KRAS, BRAF and gastric cancer

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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 (CRC) are known predictors of poor response to epidermal growth factor receptor (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 CRC 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.

Keywords: Gastric cancer (GC); KRAS; BRAF; mutation; gene amplification

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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 a 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 peri-operative chemotherapy followed by surgery (6). Thus, there is an urgent clinical need to identify new potential drug targets in order to 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 International Union Against Cancer (UICC) (12), American Joint Committee on Cancer (AJCC) (13) or Japanese Gastric Cancer Association (JGCA) (14) Tumor Node Metastasis (TNM) staging system which follow same principals but have some minor variations.

Molecular aberrations are known to play an important role in the development of GC. In addition to mutations in oncopgenes, 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 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 GC patient treatment 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).

Eligibility to be included in the current review was restricted to original articles reporting 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 (GI) stromal tumors, and cell line studies were excluded. The reference lists of publications 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 MMR status and MSI, and DNA ploidy was noted.
Results

The initial database searches found 1,369 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.

The 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 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 (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 GC**

**KRAS mutations**

The first report of a KRAS mutation in a single 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 Tables 2,3). 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.

**GC 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 (37-39,41,42,44,45,47,48,50-52) embedded tissue samples (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 (38,39,44,46,47,50-52,54,60-64,67,68) from resection specimens (70,71,73-76,78-82,84,88-91,93,95-98), ten (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 eight (36%) studies investigated locally advanced GC (40-44,61,62,82), four (18%) studies metastatic and advanced GC (48,49,81,94), three (14%) studies early GC (45,65,84), two (9%) studies metastatic GC (66,90), two (9%) studies compared early with advanced GC (46,93), one (5%) study intestinal GC (47), one (5%) study MSI GC (85) and one study (5%) investigated GC with comitant 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
### Table 2 Published literature on KRAS mutation status in gastric cancer excluding studies testing chemotherapeutic agents

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Origin</th>
<th>Total, n</th>
<th>mut KRAS, n [%]</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Victor et al.</td>
<td>1990</td>
<td>South Africa</td>
<td>11</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>Kihana et al.</td>
<td>1991</td>
<td>Japan</td>
<td>35</td>
<td>3 [9]</td>
<td>Three of seven adenoma had mut KRAS; mut KRAS in well diff GC only</td>
</tr>
<tr>
<td>Miki et al.</td>
<td>1991</td>
<td>Japan</td>
<td>31</td>
<td>4 [13]</td>
<td>mut KRAS only found in intestinal-type GC</td>
</tr>
<tr>
<td>Ranzani et al.</td>
<td>1993</td>
<td>Europe</td>
<td>32</td>
<td>3 [9]</td>
<td>One mut KRAS also had allelic losses</td>
</tr>
<tr>
<td>Craanen et al.</td>
<td>1995</td>
<td>Europe</td>
<td>45</td>
<td>0</td>
<td>Only early GC tested</td>
</tr>
<tr>
<td>Hongyo et al.</td>
<td>1995</td>
<td>Europe</td>
<td>34</td>
<td>7 [21]</td>
<td>Only intestinal-type GC tested; no mut KRAS in stage III</td>
</tr>
<tr>
<td>Lee et al.</td>
<td>1995</td>
<td>South Korea</td>
<td>140</td>
<td>11 [8]</td>
<td>mut KRAS more common in DNA aneuploid and in upper third GC</td>
</tr>
<tr>
<td>Iwaya et al.</td>
<td>1998</td>
<td>Japan</td>
<td>5</td>
<td>1 [20]</td>
<td>Synchronous primary cancers of the esophagus and other organs</td>
</tr>
<tr>
<td>Russo et al.</td>
<td>2001</td>
<td>Europe</td>
<td>63</td>
<td>5 [8]</td>
<td>mut KRAS not related to DNA ploidy</td>
</tr>
<tr>
<td>Lee et al.</td>
<td>2002</td>
<td>South Korea</td>
<td>71</td>
<td>1 [1]</td>
<td>–</td>
</tr>
<tr>
<td>Yoo et al.</td>
<td>2002</td>
<td>South Korea /US</td>
<td>104</td>
<td>10 [10]</td>
<td>mut KRAS related to intestinal-type GC and higher pT</td>
</tr>
<tr>
<td>Hiyama et al.</td>
<td>2002</td>
<td>Japan</td>
<td>48</td>
<td>4 [8]</td>
<td>mut KRAS related to well diff histology type, younger age and H. pylori infection</td>
</tr>
<tr>
<td>Lee et al.</td>
<td>2003</td>
<td>South Korea</td>
<td>319</td>
<td>9 [3]</td>
<td>mut KRAS related to advanced GC</td>
</tr>
<tr>
<td>Brennetot et al.</td>
<td>2003</td>
<td>Europe</td>
<td>82</td>
<td>10 [12]</td>
<td>mut KRAS only seen in MSI not in MSS GC</td>
</tr>
<tr>
<td>Wu et al.</td>
<td>2004</td>
<td>Japan</td>
<td>62</td>
<td>1 [2]</td>
<td>mut KRAS GC related to MSI; KRAS and BRAF mutations were exclusive</td>
</tr>
<tr>
<td>Zhao et al.</td>
<td>2004</td>
<td>China</td>
<td>94</td>
<td>8 [9]</td>
<td>Seven of eight GC with mut KRAS were MSI. All mut KRAS in GC from antrum</td>
</tr>
<tr>
<td>Tajima et al.</td>
<td>2006</td>
<td>Japan</td>
<td>133</td>
<td>7 [5]</td>
<td>Only early GC tested; no KRAS mutation in 63 gastric adenoma</td>
</tr>
<tr>
<td>Gylling et al.</td>
<td>2007</td>
<td>Japan</td>
<td>59</td>
<td>4 [7]</td>
<td>mut KRAS only seen in MSI not in MSS GC</td>
</tr>
<tr>
<td>Tajima et al.</td>
<td>2007</td>
<td>Japan</td>
<td>134</td>
<td>8 [6]</td>
<td>Only differentiated GC tested</td>
</tr>
</tbody>
</table>

Table 2 (continued)
Table 2 (continued)

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Origin</th>
<th>Total, n</th>
<th>mut KRAS, n [%]</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liu et al.</td>
<td>2009</td>
<td>China</td>
<td>52</td>
<td>5 [10]</td>
<td>mut KRAS only seen in males</td>
</tr>
<tr>
<td>Mita et al.</td>
<td>2009</td>
<td>Japan</td>
<td>86</td>
<td>0</td>
<td>5% KRAS amp</td>
</tr>
<tr>
<td>Betge et al.</td>
<td>2011</td>
<td>Austria</td>
<td>12</td>
<td>1 [8]</td>
<td>GC with concomitant renal cancer</td>
</tr>
<tr>
<td>Liu et al.</td>
<td>2011</td>
<td>China</td>
<td>58</td>
<td>6 [10]</td>
<td>mut KRAS only seen in males</td>
</tr>
<tr>
<td>Corso et al.</td>
<td>2011</td>
<td>Europe</td>
<td>63</td>
<td>11 [17]</td>
<td>Only MSI GC tested; mut KRAS more common in elderly patients</td>
</tr>
<tr>
<td>Chen et al.</td>
<td>2011</td>
<td>China</td>
<td>123</td>
<td>12 [10]</td>
<td>KRAS tested in blood</td>
</tr>
<tr>
<td>Saxena et al.</td>
<td>2012</td>
<td>India</td>
<td>62</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>Deng et al.</td>
<td>2012</td>
<td>Singapore</td>
<td>139</td>
<td>1 [1]</td>
<td>9% KRAS amp</td>
</tr>
<tr>
<td>Kim et al.</td>
<td>2013</td>
<td>South Korea/Japan</td>
<td>30</td>
<td>2 [7]</td>
<td>mut KRAS associated with CIMP</td>
</tr>
<tr>
<td>Warneke et al.</td>
<td>2013</td>
<td>Europe</td>
<td>475</td>
<td>17 [4]</td>
<td>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</td>
</tr>
<tr>
<td>Kim et al.</td>
<td>2014</td>
<td>South Korea</td>
<td>89</td>
<td>3 [3]</td>
<td>Only metastatic GC tested. KRAS amp in two cases; one case had increased copy number</td>
</tr>
<tr>
<td>Qian et al.</td>
<td>2014</td>
<td>China</td>
<td>131</td>
<td>8 [6]</td>
<td>mut KRAS and KRAS amp (5%) mutually exclusive; associated with different outcomes</td>
</tr>
<tr>
<td>Ali et al.</td>
<td>2015</td>
<td>USA</td>
<td>116</td>
<td>12 [10]</td>
<td>6% KRAS amp. Includes 36 samples from metastatic sites</td>
</tr>
<tr>
<td>Lu et al.</td>
<td>2015</td>
<td>China</td>
<td>156</td>
<td>7 [4]</td>
<td>mut KRAS associated with pN0 GC</td>
</tr>
<tr>
<td>Cristescu et al.</td>
<td>2015</td>
<td>South Korea</td>
<td>223</td>
<td>18 [8]</td>
<td>8% KRAS amp</td>
</tr>
<tr>
<td>Yoda et al.</td>
<td>2015</td>
<td>Japan</td>
<td>50</td>
<td>4 [8]</td>
<td>8% KRAS amp</td>
</tr>
</tbody>
</table>

mut KRAS, mutant KRAS; GC, gastric cancer; pT, tumor invasion depth; MSI, microsatellite instability; MSS, microsatellite stable; well diff, well differentiated; pN, lymph node metastasis; CIMP, CpG island methylator phenotype; KRAS amp, KRAS amplification.
Table 3 Published literature on KRAS mutation status in gastric cancer studies investigating chemotherapeutic agents

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Origin</th>
<th>Total, n</th>
<th>mut KRAS, n [%]</th>
<th>Stage of disease</th>
<th>Treatment</th>
<th>Sample type used for KRAS testing</th>
<th>Mutant KRAS relationship to survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pinto et al.</td>
<td>2009</td>
<td>Europe</td>
<td>32</td>
<td>3 [9]</td>
<td>Advanced unresectable. Include some junctional cancer</td>
<td>Cetuximab + cisplatin and docetaxel</td>
<td>Not specified</td>
<td>Not reported (no association with ORR)</td>
</tr>
<tr>
<td>Han et al.</td>
<td>2009</td>
<td>South Korea</td>
<td>38</td>
<td>0</td>
<td>Recurrent metastatic</td>
<td>Cetuximab + oxaliplatin/leucovorin/5-fluorouracil</td>
<td>Not specified</td>
<td>No mut KRAS</td>
</tr>
<tr>
<td>Park et al.</td>
<td>2010</td>
<td>South Korea</td>
<td>30</td>
<td>4 [13]</td>
<td>Metastatic</td>
<td>Cetuximab + chemotherapy</td>
<td>Primary tumor</td>
<td>No association with PFS and OS</td>
</tr>
<tr>
<td>Lordick et al.</td>
<td>2010</td>
<td>Europe</td>
<td>52</td>
<td>1 [2]</td>
<td>Metastatic or locally advanced unresectable</td>
<td>Cetuximab + oxaliplatin/leucovorin/5-fluorouracil</td>
<td>Not specified</td>
<td>Not reported</td>
</tr>
<tr>
<td>Moehler et al.</td>
<td>2011</td>
<td>Europe</td>
<td>29</td>
<td>0</td>
<td>Advanced</td>
<td>Sunitinib monotherapy</td>
<td>Not specified</td>
<td>No mut KRAS</td>
</tr>
<tr>
<td>Rohrberg et al.</td>
<td>2011</td>
<td>Europe</td>
<td>7</td>
<td>2 [29]</td>
<td>Advanced</td>
<td>Erlotinib + bevacizumab</td>
<td>Not specified</td>
<td>No association with PFS, OS and DC</td>
</tr>
<tr>
<td>Woll et al.</td>
<td>2011</td>
<td>Europe</td>
<td>13</td>
<td>0</td>
<td>Metastatic or locally advanced unresectable</td>
<td>Oxaliplatin, irinotecan + cetuximab</td>
<td>Biopsies/resected primary tumor</td>
<td>No mut KRAS</td>
</tr>
</tbody>
</table>

mut KRAS, mutant KRAS; PFS, progression free survival; OS, overall survival; DC, disease control; ORR, objective response rate; RR, response rate.
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, six 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 two of the seven 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 a 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 (19,37,38,40,46,47,50-54,56,60,62-64) and one or more clinicopathological variables (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 found 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), three (16%) studies
reported that KRAS mutations were exclusively found in males (53,54,63) whereas four (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 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 (pT2-4) 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 2,583 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 (19,37,38,40,56,60,62,63,68,72,75,76,88,91,93).

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).

**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 3). Four studies (40%) did not find any association between *KRAS** mutation status and progression free survival (PFS) or overall survival (OS) (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 MMR deficiency/MSI (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).
**The 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 splicing variants, 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 GC**

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% (38,39,42,43,48,50,52,61,62,68,70,75,81,88,93,109-112). Only six of the *BRAF* mutations identified were in exon 15 (38,40,70,110,112). Six mutations were found in codon 396 and four mutations in codon 608 of exon 15 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 MSI 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 GC

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

The current literature reporting on 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 OS 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 (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 ten 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 affect 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). 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 GC, small bowel and colorectal cancer (CRC)

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% based on data from four studies investigating each less than 100 patients and is therefore partly comparable to that of GCs and partly similar to CRCs (51,119-121).

In contrast to GC, in CRC many studies have reported a significant association between BRAF mutation and either deficient MMR status or MSI (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 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%) investigated 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 potential 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 GC could potentially be a promising treatment option in the future.

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Footnote
Conflicts of Interest: The authors have no conflicts of interest to declare.

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