic as results of new clinical trials change OGCa patient management meaning that patients are no longer treated by surgery alone.

COMPLEXITY OF OESOPHAGOGASTRIC CANCER

As described in the introduction, OGCa is a complex disease with respect to molecular and histological characteristics, as well as inter- and intratumour heterogeneity.

Heterogeneity of the tumour epithelium

With advances in high-throughput molecular technology, characterisation of the epithelial component of OGCa has resulted in a relatively large number of proposed molecular based OGCa subtypes probably reflecting the molecular intertumour heterogeneity at the genetic and epigenetic level. In gastric cancer (GC), these studies include The Cancer Genome Atlas (TCGA) (25), the Singapore-Duke Group (26) The Asian Cancer Research Group (ACRG) (9), as well as studies from our own research group (16-18). Molecular characterisation of oesophageal cancer (OeC) by TCGA showed a clear molecular distinction between the two histological phenotypes of oesophageal cancer: adenocarcinoma and squamous cell carcinoma (27). On the other hand, TCGA data suggests that oesophageal adenocarcinoma and gastric adenocarcinoma share many molecular characteristics (27). Despite the reported similarities between oesophageal adenocarcinoma and gastric adenocarcinoma, in **chapter 4**, we identified a difference in the incidence of Epstein-Barr virus (EBV) associated cancers as well as in the incidence of microsatellite instability (MSI) between these two tumour types. The underlying biological mechanisms for the non-existence of EBV associated oesophageal

adenocarcinoma is unclear. MSI is extremely rarely detected in oesophageal adenocarcinoma most likely related to the fact that this disease develops along the chromosomally instable (CIN) pathway (27).

It has been proposed that molecular intratumoural heterogeneity in cancer is the result of a combination of mutational and chromosomal alterations (28). GC has one of the highest levels of molecular intratumour heterogeneity of all cancer types (28). In oesophageal adenocarcinoma, copy number alterations and large scale rearrangements seem to be the main contributors to intratumour heterogeneity (29). Molecular intratumour heterogeneity has been associated with a decreased response to neoadjuvant chemotherapy (30) and intratumoral HER2 heterogeneity has been related to poor prognosis (31) in oesophageal adenocarcinoma.

In **chapter 3** we identified a relationship between *KRAS* mutation and mucinous phenotype suggesting that molecular features may influence the histological phenotype. A relationship between molecular alterations and histological features has also been suggested for lung cancer (32). We also found that more than 50% of GCs from the East and the West had more than one histological phenotype, indicating a high level of intratumour morphological heterogeneity. In Japan, intratumour morphological heterogeneity is recorded in the routine Japanese gastric cancer classification scheme by semiquantitative estimation of different histological phenotypes (33). Morphological intertumour heterogeneity is reflected in the numerous proposed classification systems in GC (34-41).

Heterogeneity of the intratumoural stroma

Whilst there are studies reporting on the heterogeneity of molecular and/or histological characteristics of the epithelial component of OGCa, studies characterising the stromal component of OGCa are limited. Our group showed that the expression of stroma-related gene sets and the morphometric quantification of the tumour-stroma proportion on routine Haematoxylin/Eosin (HE) stained slides were related to prognosis in GC (42). More recently, a tumour microenvironment related gene signature was shown to have prognostic value and appeared to be related to immunotherapy response in GC. (43). We showed recently in Japanese GC that the prognostic relevance of the intra-tumour stroma varies by histological subtype and is related to the level of intra-tumour leucocyte infiltration (44). Furthermore, we were the first to demonstrate that the quantity of intra-tumoural stroma in the pre-treatment biopsy predicts benefit from neoadjuvant chemotherapy in patients recruited into the Oe02 trial (45). Other investigators have shown a relationship between tumour-stroma ratio and survival, with stroma-rich tumours related to a poor prognosis in OGCa (46-49).

The level of inter- and intra-heterogeneity of the intratumour stroma component in OGCa is not clear from the literature. To address this question, we reanalysed our previously published OE02 biopsy data (45) to quantify the degree of heterogeneity of the stroma **(chapter 5)**. Our results suggest that degree of intratumour heterogenity of the tumour stroma ratio

may predict survival benefit from neoadjuvant chemotherapy. OeC patients with a low level heterogeneity of the tumour stroma in the pre-treatment biopsy had a greater benefit from cytotoxic chemotherapy than patients with high level heterogeneity.

Based on these findings we were interested to know which components of the stroma are contributing to its relationship with chemotherapy response. Studies in GC including our own (50) suggested a clinical value of TILs with respect to patient prognosis (51, 52), thus we selected TILs as our initial focus of investigation. As there was uncertainty about the influence of factors such as ulceration, diet and microbiome on TILs, we opted to initially use resection specimens instead of pre-treatment endoscopic biopsies and selected the area with the highest tumour cell density in the resected tumour. In order to distinguish between prognostic and predictive biomarkers and minimise bias on patient selection, this study needed to be performed in material from a clinical trial with an untreated (no chemotherapy) control group. Thus, material from the resection specimens from the Korean CLASSIC trial which compared surgery alone versus surgery plus adjuvant therapy in GC patients was thought to be optimal to investigate the relationship of TILs with patient prognosis and response to chemotherapy. Following the recommendation from the International TILs Working Group on breast cancer we opted to quantify TIL density in haematoxylin and eosin (HE) stained sections (22-24, 53-57). Whilst breast cancer studies quantify TILs in the stroma (58), we measured the total lymphocyte count per tumour area as a surrogate of a tumour's overall immunogenicity. This decision was based on observations that TILs show substantial heterogeneity in number and location within an individual GC and between GC from different patients. Borders between the tumour and stroma were often blurred (Figures 1 and 2) thus it would not have been feasible to score TILs on tumour epithelium and TILs in the intratumoural stroma separately, neither manually/visually nor via image analysis software. Moreover, tumour infiltration by immune cells is a dynamic process and we are looking at it in a static image.

Our TIL results **(chapter 6)** were concordant with other studies (51, 52) showing that GC patients with high TILs have a significantly better prognosis compared to patients with low TILs. Furthermore, we showed for the first time that HE-based TIL density can predict survival benefit from adjuvant chemotherapy in GC, confirming the results of the RNA-based study performed in the same CLASSIC trial patients (59) and our immunohistochemistry-based study in an independent series (50). We were the first to demonstrate that patients with high levels of TILs represent a subgroup of GC patients deriving little or no survival benefit from adjuvant chemotherapy. These patients are potential candidates for treatment de-escalation, thereby avoiding unnecessary toxicity and costs. Despite a recent gene expression based study showing a complex landscape of TIL interactions (43), our results indicate that the number of TILs per tumour area is important for patient prognosis and predicting response to chemotherapy, irrespective of the proportion of immune cell subtypes, level of immune cell activation or immune cell location. Our data requires validation in other cohorts for which grant funding from Cancer Research UK (CRUK) has been obtained recently.

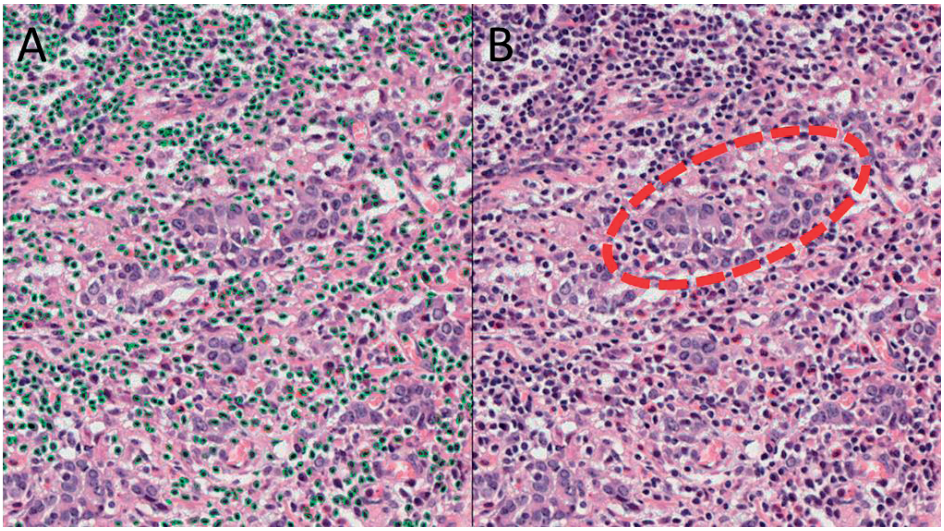

Figure 1 | Representative haematoxylin and eosin stained image from gastric cancer CLASSIC trial illustrating intratumoural lymphocyte detection using image analysis.
A | Gastric cancer with a relatively large number of diffusely distributed, green encircled intratumoral lymphocytes.
B | The same image with a manually added red dashed line encircling a tumour gland. The tumour gland is barely visible under the densely infiltrating lymphocytes and shows morphological signs of cell destruction.

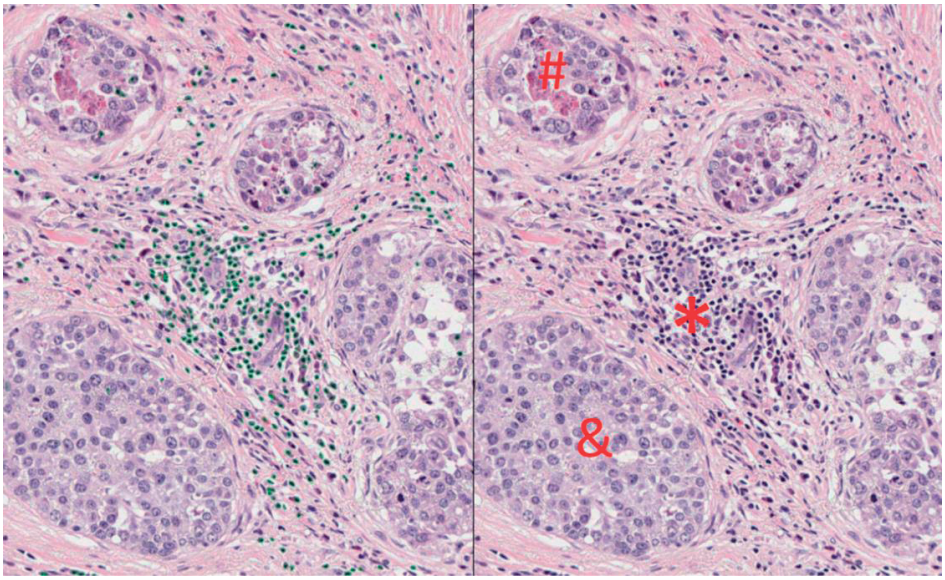

Figure 2 | Representative haematoxylin and eosin stained image from gastric cancer CLASSIC trial showing a few residual tumour cells in the centre (*) heavily surrounded by TILs (green circles, image on the left). For this 'tumour under lymphocyte attack' it would be difficult to identify where the tumour epithelium ends and the intra-tumoural stroma begins. Adjacent tumour islands are either centrally necrotic (#) with minimal TIL infiltration or appear morphologically fully viable (&) also with minimal TIL infiltration.

BIOMARKERS FOR TARGETED THERAPY AND IMMUNE THERAPY IN OESPHAGOGASTRIC CANCER

The focus of this thesis was on OGCa patients with locally advanced resectable disease, treated by surgery and chemotherapy. With the exception of the ongoing EORTC 1203 Innovation trial randomising OGCa patients with HER2-positive cancers (60) and RAMSES/FLOT7 evaluating ramucirumab in combination with FLOT in unselected patients, there is currently no other trial ongoing in patients with locally advanced resectable OGCa investigating targeted therapies.

Targeted therapies in unresectable or metastatic oesophagogastric cancer

For patients with unresectable or metastatic OGCa at the time of diagnosis there is a growing list of studies using targeted therapies. The life expectancy of these patients is usually less than 12 months if treated with cytotoxic chemotherapy (61). Due to the very poor patient outcome, new therapeutic approaches are usually tested in this setting, albeit with limited success to date (see table 2). Only a limited number of targeted therapy approaches have been approved for OGCa patients with unresectable/metastatic disease: trastuzumab for HER2 positive disease in the metastatic setting (62), ramacirumab, a VEGFR-2 antagonist, without biomarker based patient selection (63, 64) and pembrolizumab if the cancer is MSI or mismatch repair (MMR) deficient or expresses a certain level of programmed death-ligand 1 (PD-L1) (65, 66). All other recent clinical trials in OGCa with targeted therapies have failed to significantly improve patient survival (11, 12, 67-71).

In contrast to colorectal cancer, where routine testing for *KRAS* mutation is implemented as a predictor of response to EGFR therapy (82), the REAL3 trial showed no survival benefit of anti-epidermal growth factor receptor (EGFR) therapy in *KRAS* mutant OGCa (74). In this study, patients received a reduced dose of cytotoxic chemotherapy due to toxicities when combined with panitumumab (74). We have shown that OeC patients with a mucinous phenotype are particularly sensitive to neoadjuvant cytotoxic chemotherapy (83). The histological phenotypes of the REAL3 trial are not known yet, but if there was for example a relatively high proportion of mucinous cancers, the failure of the REAL3 trial might be due to the reduced chemotherapy dose rather than the EGFR targeted treatment. The relationship between *KRAS* amplification and anti-EGFR therapy has not been studied in GC despite its higher frequency compared with *KRAS* mutation **(chapter 3)**. Interestingly, despite the difference in response to therapy between molecular subtype and histological phenotype, the relationship between *KRAS* mutation and histological phenotype appears to be consistent across different cancers **(chapter 3).**

Table 2 | Clinical trials using targeted therapy in patients with metastatic oesophagogastric cancer

Target	Trial/ registry No./ authors	Cancer type	Regimen
HER2	ToGA (62)	GC or GOJ	FP/XP vs. FP/XP + trastuzumab
	LOGiC (12)	GC or OeC	CapeOx + placebo vs. CapeOx + lapatinib
	TyTAN (72)	GC	PTX vs. PTX + lapatinib
	GATSBY (73)	GC or GOJ	Docetaxel or paclitaxel vs. TDM-1
EGFR	EXPAND (70)	GC	XP vs. XP + cetuximab
	REAL-3 (74)	GC or OeC	EOC vs. EOC + panitumumab
	JapicCTI-090849 (75)	GC	Irinotecan vs. Irinotecan + nimotuzumab
VEGF	AVAGAST (11)	GC	XP + placebo vs. XP + bevacizumab
VEGFR2	REGARD (63)	GC or GOJ	Placebo vs. Ramucirumab
	RAINBOW (64)	GC or GOJ	Paclitaxel + placebo vs. Paclitaxel + ramucirmab
	Li et al. (76)	GC or GOJ	Placebo vs. Apatinib
	RAINFALL (77)	GC or GOJ	XP (or FP) vs. XP (or FP) + ramucirmab
VEGFR, RET, RAF	INTEGRATE (78)	GC	Placebo vs. Regorafenib
HGF	RILOMET-1 (68)	GC or GOJ	ECX + placebo vs.ECX + rilotumumab
MET	METGastric (69)	GC or OeC	mFOLFOX + placebo vs. mFOLFOX + onartuzumab
mTOR	GRANITE-1 (67)	GC	Placebo vs.Everolimus
Claudin 18.2	FAST (79)	GC or GOJ	EOX vs.EOX + claudiximab (extended by an arm3; EOX + high dose claudiximab)
MMP-9	GAMMA-1 (80)	GC or GOJ	mFOLFOX + placebo vs. mFOLFOX + andecaliximab

Abbreviations: GC, gastric cancer; GOJ, gastro-oesophageal cancer; OeC, oesophageal cancer; OS, overall survival; PFS, progression free survival; HR, hazard ratio; CI, confidence interval; XP, capecitabine and cisplatin; FP, 5-fluorouracil and cisplatin; Capeox, capecitabin + oxaliplatin; EOC/EOX, epirubicin + oxaliplatin + capecitabine;

Phase	Line	No. of patients	Median OS (months)	Median PFS (months)
3	1st	594	11.1 vs.13.8; HR = 0.74; 95%CI: 0.60-0.91; P = 0.0046	5.5 vs. 6.7; HR = 0.71; 95%CI: 0.59-0.85; P = 0.0002
3	1st	545	10.5 vs.12.2; HR = 0.91; 95%CI: 0.73-1.12; P = 0.3492	5.4 vs. 6.0; HR = 0.82; 95%CI: 0.68-1.00; P = 0.0381
3	2nd	261	8.9 vs. 11.0; HR = 0.84; 95%CI: 0.64-1.11; P = 0.1044	4.4 vs. 5.4; HR = 0.85; 95%CI: 0.63-1.13; P = 0.2241
3	2nd	345	8.6 vs. 7.9; HR = 1.15; 95%CI: 0.87-1.51; P = 0.8589	2.9 vs. 2.7; HR = 1.13; 95%CI: 0.89-1.43; P = 0.3080
3	1st	904	10.7 vs. 9.4; HR = 1.00; 95%CI: 0.87-1.17; P = 0.95	5.6 vs. 4.4; HR = 1.09; 95%CI: 0.92-1.29; P = 0.32
3	1st	553	11.3 vs. 8.8; HR = 1.37; 95%CI: 1.07-1.76; P = 0.013	7.4 vs. 6.0; HR = 1.22; 95%CI: 0.98-1.52; P = 0.068
2	2nd	83	7.7 vs. 8.4; HR = 0.994; 95%CI: 0.618-1.599; P = 0.9778	2.9 vs. 2.4; HR = 0.860; 95%CI: 0.516-1.435; P = 0.5668
3	1st	774	10.1 vs. 12.1; HR = 0.87; 95%CI: 0.73-1.03; P = 0.1002	5.3 vs. 6.7; HR = 0.80; 95%CI: 0.68-0.93; P = 0.0037
3	2nd	355	3.8 vs. 5.2; HR = 0.776; 95%CI: 0.603-0.998; P = 0.047	1.3 vs. 2.1; HR = 0.483; 95%CI: 0.376-0.620;P < 0.0001
3	2nd	665	7.36 vs 9.63; HR = 0.807; 95%CI: 0.678-0.962; P = 0.0169	2.86 vs. 4.4; HR = 0.635; 95%CI: 0.536-0.752; P < 0.0001
3	3rd	267	4.7 vs. 6.5; HR = 0.709; 95%CI: 0.537-0.937; P = 0.0149	1.8 vs. 2.6; HR = 0.444; 95%CI: 0.331-0.595; P < 0.001
3	1st	645	10.7 vs. 11.2; HR = 0.962; 95%CI: 0.801–1.156; p=0·6757	Investigator-assessed: 5.4 *vs.* 5.7;HR = 0.753; 95% CI: 0.607–0.935; p=0.0106 Sensitivity analysis: HR 0.961; 95% CI: 0.768–1.203; p=0.74
2	2nd or 3rd	147	4.5 vs. 5.3; HR = 0.74; 95%CI: 0.51-1.08; P = 0.147	0.9 vs. 2.6; HR = 0.40; 95%CI: 0.28-0.59; P < 0.001
3	1st	609	9.6 vs. 11.5; HR = 1.36; P = 0.021	2.86 vs. 4.4; HR = 1.27; P = 0.025
3	1st	562	11.3 vs. 11.0; HR = 0.82; 95%CI: 0.59-1.15; P = 0.24	6.8 vs. 6.7; HR = 0.90; 95%CI: 0.71-1.16; P = 0.43
3	2nd or 3rd	656	4.34 vs. 5.39; HR = 0.90; 95%CI: 0.75-1.08; P = 0.1244	1.41 vs. 1.68; HR = 0.66; 95%CI: 0.56-0.78; P < 0.0001
2	1st	161 (+85)	8.4 vs. 13.4; HR = 0.51; 95%CI: 0.36-0.73; P < 0.001	4.8 vs. 7.9; HR = 0.47; 95%CI: 0.31-0.70; P = 0.0001
3	1st	432	11.8 vs. 12.5; HR 0.93; 95%CI; 0.74- 1.18; p=0.56	7.1 vs. 7.5; HR = 0.84; 95%CI: 0.672-1.038; p=0.10

ECX, epirubicin + cisplatin + capecitabine; FOLFOX, fluorouracil + leucovorin + oxaliplation; TDM-1, tratuzumab-emtansine.
Adapted from (81).

Targeted therapy: Receptor tyrosine kinase pathway

Many targeted therapies used in OGCa clinical trials were directed against genes involved in the receptor tyrosine kinase (RTK) pathway (11, 67-70, 77, 84) because activation of the RTK pathway is one of the characteristics of the CIN GC and OeC subtypes (25). Despite the CIN subgroup comprising 50% of GC patients in TCGA study (25), our literature review in **chapter 2** found mutations in *KRAS* are extremely rare in GC (6.5%) and are not associated with survival **(chapter 3).** In contrast, the frequency of *KRAS* amplification in GC seems to be higher and associated with a worse survival in some studies (16, 85). Although we were the first to describe that up to 37% of GC exhibit RTK/RAS alterations, identifying RTKs as promising treatment targets in GC (16), there is a growing list of negative OGCa trials for drugs targeting the RTK pathway (11, 67-70, 77, 84). This could be related to co-occurrence of gene amplification in the RTK pathway and/or gene amplification heterogeneity between primary tumour and lymph node metastasis (86). Recently, i*n vitro* and *in vivo* studies have shown that wild-type *KRAS* amplified GC was only sensitive to RTK pathway blockade by inhibition of multiple genes; MEK in combination with SOS or SHP2 (85). This suggests that combination strategies are needed to target the RTK pathway in GC.

EBV and MSI have been proposed as a surrogate marker for tumour immunogenicity to predict potential response to immunotherapy in OGCa. In our multicentre study **(chapter 4),** the frequency of MMR deficiency/MSI was <1% and 10% in OeC and GC, respectively. The frequency of EBV was also low (none of the OeCs were EBV positive compared to 5% in GC). There was one patient with overlap between EBV positivity and MMR deficiency. Thus, the number of OGCa patients eligible for these new therapies based on these markers is very low (15%). In a separate study investigating TIL density in GC patients from the CLASSIC trial **(chapter 6)** we found high TIL density was associated with EBV and MSI, confirming the results of other studies (87-89). However, in concordance with a gene expression based study (29) we found that 10 (24%) EBV positive GC and 13 (38%) GC with MSI showed low TIL density whereas 197 (39%) EBV negative GC and 197 (41%) GC with MSI low/MSS showed high TIL density. This suggests that TIL density status might be a better biomarker than EBV and MSI for assessing tumour immunogenicity to guide immunotherapy based treatment decisions. In **chapter 3** we confirmed the results of other studies in GC that *KRAS* mutation is related to MSI. However, we were unable to relate TIL density to *KRAS* activation in the CLASSIC trial **(chapter 6)** as *KRAS* mutation or amplification data were not available.

In summary the success of targeted and immune therapies in unresectable or metastatic OGCa has been limited. A combination treatment approach against multiple targets may be needed. Studies using targeted therapies in locally advanced resectable OGCa are ongoing.

EMERGING TECHNOLOGIES

A limitation of many proposed biomarkers in OGCa is the loss of spatial information when extracting RNA or DNA from tissue. This approach does not allow the result to be related to back to individual cell types. Emerging technologies may be used in the future to overcome this issue. In the field of digital pathology, we have performed image analysis on HE stained tissue microarray images **(chapter 6).** Recently, deep learning methods such as convolutional neural networks (CNNs) have been used to predict patient outcome from HE stained whole slide images of gliomas and predict colorectal cancer outcome based on HE stained tissue microarray (TMA) images (90, 91). We have applied CNN to predict MSI status in gastrointestinal cancer based on HE stained images (92). Studies in lung cancer and melanoma used CNN to predict mutation status from the HE (93, 94). CNN has also been used to distinguish tumour from non-tumour on HE stained whole tissue sections from multiple different tumour types (95).

With deep learning methodology, time consuming and tedious tasks such as cell detection and classification can be efficiently automated via computational solutions. Traditionally, pathologists estimate quantities of different cell types by counting them in selected fields of views and extrapolating these numbers to slide level. Classification systems such as grading, scoring, and tumour sub-typing for prognostication suffer from subjectivity and may be biased by prior knowledge. In collaboration with Definiens (Munich, Germany), we have recently used CNN in an end-to-end weakly supervised scheme (deep learning method) to predict cancer recurrence free survival risks in a cohort of 248 Japanese gastric cancer patients. The data and high-level workflow overview is shown in Figure 3. Overall, the learning process to define the risk of dying is guided by a specific method, so called "specific loss function", which takes into account the time-to-event and censoring characteristics of survival data. The machine network was trained to run for each stain separately. We analysed IHC stainings for CD8 (cytotoxic T cells), CD20 (B cells), CD68 (pan-macrophages) and Ki67 (proliferating cells) in addition to HE. We evaluated the prognostic value of the obtained tile-based risk scores in terms of their ability to stratify the cohort into a low-risk and a high-risk group. Using Kaplan-Meier and log-rank test, we obtained significant p-values for risks associated with CD20 (Cox loss, p=0.0159) and CD68 (Cox loss, p=0.02), whilst CD8 and Ki67 as single markers turned out to be non-significant. Moreover, combining risks from two stains consistently improved the power of stratification. Figure 4 shows the risk stratification for CD20 and CD68 combined. In multivariate analyses including clinical covariates, combining risks were independent prognostic factors (Ki67+CD20: HR=1.364, p-value=0.013, CD20+CD68: HR=1.338, p-value=0.009, Ki67+CD68: HR=1.473, p-value=0.002; all for Logrank loss). Thus, our deep learning based risk scores derived from immune cell subtyping appear to provide additional prognostic information to the TNM staging system.

We used risk heat maps to visualize structures in the images which the CNN associated with specific risks (see figure 5). During prediction, each TMA core was tiled into patches and each patch was forwarded through the network. The final layer returned the risk of the respective patch/tile, which was then used for a color-coded transparent overlay on top of the original image (ranging from green for low risks to red for high risks).

Although this technology is still in its infancy, this study shows the additional value that deep learning in combination with a panel of traditional IHC markers, including immune based markers, may bring to improving patient outcome in the future.

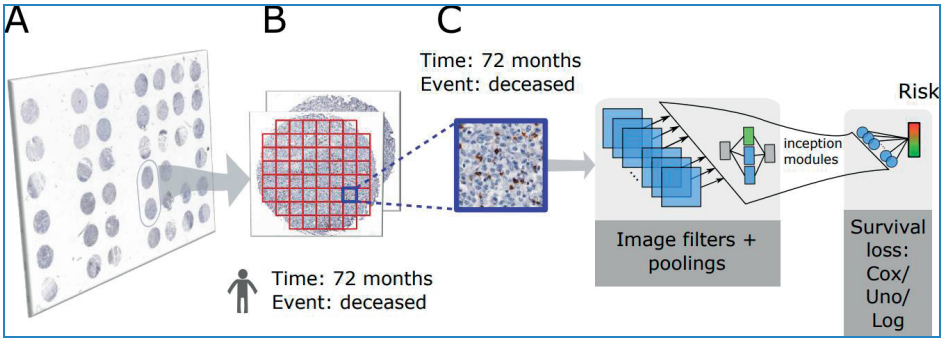

Figure 3 | A) Tissue micro array images acquired from a Japanese gastric cancer cohort. B) All cores of a given patient are tiled into patches. Both survival time and event are forwarded from the patient to the patch level. C) A convolutional neural network is trained to predict survival risks from a given input patch. Parameter estimation is guided by one of three survival loss functions: Cox, Uno or Logrank loss.

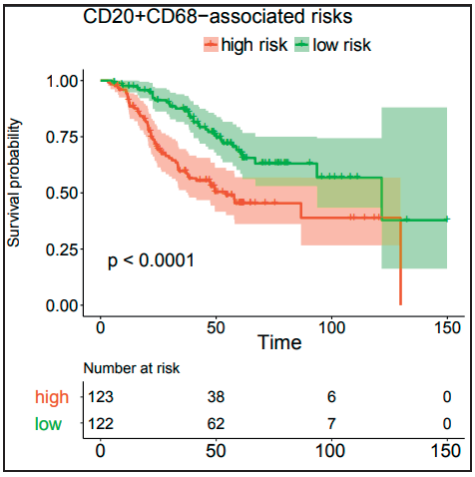

Figure 4 | Kaplan-Meier curve (Uno loss) for CD20 and CD68 combined showing stratifications of the cohort into a low and a high risk arm. The groups were retrieved by thresholding the respective feature based on the cohort median.

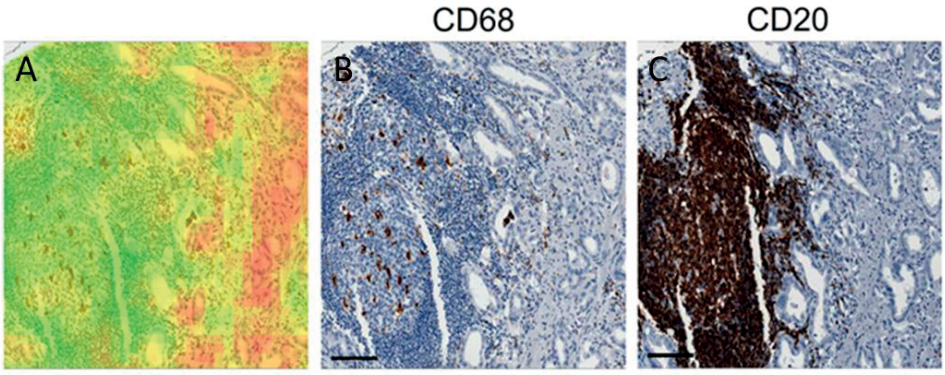

Figure 5 | Risk map and immunohistochemistry staining in GC. (A) Representative risk map for CD68, with low and high risks indicated in green and red, respectively. (B) Immune cell clusters that contain CD68(+) cells are associated with low risks. (C) Visual inspection of the corresponding core stained for CD20 reveals B cell clusters.

FUTURE PERSPECTIVES AND CONCLUSION

We have shown that characteristics of the stroma may represent a clinically useful biomarker for patient treatment stratification. Our TIL density results **(chapter 6)** were used as pilot data for a grant application to validate our findings (prognostic and predictive value of TILs) in seven randomised phase III trials including OGCa patients from the East and the West. Funding from CRUK was successfully obtained for this project, thus we will perform the required validation studies to translate this finding into the routine clinical setting in the near future.

The frequency of tumour based molecular alterations including *KRAS*, EBV and MSI markers is low in OGCa. Histological classification of the desmoplastic stroma in colorectal cancer has been shown to have prognostic value (96). Thus, the tumour based approach of subtyping may need to be combined with subtyping of the intratumoural stroma in order to improve OGCa patient management. From our *KRAS* activation study in **chapter 3** we now have detailed histological classification of the TCGA series. Combined with the extensive molecular information available from this series, our morphological phenotyping study forms the basis of future studies of the relationship between genotype and phenotype in GC.

In OGCa there remains an urgent clinical need to identify prognostic and predictive biomarkers to individualise and improve patient management. In the past decade there have been significant advances in the understanding of the biology of OGCa through molecular characterisation of tumours. It is now time to validate these findings in order to implement them in the clinical routine for the benefit for the patient.

In conclusion, identification and translation of tumour based prognostic and predictive biomarkers in OGCa to the clinic remains challenging due to the complexity of the disease and relative lack of appropriate patient cohorts for validation studies. We have identified promising stromal based markers and will perform validation studies in the near future to fully assess the prognostic and predictive value of TILs.

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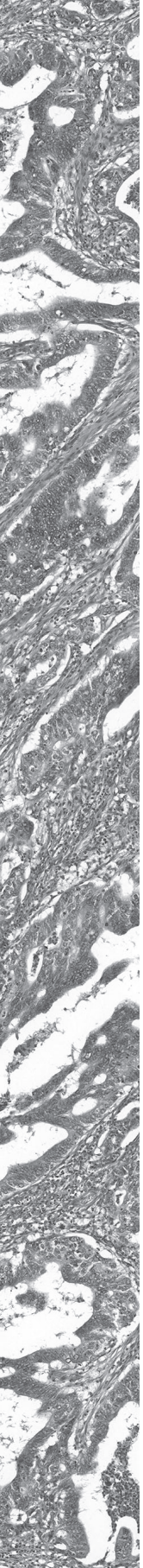


Summary

Globally, oesophagogastric cancer (OGCa) remains a major health problem with an estimated 1,407,000 new cases and 1,123,000 deaths each year. Patients with early stage OGCa are often asymptomatic. Due to the absence of an OGCa screening programme, patients in Western countries most commonly present with locally advanced disease at the time of diagnosis. Currently patient prognosis and treatment decisions in OGCa are based on TNM stage, patient's performance status and patient's preferences. However, OGCa patients with the same TNM stage can have very different outcomes. OGCa patients have a poor prognosis with a 5-year survival in Europe of 45-47% when diagnosed at a disease stage where the tumour is resectable and is treated with neoadjuvant/peri-operative chemo(radio)therapy and surgery. The survival benefit from neoadjuvant/peri-operative chemotherapy is modest at 6-14% improved 5-year survival compared to treatment by surgery alone, suggesting that only a subset of patients benefits from chemotherapy. Thus, there remains an urgent clinical need to identify biomarkers to individualise and improve OGCa patient management.

The **aim** of this thesis was to investigate prognostic and predictive biomarkers in locally advanced resectable OGCa. We first focussed on the molecular characterisation of the tumour cells and thereafter on the characterisation of the tumour microenvironment.

As *KRAS* and *BRAF* mutations in colorectal cancer are known predictors of poor response to EGFR targeting agents, in **chapter 2** we performed a literature review to analyze and summarize the current literature on *KRAS* and *BRAF* mutations, including *KRAS* amplifications in gastric cancer (GC). We included a total of 69 studies and found the current knowledge on *KRAS* and *BRAF* in GC to be limited due to small sample size of investigated tumours and the use of a variety of different methodologies, making any comparisons between studies difficult. The frequency of *KRAS* mutation and *KRAS* amplification is low (<10%) in GC. In particular, the frequency of *KRAS* mutations in GC is much lower than that in colorectal cancer. *KRAS* mutations and *KRAS* amplifications seem to be mutually exclusive, suggesting the potential need to screen GC patients for both genetic aberrations when searching for *KRAS* activation. *BRAF* V600E mutations are extremely rare in GC. So far, all clinical studies in unselected patients with metastatic GC have failed to show a significant benefit for EGFR targeting therapy. Post hoc analysis of the REAL3 trial showed no relationship between *KRAS* mutation status and EGFR treatment effect.

Studies in lung and ovarian cancer suggest a relationship between *KRAS* activation and histological phenotype. Therefore, we investigated whether *KRAS* mutation and/or *KRAS* amplification (collectively called *KRAS* activation) are also related to the histological phenotype in GC which could then potentially indicate whether KRAS activation is an early or late event in gastric cancer carcinogenesis **(chapter 3)**. Digitized Haematoxylin/Eosin stained slides from 1282 GC resection specimens were classified according to Japanese Gastric Cancer Association (JGCA) and the Lauren classification by at least two observers. *KRAS* mutation and *KRAS* amplification were found in 68 (5%) and 47 (7%) GCs, respectively. We confirmed a relationship between presence of *KRAS* mutation and mucinous phenotype in

GC as described in ovarian cancer and lung cancer. Interestingly, 724 GCs (57%) showed more than one histological phenotype. This relatively high level of intratumour morphological heterogeneity could reflect *KRAS* mutation heterogeneity, which may explain the failure of anti-EGFR therapy in GC.

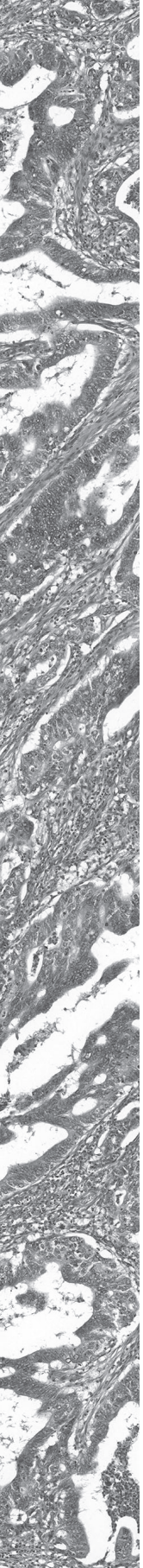
Immune checkpoint targeting therapy has recently shown promise in several cancer types. Proposed biomarkers to predict potential response to immune checkpoint inhibitors include DNA mismatch repair (MMR) and/or Epstein-Barr virus (EBV) status. Therefore, in **chapter 4,** we determined the frequency of EBV and MMR in a large multicentre series of 988 oesophageal cancer (OeC) and 1213 GC using EBV-encoded RNA *in situ* hybridisation and MMR protein expression by immunohistochemistry (IHC), respectively. In a large subset of OeC, we tested microsatellite instability (MSI) in parallel with MMR IHC. The frequency of MMR deficiency and MSI was very low in OeC (0.8% and 0.6%, respectively) and much lower than in GC (10.3%). None of the OeCs were EBER positive in contrast to 4.8% EBER positive GC. This is the largest study to date demonstrating that in contrast to GC, EBV and MMR deficiency do not play a role in OeC carcinogenesis. Thus, the potential clinical usefulness of determining MMR deficiency/EBV status to screen patients for eligibility for immune checkpoint targeting therapy differs between OeC and GC patients.

Whilst many OGCa studies have focused on the characterisation of tumour epithelial cells, there is a growing interest in the role of the tumour microenvironment in cancer development and progression. Therefore, in **chapter 5,** we investigated whether the intratumour heterogeneity of the tumour/stroma content in the diagnostic biopsy of OeC patients is related to survival after neoadjuvant chemotherapy. Firstly, we established a new method using a statistical mixed effect model (MEM) to measure intratumour heterogeneity of the proportion of tumour (IHPoT). We used the newly developed method to estimate IHPoT (variation of the proportion of tumour in haematoxylin/eosin stained pre-treatment biopsy pieces from the same patient) in the pre-treatment biopsies from 218 OeC OE02 trial patients. We found that patients with a low IHPoT index (biopsies from the same tumour have a similar proportion of tumour) had a survival benefit from cytotoxic chemotherapy. This is the first study suggesting that IHPoT measured in the pre-treatment biopsy can predict chemotherapy survival benefit in OeC patients. IHPoT may represent a clinically useful biomarker for patient treatment stratification.

Based on these biopsy findings we were interested to know which components of the stroma (including fibroblasts, extracellular matrix, vessels and immune cells) are contributing to its relationship with chemotherapy response. Studies in GC suggested a clinical value of tumour infiltrating lymphocytes (TILs) with respect to patient prognosis (52, 53), thus we selected TILs as our initial focus of investigation of the stroma components **(chapter 6)**. We analysed the number of lymphocytes per area (so called TIL density) in patients with resectable, stage II-III GC from the Korean phase III CLASSIC trial. We used image analysis software (MIM from HeteroGenius, UK) to build a colour model for the identification of

lymphocytes. We calculated the TIL density using digital haematoxylin and eosin (HE) stained tissue microarrays constructed from GC resection specimens from 629 CLASSIC trial patients. TIL density proved to be an independent prognostic and predictive biomarker for survival benefit from adjuvant chemotherapy. Patients with high TIL density GC had a significantly improved survival and derived little or no benefit from adjuvant chemotherapy (Xelox) compared with patients with low TIL density GC. Patients with low TIL had a significant benefit from adjuvant chemotherapy. We concluded that TIL density measured on routine HE stained tissue sections may represent a new clinically useful biomarker identifying GC patients who may not require adjuvant chemotherapy and for whom treatment could be de-escalated. Validation of these results following the biomarker roadmap principle is ongoing.

In **Chapter 7,** we discuss the implications of our research in the context of the current literature. We also critically discuss the problems and shortcomings of current OGCa prognostic and predictive biomarker studies. To address one aspect of this, we outline plans for validation studies in the near future to fully assess the prognostic and predictive value of TILs. We also discuss the potential role of emerging technologies in the clinical management of OGCa patients in the future.



Valorisation

Oesophagogastric cancer (OGCa) remains a major public health issue with an estimated 1,407,000 new cases and 1,123,000 deaths worldwide in 2012 (1). This is despite the decline in newly diagnosed gastric cancer (GC) cases in recent years (1). OGCa is often asymptomatic and patients usually present with advanced stage disease. The standard of care treatment for locally advanced resectable disease is neoadjuvant/peri-operative chemo(radio)therapy and surgery. Survival remains poor, with 5-year overall survival up to 47% (2). Patients presenting with metastatic disease have a median life expectancy of less than 12 months if treated with cytotoxic chemotherapy (3). Thus, OGCa represents a substantial burden to patients in terms of morbidity and mortality.

Cancer-related health care costs have increased over the past decades (4), with OGCa having one of the largest expenditures in cancer care during the first 12 months after initial diagnosis (5). The estimated national cost of OGCa healthcare in the US was 3.15 billion USD in 2010 (5). In the Netherlands, €121 million was spent on OGCa patient health care in 2011 (6). With the use of emerging technologies such as advanced endoscopic imaging and deep-sequencing based technologies, and the high costs of new targeted therapies, including immune checkpoint targeting therapy, the already considerable economic burden related to OGCa is predicted to rise.

The prognosis prediction and treatment decisions for OGCa patients are currently based on TNM staging (7). As the cost of OGCa patient care increases with disease stage (8, 9), early detection is an important factor in reducing the economic burden. However, population screening by endoscopy is only cost effective in areas with high incidence (10, 11). New, potentially cheaper methods of screening, such as the cytosponge are currently under investigation (12). Furthermore, there are currently no biomarkers implemented in the clinic that could be measured in the blood, urine or tissue with sufficient sensitivity and specificity for early detection of OGCa (13). As patients with the same stage of disease can have very different outcomes, there remains a need to individualise and improve OGCa patient management to benefit the patient and improve efficiency in healthcare expenditure.

There are currently no prognostic or predictive biomarkers used in clinical practice for the management of OGCa patients. A prognostic biomarker provides information on clinical outcome. A predictive biomarker indicates the likely benefit of a treatment. Both likely prognosis and likely benefit from a particular treatment, together with patient's wishes, are used to guide patient management decisions (14). In this thesis, we investigated prognostic and predictive biomarkers in the epithelial tumour cells **(chapter 2, 3 and 4)** and tumour microenvironment **(chapter 5 and 6)** of patients with locally advanced resectable OGCa.

In **chapter 4**, we investigated the frequency of Epstein-Barr virus (EBV) and mismatch repair (MMR) deficiency in OGCa as they have been suggested as potential biomarkers for patient selection for immunotherapy or adjuvant cytotoxic therapy. We found the frequency of EBV and MMR is extremely low in OGCa, thus a large number of patients would need to be screened to identify the few patients with 'positive' tumours. Hence, we concluded that it

may not be economically feasible to screen patients for these tumour based molecular markers. We have recently used deep learning to predict MSI status (a surrogate marker for MMR deficiency) based on HE stained images (not part of this thesis) (15). Whilst this may offer a cost effective solution, the results from this study require validation in independent datasets which are ongoing. However, the challenge remains, that EBV and MSI/MMR deficiency status would only be able to influence the management in a minority of OGCa patients.

Similarly, the frequency of *KRAS* mutation **(chapter 2)** and *KRAS* amplification **(chapter 3)** is low in GC. Aside from the low frequency and economic feasibility of *KRAS* testing in OGCa, anti-epidermal growth factor receptor (EGFR) therapy in *KRAS* mutant OGCa does not appear to be effective (3). This is in contrast to colorectal cancer, where routine testing for *KRAS* mutation is implemented as a predictor of response to EGFR therapy (16). Thus, there is a clinical need to understand the biological differences in response to EGFR therapy between colorectal cancer and GC.

In subsequent chapters we expanded our work to the tumour microenvironment. In **chapter 5** we stratified oesophageal cancer (OeC) patients according to survival benefit from neoadjuvant chemotherapy based on the proportion of tumour/stroma heterogeneity between OeC biopsy pieces from the same patient. Patients with a low level of morphological heterogeneity had a survival benefit from cytotoxic chemotherapy. This was an exploratory, hypothesis generating image analysis based study which requires validation. If validated, future studies need to assess whether proportion of tumour/stroma heterogeneity can be assessed by a pathologist on routine haematoxylin and eosin (HE) stained slides.

In a separate study using tissue from 629 patients recruited into the Korean CLASSIC trial, we showed for the first time that tumour infiltrating lymphocyte (TIL) density measured on haematoxylin and eosin (HE) stained resection specimens may be used as a biomarker to predict survival benefit from adjuvant chemotherapy in GC patients **(chapter 6)** . Patients with high TIL density had little or no survival benefit from adjuvant cytotoxic chemotherapy and may therefore be potential candidates for treatment de-escalation. The results of this study also require validation but may have the potential to reduce patient morbidity due to (unnecessary) chemotherapy as well as reducing the healthcare costs normally related to the treatment of OGCa patients with adjuvant chemotherapy. From the patient perspective, the use of HE based TIL density as a predictive biomarker may offer more certainty about the potential success of chemotherapy. This may help to reduce the impact of unnecessary physical and psychological side effects of chemotherapy (either temporary or permanent), enabling patients to return to work earlier, thus reducing the financial impact of their illness on themselves and their families. Ultimately, predictive biomarkers may be able to improve outcomes and quality of life for OGCa patients. From the economic perspective, predictive biomarkers in OGCa to stratify patients for treatment with cytotoxic chemotherapy has the potential to improve the efficiency of the treatment and make it more cost-effective as only those patients who benefit and require chemotherapy would be treated. Importantly, for

prognostic and predictive biomarkers to reduce healthcare costs, the investment in technology should not offset the savings. As we performed TIL density using routine diagnostic HE stained slides produced at the time of pathological evaluation of the resection specimen and image analysis software, the costs are minimal and this test could be introduced relatively quickly into the routine setting, after appropriate validation. Studies in breast cancer have shown that TIL density on the HE can be assessed manually, thus reducing the cost even further. The results of this pilot work in the CLASSIC trial patients allowed us to obtain a Cancer Research UK project grant for validation and assessment of introduction of HE based TIL density into the routine clinic.

In conclusion, the knowledge generated in this thesis is not only of scientific importance, but will likely have societal and economic impact in the future. If validated, as described in the future perspectives **(chapter 7)**, HE based TIL density has the potential to improve the clinical management of GC patients while reducing expenditure on expensive chemotherapeutic drugs by ensuring only those patients benefiting from the drugs will be treated.

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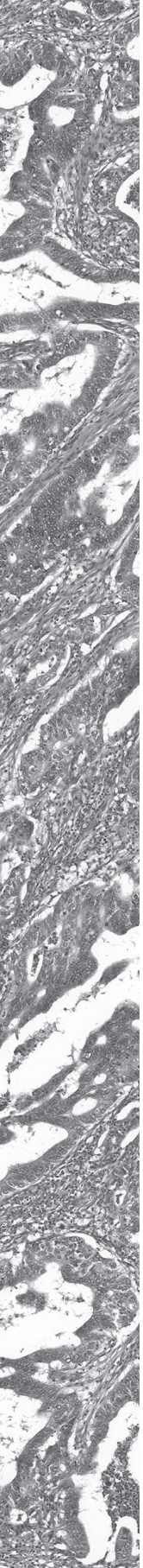

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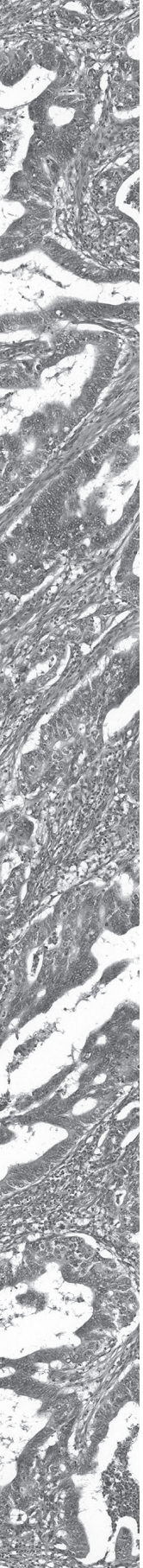

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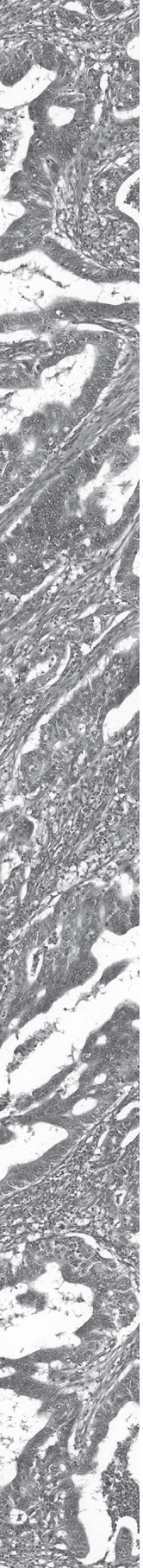
Lindsay Charlotte Hewitt (née Ward) was born on 10th August 1983 in Bradford, UK. Her interest in science began at school and she studied for her Bachelor degree in Molecular Cell Biology at the University of York, UK. After graduating in 2004, Lindsay worked in the histology and cytology department at IDEXX, an animal diagnostic laboratory. In 2006, whilst continuing to work, she began studying part-time for her Master's degree in Pathological Sciences at Sheffield Hallam University, UK. After graduating in 2008, Lindsay wanted to pursue a career in cancer research and began working as a research technician at the Faculty of Medicine and Health, Section of Pathology and Tumour Biology, University of Leeds, based at St James's University Hospital, UK. Lindsay provided pathological and molecular laboratory support, and was involved in the retrospective collection of tissue samples in the multicentre randomised controlled clinical trial of pre-operative chemotherapy in resectable oesophageal cancer (Oe02) and biological sample collection (blood and tissue) from patients entering the multicentre randomised controlled OE05 and ST03 clinical trials. In 2011**,** she was promoted to senior research technician. In March 2015, Lindsay moved to The Netherlands to start work as a PhD student in the Pathology Department, Maastricht University Medical Center+, under the supervision Prof. dr Grabsch and Dr Melotte, investigating prognostic and predictive biomarkers in oesophagogastric cancer.

In October 2019 Lindsay started working as Clinical Safety Specialist at Medtronic Bakken Research Center in Maastricht.



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