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Original article

Histological intratumoral heterogeneity in pre-treatment oesophageal cancer biopsies predicts survival benefit from neoadjuvant chemotherapy – results from the UK MRC OE02 trial

Running title

Heterogeneity in oesophageal cancer

Naser Davarzani*[1,2], Lindsay C. Hewitt*[1,3], Matthew D. Hale[3], Veerle Melotte[1,4], Matthew Nankivell[5], Gordon G. A. Hutchins[3], David Cunningham[6], William H. Allum[7], Ruth E. Langley[5], Shahab Jolani#[8], Heike I. Grabsch#[1,3]

1. Department of Pathology, GROW School for Oncology and Developmental Biology, Maastricht University Medical Center, Maastricht, The Netherlands.
2. Biosystems Data Analysis, Swammerdam Institute for Life Sciences, Amsterdam University, Amsterdam, The Netherlands
3. Division of Pathology and Data Analytics, Leeds Institute of Medical Research at St James's, University of Leeds, Leeds, UK
4. Department of Clinical Genetics, University of Rotterdam, Erasmus University Medical Center, Rotterdam, The Netherlands
5. Medical Research Council Clinical Trials Unit at University College, London, UK
6. Gastrointestinal and Lymphoma Unit, Royal Marsden Hospital, London, UK
7. Department of Surgery, Royal Marsden Hospital, London, UK.

8. Department of Methodology and Statistics, CAPHRI, Maastricht University, Maastricht, The Netherlands.

* joint first authors

joint last authors

Corresponding author:

Prof Heike I Grabsch

Department of Pathology

Maastricht University Medical Center+

P. Debyelaan 25

6229 HX Maastricht

The Netherlands

Phone: +31433874610

Fax: +31433874616

Email: H.Grabsch@maastrichtuniversity.nl

Abstract

Aim

Despite the use of multimodal treatment, survival of oesophageal cancer (OeC) patients remains poor. One proposed explanation for the relatively poor response to cytotoxic chemotherapy is intratumour heterogeneity. The aim was to establish a statistical model to objectively measure intratumour heterogeneity of the proportion of tumour (IHPoT) and to use this newly developed method to measure IHPoT in the pre-treatment biopsies from OeC OE02 trial patients.

Methods

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

Results

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

Conclusions

This is the first study suggesting that IHPoT measured in the pre-treatment biopsy can predict chemotherapy survival benefit in OeC patients. IHPoT may represent a

clinically useful biomarker for patient treatment stratification. Future studies should determine if pathologists can reliably estimate IHPoT.

Key words: oesophageal cancer, histological heterogeneity, neoadjuvant chemotherapy, proportion of tumour, pre-treatment biopsy

INTRODUCTION

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

Decisions about OeC patient treatment are made at the time of diagnosis after confirming the presence of cancer in the endoscopic biopsy and clinical staging of the disease. We recently quantified the relative tumour content (proportion of tumour per area (PoT)) as continuous measurement values on Haematoxylin/Eosin stained digital slides in the pre-treatment biopsies of OeC patients using a well-established morphometric method called point counting and were able to demonstrate that PoT can predict survival benefit from cytotoxic chemotherapy [7]. Importantly, our previous study was the first to show that the relationship between PoT and chemotherapy benefit was non-linear: only patients with a mean PoT of all tumour containing biopsies between 40% and 70% derived benefit from chemotherapy whereas patients with mean PoT < 40% or mean PoT > 70% did not benefit from chemotherapy. During this previous study, we noticed that the PoT value can vary considerably between biopsy pieces from the same patient.

Considering that not only the absolute mean PoT value of all tumour containing biopsies per patient [7] but also the difference of the PoT value between biopsy pieces

from the same patient (intratumour heterogeneity of the proportion of tumour (IHPoT)) might influence chemotherapy survival benefit, we hypothesized that OeC patients with relatively low IHPoT (e.g. similar PoT values in different biopsies from the same patient) will have greater survival benefit from neoadjuvant 5-fluoruracil/cisplatin chemotherapy compared to those with high IHPoT.

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

MATERIAL AND METHODS

Study population

The UK Medical Research Council (MRC) OE02 trial randomized 802 patients with locally advanced resectable oesophageal cancer to surgery alone or 2 cycles of 5-fluoruacil plus cisplatin chemotherapy followed by surgery [3, 8]. Absolute tumour content per biopsy area (proportion of tumour, PoT) of each pre-treatment biopsy piece was available from 281 OE02 trial patients (140 patients treated with chemotherapy followed by surgery (chemo+surgery) and 141 patients treated with surgery alone) from our previous study [7].

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

Calculating intratumoural heterogeneity of the proportion of tumour

Of the 281 patients with existing pre-treatment biopsy PoT value from our previous study [7], 218 patients (S patients n=115, CS patients n=103) had PoT values from two or more tumour containing biopsies. Although a large number of studies in the literature uses the term 'tumour heterogeneity', it is not clear under what conditions samples/values from the same tumour should be classified as 'heterogeneous'. We set out to establish a statistical method to calculate an intratumoural heterogeneity index of PoT and to explore its predictive and prognostic value in patients with oesophageal cancer recruited to the OE02 trial. The statistical method considers the number of available biopsy pieces and percentage of tumour (PoT) value and calculates an index which is a measure of the variation between the PoT values of the biopsy pieces.

In the field of multilevel data analysis, the mixed effects model (MEM) has been proposed as an appropriate model to analyse different quantities measured from the same individual [9-11], e.g., in our case the PoT values from different biopsy pieces of the same patient. We applied the R package "lme4" [12, 13] to build the MEM, which provides a value describing the level of variation (heterogeneity) between PoT values of the same patient. Theoretically, the obtained heterogeneity index can range from zero (no heterogeneity) to infinity (maximal heterogeneity). Details of the statistical methodology including data structure can be found in the supplemental information: text S1 and table S1. The error in estimating the intratumoural heterogeneity index of PoT (IHPoT index) using MEM was measured by performing a simulation study, see supplemental text S2 for methodology.

Statistical analyses

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

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

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

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

the same patient. For the patients with low IHPoT index, a multivariate analysis using Cox model adjusted by age, sex, tumor location and histological tumor type, has been performed, too.

RESULTS

The median number of biopsy pieces per patient was 3 (range: 2 to 12 pieces). In total, PoT values from 775 individual biopsy pieces from 218 patients were available for analysis. The majority of patients (n=77, 35.3%) had two biopsies, 56 (25.7%) patients had 3 biopsies, 40 (18.3%) patients had 4 biopsies, 13 (6%) patients had 5 biopsies, 16 (7.3%) patients had 6 biopsies and16 (7.3%) patients had 7 or more biopsies with PoT, figure 1.

The median IHPoT index was 0.1638 (range: 0 to 3.17). Based on Q-statistics (see material and methods) we used a cut off of 0.2030 for the IHPoT index to classify the heterogeneity of tumours. Tumours from 97 (44.5 %) OeC patients (48 (41.7%) surgery patients, 49 (47.6%) chemo+surgery patients) were classified as showing relatively high IHPoT (IHPoT index >0.2030). Tumours from 121 (55.5%) OeC patients (67 (58.3%) surgery patients, 54 (52.4%) chemo+surgery patients) were classified as showing relatively low IHPoT (IHPoT index ≤0.2030). There was no linear relationship between the number of biopsies per patient and IHPoT index per patient, see figure 1. Moreover, our simulation study showed that the error in calculating the IHPoT index using MEM was very small (close to zero) regardless of the number of biopsy pieces, see figure S1.

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

Intratumoural heterogeneity of the proportion of tumour and survival

Chemo+surgery patients with low IHPoT index in the pre-treatment biopsy had a significantly longer survival compared to surgery patients with low IHPoT index in the univariate analysis (hazard ratio (HR) =1.81, 95% confidence interval (CI): 1.20-2.75, *P*=0.005, figure 2), and in the multivariate analysis (hazard ratio (HR) = 1.9, 95% confidence interval (CI): 1.24-2.98, P=0.003).

There was no significant difference in survival when comparing chemo+surgery patients with high IHPoT index in the pre-treatment biopsy to surgery patients with high IHPoT index (HR=1.15, 95%CI: 0.72-1.81, *P*=0.566, figure 2).

As we previously found that patients with a mean absolute biopsy PoT value between 40% and 70% had a survival benefit from pre-operative chemotherapy, we additionally explored whether the improved OS in this particular patient subgroup is related to the degree of intratumour heterogeneity of PoT. In chemo+surgery and surgery patients, 84 (55.6%) patients with a mean absolute biopsy PoT value between 40% and 70% had a low IHPoT index compared to 67 (44.4%) patients with mean absolute PoT values < 40% or > 70%, p=0.956. The survival benefit from pre-operative

chemotherapy seemed to be even higher in the subgroup of chemo+surgery patients with a mean absolute biopsy PoT value between 40% and 70% and low IHPoT index (*n*=36, HR=2.71, 95%CI: 1.60-4.61, *P*<0.001, figure 2), which has been also confirmed by the multivariate analysis (HR=3.13, 95%CI: 1.77-5.55, P<0.001). In contrast, patients with a mean absolute biopsy PoT value between 40% and 70% and high IHPoT index did not have a survival benefit from chemotherapy (figure 2). In exploratory analysis, patients with mean absolute PoT <40 % or > 70%, did not seem to have a survival benefit from chemotherapy irrespective of the IHPoT index (Figure S2).

There was neither a significant difference in survival of S patients comparing high versus low IHPoT index (HR=0.76, 95%CI: 0.50-1.15, P=0.19) nor within the CS patients (HR=1.19, 95%CI: 0.75-1.90, *P*=0.45), figure 3.

DISCUSSION

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

In this exploratory, hypothesis generating study using a MEM, we found that patients with a low IHPoT index in the pre-treatment biopsy (e.g. the proportion of tumour per biopsy piece from the same patient was very similar) had a survival benefit from cytotoxic chemotherapy. We have previously shown that patients with a mean absolute PoT of 40%≤PoT≤70% had a survival benefit from pre-operative chemotherapy [7]. We

can now demonstrate that patients with tumours with a mean absolute PoT value between 40% and 70% and low IHPoT index at the same time had the most survival benefit from pre-operative chemotherapy. In contrast, patients with a high IHPoT index (e.g. large variation in the PoT values between biopsy pieces) derived little or no survival benefit from chemotherapy.

Recently, image analysis of haematoxulin/eosin stained sections from lung cancer was found to be predictive of mutation status [17], providing evidence that the morphological phenotype of the tumour is reflective of its molecular phenotype. Studies in oesophageal, head and neck and colon cancer have investigated 'molecular intratumoural heterogeneity' without providing a definition for intratumour heterogeneity as such. Existing data relating to 'intratumoural heterogeneity' are therefore difficult to interpret and cannot be compared with each other or with our current study which investigated histological intratumoural heterogeneity [18-27] .

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

To the best of our knowledge this is the first study that has used a statistical method to objectively measure and clearly define intratumour heterogeneity. Results of our study suggest that intratumoural heterogeneity of the relative tumour content per tissue area is a potential useful biomarker for clinical decision making in oesophageal cancer

patients. Based on the results of our simulation study, we propose that the minimum number of biopsy pieces required to measure IHPoT index is 2. As implementation of MEM for IHPoT index reporting in routine pathology might not be feasible, future studies should determine whether IHPoT in OeC biopsies can be reliably estimated by pathologists.

Limitations of our study include that this is a retrospective ad hoc analyses of a subset of available pre-treatment biopsies from OE02 trial patients containing 2 or more tumour containing biopsy pieces. In our study, we measured intratumoural heterogeneity between biopsy pieces from the same patient. Intratumoural heterogeneity within individual biopsy pieces was not considered but may have an influence on our results. It was unfeasible to perform multivariate analyses, including known prognostic factors such as depth of invasion and lymph node status, for two reasons. Firstly, detailed pre-treatment staging data were not collected in this trial [8]. Secondly, using the pathological stage derived after surgery may not be representative of the stage in the biopsies from patients treated with neoadjuvant chemotherapy due to chemotherapy induced pathological changes. It was also not feasible to perform analyses based on histological subtype due to small sample size and a lack of statistical power. Furthermore it is not clinically relevant since patients with oesophageal squamous cell carcinoma and adenocarcinoma receive the same treatment.

CONCLUSIONS

In the era of whole genome sequencing and NGS, the increasing complexity of intratumoural heterogeneity in cancer is becoming evident. However, the predictive

value of molecular heterogeneity in response to therapy remains to be clarified and has not been implemented into clinical routine. We have shown that estimating intratumoural heterogeneity of a histological factor such as proportion of tumour using digitized haematoxylin/eosin stained pre-treatment biopsy slides and a mixed effect model is predictive of survival benefit to cytotoxic chemotherapy in OeC patients from the Oe02 trial and may represent a clinically useful biomarker for patient treatment stratification.

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Conflicts of Interest

DC has received National Institute for Health funding from The Royal Marsden and Institute of Cancer Research Biomedical research Centre. HG has received funding from The Pathological Society of Great Britain and Ireland, Yorkshire Cancer Research and Sasakawa Foundation UK. All remaining authors have declared no conflicts of interest.

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Figure Legends

Figure 1. Range of intratumoural heterogeneity of the proportion of tumour for patients with different number of biopsy pieces

Figure 2. Five years overall survival of patients treated with chemotherapy plus surgery (CS) versus surgery (S) alone stratified by intratumoural heterogeneity of the proportion of tumour (IHPoT) index and mean absolute PoT value.

(A) Patients with low IHPoT index (< 0.2030): CS patients survived significantly longer than S patients (HR=1.81, 95%CI: 1.20-2.75, P=0.005).

(B) Patients with high IHPoT index (> 0.2030): There is no significant difference in survival between CS patients and S patients (HR=1.15, 95%CI: 0.72-1.81, P=0.566).

(C) Patients with low IHPoT index and 40% ≤PoT≤ 70%: CS patients survived significantly longer than S patients (HR=2.71, 95%CI: 1.60-4.61, P<0.001),

(D) Patients with high IHPoT index and 40% ≤PoT≤ 70%: There is no significant difference in survival between CS patients and S patients (HR=1.52, 95%CI: 0.85-2.70, P<0.153).

Figure 3. Five years overall survival of patients with high versus low intratumoural heterogeneity of the proportion of tumour (IHPoT) index within each treatment group.

(A) There is no significant difference between the survival of S patients with high IHPoT index versus low IHPoT index (HR=0.76, 95%CI: 0.50-1.15, P=0.19)

(B) There is no significant difference between the survival of CS patients with high IHPoT index versus low IHPoT index (HR=1.19, 95%CI: 0.75-1.90, P=0.45).

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

	Chemotherapy + surgery			Surgery alone		
	Low IHPoT *n* (%)	High IHPoT *n* (%)	p-value	Low IHPoT *n* (%)	High IHPoT *n* (%)	*p-value*
Age (years)						
≤ 65	32 (57)	24 (43)	0.477	39 (57)	29 (43)	0.883
> 65	22 (50)	22 (50)		28 (56)	22 (44)	
Gender						
Female	10 (46)	12 (56)	0.363	17 (50.0)	17 (50.0)	0.344
Male	4 4(56)	34 (44)		50 (59.5)	34 (40.5)	
Depth of invasion ((y)pT)*						
T0/Tis	2 (67)	1 (33)	0.055	0	0	0.353
T1	3 (33)	6 (67)		6 (50)	6 (50)	
T2	9 (82)	2 (18)		5 (83)	1 (17)	
T3	33 (57)	25 (43)		42 (58)	30 (42)	
T4	0	3 (100)		0	1 (100)	
Lymph node status ((y)pN)*						
N0	20 (51)	19 (49)	0.422	20 (59)	14 (41)	0.985
N1	27 (60)	18 (40)		34 (59)	24 (41)	
(y)pTNM stage*						
0	2 (67)	1 (33)	0.706	0	0	0.361
I	2 (33)	4 (67)		4 (44)	5 (56)	
II	19 (56)	15 (44)		21 (68)	10 (32)	
III	24 (59)	17 (42)		28 (55)	23 (45)	
Mandard tumour regression grade						
1	2 (67)	1 (33)	0.788	Not applicable		
2	1 (50)	1 (50)				
3	7 (70)	3 (30)				
4	13 (48)	14 (52)				
5	24 (59)	17 (42)				
Histological tumour type						
Squamous cell carcinoma	11 (50)	11 (50)	0.791	10 (46)	12 (55)	0.346
Adenocarcinoma	33 (57)	25 (43)		41 (62)	25 (38)	
others	1 (100)	0		2 (67)	1 (33)	
Resection margin status						
Positive	14 (50)	14 (50)	0.661	20 (61)	13 (39)	0.629
Negative	33 (55)	27 (45)		31 (55)	25 (45)	
Tumour location						
Lower	31 (46)	36 (54)	**0.010**	50 (62)	31 (38)	0.256
Middle	12 (57)	9 (43)		12 (46)	14 (53)	
Upper	11 (92)	1 (8)		5 (46)	6 (43)	
*No data is available for patients who did not proceed to surgery, *n*=43.						

IHPoT, intratumoural heterogeneity of the proportion of tumour