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Mutually exclusive *FGFR2*, *HER2*, and *KRAS* gene amplifications in gastric cancer revealed by multicolour FISH

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ABSTRACT

Gastric cancer (GC) is a major cause of global cancer mortality. Previous genomic studies have reported that several RTK-RAS pathway components are amplified in GC, with individual tumours often amplifying one component and not others ("mutual exclusivity"). Here, we sought to validate these findings for three RTK/RAS components (*FGFR2, HER2, KRAS*) using fluorescence in situ hybridisation (FISH) on a series of gastric tumours, cell lines and patient-derived xenografts. Applying dual-colour FISH on 137 gastric tumours (89 FFPE surgical resections and 48 diagnostic biopsies), we observed *FGFR2* amplification in 7.3% and *HER2* amplification in 2.2% of GCs. GCs exhibiting *FGFR2* amplification were associated with high tumour grade ($p = 0.034$). In FISH positive tumours, striking differences in copy number levels between cancer cells in the same tumour were observed, suggesting intra-tumour heterogeneity. Using a multicolour FISH assay allowing simultaneous detection of *FGFR2, HER2*, and *KRAS* amplifications, we confirmed that these components exhibited a mutually exclusive pattern of gene amplification across patients. The FISH data were also strongly correlated with Q-PCR levels and at the protein level by immunohistochemistry. Our data confirm that RTK/RAS components are mutually exclusively amplified in GC, and demonstrate the feasibility of identifying multiple aneuploidies using a single FISH assay. Application of this assay to GC samples, particularly diagnostic biopsies, may facilitate enrollment of GC patients into clinical trials evaluating RTK/RAS directed therapies. However, the presence of intra-tumour heterogeneity may require multiple biopsy samples to be obtained per patient before a definitive diagnosis can be attained.

Introduction

Gastric cancer (GC) is the second leading cause of cancer death worldwide with Eastern Asia having the highest incidence rate [1]. Due to lack of symptoms at early stages of GC, most patients are diagnosed at advanced disease stages with median survival times of 8–12 months [2]. Besides environmental factors such as *Helicobacter pylori* infection, multiple genetic and epigenetic alterations can contribute to the progression and development of GC [3–5]. Improving the survival of GC patients will require the development of new diagnostic methods and anti-tumour treatment modalities.

Previous studies have shown that several components of the RTK-RAS signalling pathway can exhibit genomic amplification in GC. Examples of such factors include fibroblast growth factor receptor 2 (*FGFR2*; 10q), the human epidermal growth factor receptor 2 oncogene (*HER2*; 17p) and the Kirsten-Ras oncogene (*KRAS*; 12p) [6]. We recently performed a genome-wide study employing single nucleotide polymorphism (SNP) arrays and found that these three genes (and also other RTK/RAS components) were amplified in a mutually exclusive manner across different GCs [7] that is, individual GCs exhibiting amplification of one factor rarely amplified the other factors. Similar patterns of exclusivity have been observed in other cancers, including *EGFR* and *KRAS* mutations in lung cancer [8], or *KRAS* and *BRAF* mutations in colon cancer [9]. It has been proposed that such patterns may be due to the different factors resulting in activation of the same "core" oncogenic pathway in tumours.

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Mutually exclusive patterns of genomic and mutational aberration are also of clinical relevance, by providing a framework to stratify patients into different subgroups that might be treated with distinct therapies.

FGFR2 is a receptor tyrosine kinase (RTK) regulating cell growth and development. *FGFR2* amplifications have been reported in 4–10% of GC, and associated with poor prognosis and diffuse-type GC [10,11], where FGFR2 protein overexpression has also been reported [12,13]. *FGFR2* gene amplifications and protein overexpression have also been demonstrated in small subsets of breast cancer [14–16]. *HER2*, another RTK, is amplified and over expressed in breast cancer, colorectal and GC [17,18]. In GC, protein overexpression of membranous HER2 has been observed in 8–53% of tumours by immunohistochemistry (IHC) [19,20] and 7.1% of GC tumours by both FISH and IHC [18]. Trastuzumab, a monoclonal antibody targeting *HER2*, was recently shown to improve overall survival specifically in patients with *HER2* positive GC when combined with standard chemotherapy [21,22].

KRAS encodes a membrane bound 21 kDa small GTPase protein. Activating mutations in *KRAS* gene have been reported in 25–30% of almost all solid tumours [23]. However, in certain tumour types such as GC, the *KRAS* mutation rate is particularly low [24], suggesting the existence of other mechanisms besides mutation that may activate this important oncogene. Recent studies have suggested that genomic amplification of wild-type *KRAS* may be one such mechanism [25]. In GC, the *KRAS* amplification rate has been reported as 9% [7], and *KRAS* amplifications have been observed in ~15% non-small cell lung cancers (NSCLC) [26]. *KRAS* amplifications have also been observed in endometrial cancer, where they are associated with poor outcome and an aggressive phenotype [27].

At present, a variety of different technology platforms are available to quantitate genomic amplifications in cancer samples, such as SNP arrays, quantitative PCR, MLPA, and next-generation sequencing (NGS). However, in most routine diagnostic pathology laboratories, FISH remains the gold standard for identifying genomic amplifications. Advantages of FISH, compared to other technologies, is based on its ability to precisely count copy number levels in individual nuclei (*HER2* in breast cancer), and also for identifying gene fusions particularly in situations where the multiple fusion partners may be involved (*BCR-Abl* in leukaemias, *ALK-EML4* in non-small cell lung cancers) or the breakpoint is variable. In the present study, we sought to develop a robust multi-colour FISH assay that would permit the simultaneous detection of three RTK-RAS pathway genes (*FGFR2, HER2* and *KRAS*) in a variety of GC-related samples, including surgical resection specimens, diagnostic biopsies, and cell lines. Using this assay, we confirmed the mutually exclusive pattern of RTK/RAS gene amplification and investigated overall levels of gene heterogeneity in gene amplification amongst FISH-positive GCs.

Materials and methods

Patient demography

A total of 137 GC cases diagnosed at the National Cancer Centre from 1997 to 2012 that had consented to the use of their tumours for research purpose were included in this study. All GC specimens (89 primary tumours and 48 biopsies) were acquired in compliance with the guidelines of the Institutional Review Board (NUS-IRB-1508 and 2009/907/B). Table 1 shows the patient characteristics. Histopathological staging was based according to the American Joint Committee on Cancer classification, 6th edition [28]. Gastric tumour types were classified according to Lauren's classification [29] and tumour grade according to WHO classification [30]. Patients were followed up from the date of surgery until death for the survival analysis. The patients with primary tumours were surgically treated but 15 of the 48 biopsy patients did not undergo surgery. Patients who were lost to follow-up or died from other causes at the time of analysis were censored at their last date of follow up. GC xenografts (n = 2) were generated using GC cells isolated from malignant ascites of patients that had undergone surgery for signet ring cell carcinoma. These xenografts were shown to be *FGFR2* amplified by next generation exome sequencing analysis using allele specific copy number analysis (ASCAT2.0) software.

Labelling and validation of locus specific BAC probes

We designed *FGFR2* and *KRAS* FISH probes based on the locations of amplified genomic regions from our previous study [7]. Bacterial artificial chromosome (BAC) clones RP11-621P20 (*FGFR2*) and RP11-295I5 (*KRAS*) (CHORI, Oakland, CA, USA) were streaked, cultured and DNA isolated using a QIA maxi-plasmid kit (Qiagen, Venlo,

Table 1
FISH data correlated with clinicopathological parameters.

Clinicopathological features	Number of cases	FGFR2 amplification	FGFR2 deletion	FGFR2 diploid	p value	HER2 amplification	HER2 deletion	HER2 diploid	p value
Gender	N = 137								
Male	79	4	5	70	0.492	2	19	58	0.434
Female	58	6	3	49		1	9	48	
Age (years)									
<65 years	61	6	5	50	0.314	1	12	48	0.901
≥65 years	76	4	3	69		2	46	58	
Tumour stage	N = 100								
pT1/pT2	54	4	4	46	0.940	1	12	41	0.774
pT3/pT4	46	4	4	38		1	13	32	
N stage									
Negative	26	1	1	24	0.406	2	8	16	0.034*
Positive	74	7	7	60		0	17	57	
M stage									
M0	71	5	5	61	0.563	2	19	50	0.812
M1	29	4	2	23		1	6	22	
Grade									
Well and Mod	34	1	0	33	0.034*	2	9	23	0.117
Poor and Undiff	66	8	7	51		0	15	51	
Lauren classification	N = 84								
Intestinal	39	3	2	34	0.486	1	9	29	0.969
Diffuse	33	4	2	27		1	9	23	
Mixed	12	0	2	10		0	3	9	

FGFR2 and *HER2* copy number alterations (gene amplifications and deletions) and diploid status are correlated with clinicopathological features in FFPE primary and biopsy GC tissues [clinicopathological information such as the TNM status was available only for 100 GC patients and Lauren's classification for 84 patients due to no surgical record in most of the biopsy cases]. P values of the correlation between copy numbers and clinicopathological variables are given.
Mod, moderately differentiated; Undiff, undifferentiated.
* p value < 0.05 is significant.