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Original Article

Histological intratumoral heterogeneity in pretreatment esophageal cancer biopsies predicts survival benefit from neoadjuvant chemotherapy: results from the UK MRC OE02 trial

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SUMMARY. **Despite the use of multimodal treatment, survival of esophageal cancer (EC) patients remains poor. One proposed explanation for the relatively poor response to cytotoxic chemotherapy is intratumor heterogeneity. The aim was to establish a statistical model to objectively measure intratumor heterogeneity of the proportion of tumor (IHPoT) and to use this newly developed method to measure IHPoT in the pretreatment biopsies from from EC patients recruited to the OE02 trial. A statistical mixed effect model (MEM) was established for estimating IHPoT based on variation in hematoxylin/eosin (HE) stained pretreatment biopsy pieces from the same individual in 218 OE02 trial patients (103 treated by chemotherapy and surgery (chemo+surgery); 115 patients treated by surgery alone). The relationship between IHPoT, prognosis, chemotherapy survival benefit, and clinicopathological variables was assessed. About 97 (44.5%) and 121 (55.5%) ECs 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, $P = 0.566$). This is the first study suggesting that IHPoT measured in the pretreatment biopsy can predict chemotherapy survival benefit in EC patients. IHPoT may represent a clinically useful biomarker for patient treatment stratification. Future studies should determine if pathologists can reliably estimate IHPoT.**

KEY WORDS: **esophageal cancer, histological heterogeneity, neoadjuvant chemotherapy, pretreatment biopsy, proportion of tumor.**

INTRODUCTION

Esophageal cancer (EC) 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 EC 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 three drugs instead of two or increasing the number of chemotherapy cycles given preoperatively did not improve EC patient survival.[6]

Decisions about EC patient treatment are made at the time of diagnosis after confirming the presence of cancer in the endoscopic biopsy and clinical staging of

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the disease. We recently quantified the relative tumor content (proportion of tumor per area [PoT]) as continuous measurement values on hematoxylin/eosin (HE) stained digital slides in the pretreatment biopsies of EC 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 nonlinear: only patients with a mean PoT of all tumor 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 tumor containing biopsies per patient[7] but also the difference of the PoT value between biopsy pieces from the same patient (intratumor heterogeneity of the proportion of tumor [IHPoT]) might influence chemotherapy survival benefit, we hypothesized that EC 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: (i) to establish a statistical method to objectively measure intratumoral heterogeneity of the proportion of tumor (IHPoT) and (ii) to use this newly developed method to determine IHPoT in the pretreatment biopsies from esophageal 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 esophageal cancer to surgery alone or 2 cycles of 5-Fluorouracil plus cisplatin chemotherapy followed by surgery.[3, 8] Absolute tumor content per biopsy area (proportion of tumor, PoT) of each pretreatment 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 intratumoral heterogeneity of the proportion of tumor

Of the 281 patients with existing pretreatment biopsy PoT value from our previous study,[7] 218 patients (surgery patients $n = 115$, chemo + surgery patients $n = 103$) had PoT values from two or more tumor-containing biopsies. Although a large number of studies in the literature use the term 'tumor heterogeneity', it is not clear under what conditions samples/values from the same tumor should be classified as 'heterogeneous'. We set out to establish a statistical method to calculate an intratumoral heterogeneity index of PoT and to explore its predictive and prognostic value in patients with esophageal cancer recruited to the OE02 trial. The statistical method considers the number of available biopsy pieces and the percentage of tumor (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 effect model (MEM) has been proposed as an appropriate model to analyze 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 intratumoral 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 cutoff 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 tumor regression grade,[16] histological tumor type, resection margin status, and tumor location) was 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–

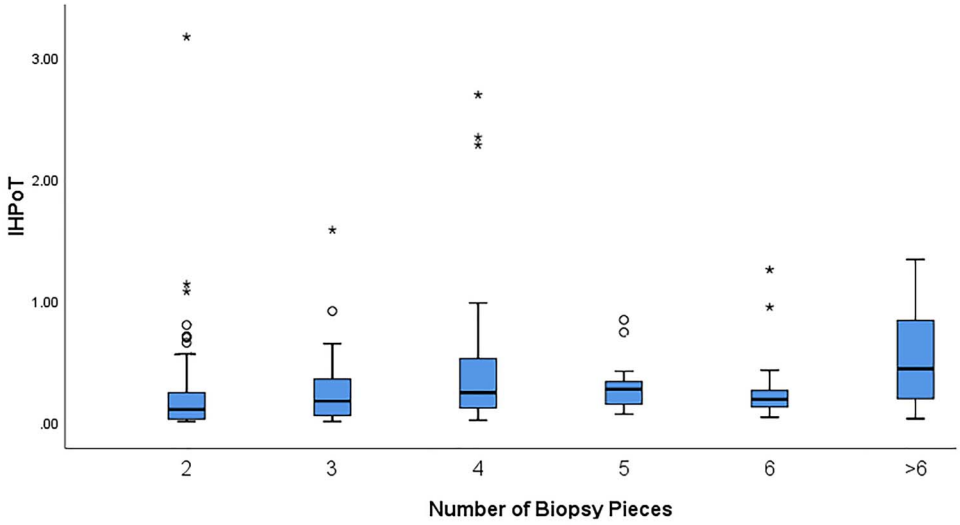

Fig. 1 Range of intratumoral heterogeneity of the proportion of tumor for patients with different number of biopsy pieces.

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 preoperative 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 was performed.

RESULTS

The median number of biopsy pieces per patient was 3 (range 2–12 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, and 16 (7.3%) patients had 7 or more biopsies with PoT (Fig. 1).

The median IHPoT index was 0.1638 (range 0–3.17). Based on Q-statistics (see Material and Methods), we used a cutoff of 0.2030 for the IHPoT index to classify the heterogeneity of tumors as high versus low. Tumors from 97 (44.5%) EC patients (48 [41.7%] surgery patients, 49 [47.6%] chemo+surgery patients) were classified as high IHPoT (IHPoT index >0.2030). Tumors from 121 (55.5%) EC patients (67 [58.3%] surgery patients, 54 [52.4%] chemo+surgery patients) were classified as low IHPoT (IHPoT index ≤0.2030). There was no linear relationship between the number of biopsies per patient and IHPoT index per patient (see Fig. 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 Fig. S1).

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

Intratumoral heterogeneity of the proportion of tumor and survival

Chemo+surgery patients with low IHPoT index in the pretreatment biopsy had a significantly longer survival compared to surgery patients with low IHPoT index in univariate analysis (HR = 1.81, 95% CI: 1.20–2.75, $P = 0.005$ [Fig. 2]) and in multivariate analysis (HR = 1.9, 95% 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 pretreatment biopsy to surgery patients with high IHPoT index (HR = 1.15, 95% CI: 0.72–1.81, $P = 0.566$ [Fig. 2]).

As we previously found that patients with a mean absolute biopsy PoT value between 40% and 70% had a survival benefit from preoperative chemotherapy, we additionally explored whether the improved OS in this particular patient subgroup is related to the degree of intratumor heterogeneity of PoT. In

Table 1 Patient characteristics according to intratumoral heterogeneity of the proportion of tumor 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 tumor 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 tumor 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)	
Tumor 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)	

IHPoT, intratumoral heterogeneity of the proportion of tumor.
*No data is available for patients who did not proceed to surgery, $n = 43$.

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 preoperative 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, $P < 0.001$ [Fig. 2]), which has been also confirmed by 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 (Fig. 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 (Fig. S2).

There was neither a significant difference in survival of surgery patients comparing high versus low IHPoT index (HR = 0.76, 95% CI: 0.50–1.15, $P = 0.19$) nor within the chemo+surgery patients (HR = 1.19, 95% CI: 0.75–1.90, $P = 0.45$) [Fig. 3].

DISCUSSION

This is the first study to measure intratumoral heterogeneity of the proportion of tumor (IHPoT) in routine hematoxylin/eosin stained pretreatment endoscopic biopsies from esophageal cancer (EC) patients from the randomized UK MRC OE02 trial. We used a mixed effect model (MEM) to estimate the IHPoT level by modeling the probability of being tumor for each measurement point in the biopsy pieces.

In this exploratory, hypothesis-generating study using a MEM, we found that patients with a low

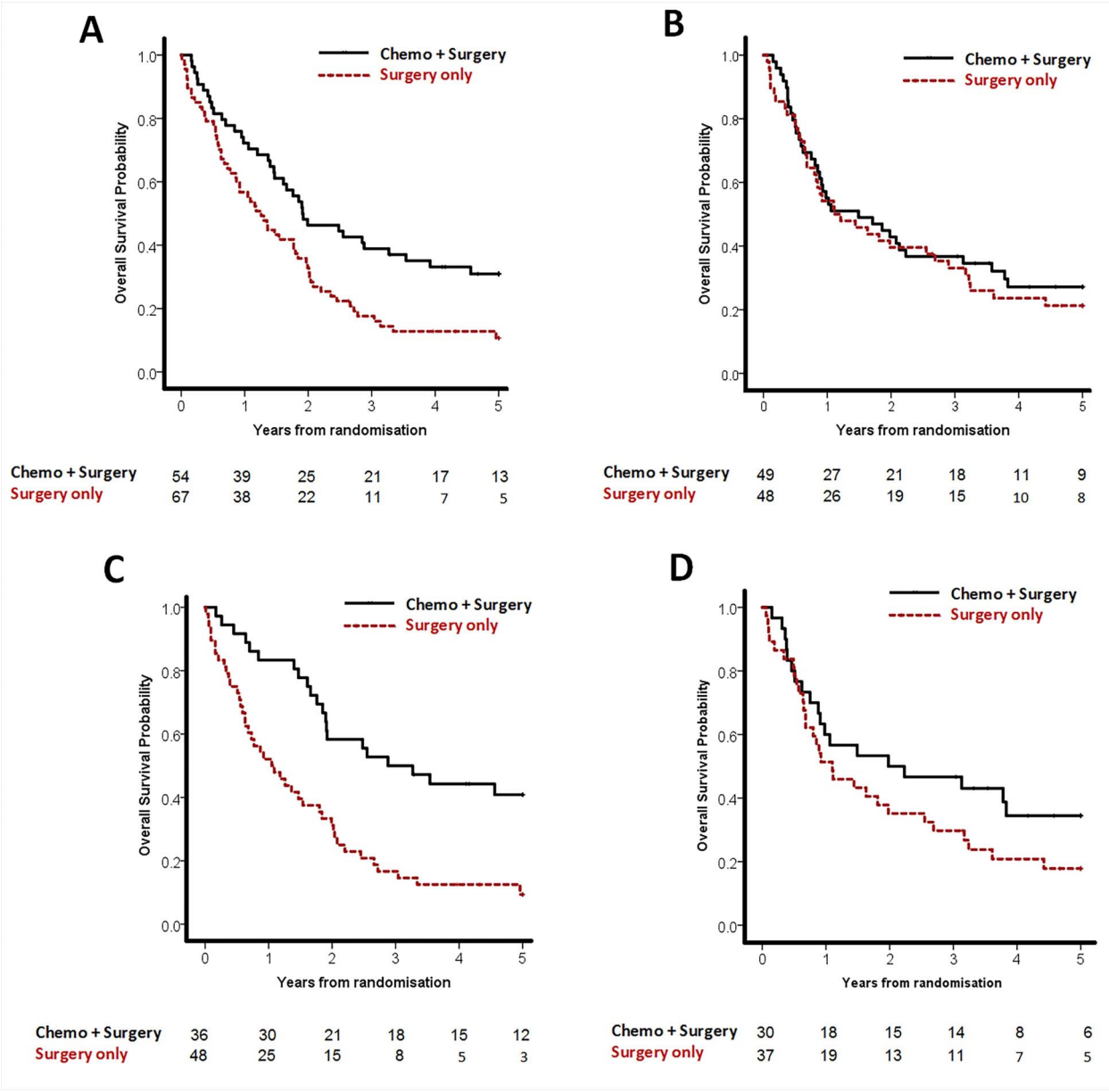

Fig. 2 Five-year overall survival of patients treated with chemotherapy plus surgery versus surgery alone stratified by intratumoral heterogeneity of the proportion of tumor (IHPoT) index and mean absolute PoT value. (A) Patients with low IHPoT index (<0.2030): chemo+surgery patients survived significantly longer than surgery 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 chemo+surgery patients and surgery patients (HR = 1.15, 95% CI: 0.72–1.81, $P = 0.566$). (C) Patients with low IHPoT index and $40\% \leq \text{PoT} \leq 70\%$: chemo+surgery patients survived significantly longer than surgery patients (HR = 2.71, 95% CI: 1.60–4.61, $P < 0.001$). (D) Patients with high IHPoT index and $40\% \leq \text{PoT} \leq 70\%$: there is no significant difference in survival between chemo+surgery patients and surgery patients (HR = 1.52, 95%CI: 0.85–2.70, $P < 0.153$).

IHPoT index in the pretreatment biopsy (e.g. the proportion of tumor 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 $40\% \leq \text{PoT} \leq 70\%$ had a survival benefit from preoperative chemotherapy.[7] We can now demonstrate that patients with tumors with a mean absolute PoT value between 40% and 70% and low IHPoT index at the same time had the most survival benefit from preoperative 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 hematoxylin/eosin stained sections from lung cancer was found to be predictive of mutation status,[17] providing evidence that the morphological phenotype of the tumor is reflective of its molecular phenotype. Studies in esophageal, head and neck, and colon cancer have investigated 'molecular intratumoral heterogeneity' without providing a definition for intratumor

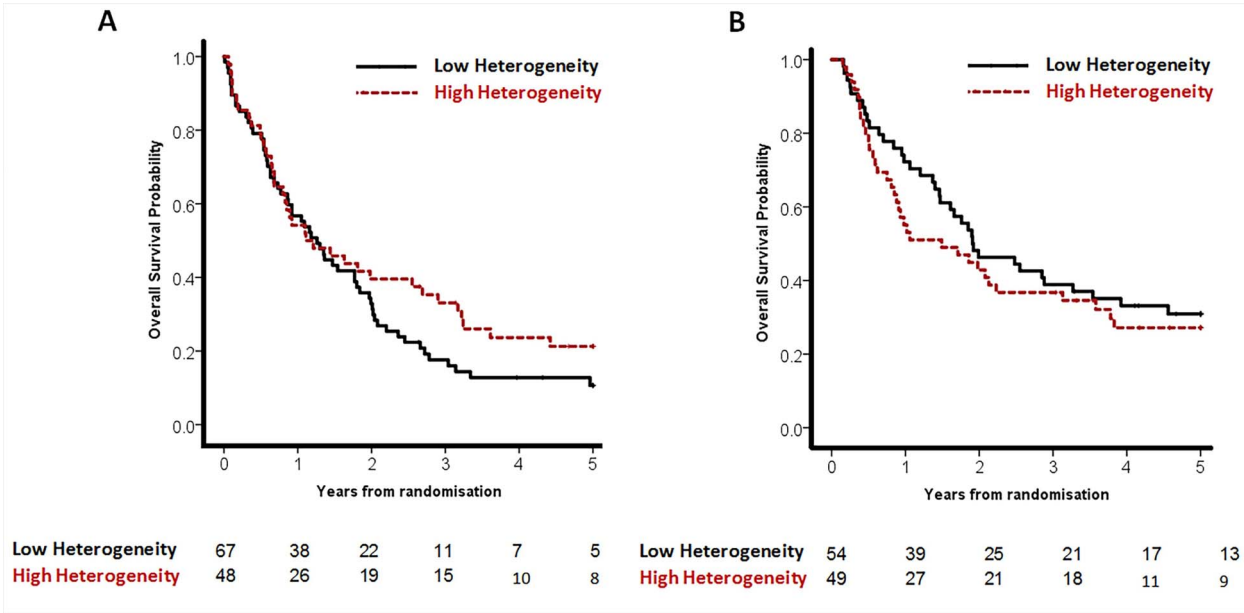

Fig. 3 Five-year overall survival of patients with high versus low intratumoral heterogeneity of the proportion of tumor (IHPoT) index within each treatment group. (A) There is no significant difference between the survival of surgery patients with high IHPoT index versus low IHPoT index (HR = 0.76, 95% CI: 0.50–1.15, $P = 0.19$). (B) There is no significant difference between the survival of chemo+surgery patients with high IHPoT index versus low IHPoT index (HR = 1.19, 95% CI: 0.75–1.90, $P = 0.45$).

heterogeneity as such. Existing data relating to 'intratumoral heterogeneity' are therefore difficult to interpret and cannot be compared with each other or with our current study which investigated histological intratumoral 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 EC patients, multiregion exome sequencing showed that 'intratumor 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 randomized 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 intratumor heterogeneity. Results of our study suggest that intratumoral heterogeneity of the relative tumor content per tissue area is a potential useful biomarker for clinical decision making in esophageal 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 EC biopsies can be reliably estimated by pathologists.

Limitations of our study include that this is a retrospective ad hoc analysis of a subset of available pretreatment biopsies from OE02 trial patients containing two or more tumor-containing biopsy pieces. In our study, we measured intratumoral heterogeneity between biopsy pieces from the same patient. Intratumoral 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 pretreatment 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 esophageal squamous cell carcinoma and adenocarcinoma receive the same treatment.

CONCLUSIONS

In the era of whole genome sequencing and next generation sequencing, the increasing complexity of intratumoral 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 intratumoral heterogeneity of a histological factor such as proportion

of tumor using digitized hematoxylin/eosin stained pretreatment biopsy slides and a mixed effect model is predictive of survival benefit to cytotoxic chemotherapy in EC patients from the Oe02 trial and may represent a clinically useful biomarker for patient treatment stratification.

SUPPLEMENTARY DATA

Supplementary data mentioned in the text are available to subscribers in *DOTESO* online.

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CONFLICTS OF INTEREST

All authors declared no conflicts of interest.

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