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Original Research

Clonal diversity of *MYC* amplification evaluated by fluorescent *in situ* hybridisation and digital droplet polymerase chain reaction in oesophagogastric cancer: Results from a prospective clinical trial screening programme

Michael Davidson [a,1], Lauren I. Aronson [b,1], Julie Howard-Reeves [a], Hanna Bryant [b], Rosalind J. Cutts [b], Sanna Hulkki-Wilson [a], Kyriakos Kouvelakis [a], Eftheleria Kalaitzaki [a], David Watkins [a], Naureen Starling [a], Sheela Rao [a], Marta Llorca Cardenosa [b], Ruwaida Begum [a], Isma Rana [a], Retchel Lazaro-Alcausi [a], Monica Terlizzo [a], Andrew Wotherspoon [a], Gina Brown [a], John Swansbury [a], Christopher J. Lord [b,c], David Cunningham [a], Ian Chau [a,*], Irene Y. Chong [a,b,**]

[a] *Royal Marsden NHS Foundation Trust, Downs Road, Surrey, SM2 5PT, UK*
[b] *Institute of Cancer Research, 237 Fulham Road, London, SW3 6JB, UK*
[c] *The Breast Cancer Now Toby Robins Research Centre, 237 Fulham Road, London, SW3 6JB, UK*

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Abstract ***Introduction:*** The *MYC* proto-oncogene is among the most commonly dysregulated genes in human cancers. We report screening data from the i*MYC* trial, an ongoing phase II study assessing ibrutinib monotherapy in advanced pretreated *MYC*- and/or *HER2*-amplified oesophagogastric cancer, representing the first attempt to prospectively identify *MYC* amplifications in this tumour type for the purposes of therapeutic targeting. ***Methods:*** Screening utilising a fluorescent *in situ* hybridisation (FISH) assay for assessment of tumour *MYC* amplification has been instituted. An experimental digital droplet polymerase

* *Corresponding author.*
** *Corresponding author.* Royal Marsden NHS Foundation Trust, Downs Road, Surrey, SM2 5PT, UK.
E-mail addresses: ian.chau@rmh.nhs.uk (I. Chau), irene.chong@icr.ac.uk (I.Y. Chong).
[1] These authors contributed equally to the work.

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Digital PCR

chain reaction (ddPCR) assay to assess *MYC* amplification in both tumour and circulating-tumour (ct)DNA has been developed and investigated.

Results: One hundred thirty-five archival tumour specimens have undergone successful FISH analysis with 23% displaying evidence of *MYC* amplification. Intertumour heterogeneity was observed, with the percentage of cancer cells harbouring *MYC* amplification ranging widely between samples (median 51%, range 11–94%). Intratumoural clonal diversity of *MYC* amplification was also observed, with a significant degree of variance in amplification ratios (Bartlett's test for equal variance $p < 0.001$), and an association between greater variance in *MYC* amplification and improved outcome with prior first-line chemotherapy. ddPCR was most accurate in quantifying *MYC* amplification in tumour-derived DNA from cases with a high proportion (>70%) of amplified cells within the tumour specimen but was not reliable in samples containing a low proportion of amplified cells or in ctDNA.

Conclusions: Our results illustrate the utility of FISH to assess *MYC* amplification prospectively for a biomarker-selected trial by providing reliable and reproducible results in real time, with a high degree of heterogeneity of *MYC* amplification observed. We show that ddPCR can potentially detect high-level *MYC* amplifications in tumour tissue.

1. Introduction

The prognosis for patients with advanced oesophagogastric (OG) cancer is poor, with a median overall survival (OS) of less than 12 months in most clinical trials [1]. There is utility for the targeted agents trastuzumab and ramucirumab in *HER2*-positive first-line and unselected second-line settings, respectively [2–4]. Further studies evaluating targeted agents inhibiting *EGFR*, *MET*, *PI3K/mTOR* and *PARP* have been disappointing, all failing to improve overall survival [5–9]. Therefore, effective biomarker-driven treatment approaches are urgently required to address this area of unmet clinical need.

The *MYC* proto-oncogene is among the most commonly dysregulated genes in human cancer, controlling the transcription of genes involved in multiple oncogenic pathways [10,11]. Effective methods for targeting *MYC* represent an attractive potential therapeutic strategy. As it is a transcription factor that lacks enzymatic activity, *MYC* cannot be directly targeted by small molecule inhibitors or antibody-based therapies [12]. Strategies based on the concept of synthetic lethality, whereby disruption of two or more genes in combination result in a deleterious phenotype, have been investigated to indirectly target tumours displaying *MYC* overactivity [13,14]. Using functional genomic screening of oesophageal cell lines, we previously observed selective decreased cell viability following silencing of Bruton's tyrosine kinase (*BTK*) in *MYC*-amplified cell lines. This was further validated using ibrutinib, a clinical irreversible BTK inhibitor that is also known to target *HER2* [15,16]. In *MYC*-amplified cells, ibrutinib downregulated levels of MYC protein and downstream effectors, and elicited G1 cell cycle arrest and apoptosis. BTK signalling occurs partly via the canonical RAS-RAF-MEK-ERK pathway. ERK is a known mediator of MYC phosphorylation, and a putative mechanism of action for *MYC* and *BTK* interaction was identified through observation of BTK-dependent, ERK-mediated, MYC phosphorylation [16].

As a result of our preclinical observations, we initiated i*MYC* (NCT02884453), an ongoing phase II non-randomised study to assess the efficacy of ibrutinib monotherapy in advanced pretreated OG cancer that represents the first attempt at targeting *MYC* amplification clinically in this tumour type [17]. As there are no established definitions of *MYC* 'positivity' in solid organ tumours, previous studies investigating *MYC* amplification have used fluorescent *in situ* hybridisation (FISH)-based techniques which defined positivity based on established cutoffs derived from *HER2* FISH testing [18]. In parallel, we also developed a novel digital droplet polymerase chain reaction (ddPCR) assay to assess *MYC* amplification in both tissue and circulating-tumour (ct)DNA.

Here we give the first report of the frequency, pattern and heterogeneity of *MYC* amplification in OG cancer from a prospective screening study with clinical outcome correlation. We describe the development of a ddPCR assay using two independent reference probes and assess the correlation of *MYC* amplification detected by FISH and ddPCR in both primary tumour tissue and ctDNA samples.

2. Materials and methods

2.1. Patient samples

Patients treated at the Royal Marsden Hospital, UK, with OG cancer of either squamous cell carcinoma (SCC) or adenocarcinoma (AC) histological subtype being considered for or undergoing systemic anticancer therapy were eligible for screening. Informed written

consent was obtained from all patients within the context of the i*MYC* trial (NCT02884453). At the time of consent, an archival diagnostic formalin-fixed paraffin-embedded (FFPE) tumour sample was obtained for analysis and blood samples were collected in Streck Cell-Free DNA blood collection tubes.

2.2. DNA extraction

FFPE tissue was cut into 5–8 micron sections and macrodissected to ensure >70% tumour content. DNA extraction was performed with the QIAamp DNA FFPE tissue kit (Qiagen) as per manufacturer's instructions. ctDNA was isolated from 4 to 5 mL of plasma using the QIAamp Circulating Nucleic Acid kit (Qiagen) as per manufacturer's instructions. Tumour and ctDNA was quantified using the Qubit dsDNA HS assay (Thermo Fisher Scientific).

2.3. Fluorescent in situ hybridisation

A dual-probe FISH assay was developed and validated to diagnostic quality for the purposes of the study. Screening was performed using a combination of a reference probe mapping to the centromere of chromosome 8 (CEP8) and a *MYC* probe mapping to chromosome 8q24, covering the entire coding region of the *MYC* gene from exons 1–3. Dual-label FISH was performed on each tissue section using standard techniques, and results were reported in a standardised manner, recording both the range and modal ratio of CEP8 and *MYC* signals and, in the case of amplification, the proportion of cells displaying an amplified signal. The results were deemed to be amplified if *MYC*:CEP8 ratio ≥2.5.

2.4. Digital droplet PCR

A challenge in using ddPCR for the detection of gene amplifications is the reliable quantification of changes in the abundances of germline reference loci [19]. Suitable reference genes were identified through analysis of publically available copy number data to calculate copy number ratios of *MYC* versus all possible chromosome 8 reference genes in *MYC*-amplified and non-amplified cancers. For the final analysis, two reference genes were chosen: a chromosome 8 reference gene, *CEBPD*, was selected on the basis of high sensitivity for *MYC* amplification and a chromosome 14 reference gene, *RPPH1*, frequently used in assessment of copy number variation. Data were analysed with respect to each control gene individually and the average ratio of *MYC*:*RPPH1* and *MYC*:*CEBPD* reported for each sample (see supplementary material S1 for additional description of ddPCR assay development and methodology).

2.5. Statistical analysis

Statistical analysis for ddPCR reference probes was carried out using the R statistical package (available at http://www.R-project.org); all further statistical analysis was carried out using PRISM version 7 (available at https://www.graphpad.com/scientific-software/prism). Categorical variables were compared using χ^2 test; continuous variables by Mann–Whitney *U*-test; progression-free survival (PFS) and OS by Kaplan–Meier method. Significance level for statistical tests was set at 0.05.

3. Results

3.1. Prospective patient cohort

Between July 2016 and January 2018, 162 patients were consented for prospective screening as part of the *iMYC* study. Eighty-six percent (139/162) of cases were of AC histological subtype and 92% (149/162) had evidence of distant metastases at the time of enrolment. Of these, 88% of patients had received platinum-based first-line chemotherapy treatment (Table 1).

Table 1
Patient characteristics of i*MYC* prescreening population.

Total number of patients	n = 162
Age in years (median, range)	
At diagnosis	61.2 (36.4–85.5)
At time of ctDNA analysis	62.2 (37.1–85.8)
Sex	
Male	131 (81%)
Female	31 (19%)
Site of primary tumour	
Oesophagus/OGJ	127 (78%)
Stomach	35 (22%)
Histological subtype	
Adenocarcinoma	139 (86%)
Squamous cell carcinoma	23 (14%)
HER2 status*	
Positive	27 (17%)
Negative	135 (83%)
Disease status at enrolment	
Locally advanced	13 (8%)
Metastatic	149 (92%)
First-line treatment received (metastatic patients only)	
Platinum based	131 (88%)
Taxane based	9 (6%)
Other	7 (5%)
No treatment	2 (1%)
ctDNA analysis performed	127 (78%)

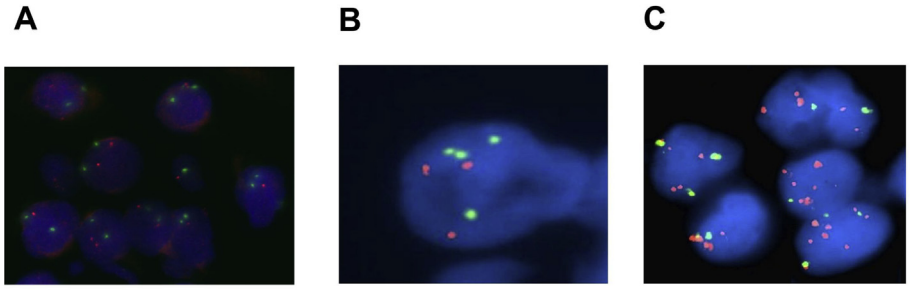
OGJ, oesophagogastric junction.
* Defined as *HER2*:*CEP17* ratio ≥2 by standard DDISH testing.

3.2. Evaluation of *MYC* amplification by FISH analysis of primary tumour FFPE samples

Samples were categorized as follows: 'diploid, copy number neutral' (displaying 2 *MYC* and 2 CEP8 signals per cell) (Fig. 1A), 'polysomatic' (additional signals of both *MYC* and CEP8) (Fig. 1B), '*MYC* amplified' (increased ratio of *MYC* to CEP8 signals) (Fig. 1C) +/− polysomy. Where a range of signal patterns was seen, the most prevalent (modal) pattern within the sample was recorded. Of 135/162 (85%) successfully analysed samples, a 'diploid, copy number neutral' signal pattern was seen in 24 (18%) cases (Fig. 1D). The most commonly observed pattern was polysomy without *MYC* amplification, seen in 80 (59%) cases. Amplification with no evidence of polysomy was observed in 16 (12%) samples and amplification with polysomy was seen in 15 (11%) samples. Intertumour heterogeneity was observed, with the percentage of cancer cells harbouring *MYC* amplification ranging widely between samples (median 51%, range 11–94%) (Fig. 2A). Intratumoural diversity, as manifested by the range of *MYC* amplification present within each specimen, was observed with 22/31 (71%) amplified samples showing a range of amplification ratios present (Fig. 2B–C) and a significant variance in amplification ratios (Bartlett's test for equal variance $p < 0.001$).

3.3. Clinical correlates of *MYC* amplification

We went on to assess association of *MYC* amplification with further histopathological features and response to



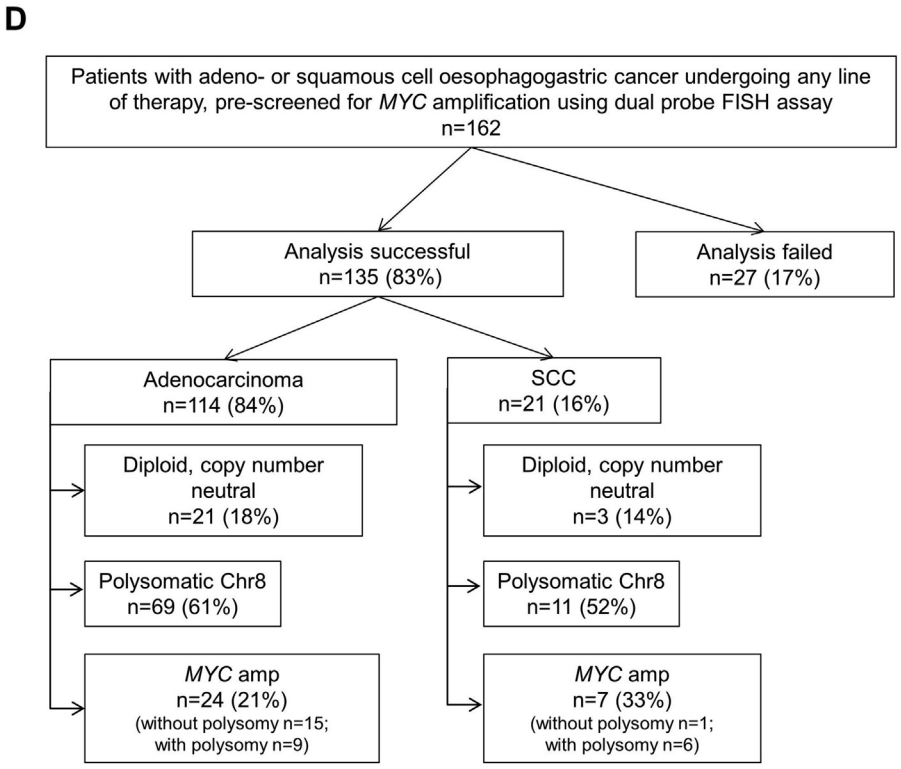


Fig. 1. Patterns of *MYC* amplification in primary oesophagogastric tumour samples detected by FISH (red = *MYC*; green = CEP8). (A) Normal diploid pattern (2 *MYC* and 2 CEP8 signals per cell). (B) Polysomic pattern (additional signals of both *MYC* and CEP8). (C) *MYC*-amplified pattern (increased ratio of *MYC* to CEP8 signals). (D) Flow diagram indicating the results of *MYC* FISH assessment in i*MYC* trial samples, separated based on histology. FISH, fluorescent *in situ* hybridisation.

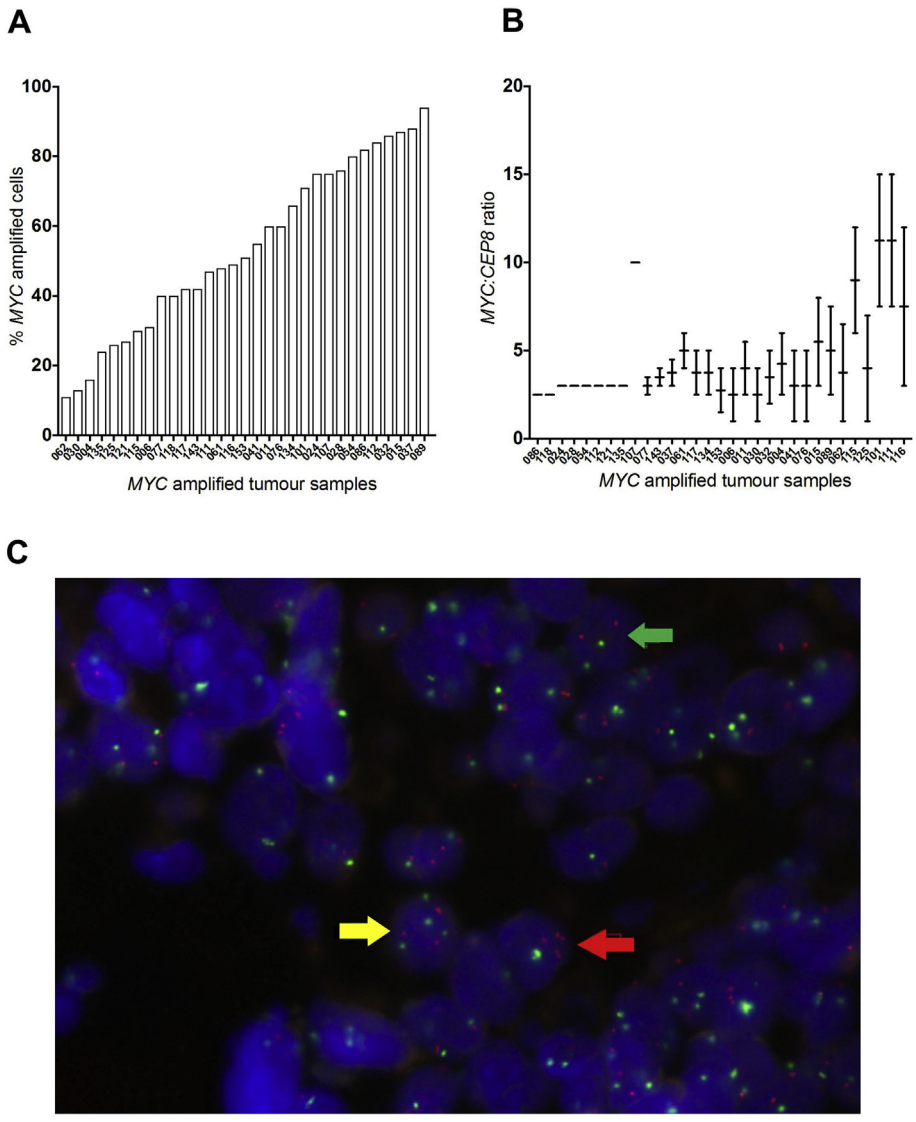


Fig. 2. Intertumoural and intratumoural heterogeneity of *MYC* amplification. (A) Bar chart illustrating proportion of cells displaying *MYC* amplification within each amplified specimen; (B) line chart demonstrating range of *MYC* amplification ratios seen within each amplified specimen; (C) individual sample showing intratumoural heterogeneity of *MYC* amplification patterns; green arrow = normal diploid; yellow arrow = polysomatic; red arrow = *MYC* amplified.

prior systemic therapy. We observed that, although *MYC* amplification was identified in a higher percentage of SCC histological than AC subtypes, the difference was not statistically significant (33 vs 21%; $p = 0.22$ χ^2). We found no difference in *HER2* status, tumour differentiation or presence of signet cells between *MYC*-amplified and non-amplified tumours (Table S1). When considering the potential influence of *MYC* amplification on clinical outcome in patients with advanced disease treated with any first-line systemic therapy (n = 125), we found no significant difference in response rates (68 vs 55%; $p = 0.22$ χ^2), median PFS (22.9 vs 22.1 weeks; $p = 0.55$ log-rank; Fig. 3A) or OS (61.6 vs 63.3 weeks; $p = 0.13$ log-rank; Fig. 3B) between *MYC*-amplified and non-amplified tumours. Given the differences within the screening population in terms of first-line treatment received, we then selected the patient group who had received a standard chemotherapy combination comprising a fluoropyrimidine and platinum agent with or without the addition of an anthracycline (n = 84). For these patients, there was a trend towards improved overall response rates in the *MYC*-amplified cohort (64 vs 45%, $p = 0.09$ χ^2), but amplification status again did not affect median PFS or OS. In an exploratory analysis, we assessed the impact of degree of clonal diversity in *MYC* amplification on survival outcome by undertaking tertile analysis based on the range of *MYC:CEP8* ratios observed within the amplified tumour specimens. We observed that tumour samples with the highest variance in *MYC* amplification

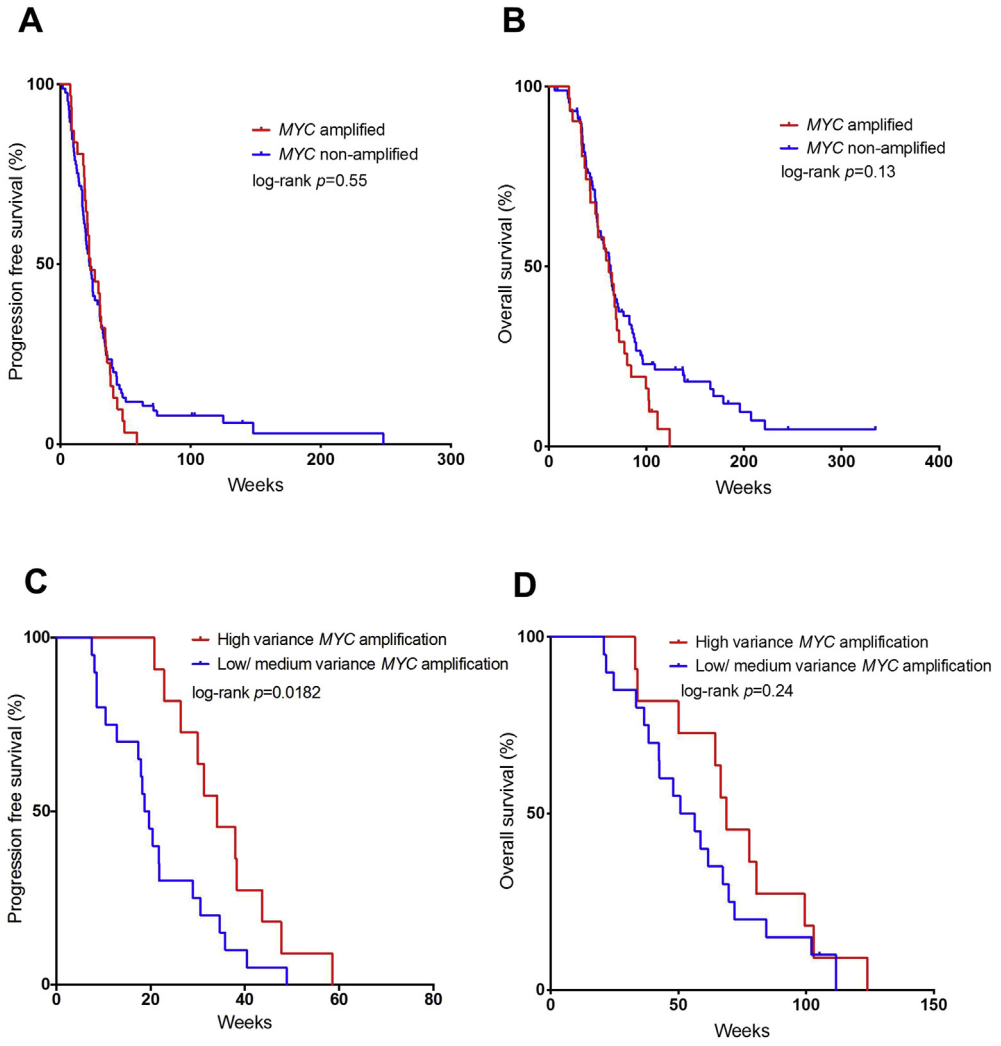


Fig. 3. Influence of *MYC* amplification status on survival. (A) Progression-free survival on first-line systemic treatment for *MYC*-amplified versus non-amplified tumours by FISH (22.9 vs 22.1 weeks; $p = 0.55$ log-rank). (B) Overall survival for *MYC*-amplified versus non-amplified tumours by FISH (61.6 vs 63.3 weeks; $p = 0.13$ log-rank). (C) Progression-free survival for high variance (defined as top tertile of tumours by range of *MYC* amplification observed within specimen) versus low/medium variance (low- and mid-tertile tumours by range of *MYC* amplification observed within specimen) tumours (34.1 vs 18.7 weeks; $p = 0.0182$ log-rank). (D) Overall survival of high variance versus low/medium variance *MYC*-amplified tumours (68.7 vs 50.7 weeks; $p = 0.24$ log-rank). FISH, fluorescent *in situ* hybridisation.

were associated with a significantly longer PFS after first-line treatment (34.1 vs 18.7 weeks; log-rank $p = 0.0182$; Fig. 3C) but no difference in OS was seen (68.7 vs 50.7 weeks; $p = 0.24$ log-rank; Fig. 3D). The proportion of amplified cells within each tumour sample did not appear to influence survival.

3.4. *MYC* amplification assessment by ddPCR in patient samples from the iMYC trial

We then analysed patient samples retrieved as part of the i*MYC* study and, as of October 2018, 105 archival FFPE tumour samples have undergone DNA extraction. Of these, ddPCR analysis was successful in 98 (93%) (Fig. 4A). When comparing *MYC* amplification status as assessed by FISH and ddPCR, we observed a higher median tumour ddPCR ratio in *MYC*-amplified as compared to non-amplified tumours (1.417 vs 1.246; $p = 0.017$ Mann–Whitney *U*-test; Fig. 4B). However, the ddPCR ratios detected in OG tumours were lower than expected compared with cell line data (Fig. S3C), highlighting the difference between cell line and primary samples. Considering that we had observed significant intertumoural and intratumoural heterogeneity of *MYC* amplification, we hypothesised that ddPCR would have greater sensitivity in more highly amplified cases. To address this, we undertook a tertile analysis based on the proportion of amplified cells within the sample. Consistent with our hypothesis, we observed no significant difference in ddPCR ratio between amplified samples containing greater or less than 35% *MYC*-amplified cells (1.432 vs 1.387; $p = 0.3111$ Mann–Whitney *U*-test;

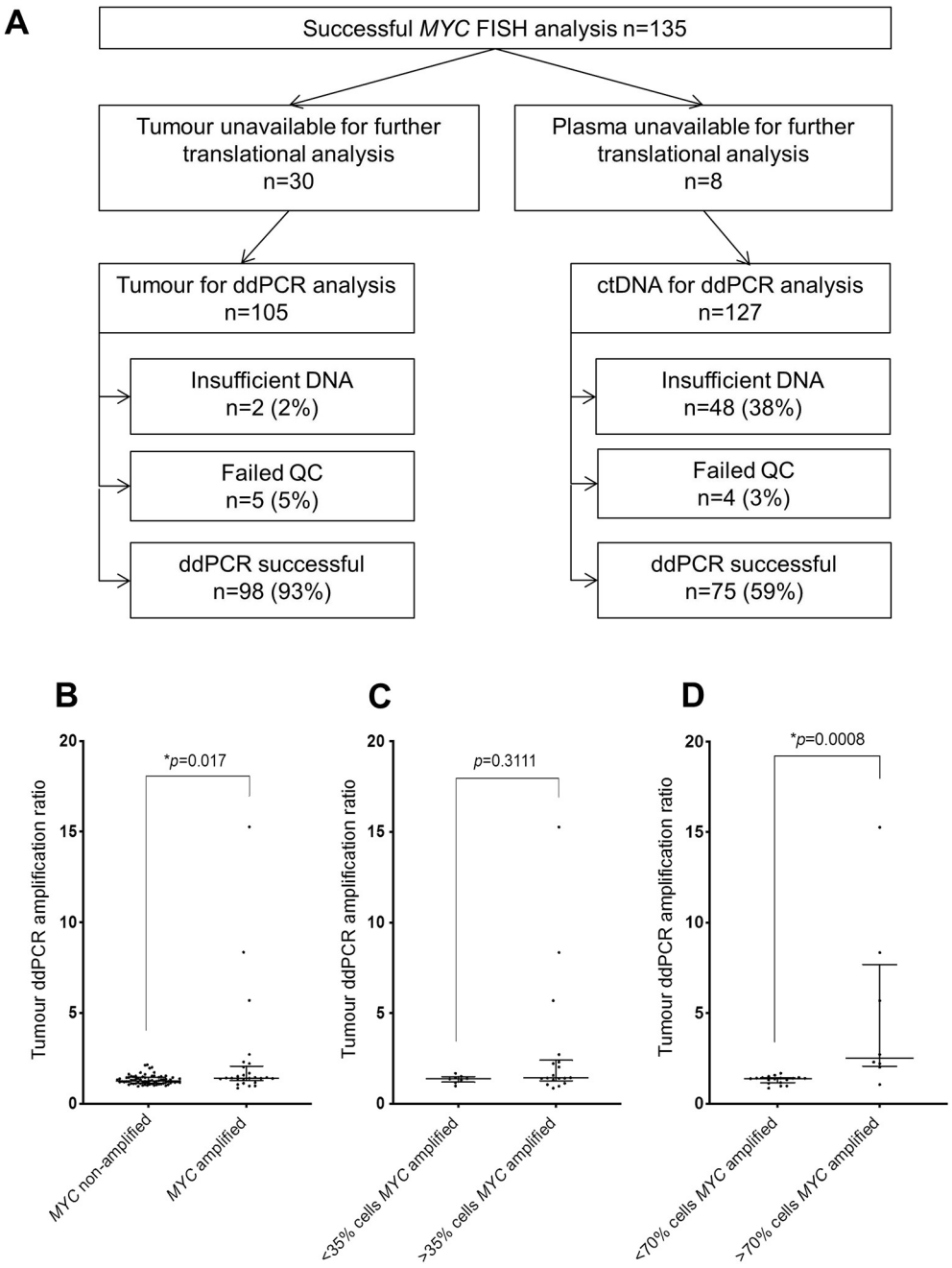


Fig. 4. *MYC* amplification assessment by ddPCR in patient samples from the i*MYC* trial. (A) Flow diagram of tumour and ctDNA ddPCR sample analysis from patients entered into the i*MYC* trial; scatterplots showing (B) significant difference in tumour ddPCR ratio between *MYC*-amplified and non-amplified samples assessed by FISH ($p = 0.017$ Mann–Whitney *U*-test); (C) no difference in tumour ddPCR ratio between *MYC*-amplified samples with< 35% and >35% amplified cells, respectively ($p = 0.3111$ Mann–Whitney *U*-test); (D) a significant difference in tumour ddPCR ratio between *MYC*-amplified samples with <70% and >70% amplified cells, respectively ($p = 0.008$ Mann–Whitney *U*-test). ddPCR, digital droplet polymerase chain reaction; FISH, fluorescent *in situ* hybridisation.

Fig. 4C). By contrast, for samples containing greater or less than 70% amplified cells, the ddPCR ratio was found to be significantly higher in these more amplified samples (2.512 vs 1.396; $p = 0.0008$ Mann–Whitney *U*-test; Fig. 4D), with a receiver operator area under the curve of 0.8958 (95% CI 0.7026–1.089; $p = 0.0015$). Using a ddPCR cutoff ratio of 2.0 resulted in a sensitivity of 87.5% and specificity of 100% in identifying these highly (>70% cells) amplified cases.

ddPCR analysis using ctDNA was possible in 75/127 (59%) plasma samples, as the remainder had insufficient DNA for analysis (Fig. 4A). We observed no difference in plasma ddPCR ratios between *MYC*-amplified and non-amplified tumours (1.547 vs 1.450; $p = 0.5705$

Mann–Whitney *U*-test), and the percentage of cells harbouring *MYC* amplification in the primary tumour did not influence the ddPCR ratio.

4. Discussion

Based on preclinical work demonstrating a synthetically lethal gene interaction between *BTK* and *MYC* [16], we have prospectively assessed *MYC* amplification for the first time in this tumour type for the purposes of therapeutic targeting with the BTK-inhibitor ibrutinib within a biomarker-selected clinical trial. Our screening results illustrate the utility of cytogenetic analysis by FISH to assess *MYC* amplification in this context by providing reliable and reproducible results in real time. We found *MYC* amplification in OG tumours assessed by FISH to be a relatively common event, occurring in 33% of SCC and 21% of AC tumours, respectively. This is consistent with *MYC* amplification frequencies reported in a recent analysis of oesophageal AC and SCC undertaken by the Cancer Genome Atlas Research Network [20]. We observed a high degree of heterogeneity in amplification patterns, which has previously been described in a retrospective study in this tumour type [21]. Intratumoural heterogeneity in OG cancer, comprising both spatial heterogeneity and temporal heterogeneity along progression from primary to recurrent or advanced disease, is increasingly recognised [22], and heterogeneity of expression of *ERBB2* and *FGFR* genes has been associated with differential responses to targeted therapies in gastric cancer [23,24]. Whether the heterogeneity of *MYC* amplification will impact upon the efficacy of its targeting within our current ongoing clinical trial remains to be seen; however, this will be an important consideration in future efforts to target *MYC* from a clinical perspective.

We observed a trend towards improved response to first-line platinum/fluoropyrimidine-based chemotherapy in *MYC*-amplified tumours, consistent with previously described associations between *MYC* amplification and favourable chemotherapy response in breast and ovarian cancer [25–27]. Although *MYC* status did not influence survival outcomes overall, we identified an association with improved PFS in cases with a higher variance in observed amplification ranges within the specimen. *MYC* amplification has been associated with a tumour-hypoxic molecular signature indicating increased underlying genomic instability [11]. Genomic instability has also been correlated with improved outcomes to platinum-based chemotherapy [28], and it is possible that clonal diversity of *MYC* amplification may be a surrogate for this. However, further work is necessary to clarify the role of *MYC* in mediating platinum response in this disease, and validation of our findings in a larger independent data set will be required.

Given the heterogeneity seen, identifying amplifications from pooled DNA as compared to single cell-based analysis such as FISH is likely to be challenging. Our novel ddPCR assay was developed based on robust identification of suitable reference genes. Although a statistically significant difference in tumour ddPCR ratio was seen between *MYC*-amplified and non-amplified tumours as assessed by FISH, the absolute difference was small. However, ddPCR was able to identify more highly amplified tumour samples (containing >70% amplified cells). Thus, the ability of ddPCR to detect *MYC* amplifications may be limited to those tumour samples displaying a homogenous high-level pattern of *MYC* amplification only. Its application to ctDNA was limited by the relatively small numbers of samples where adequate DNA could be extracted and successfully analysed. The high failure rate encountered is likely to be due, in part, to the nature of the i*MYC* prescreening cohort as patients could have blood taken at any time before, during, or after a line of treatment. Effective systemic treatment can potentially reduce absolute ctDNA levels [29], and thus the yield of extracted ctDNA from plasma was potentially influenced by the clinical context at the time point when the blood was taken. The accuracy of ctDNA ddPCR in detecting genomic amplifications is known to be lower than tumour ddPCR [30], and the high heterogeneity of *MYC* amplification may have contributed to the lower concordance seen [21]. Furthermore, *MYC* amplification has been associated with both aneuploidy and increased intratumoural heterogeneity in other solid tumours and may be an acquired event in tumour evolution from primary to metastatic disease, potentially affecting equivalence of results between primary tumour specimens and ctDNA from blood samples taken at differing time points of disease progression [31,32].

As research into effective *MYC*-targeting treatment continues, robust and reproducible methods of biomarker detection will be necessary, and we show that prospective screening for *MYC* amplification in OG cancer for the purposes of therapeutic targeting is feasible. The FISH assay used has revealed *MYC* amplification to be a common event in this tumour type, with a high degree of heterogeneity of *MYC* amplification patterns observed. We show that ddPCR can potentially be used to detect *MYC* amplifications in tumour samples with a high proportion of amplified cells; however, further work is necessary to optimise this technique in ctDNA. Although a trend towards improved chemotherapy response rates in *MYC*-amplified patients was noted, no clear association between *MYC* status and survival was seen. The clinical implications of *MYC* alterations in OG cancer are therefore likely to lie in the potential to harness *MYC* as a therapeutic target, and the clinical efficacy results of our biomarker-selected trial are awaited.

Statement of author contributions

Study concept and design of the article were performed by MD, LA, IYC and IC. Data acquisition was performed by MD, RLA, RB, DC, DW, NS, SR and IC. Quality control of data and algorithms was performed by MD, IYC, KK and EK. Data analysis and interpretation were carried out by MD, IYC, LA, HB, JHR, RC, SHK, KK, EK, MLC, MT, AW, GB, JS and CJL. Statistical analysis was performed by MD, RC, KK and EK. Manuscript preparation was carried out by MD, LA and IYC. Manuscript editing was carried out by MD. Manuscript review was performed by all authors. All authors were involved in preparation of the manuscript and had final approval of the submitted version.

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Conflict of interest statement

NS has received Research funding from AstraZeneca, BMS and Merck and received honoraria from AstraZeneca. DC has received funding from Amgen, AstraZeneca, Bayer, Celgene, Merck-Serono, Medimmune, Merrimack, Novartis, Roche and Sanofi. IC has served on advisory boards for Eli-Lilly, Bristol Meyers Squibb, MSD, Bayer, Roche, Merck-Serono, Five Prime Therapeutics, AstraZeneca, Oncologie International and Pierre Fabre; received research funding from Eli-Lilly, Janssen-Cilag, Sanofi Oncology and Merck-Serono; received honorarium from Eli-Lilly. IYC has received from research funding from Merck, KGaA and Janssen-Cilag.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2019.09.003.

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