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Frequent co-amplification of receptor tyrosine kinase and downstream signalling genes in Japanese primary gastric cancer and conversion in matched lymph node metastasis.

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Competing Interests: J Coffa, I Zondervan and S Savola are employed by MRC-Holland b.v., manufacturer of commercially available MLPA probemixes. All other authors declare no competing interests.

Running Head: Gene Co-amplifications and Conversion in Japanese Gastric Cancer

INTRODUCTION

Despite a decline in incidence over the last decade, gastric cancer (GC) remains a major cause of global cancer mortality[1]. GC is particularly common in Asian countries[2] with the highest incidences reported in South Korea and Japan[3]. Amplification of receptor tyrosine kinases (RTKs) such as *EGFR*, *HER2*, *FGFR2* and *MET* has been associated with GC pathogenesis and tumour progression[4-7]. Recent studies including our own seem to indicate that RTK gene amplification in primary GC (primGC) occurs in a mutually exclusive manner suggesting that targeting an individual RTK may be an effective treatment[8-10]. The ToGA trial demonstrated benefit from HER2 targeted treatment using trastuzumab in patients with HER2 positive metastatic GC[11 12]. However, all other currently available HER2, EGFR and MET targeted drugs (lapatinib, T-DM1, cetuximab, panitumumab, rilotumumab, and onartuzumab) have been less successful in GC patients raising the question about potential mechanisms of resistance against RTK targeted therapy[13 14].

Based on recent studies in colorectal cancer[15 16], breast cancer[17-19] and non-small cell lung cancer cell lines[20 21], one suggested potential resistance mechanism could be co-existing amplifications of genes located downstream of RTK such as *KRAS*, *PIK3CA*, *CCNE1* and *MYC* (collectively called downstream signalling (DSS) genes from hereon)[15-20 22]. In GC cell lines, co-amplification of *MET* and *KRAS* was related to resistance to MET inhibitors and *MET* amplification to resistance to the HER2 and EGFR targeted drug lapatinib[23]. Furthermore, co-amplification of *EGFR* and *HER2* has been associated with HER2 targeted therapy resistance in GC[24].

A second potential resistance mechanism could be related to genetic heterogeneity resulting in a discordant gene copy number status between primary cancer and metastatic site[25] as reported recently in cancers of breast[26], colorectal[27], renal[28], larynx/pharynx[29] and liver[30]. In GC, up to 10% discordance of *HER2* gene copy number status between primGC and matched lymph node metastasis has been reported[31 32] and was related to poorer prognosis[33]. However, the concordance of the gene copy number status between primGC and matched lymph node metastasis (LNmet) has not been investigated for other RTKs or DSSs and there are no studies evaluating the presence of RTK and DSS gene co-amplifications in this context.

We hypothesised that (1) co-amplification of RTK (*HER2*, *EGFR*, *MET*, *FGFR2*) and DSS (*PIK3CA*, *KRAS*, *CCNE1*, *MYC*) is a common phenomenon in primGC, (2) patients with co-amplification of RTK and DSS in the primGC have the poorest overall survival, and that (3) the RTK and DSS gene copy number status will only be discordant between primGC and their matched LNmet in a minority of patients.

The aim of this study was to develop a new multiplex ligation-dependent probe amplification (MLPA) probemix for the simultaneous assessment of RTKs (*EGFR*, *FGFR2*, *MET*, *HER2*) and DSSs (*KRAS*, *PIK3CA*, *CCNE1*, *MYC*) gene copy number status using DNA extracted from formalin fixed paraffin embedded tissue of primGC and matched LNmet and to establish the relationship between RTKs and DSSs gene copy number status with clinicopathological data and patient survival.

MATERIAL AND METHODS

Patients

After quality control, results were available from 237 patients with TNM 7th ed [34] stage II/III/IV gastric cancer who underwent potentially curative surgery at the Kanagawa Cancer Center Hospital (KCCH, Yokohama, Japan) between 2001 and 2010. 101 (43%) patients were treated by surgery alone and 136 by surgery and adjuvant cytotoxic chemotherapy. **None of the patients included in this study received targeted therapy.** Demographical and clinicopathological data including depth of invasion (pT category), lymph node status (pN category), distant metastasis (pM category), histological subtype according to Lauren classification[35] and tumour location according to the Japanese classification[36] were retrieved from local hospital records. Patient follow-up data and other parameters were obtained from histopathology reports and hospital records. Median follow-up time from surgery was 3.5 years, ranging from 0.3 to 9.5 years. This study was approved by the Local Research Ethics Committee.

Gastric cancer cell lines

DNA from 13 GC cell lines (CLS145, HS746T, KATOIII, MKN1, MKN7, NCI-N87, NUGC4, SNU16, YCC1, YCC11, YCC3, YCC7 and YCC9) with previously described gene copy number status[9] was extracted using the Qiagen genomic DNA extraction kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer and used to assess the sensitivity and specificity of the newly developed Multiplex Ligation Probe Amplification (MLPA) probemix.

DNA was extracted from eight EDTA blood samples from healthy human donors using the Qiagen DNAeasy Blood and Tissue kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer and used as reference sample for the GC cell lines in the MLPA probemix.

DNA Extraction from gastric cancer tissue samples

All Haematoxylin/Eosin stained slides from all 241 formalin fixed paraffin embedded (FFPE) gastrectomy specimens were reviewed. From all patients, a block from the primary tumour with the highest number of tumour cells per area was selected. From 128 patients with regional lymph node metastases, a block from the lymph node metastasis with the highest number of tumour cells per area was selected. DNA extraction was performed as described previously[37]. DNA concentration was measured by ND-100 Spectrophotometer (Labtech International) and adjusted to a final concentration of 100ng/μl. DNA from 12 normal FFPE tonsils was extracted using the same method, pooled and used as reference sample in the MLPA probemix.

MLPA experiment

The MLPA protocol by Schouten *et al* [38] was slightly modified by increasing the number of PCR cycles from 33 to 35. MLPA probemix and reagents (Salsa MLPA reagent kit, Salsa PCR reagents, Salsa polymerase, Ligase-65) were developed and supplied by MRC-Holland b.v., Amsterdam, NL. Each experimental run included DNA from GC cell lines, GC tissue (primary GC (primGC) and/or lymph node metastasis (LNmet)), reference sample (blood or tonsils), as well as negative controls (no DNA).

The first 90 cases were run in duplicates to assess reproducibility of results between runs. In 7 out of 720 (1%) of duplicates, there was a different result which would lead to a different gene copy number status class. Considering this high degree of reproducibility, it was felt that running samples in duplicates for the entire cohort was unnecessary.

The MLPA reaction product was sequenced on an ABI 3130 XL Sequencer (ABI Biosystems, California, USA). The output files from the sequencer (FSA files) were imported into Coffalyser.Net software version 130202.2357 [39] for analysis. Samples with a Coffalyser analysis score (CAS) of less than 50% were considered to be of poor quality and excluded from further analyses. Failed experiments were repeated at least twice before a case was finally excluded from the analyses. Four primGC and fifteen LNmet had to be excluded following Coffalyser quality control analysis (Figure 1).

Reference samples (DNA from blood or tonsils as appropriate) with a CAS of 100% were used to determine the normal range of values for the peak height recognition. Based on the results from our normal reference samples and in accordance with previously published studies[40 41], we set the thresholds as follows: a gene copy number ratio of less than 0.80 was categorised as 'loss', between 0.80-1.30 as 'normal', between 1.31-2.00 as 'low level amplification', between 2.01-5.00 as 'high level amplification' and above 5.00 as 'very high level amplification'.

MLPA assay

For further details on the MLPA probemix development, data analysis, use of competitor mixes for very highly amplified genes and studies to validate the assay, see supplementary document Table S1, S2 and Figure S1.

Statistical analyses

Statistical analyses were performed using PASW Statistics 21 (IBM Corporation, New York, USA) analysing the gene copy number status of receptor tyrosine kinases (RTKs) *EGFR*, *FGFR2*, *MET*, *HER2* and downstream signalling pathway genes (DSSs) *KRAS*, *PIK3CA*, *CCNE1* and *MYC*. All analyses described below were performed separately for primGC and LNmet. PrimGC or LNmet with low, high and very high level amplification (see MLPA experiment description above) were grouped together as 'amplification' and compared to samples with deletion or normal gene copy number status.

Co-amplification was defined as two or more genes having a gene copy number ratio >1.30. The Chi-squared test was used to analyse the relationship between gene copy number statuses of different genes. The relationship between gene copy number status and clinicopathological variables in the primGC and gene copy number status in the LNmet was analysed using the Kruskal-Wallis test.

For survival analyses, primGC or LNmet with a gene copy number ratio of all RTK genes ≤ 1.30 were classified as 'no RTK amplification', with a gene copy number ratio of a single RTK gene > 1.30 were classified as 'RTK amplification' and those with a gene copy number ratio of more than one RTK gene > 1.30 were classified as 'RTK co-amplification'. The same classification was used for DSS gene copy number status. GC with 'no DSS amplification + no RTK amplification' were compared to GC with 'no DSS amplification + RTK amplification', 'DSS amplification + no RTK amplification', as well as with 'DSS amplification + RTK amplification'.

In cases with amplifications, the status of the amplified gene(s) was compared between primGC and LNmet to identify so called 'conversion': (1) negative conversion was defined as gene amplification (copy number > 1.30) detectable in the primGC which was not detectable in the matched LNmet; (2) positive conversion was defined as gene amplification detectable in the LNmet which was not detectable in the matched primGC; (3) no conversion was defined as gene amplification detectable in both, primGC and LNmet. Univariate survival analyses were performed using the Kaplan Meier method and log rank test. Multivariate analyses was performed using Cox regression proportional hazard model including TNM stage and variable of interest in the model. P values of less than 0.05 were considered significant.

RESULTS

MLPA analysis of gene copy number status in primary gastric cancer (primGC)

Results from 237 primGC were available for final analyses (Figure 1). The median (range) age of the study cohort was 65 years (35 to 85 years), for details on patients' characteristics see Table 1.

In primGC, the most frequently amplified gene was *PIK3CA* (n=60, 25%) followed by *KRAS* (n=47, 20%), *MYC* (n=44, 19%), *HER2* (n=42, 18%), *CCNE1* (n=39, 16%), *EGFR* (n=25, 10%), *FGFR2* (n=24, 10%) and *MET* (n=11, 4%). For details on the frequency of normal gene copy number and losses see supplementary document Table S3.

MLPA analysis of gene copy number status in lymph node metastasis (LNmet)

Results from 103 LNmet were available for final analyses (Figure 1). In LNmet, the most frequently amplified gene was *MYC* (n=27, 26%) followed by *PIK3CA* (n=18, 18%), *KRAS* (n=12, 11%), *CCNE1* (n=9, 9%), *HER2* (n=7, 7%), *FGFR2* (n=7, 7%), *EGFR* (n=5, 5%) and *MET* (n=2, 2%). For details on the frequency of normal gene copy number and losses see supplementary document Table S3.

RTK and DSS gene co-amplification in primary gastric cancer

There were 86 (36%) primGC with neither DSS nor RTK amplification, 74 (31%) primGC with DSS amplification without RTK co-amplification, 25 (11%) primGC with RTK co-amplification without DSS co-amplification and 52 (22%) primGC had co-amplification of RTK and at least one DSS (Table 2).

Co-amplification of DSS genes was present in 48 (20%) primGC and was more common than RTK co-amplifications (n=21, 9%). High level co-amplification (gene copy number > 2.00) of RTK was not detected and was rare for DSS (n=4, 2%). For details about the frequency of co-amplification of individual genes see Venn diagram supplementary document Figure S2.

RTK and DSS gene co-amplification in lymph node metastasis

There were 45 (44%) LNmet with neither DSS nor RTK amplification, 39 (37%) LNmet with DSS amplification without RTK co-amplification, 9 (9%) LNmet with RTK amplification without DSS co-amplification and 10 (10%) LNmet had co-amplification of RTK and at least one DSS (Table 2). Co-amplification of DSS genes was present in 14 (14%) LNmet and was more common than RTK co-amplifications (n=2, 2%). High level co-amplification (gene copy number > 2.00) of RTK occurred in one LNmet (1%) and of DSS in two LNmet (2%).

Comparison of gene copy number status between primary gastric cancer and matched lymph node metastasis

70 (68%) of the 103 patients with results from primGC and matched LNmet showed 'conversion'. When comparing gene copy number status of individual genes between primGC and matched LNmet, there was a significant difference for *KRAS* ($p < 0.001$), *MYC* ($p < 0.001$), *CCNE* ($p < 0.001$), *HER2* ($p = 0.005$) and *FGFR2* ($p < 0.001$), whereas *PIK3CA*, *EGFR* and *MET* gene copy number status was similar (all p-values > 0.5).

Considering all RTK and DSS genes together, 15 (21%) patients had positive (primGC not amplified, LNmet amplified) and negative conversions (primGC amplified, LNmet not amplified), 38 (54%) had negative conversions only and 17 (24%) had positive conversions only.

Considering RTK genes only, a total of 33 (32%) patients had a conversion: 2 (6%) had positive and negative conversions, 25 (76%) had negative conversions only and 6 (18%) had positive conversions only.

Considering DSS genes only, a total of 56 (54%) patients had a conversion: 10 (19%) had positive and negative conversions, 30 (54%) had negative conversions only, and 16 (29%) had positive conversions only.

Frequencies of conversion per individual gene are shown in Table 3.

Relationship between gene copy number status and clinicopathological variables

EGFR, *HER2*, *MYC* and *CCNE1* amplification in the primGC was more frequent in intestinal type GC ($p=0.001$, $p=0.005$, $p=0.009$ and $p=0.001$, respectively). *MET* amplification in the primGC was more frequently seen in GC with higher TNM stage ($p=0.001$). *PIK3CA* amplification was more frequently seen in GC with higher pT category ($p=0.015$), Table 4A and 4B.

Relationship between gene copy number status and overall survival

Patients with RTK amplification in the primGC had a significantly worse survival than patients without RTK amplification ($p=0.040$, Figure 2A). However, multivariate analyses including primGC RTK gene copy number status (RTK amplification versus no RTK amplification) and TNM stage in the model showed that primGC RTK copy number status was not an independent prognostic marker (Hazard ratio:1.457 95%Confidence interval: 0.995-2.135, $p=0.053$).

There was no significant survival difference for GC patients with or without DSS amplification in the primGC ($p=0.153$, Figure 2B).

There was no significant survival difference when patients were stratified by presence or absence of DSS and RTK co-amplifications in the primGC ($p=0.150$, Figure 2C).

There was no significant survival difference for patients with or without RTK amplification, with or without DSS amplifications or with or without DSS and RTK co-amplifications in the LNmet (all p -values > 0.05 , Figure 3).

There was no significant survival difference between GC patients with or without RTK and/or DSS conversion. There was also no survival difference when patients were stratified by the type of conversion (p -value 0.296, Figure 4).

DISCUSSION

Recent studies including our own [9] suggest that approximately one third of gastric adenocarcinomas have mutually exclusive amplification of targetable receptor tyrosine kinases (*EGFR*, *HER2*, *FGFR2*, *MET*) [9 10]. Pre-clinical and clinical studies suggest that (1) co-amplification of receptor tyrosine kinase genes (RTK) and downstream signalling (DSS) genes could potentially alter targeted therapy efficacy [22 23] and that (2) gene copy number status discordance between primary tumour and metastases may influence response to RTK targeted treatment [31-33]. Currently, very little is known about the frequency of RTK and DSS co-amplification and concordance of gene copy number status between primary gastric cancer (primGC) and matched lymph node metastasis (LNmet).

As part of this study we designed a new multiplex ligation probe-dependent amplification (MLPA) probemix to allow simultaneous assessment of the gene copy number status of multiple RTKs and DSSs genes in DNA extracted from formalin fixed paraffin embedded gastric cancer specimens in a single experiment [42 43]. Our validation studies demonstrated a high level of reproducibility, specificity and sensitivity and a low 11/366 (3%) technical dropout rate suggesting that the newly developed MLPA assay could be a clinically useful routine screening method to assess RTK and DSS gene copy number status.

Our study confirmed previous reports [9 10] that approximately 30% GC have at least one RTK amplified and that the presence of RTK amplification in the primary tumour is associated with poorer survival (Figure 2A).

The co-amplification frequency of *HER2* and *EGFR*, *HER2* and *MET*, as well as of *FGFR2* and *MET* in the current study is similar to that reported previously[9 44-46]. Furthermore, our study confirmed that high level RTK co-amplifications are mutually exclusive events as reported previously[9 10]. In addition, we found that low level RTK co-amplifications can occur, which may explain some of the inconsistencies reported in the literature[9 44-46] where the definition of what constitutes an amplification varies, see supplementary document Table S4. The current study supports the previously reported finding that amplification of *EGFR*, *HER2*, *MYC* and *CCNE1* is more frequent in intestinal type GC[47] [48].

This is the first study to demonstrate that RTK and DSS co-amplification is a common event in primary GC. Importantly, this is the first study to identify a substantial subgroup of GC patients (36%) with neither RTK nor DSS amplification suggesting that there is a subgroup of GC patients where the ERK/MAPK pathway may not be the main oncogenic driver. This finding is of potential clinical relevance as patients without RTK amplification in their primary GC had better overall survival and thus might require a different therapy approach.

Finally, this is the most comprehensive study in GC investigating the concordance between the gene copy number statuses of targetable receptor tyrosine kinases and downstream signalling oncogenes in primary tumour and matched lymph node metastasis demonstrating that the gene copy number status of the majority of investigated genes differs in up to 68% GC between the primGC and the LNmet. However the conversion of RTK or DSS genes does not appear to influence survival (Figure 4).

The *HER2* gene copy number status concordance between primGC and LNmet is lower in the current study than reported in a recent meta-analysis[49]. Nevertheless, the meta-analysis also showed a higher negative than positive conversion rate as demonstrated in the current study. These different results could be related to differences in case mix, number of investigated cases per study, patient ethnicity as well as to the use of different experimental platforms to assess *HER2* gene copy number status. Whilst there is good evidence supporting similar specificity and sensitivity of the MLPA assay compared to the FISH method[40 50], the MLPA assay is unable to distinguish between polysomy and amplification which could explain our relatively higher frequencies in comparison to the current literature.

The results from selected RTK and DSS gene copy number analyses in our study support the current model hypothesis that metastatic tumour cells in the lymph node may constitute a cell population with a different genetic makeup compared to the matched primary tumour. The clinically undetected 'loss' of an amplification of a RTK gene in a metastatic site which was present in the primary tumour may potentially render the targeted drug inefficient. This issue could potentially be addressed by investigating the gene copy number status in the primary tumour as well as in the metastatic site.

This study has some limitations. This is a retrospective study and thus we were unable to assess the clinical relevance of co-amplifications of RTK and DSS with respect to resistance to RTK targeted therapy as patients in this study did not receive targeted therapy. Therefore, the predictive value of RTK/DSS co-amplification and/or genetic conversion between primary cancer and metastasis in gastric cancer patients remains to be investigated.

Furthermore, this study focussed on TNM stage II, III and IV Japanese GC and further prospective studies need to demonstrate that the results from this study can be reproduced in GC from patients with other disease stages and/or ethnicities.

On the other hand, our study demonstrated that assessment of the gene copy number status of multiple selected genes is feasible with DNA from FFPE material (e.g. routine histopathological material), in a timely fashion at relative low cost with high sensitivity, specificity and excellent reproducibility using the new developed MLPA assay.

In conclusion, the emergence of targeted therapy acquired resistance mechanisms requires the development of clinically applicable and affordable methods screening for the drug targets as well as for genes related to the same pathway or other potential mechanisms that may impact drug effectiveness. This is the first study in gastric cancer demonstrating that co-amplification of receptor tyrosine kinase (RTK) genes and downstream signalling (DSS) genes is more frequent than RTK amplification alone and that conversion of gene copy number status between primary GC and matched lymph node metastasis is more frequent than no conversion.

As patients in this study did not receive targeted therapy, we can currently only speculate that RTK and DSS co-amplification in GC as well as discordance of gene copy number status between primary tumour and lymph node metastases in GC could potentially explain the recently seen failures of EGFR and MET targeted therapy in GC. Future studies are needed to investigate whether GC patients considered for RTK targeted therapy require gene copy number assessment of receptor tyrosine kinase genes as well as downstream signalling genes from primary tumour and lymph node metastasis to identify potential responders.

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FIGURE LEGENDS

Figure 1 – Flow diagram detailing how the final number of primary gastric cancer and matched lymph node metastasis available for analyses was reached.

Figure 2. Kaplan Meier plots showing probability of overall survival stratified by receptor tyrosine kinase gene (RTK) and/or downstream signalling gene (DSS) copy number status in the primary gastric cancer.

A. Kaplan Meier survival analysis showed that gastric cancer patients without RTK gene amplification in their primary tumour survived significantly longer than patients with at least one amplified RTK gene.

B. Kaplan Meier survival analysis showed no difference in overall survival when patients were stratified by DSS gene copy number status of the primary tumour.

C. Kaplan Meier survival analysis showed no difference in overall survival when patients were stratified by combining the RTK and DSS gene copy number status of the primary tumour. Key: (+) = amplification, (-) = no amplification.

Figure 3. Kaplan Meier plots showing probability of overall survival by receptor tyrosine kinase gene (RTK) and/or downstream signalling gene (DSS) copy number status in the lymph node metastasis.

A. Kaplan Meier survival analysis showed no difference in overall survival when patients were stratified by RTK gene copy number status of the lymph node metastasis.

B. Kaplan Meier survival analysis showed no difference in overall survival when patients were stratified by DSS copy number status of the lymph node metastasis.

C. Kaplan Meier survival analysis showed no difference in overall survival when patients were stratified by combining the RTK and DSS gene copy number status of the primary tumour. Key: (+) = amplification, (-) = no amplification. Key: (+) = amplification, (-) = no amplification.

Figure 4. Kaplan Meier plots showing probability of overall survival by gene copy number conversion.

Considering the gene copy number status of all RTK and DSS genes, Kaplan Meier survival analysis showed no difference in overall survival between patients with and without gene copy number conversion between primary tumour and lymph node metastasis.

Frequent co-amplification of receptor tyrosine kinase and downstream signalling genes in Japanese primary gastric cancer and conversion in matched lymph node metastasis.

Silva ANS¹, Coffa J², Menon V³, Hewitt LC⁴, Das K⁵, Miyagi Y⁶, Bottomley D⁷, Slaney H⁸, Aoyama T⁹, Mueller W¹⁰, Arai T¹¹, Tan IB¹², Deng N¹³, Chan XB¹⁴, Lin SJ¹⁵, Tan P¹⁶, Tsuburaya A¹⁷, Sakamaki K¹⁸, Hayden JD¹⁹, Yoshikawa T²⁰, Zondervan I²¹, Savola S²², Grabsch HI²³

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Table 3: Frequency of conversion between primary gastric cancer (primGC) and matched lymph node metastasis (LNmet) by individual gene.

Table 4: Relationship between primary gastric cancer receptor tyrosine kinase (RTK) and downstream signalling (DSS) gene copy number status and clinicopathological variables.

Table 1. Clinicopathological characteristics of the gastric cancer patient cohort (n=237) and the subgroup of patients with lymph node metastasis (Lnmet, n=103)				
	Gastric cancer cohort		Gastric cancer subgroup with LNmet	
	n	%	n	%
Gender				
Male	166	70	75	73
Female	71	30	28	27
Tumour location				
Proximal	71	30	28	27
Mid	97	41	41	40
Distal	69	29	34	33
Depth of invasion (pT)				
pT1b	6	3	1	1
pT2	43	18	21	20
pT3	31	13	13	13
pT4a	157	66	68	66
Lymph node status (pN)				
pN0	37	16	0	0
pN1	58	24	22	21
pN2	63	27	29	28
pN3a	61	26	42	41
pN3b	18	7	10	10
Distant metastasis (pM)				
pM0	226	95	98	95
pM1(peritoneal)	11	5	5	5
TNM stage 7 th Edition				
II	93	39	24	23
III	133	56	74	72
IV	11	5	5	5
Histological tumour type (Lauren)				
Intestinal	111	47	58	56
Diffuse	126	53	45	44

Table 2.

Frequency of Receptor Tyrosine Kinases (RTK) and Downstream Signalling (DSS) gene amplification in primary gastric cancer (primGC) and lymph node metastasis (LNmet).

	No RTK amplification		RTK amplification	
	primGC n (%)	LNmet n (%)	primGC n (%)	LNmet n (%)
No DSS amplification	86 (54)	45 (54)	25 (32)	9 (47)
DSS amplification	74 (46)	39 (46)	52 (68)	10 (53)

Table 3.

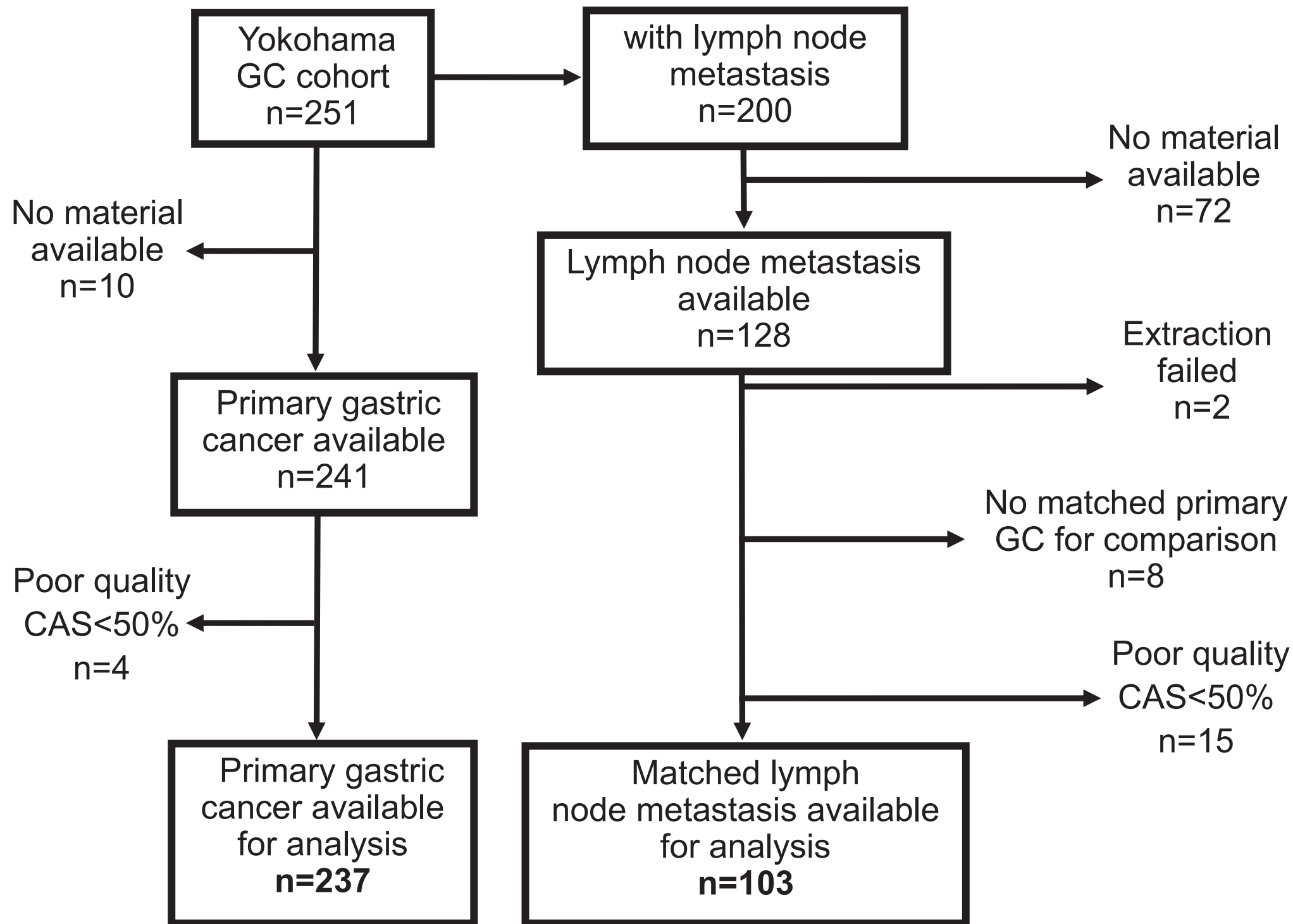
Frequency of conversion between primary gastric cancer (primGC) and matched lymph node metastasis (LNmet) by individual gene.

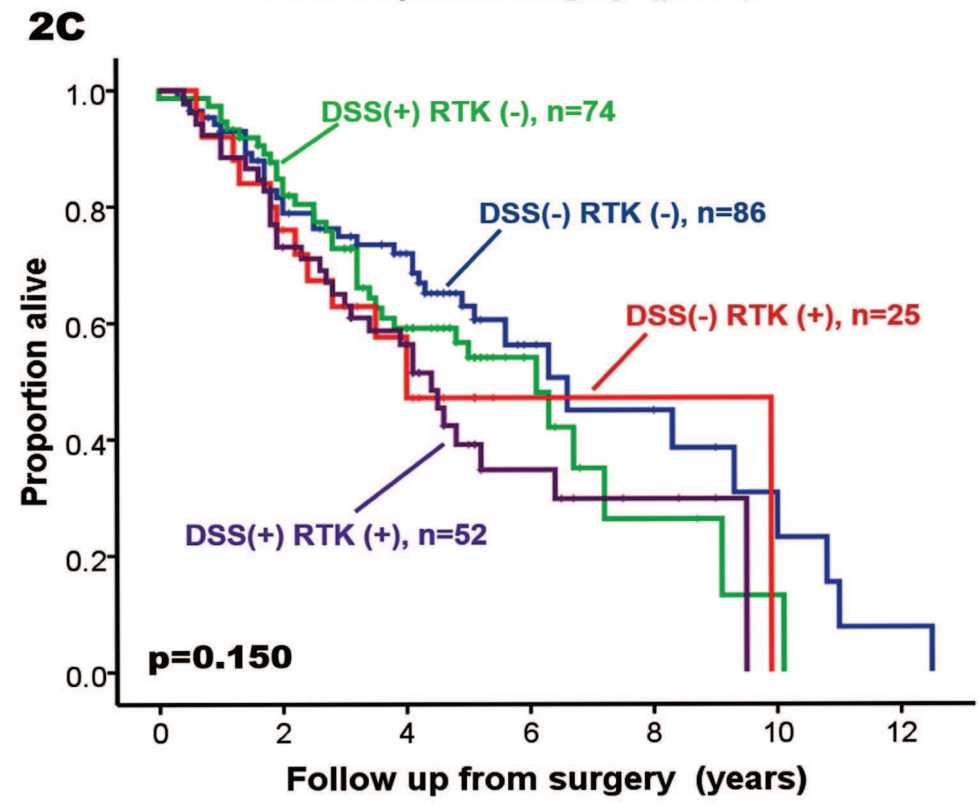
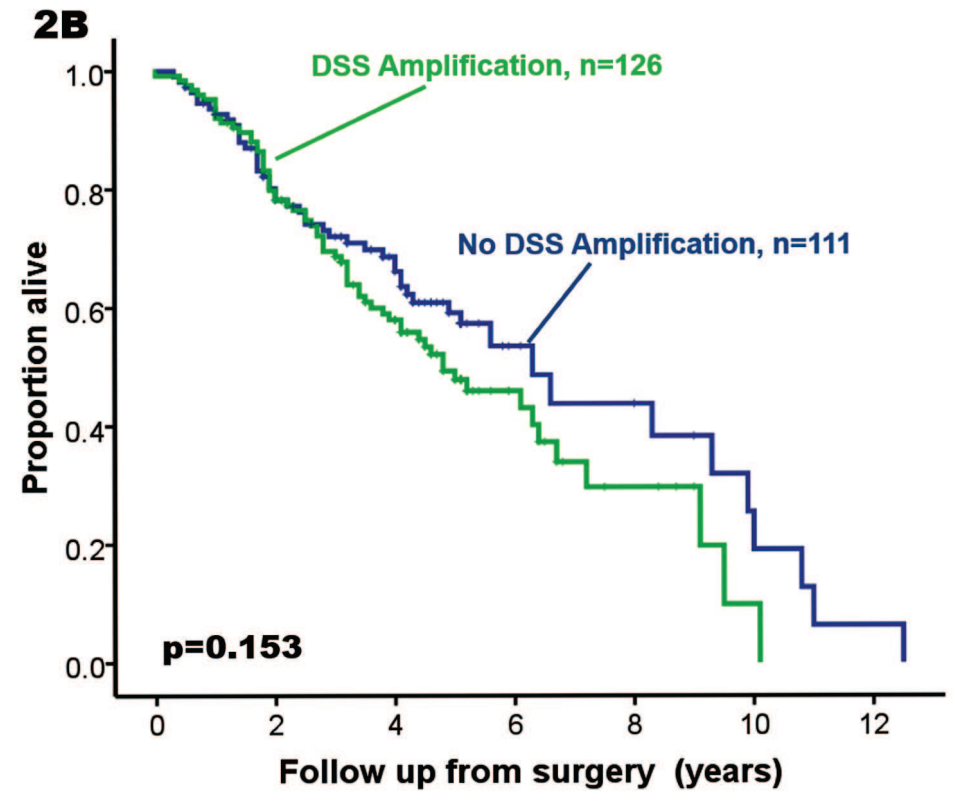
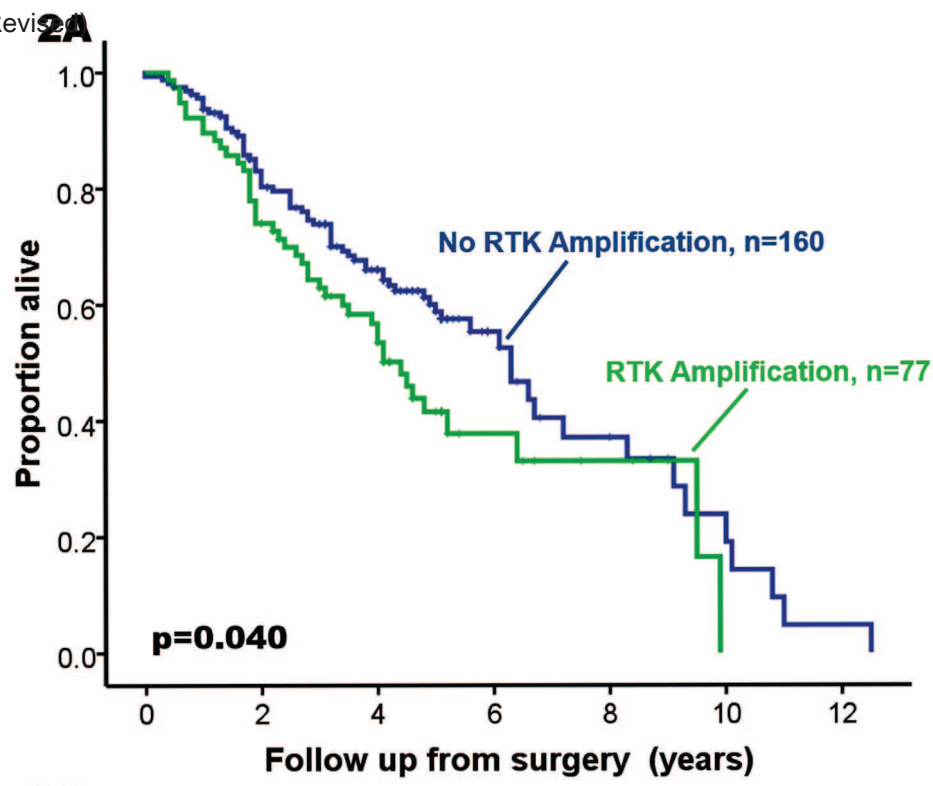
Gene	Amplified in primGC, not amplified in LNmet (negative conversion) n (%)	Amplified in primGC and amplified in LNmet (no conversion) n (%)	Not amplified in primGC and amplified in LNmet (positive conversion) n (%)
EGFR	8 (9)	2 (4)	3 (8)
HER2	17 (20)	5 (10)	2 (5)
FGFR2	7 (8)	4 (8)	3 (8)
MET	4 (5)	1 (2)	1 (3)
PIK3CA	22 (25)	9 (18)	10 (27)
KRAS	9 (10)	9 (18)	3 (8)
CCNE	14 (16)	8 (16)	1 (3)
MYC	6 (7)	13 (25)	14 (38)

Table 4A. Relationship of primary gastric cancer receptor tyrosine kinases gene copy number status and clinicopathological variables												
	RTK gene											
	EGFR		p-value	HER2		p-value	FGFR2		p-value	MET		p-value
	Amplified n (%)	Not amplified n (%)		Amplified n (%)	Not amplified n (%)		Amplified n (%)	Not amplified n (%)		Amplified n (%)	Not amplified n (%)	
Gender												
Male	20 (80)	146 (70)	0.251	30 (71)	136 (70)	0.829	18 (75)	148 (69)	0.577	7 (63)	159 (70)	0.636
Female	5 (20)	66 (30)		12 (29)	59 (30)		6 (25)	65 (31)		4 (37)	67 (30)	
Depth of invasion (pT)												
pT1b	0 (0)	6 (3)	0.225	1 (2)	5 (3)	0.981	0 (0)	6 (3)	0.501	0 (0)	6 (3)	0.918
pT2	3 (12)	40 (19)		7 (17)	36 (18)		3 (13)	40 (19)		2 (18)	41 (18)	
pT3	1 (4)	30 (14)		5 (12)	26 (13)		5 (21)	26 (12)		1 (9)	30 (13)	
pT4a	21 (84)	136 (64)		29 (70)	128 (6)		16 (66)	141 (66)		8 (73)	149 (66)	
Lymph node status (pN)												
pN0	4 (16)	33 (16)	0.998	8 (19)	29 (15)	0.913	4 (17)	33 (15)	0.257	1 (9)	36 (16)	0.102
pN1	6 (24)	52 (25)		9 (21)	49 (25)		5 (21)	53 (25)		1 (9)	57 (25)	
pN2	6 (24)	57 (27)		10 (24)	53 (27)		8 (33)	55 (26)		6 (55)	57 (25)	
pN3a	7 (28)	54 (25)		11 (26)	50 (26)		3 (12)	58 (27)		1 (9)	60 (27)	
pN3b	2 (4)	16 (7)		4 (10)	14 (7)		4 (17)	14 (7)		2 (18)	16 (7)	
Distant metastasis (pM)												
pM0	25 (100)	201 (95)	0.875	41 (98)	185 (95)	0.444	23 (96)	203 (95)	0.907	8 (73)	218 (96)	<0.001
pM1	0 (0)	11 (5)		1 (2)	10 (5)		1 (4)	10 (5)		3 (27)	8 (4)	
TNM stage												
Stage II	7 (28)	85 (40)	0.191	15 (36)	77 (40)	0.624	8 (33)	84 (39)	0.824	2 (18)	90 (39)	0.001
Stage III	18 (72)	116 (55)		26 (62)	108 (55)		15 (63)	119 (56)		6 (55)	128 (57)	
Stage IV	0 (0)	11 (5)		1 (2)	10 (5)		1 (4)	10 (5)		3 (27)	8 (4)	
Histological tumour type (Lauren)												
Intestinal	19 (80)	88 (42)	0.001	27 (66)	80 (42)	0.005	11 (46)	96 (46)	0.993	6 (55)	101 (44)	0.557
Diffuse	5 (20)	121 (58)		14 (34)	112 (68)		13 (54)	113 (54)		5 (45)	121 (56)	

Table 4B.												
Relationship of primary gastric cancer downstream signalling gene copy number status and clinicopathological variables.												
	DSS Genes											
	PIK3CA		p-value	KRAS		p-value	MYC		p-value	CCNE1		p-value
	Amplified n (%)	Not amplified n (%)		Amplified n (%)	Not amplified n (%)		Amplified n (%)	Not amplified n (%)		Amplified n (%)	Not amplified n (%)	
Gender												
Male	48 (80)	118 (66)	0.052	33 (70)	133 (70)	0.977	34 (77)	132 (68)	0.247	29 (74)	137 (69)	0.423
Female	12 (20)	59 (34)		14 (30)	57 (30)		10 (23)	61 (32)		10 (26)	61 (31)	
Depth of invasion (pT)												
pT1b	1 (2)	5 (3)	0.047	1 (2)	5 (3)	0.593	1 (2)	5 (3)	0.971	1 (3)	5 (3)	0.015
pT2	8 (13)	35 (20)		8 (17)	35 (18)		9 (20)	34 (18)		8 (21)	35 (18)	
pT3	14 (23)	17 (10)		9 (19)	22 (12)		6 (14)	25 (13)		11 (28)	20 (10)	
pT4a	37 (62)	120 (67)		29 (62)	128 (67)		28 (64)	129 (66)		19 (48)	138 (69)	
Lymph node status (pN)												
pN0	7 (12)	30 (17)	0.724	7 (15)	30 (16)	0.749	7 (16)	30 (16)	0.997	4 (10)	33 (17)	0.244
pN1	13 (22)	45 (25)		15 (32)	43 (23)		11 (25)	47 (24)		15 (38)	43 (22)	
pN2	18 (30)	45 (25)		12 (26)	51 (27)		11 (25)	52 (27)		10 (26)	53 (27)	
pN3a	18 (30)	43 (24)		10 (21)	51 (27)		12 (27)	49 (25)		8 (21)	53 (27)	
pN3b	4 (6)	14 (9)		3 (6)	15 (7)		3 (7)	15 (8)		2 (5)	16 (7)	
Distant metastasis (pM)												
pM0	59 (98)	167 (94)	0.206	43 (91)	183 (96)	0.160	42 (95)	184 (95)	0.973	37 (95)	189 (95)	0.875
pM1	1 (2)	10 (6)		4 (9)	7 (4)		2 (5)	9 (5)		2 (5)	9 (5)	
TNM stage												
Stage II	21 (35)	71 (40)	0.290	18 (38)	74 (39)	0.367	16 (36)	76 (39)	0.929	18 (46)	74 (37)	0.557
Stage III	38 (63)	96 (54)		25 (53)	109 (57)		26 (59)	108 (56)		19 (49)	115 (58)	
Stage IV	1 (2)	10 (6)		4 (9)	7 (4)		2 (5)	9 (5)		2 (5)	9 (5)	
Histological tumour type (Lauren)												
Intestinal	23 (38)	84 (48)	0.217	20 (43)	87 (47)	0.605	28 (63)	79 (42)	0.009	28 (73)	79 (41)	<0.001
Diffuse	36 (62)	90 (52)		27 (57)	99 (53)		16 (37)	110 (58)		10 (27)	116 (59)	

Figure 1 (Revised)





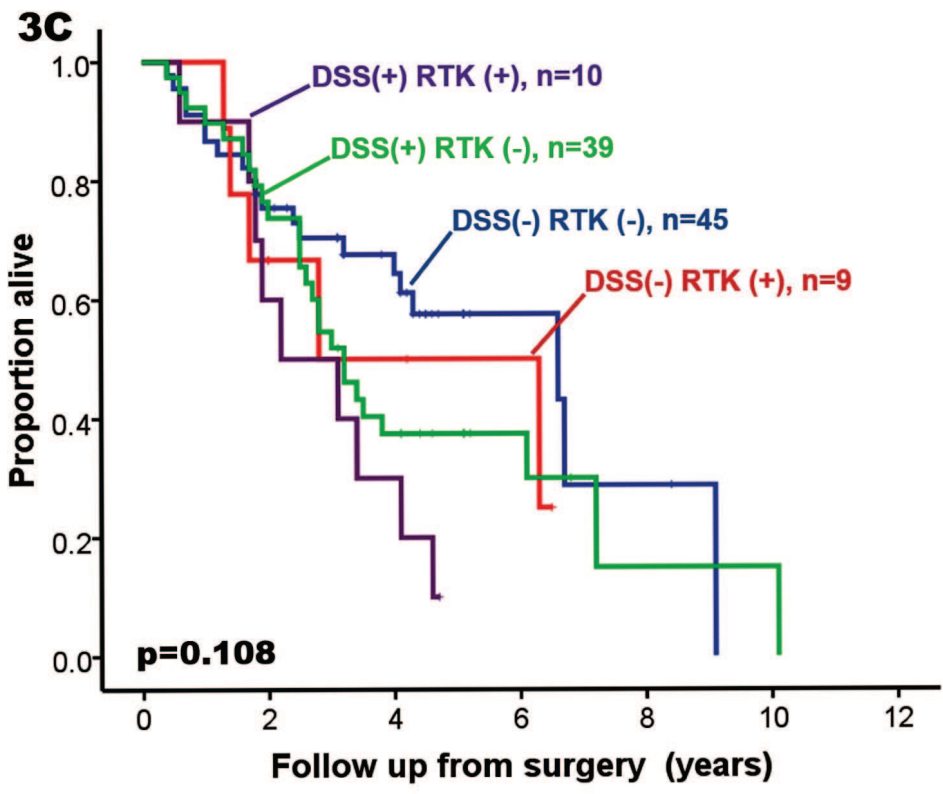
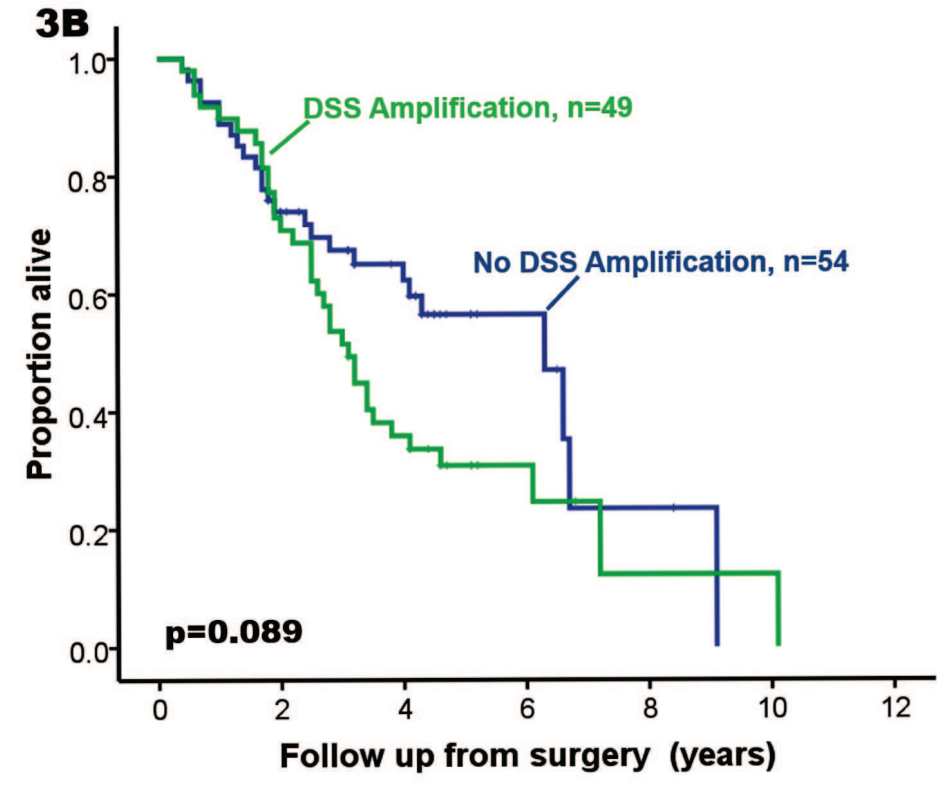
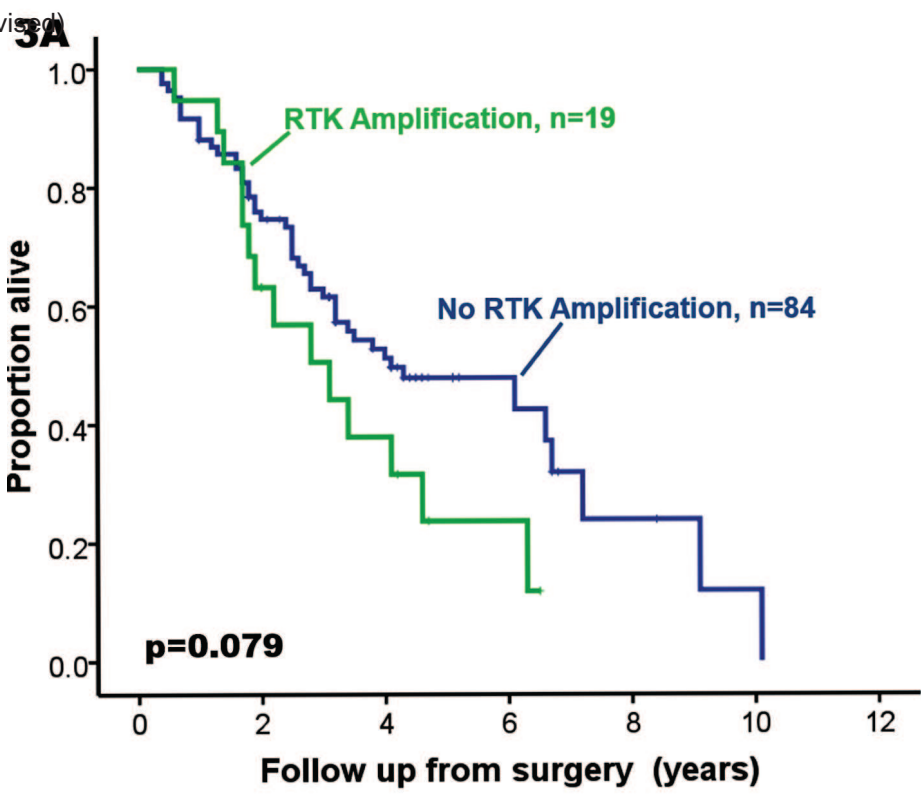
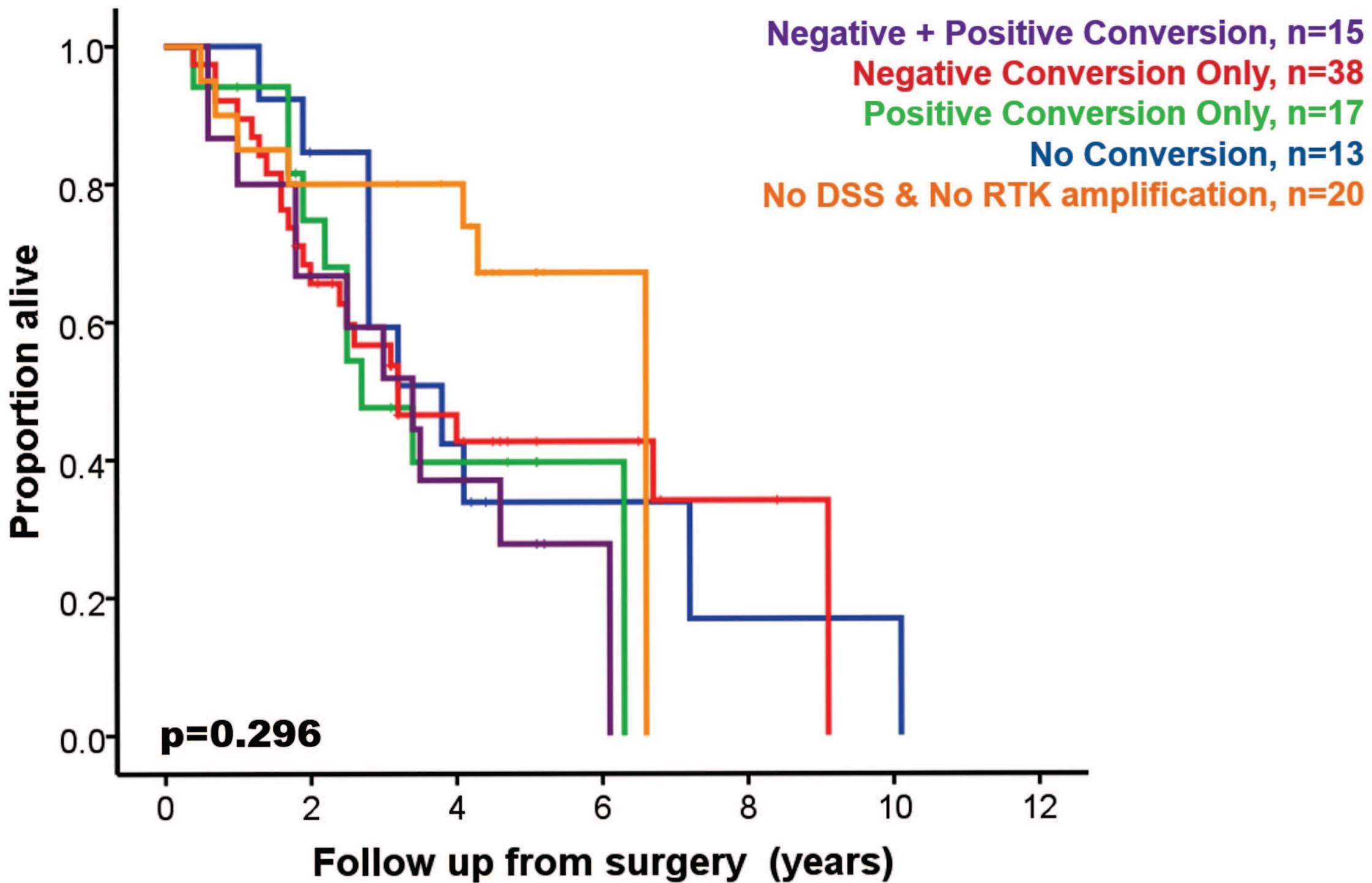


Figure 4 (Revised)



Frequent co-amplification of receptor tyrosine kinase and downstream signalling genes in Japanese primary gastric cancer and conversion in matched lymph node metastasis

Silva ANS¹, Coffa J², Menon V³, Hewitt LC⁴, Das K⁵, Miyagi Y⁶, Bottomley D⁷, Slaney H⁸, Aoyama T⁹, Mueller W¹⁰, Arai T¹¹, Tan IB¹², Deng N¹³, Chan XB¹⁴, Lin SJ¹⁵, Tan P¹⁶, Tsuburaya A¹⁷, Sakamaki K¹⁸, Hayden JD¹⁹, Yoshikawa T²⁰, Zondervan I²¹, Savola S²², Grabsch HI²³

Supplementary document

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MLPA Probe Development

Table S1: MLPA probe composition of the MRC Holland P458-A1 Gastric cancer probemix.

MLPA Validation Studies

Table S2: Comparison of DNA copy number status between single nucleotide polymorphism (SNP) array [1] and multiplex ligation dependent probe amplification (MLPA, this study) in gastric cancer cell lines.

MLPA Data (Coffalyser Software) Analysis & Competitor Mixes

Figures S1: Examples of data output files from the Coffalyser Software after comparative analysis demonstrating the effect of the competitor mixes

Figure S1A: Cell line mix with known amplifications of *FGFR2*, *KRAS*, *ERBB2* and *MYC* and no competitive inhibition

Figure S1B: Cell line mix with known amplifications of *FGFR2*, *KRAS*, *ERBB2* and *MYC* with competitive inhibition of *KRAS* and *FGFR2*

Figure S1C: Cell line mix with known amplifications of *FGFR2*, *KRAS*, *ERBB2* and *MYC* with competitive inhibition of *MYC*

Figure S1D: Cell line mix with known amplifications of *FGFR2*, *KRAS*, *ERBB2* and *MYC* with competitive inhibition of *ERBB2*

Figure S2: Venn diagrams illustrating frequency of co-amplification of receptor tyrosine kinase genes (A) and downstream signalling genes (B) in the primary tumour.

Table S3: MLPA gene copy number status in primary gastric cancer (n=237) and lymph node metastasis (n=103)

Table S4: Comparison of the reported frequency of RTK co-amplification.

MLPA probemix development

For this study, a novel multiplex ligation-dependent probe amplification (MLPA) probemix for GC (P458-A1-lot0312) was designed and optimized by MRC-Holland (IZ, JC, SS, MRC Holland, Amsterdam, The Netherlands). The probemix was designed to assess the gene copy number status of receptor tyrosine kinase (RTKs: *EGFR*, *HER2*, *FGFR2* and *MET*) and downstream signalling genes (DSSs: *PIK3CA*, *KRAS*, *MYC*, and *CCNE*) as well as some other genes. A detailed description of the probemix is provided in supplementary table 1.

The probes included in this assay were selected either from existing probes from the MRC-Holland MLPA probe database or newly designed by MRC-Holland. 13 probes detecting nine different autosomal locations, which have reportedly relatively stable gene copy number status in GC, were included in the assay as reference probes for data normalisation. In addition, the MLPA assay contained nine quality control probes to assess DNA denaturation status and DNA quantity, as well as probes specific for the X and Y chromosomes.

Table S1. MLPA probe composition of the MRC Holland P458-A1 Gastric cancer probemix					
Length	Probe	Gene	Chr. band	Exon	HG18 location
Target probes					
427	03827-L21157	PIK3CA	3q26.32	Exon 2	03-180.399605
259	16057-L21634	PIK3CA	3q26.32	Exon 7	03-180.410094
214	03826-L22080	PIK3CA	3q26.32	Exon 19	03-180.430490
418	05435-L20672	EGFR	7p11.2	Exon 2	07-055.177534
282	05960-L21637	EGFR	7p11.2	Exon 14	07-055.198927
325	05971-L21639	EGFR	7p11.2	Exon 27	07-055.236403
196	10314-L22071	MET	7q31.2	Exon 4	07-116.167344
208	10320-L10834	MET	7q31.2	Exon 10	07-116.186668
266	02577-L21635	MET	7q31.2	Exon 21	07-116.223340
166	15894-L21929	MYC	8q24.21	Exon 1	08-128.817870
156	00580-L21927	MYC	8q24.21	Exon 3	08-128.822148
331	07631-L21642	FGFR2	10q26.13	Exon 21	10-123.229418
310	07628-L07312	FGFR2	10q26.13	Exon 16	10-123.237417
160	07626-L21928	FGFR2	10q26.13	Exon 3	10-123.343235
202	17597-SP0529-L22061	KRAS	12p12.1	Exon 6	12-025.252102
382	17605-SP0543-L21602	KRAS	12p12.1	Exon 4	12-025.269833
392	09507-L22081	KRAS	12p12.1	Exon 3	12-025.271583
180	17596-L22078	KRAS	12p12.1	Exon 2	12-025.289376
145	00675-L21512	ERBB2	17q12	Exon 7	17-035.118096
339	12047-L21640	ERBB2	17q12	Exon 22	17-035.133374
357	00717-L21644	ERBB2	17q12	Exon 29	17-035.136627
171	04201-L03537	CCNE1	19q12	Exon 5	19-034.999920
399	15145-L22082	CCNE1	19q12	Exon 12	19-035.006411
Reference probes					
190	08838-L08898	DYSF	2p13.2	Exon 57	02-071.763257
137	05714-L05152	MAL	2q11.1	Exon 2	02-095.077493
475	03337-L21654	KCNIP4	4p15.31	Exon 10	04-020.343400
406	10715-L22083	PKHD1	6p12.2	Exon 36	06-051.932638
220	14933-L16666	LAMA2	6q22.33	Exon 26	06-129.678701
319	03156-L22075	MYBPC3	11p11.2	Exon 13	11-047.321628
500	14882-L21656	RPGRIP1	14q11.2	Exon 13	14-020.859956
490	09772-L21655	SPG11	15q21.1	Exon 11	15-042.705889
444	11223-L21650	POLG	15q26.1	Exon 21	15-087.662913
130	13867-L15385	ABAT	16p13.2	Exon 9	16-008.765458
371	05770-L21374	VPS35	16q11.2	Exon 13	16-045.260343
304	12619-L22074	RBM11	21q11.2	Exon 2	21-014.513892
459	06220-L21652	SLC25A18	22q11.21	Exon 1	22-016.423313

MLPA assay validation studies

This study used DNA from GC cell lines with previously published SNP array results [9] to assess the sensitivity and specificity of the assay and to demonstrate that the assay was able to detect more than one amplification and/or deletions in the same sample. Leeds GC FFPE archival material with known FISH dual RTK (*HER2* and *FGFR2*) amplification [40] was used to test the assay in DNA extracted from FFPE. Furthermore, two of the MLPA probes are located in the X and Y chromosome. The results from the gender-specific probes were compared to the reported gender and used as an additional means of quality control of the assay.

Gastric cancer cell lines with known gene copy number status

Thirteen GC cell lines with known gene copy number aberrations were investigated by the newly developed MLPA assay. The MLPA assay successfully identified 24 out of 25 gene amplifications previously detected by SNP arrays[9] (Supplementary Table 2). The previously described amplification of *PIK3CA* was not identified in cell line NUGC4. In addition, the MLPA assay identified copy number aberrations not previously described by SNP array[9] such as *MYC* amplification in CLS145, *MET* amplification in SNU16, *EGFR* and *CCNE1* amplifications in YCC3 and *PIK3CA* amplification in YCC9 GC cell line. These small differences between the MLPA assay and the previous SNP array results can probably be explained by different threshold settings during data analyses between the different experimental platforms.

Gastric cancer tissues with known gene copy number status

Three GC FFPE tumour samples with previously FISH detected co-amplification of *FGFR2* and *HER2*[40] also demonstrated co-amplification of *FGFR2* and *HER2* by the MLPA assay.

Gender identification.

The MLPA assay correctly identified the gender of all 237 patients in this cohort.

Table S2.		
Comparison of gene copy number status between single nucleotide polymorphism (SNP) array [1] and multiplex ligation dependent probe amplification (MLPA, this study) in gastric cancer cell lines.		
Cell Line	SNP amplified genes (SNP copy number)[1]	MLPA gene copy number ratios (this study)
HS746T	MET (1.57)	>5.00
KATOIII	FGFR2 (2.15)	>5.00
SNU16	MYC (1.08), FGFR2 (1.73)	>5.00, >5.00
YCC1	KRAS (1.91)	>5.00
CLS145	CCNE1 (0.80), ERBB2 (0.45), EGFR (0.45)	>3.00, 1.87, 2.17
MKN1	KRAS (1.36), MYC (0.43)	>5.00, 2.12
MKN7	ERBB2 (1.63), CCNE1 (1.21)	>5.00, >3.00
NUGC4	EGFR (0.87), ERBB2 (0.64), PIK3CA (0.76)	>2.00, 2.29, 1.21
YCC11	EGFR (1.66), MYC (1.41), CCNE1 (0.43)	>5.00, >3.00, 1.43
NCI-N87	ERBB2 (2.08), EGFR (0.58), MYC (0.44)	>5.00, 1.99, 1.40
YCC9	ERBB2 (2.37), MYC (0.59)	>5.00, 1.75
YCC7	ERBB2 (0.61)	1.99
YCC3	ERBB2 (0.55)	2.30

MLPA Coffalyser Analyses

After quality control, a comparative analysis with three normalisation cycles was performed using Coffalyser.Net software version 130202.2357. In short, in every sample, for every probe, a tumour to normal gene copy number ratio was obtained by dividing the relative peak height for each probe in the tumour by the relative value of the same peak in the reference sample (e.g. normal tonsil or normal blood) DNA. The median peak height of all probes per gene was used for final analyses. For the analysis of the gene copy number ratio in cases with gene copy number ratio >5, the gene copy number ratio of the very highly amplified gene was used together with the gene copy number ratios of the other genes following competitive inhibition of the very highly amplified gene (See Figures S1A-S1D).

MLPA 'competitor' mixes for very high level gene amplification

High level amplification of a gene targeted by a MLPA probe can result in potential 'false' low level signals from the other probes due to probes competing for the universal primer pair present in the PCR reaction mix. To address this potential problem, so called 'competitor mixes' were developed by MRC-Holland for all target genes in the MLPA probemix. These competitor mixes contained oligonucleotides identical to the 5' end of the original MLPA probe but only contained part of the original PCR primer sequence. The gene specific competitor mix was added to the reaction mix at the start of the experiment resulting in lower amplification efficiency of the highly amplified probes during the PCR reaction, thus increasing the amplification efficiency of all other probes. All samples that showed a MLPA gene copy number ratio >5 were reanalysed with the appropriate competitor mix. The effect of including the competitor mix on the MLPA reaction is shown in supplementary figures 1A-1D.

Figure S1A

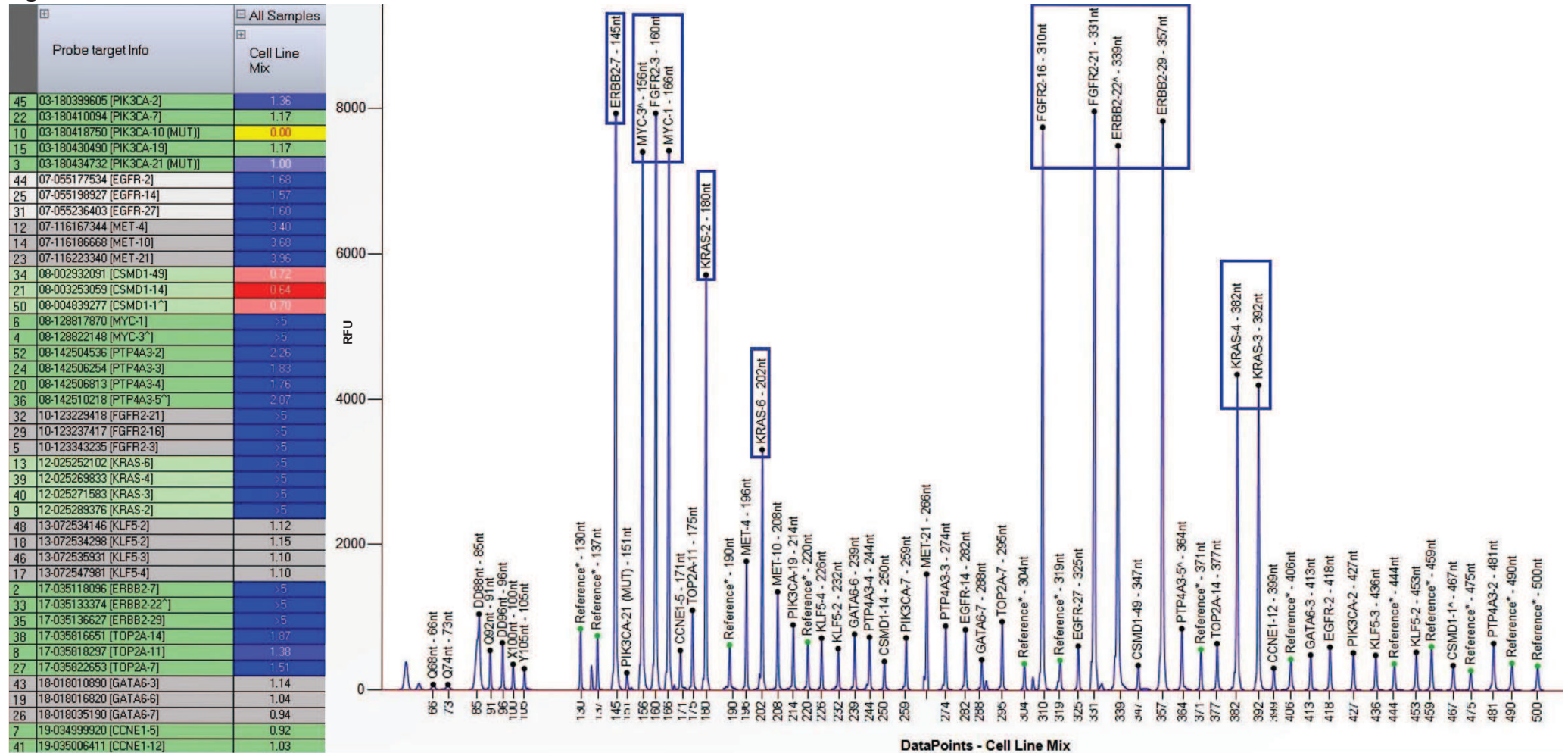


Figure S1A – Data output from the GC cell line mix following comparative analysis in Coffalyser Software. The column contains the sample name and the row has the information on copy number status per probe. The table key is as follow: yellow – deletion/probe inhibited/not available; red – deletion (<0.80); green or grey - normal copy number (0.8-1.3) and blue: amplification (>1.30). The graph represents the data shown in the table. X-axis – fragment length; Y-axis - relative fluorescence units (RFU). There are 6 blue boxes around the probes with very high signals. The median value of all probes per gene was used for statistical analysis.

Figure S1B

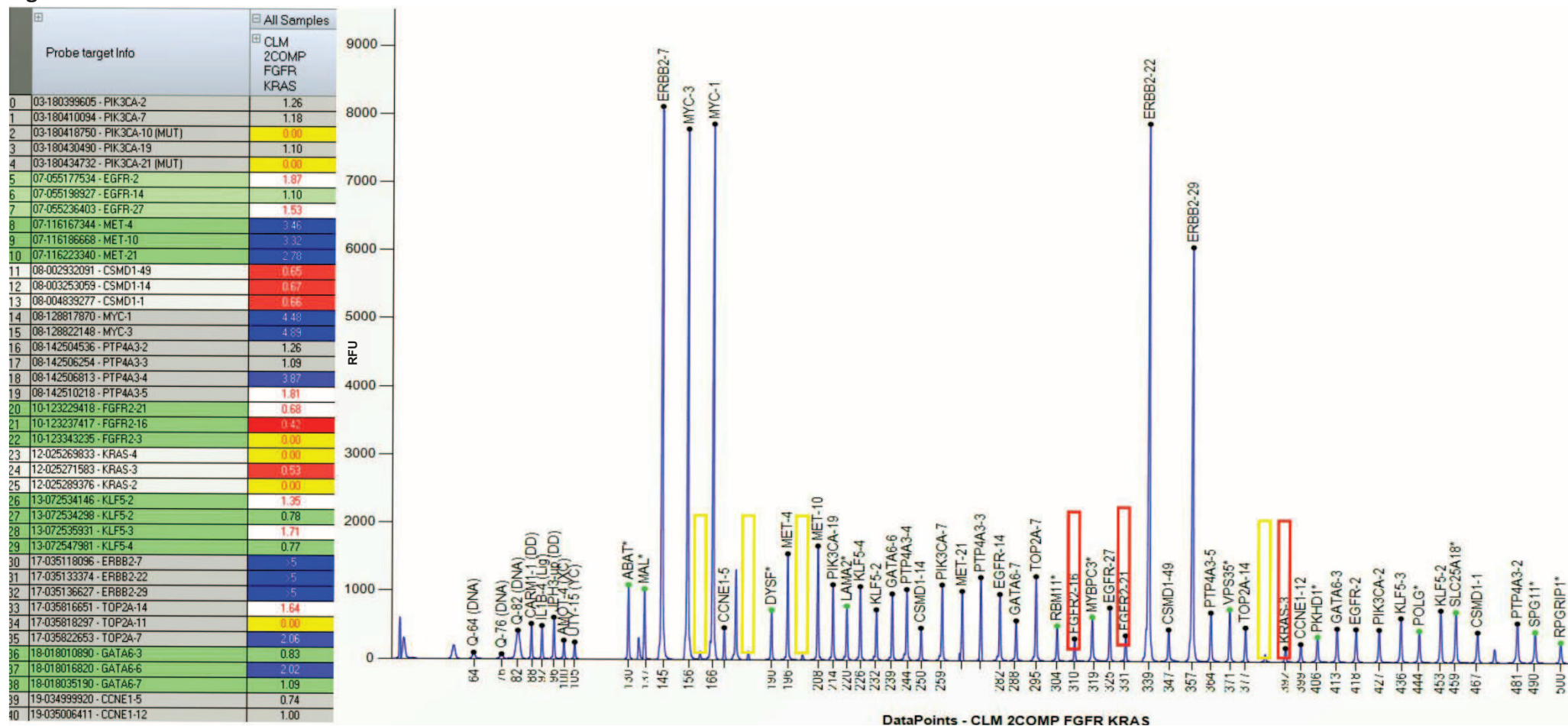


Figure S1B – Data output of the same GC cell line mix used in figure 1A after the use of the competitor mixes following comparative analysis in Coffalyser Software. The column contains the sample name and the row has the information on copy number status per probe. Competitor mixes were used for *KRAS* and *FGFR2*. The table key is as follow: yellow –probe fully inhibited/not available; red – deletion (<0.80); green or grey - normal copy number (0.8-1.3) and blue: amplification (>1.30). The graph represents the data shown in the table. X-axis – fragment length; Y-axis - relative fluorescence units (RFU). There was competitive inhibition of the probes that were previously very highly amplified (see supplementary Image 1A). There are 4 yellow boxes around probes that were fully inhibited and therefore do not have readable signal (represented in yellow in the table) and 3 red boxes around probes which have a very low signal (represented in red in the table). The median value of all probes per gene was used for statistical analysis.

Figure S1C

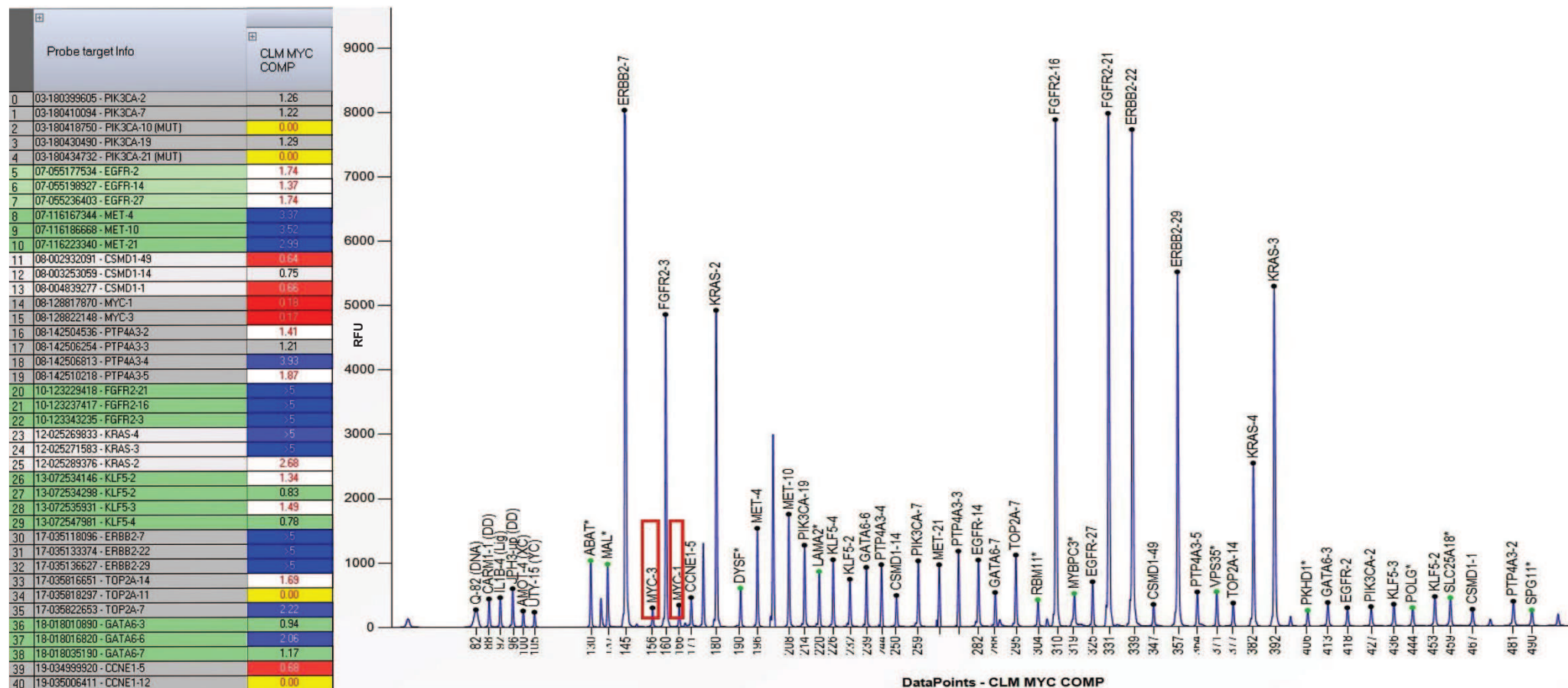


Figure S1C – Data output of the same GC cell line mix used in figure 1A after the use of the competitor mixes following comparative analysis in Coffalyser Software. The column contains the sample name and the row has the information on copy number status per probe. A competitor mix was used for MYC. The table key is as follow: yellow –probe fully inhibited/not available; red – deletion (<0.80); green or grey - normal copy number (0.8-1.3) and blue: amplification (>1.30). The graph represents the data shown in the table. X-axis – fragment length; Y-axis - relative fluorescence units (RFU). There was competitive inhibition of the probes that were previously very highly amplified (see supplementary image 1A). There are 2 red boxes around probes which have a very low signal (represented in red in the table). The median value of all probes per gene was used for statistical analysis.

Figure S1D

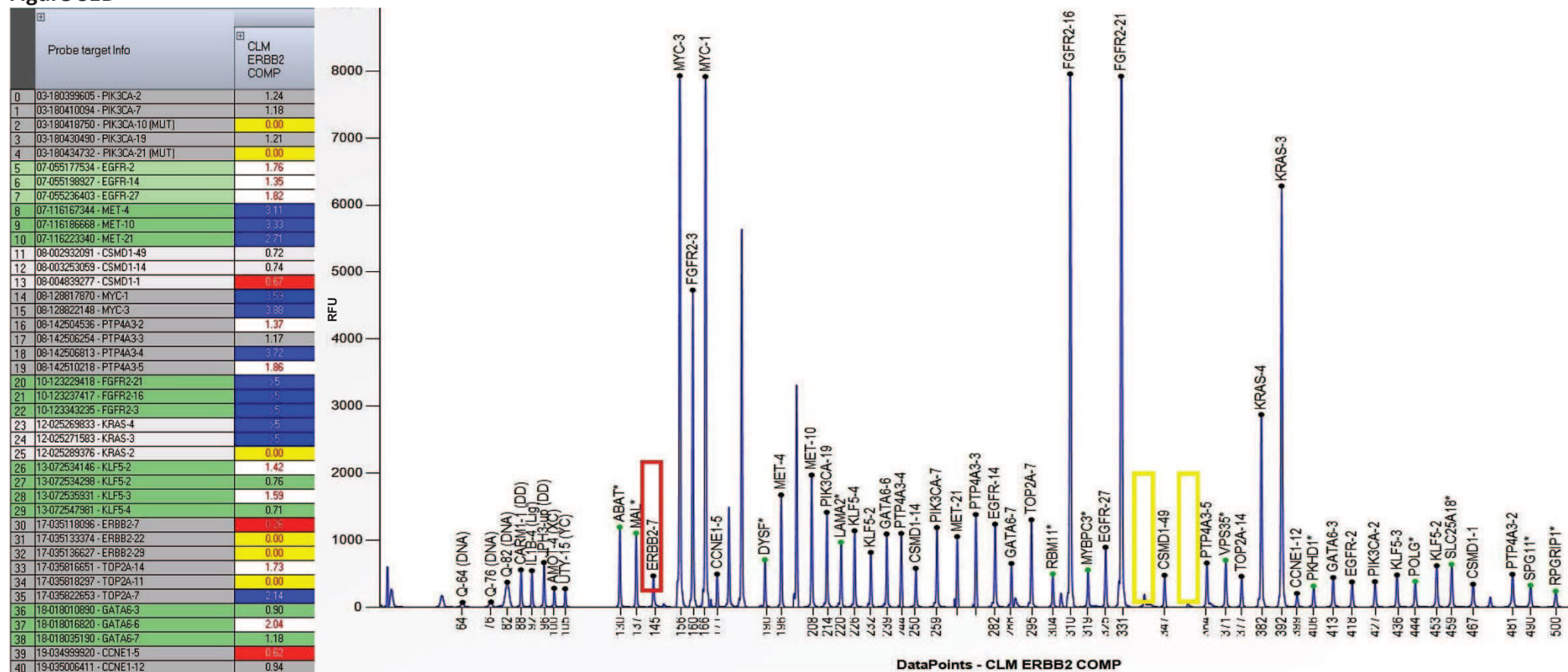
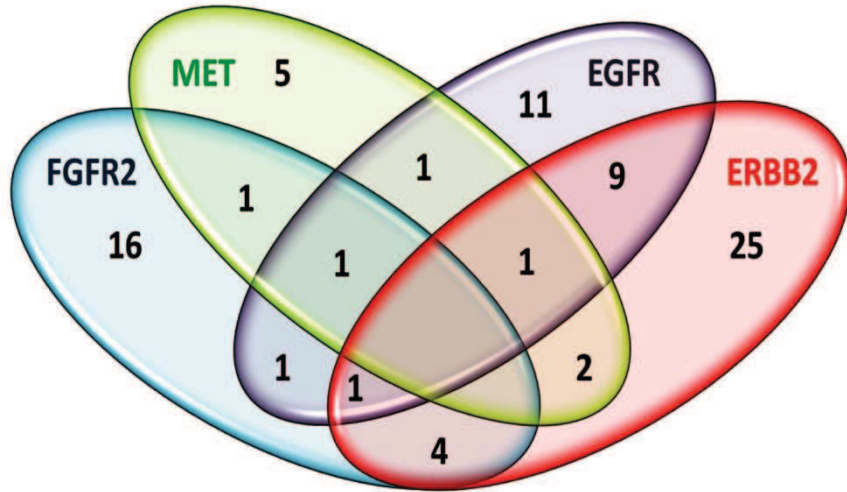


Figure S1D – Data output of the same GC cell line mix used in figure 1A after the use of the competitor mixes following comparative analysis in Coffalyser Software. The column contains the sample name and the row has the information on copy number status per probe. A competitor mixes was used for *ERBB2*. The table key is as follow: yellow –probe fully inhibited/not available; red – deletion (<0.80); green or grey - normal copy number (0.8-1.3) and blue: amplification (>1.30). The graph represents the data shown in the table. X-axis – fragment length; Y-axis - relative fluorescence units (RFU). There was competitive inhibition of the probes that were previously very highly amplified (See Supplementary Image 1A). There are 2 yellow boxes around the probes that were fully inhibited (represented in yellow in the table) and 1 red box around the probe with a very low signal (represented in red in the table). The median value of all probes per gene was used for statistical analysis.

Figure S2

A



B

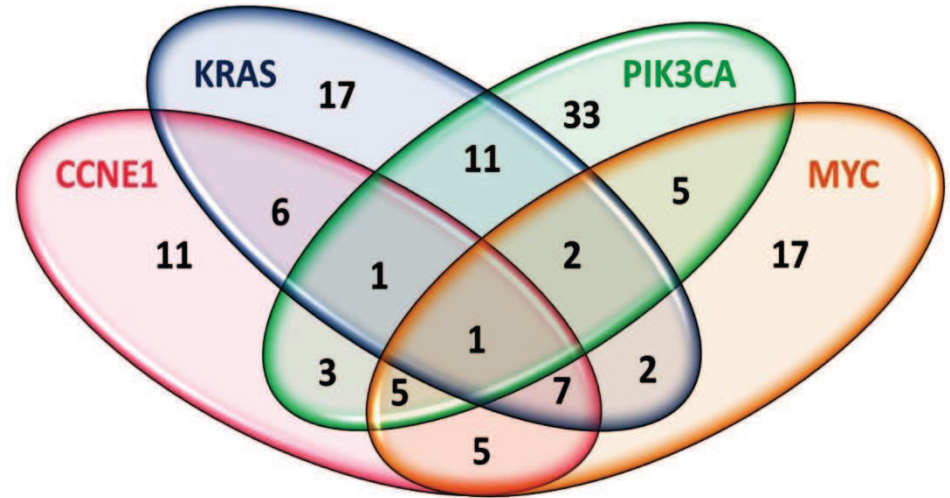


Figure 2. Venn diagrams illustrating frequency of co-amplification of receptor tyrosine kinase genes (A) and downstream signalling genes (B) in the primary gastric cancer. Name of the gene at the top of each ellipse, frequency of gastric cancers with co-amplifications shown in the area where the ellipses intercept.

Table S3.
MLPA gene copy number status in primary gastric cancer tissue (n=237) and lymph node metastasis (n=103)

		Primary GC	LNmet	Primary GC	LNmet	Primary GC	LNmet	Primary GC	LNmet
		Deletion n (%)	Deletion n (%)	Normal n (%)	Normal n (%)	Low level amplification n (%)	Low level amplification n (%)	High level amplification n (%)	High level amplification n (%)
R T K	EGFR	4 (2)	1 (1)	208 (88)	97 (94)	20 (8)	2 (2)	5 (2)	3 (3)
	ERBB2	42 (18)	1 (1)	153 (64)	95 (92)	28 (12)	3 (3)	14 (6)	4 (4)
	FGFR2	2 (1)	1 (1)	211 (89)	95 (92)	19 (8)	4 (4)	5 (2)	3 (3)
	MET	42 (18)	6 (6)	184 (78)	95 (92)	10 (4)	2 (2)	1 (0)	0 (0)
D S S	PIK3CA	0 (0)	0 (0)	177 (75)	84 (82)	59 (25)	17 (17)	1 (0)	1 (1)
	KRAS	8 (3)	3 (3)	182 (77)	88 (86)	38 (16)	5 (4)	9 (4)	7 (7)
	MYC	14 (6)	1 (1)	179 (75)	75 (73)	38 (16)	25 (24)	6 (3)	2 (2)
	CCNE1	37 (16)	5 (5)	161 (68)	89 (86)	22 (9)	2 (2)	17 (7)	7 (7)

Table S4.
Comparison of the reported frequency of receptor tyrosine kinase co-amplification

Co-amplification	Current study n (%)	Yk et al ⁴⁵ n (%)	Liu et al ⁴⁶ n (%)	Nagatsuma et al ⁴⁷ n (%)	Deng et al ⁹ n (%)
ERBB2 and EGFR	11 (5%)	3 (4%)	-	3 (0.3%)	exclusive
ERBB2 and MET	3 (1%)	-	3 (2%)	-	exclusive
ERBB2 and FGFR2	6 (3%)	-	exclusive	-	exclusive
FGFR2 and MET	2 (1%)	-	1(1%)	-	exclusive
EGFR and MET	3 (1%)	-	-	3 (0.3%)	exclusive

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List of Specific Contributions:

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3. Varsha Menon – DNA Dilution, MLPA Data Collection and Analysis, Writing Manuscript
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