Oral Oncology 126 (2022) 105756

Contents lists available at ScienceDirect

Oral Oncology

journal homepage: www.elsevier.com/locate/oraloncology

Biological properties of hypoxia-related gene expression models/signatures on clinical benefit of anti-EGFR treatment in two head and neck cancer window-of-opportunity trials

Introduction

In head and neck squamous cell carcinoma (HNSCC) patients, the use of drugs acting against the epidermal growth factor receptor (EGFR) [1], has shown to be beneficial in curative and palliative settings. Treating HNSCC patients through biomarkers able to predict response and linked to the biological effect of treatments should be advisable, but no biomarkers other than PD-L1 expression have entered into clinical practice so far. This purpose could be achieved through window-of-opportunity (WOO) trials [2], in which non-pretreated patients receive neo-adjuvant drugs before standard-of-care therapy. The aim of these studies is the evaluation of the biological activity, which is variously defined according to the drug under study.

Previous gene-expression (GE) analyses of baseline samples revealed that Cluster 3 and Basal cluster are predictive of EFGR inhibitor response [3].

Herein, we report additional biomarker analyses to predict the response to EGFR-inhibitors based on two WOO trials (with Cetuximab [4] (CET) and Afatinib [5] (AFA) (NCT00714649 and NCT01538381). Our aims were to assess the post-treatment variations of available models/signatures predictive of EGFR inhibitor response and their modifications in relation to the microenvironment.

Patients and study design

Pre- and post-treatment GE data belonging to two clinical studies were retrieved from GEO repository: GSE109756 [4]; GSE192464 [5]. The primary outcome was the reduction in the tumor uptake volume as assessed by FDG-PET. The response to therapy was expressed as a continuous trait representing the change in SUVmax between the pre- and post-treatment as: $\Delta SUV = (SUVmax\ post - SUVmax\ pre)/SUVmax\ pre$. The two trials presented differences in: (i) clinical characteristics, such as the percentage of stage III-IV cases; (ii) experimental aspects of GE data generation such as origin of tumor samples (frozen for CET and formalin-fixed paraffin-embedded for AFA) and microarray platform Chip Affymetrix for CET and DASL Illumina for AFA). Due to these differences, we decided to harmonize the datasets. To achieve data harmonization, the genes uniquely shared between platforms were considered for the analyses, and two data matrices were generated: (i) 18 CET and 19 AFA patients with GE at baseline; (ii) 14 CET and 19 AFA patients with paired pre-post GE. The main clinical features of the present case materials are shown in Fig. 1A. A literature survey highlighted a number of hypoxia/microenvironment models and signatures already defined as prognostics in various HNSCC datasets. GE hypoxia microenvironment models/signatures were applied to data matrices following the methods in the original papers. In details models/signatures associated to: hypoxia were Cl3-Hypoxia [6], Basal cluster (BA) [7], Winter [8], Buffa [9] and Eustace [10]; immune tumor microenvironment were immune and microenvironment scores inferred by xCell [11]; IFNγ [12]. Correlation is imputed as Spearman's rank correlation coefficient and reported along with 95% confidence intervals. Statistical analyses and plotting were carried out in R v. 41.0. The significance level was set to 0.05. Three-dimensional plots were generated with the R package 'plot3D' (http://www.rforscience.com/rpackages/visualisation/plot3d/), implementing the 'scatter3D' function.

Results

Gene-expression based biomarkers in pre-treatment samples

Table 1A reports the association between ΔSUV and the models/signatures tested in pre-treatment specimens. Considering the hypoxia tumor properties, a significant inverse correlation between ΔSUV and Cl3 or BA models was observed in both CET and AFA studies (i.e. high hypoxia GE scores were associated with better response), while the inverse correlation was less consistent once the three signatures (i.e. Winter, Buffa, Eustace) were applied. When tumor microenvironment is assessed, only AFA treatment disclosed a significant positive correlation between ΔSUV and immune score or IFNγ signature (i.e. low immune scores were associated with better response). Pre-treatment hypoxia and immune score were inversely correlated in both CET and AFA samples (Fig. 1B), meaning that a higher CL3 score is associated to a lower immune score. Exploring the integrated relationships among ΔSUV, hypoxia models and immune score in a primarily sequential 3D structure, a significant inverse correlation between hypoxia and immune score was found to be correlated to ΔSUV (Fig. 1C). This means that a deep clinical response was associated with higher hypoxia and low immune score.

https://doi.org/10.1016/j.oraloncology.2022.105756
Received 23 December 2021; Accepted 26 January 2022
Available online 2 February 2022

A

Variable	Treatment received		p-value[2]
	cetuximab, N = 18[1]	afatinib, N = 19[1]	
Gender			>0.9
female	0 (NA%)	6 (32%)	
male	0 (NA%)	13 (68%)	
Age (years)	58 (47, 68)	58 (52, 65)	0.7
Tumor site			0.054
larynx	3 (17%)	0 (0%)	
oral cavity	15 (83%)	16 (84%)	
oropharynx	0 (0%)	3 (16%)	
TNM stage			<0.001
I	2 (11%)	0 (0%)	
II	11 (61%)	3 (16%)	
III	3 (17%)	3 (16%)	
IV	2 (11%)	13 (68%)	
Δ SUV	-55 (-59, -48)	-34 (-51, -15)	0.001

[1] n (%); Median (IQR)

[2] Fisher's exact test (gender, tumor site, TNM stage), Wilcoxon rank sum exact test (age, delta SUV)

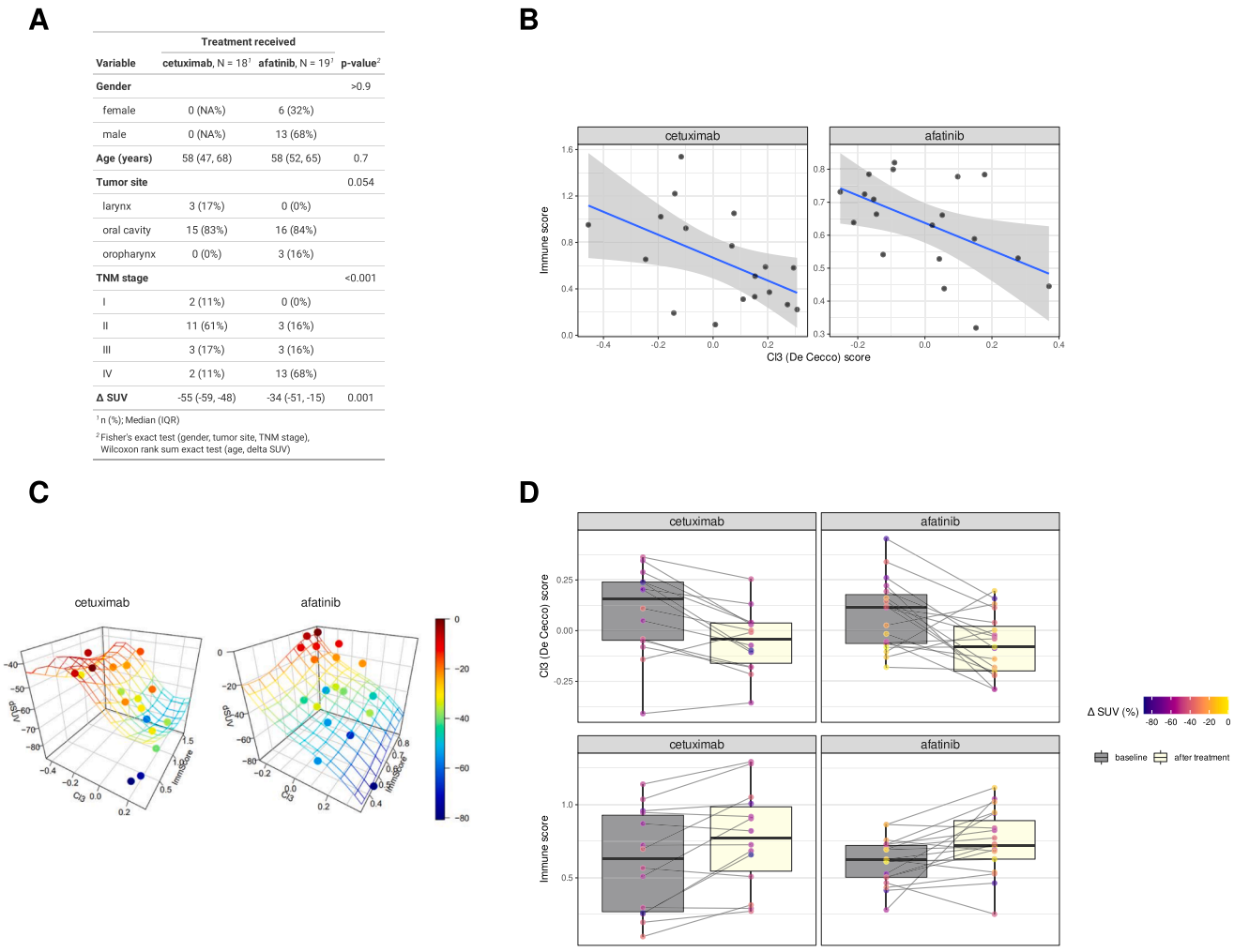

Fig. 1. Predictive biomarkers in response to EGFR inhibitors. (A) Clinical characteristic of CET and AFA pre-treatment patients. (B) Biological interplay between Cl3-hypoxia and immune score in pre-treatment samples. The scatter plots depict the correlation between Cl3-hypoxia and immune score in both CET and AFA. **(C) Plot view in scatter 3D color among three attributes (Cl3-hypoxia model, immune score, and ΔSUV) in pre-treatment samples:** it is appreciated an inverse correlation between hypoxia/immune tumor properties reaching correlation −0.47, p-value 0.024 and correlation −0.45, p-value 0.026 for CET and AFA, respectively. Statistical analyses and plotting were carried out in R v. 41.0. Three-dimensional plots were generated with the R package 'plot3D', implementing the 'scatter3D' function. Correlation is imputed as Spearman's rank correlation coefficient. **(D) Paired Pre-post treatment GE analysis of Cl3 and immune score**: it is shown the expression of Cl3 (De Cecco) in CET and AFA in pre-and post-treatment tumor samples with a significant p-value, $p = 0.00135$ and $p = 0.00570$, respectively and the expression of immune score in CET and AFA pre- and post-treatment tumor samples with a significant p-value, $p = 0.00698$ and $p = 0.01820$ respectively. P-value was calculated by paired samples t-tests.

Biomarkers expression changes after neoadjuvant EGFR inhibitors treatment

Paired pre- post-treatment GE profiles confirmed the significance of the analyzed signatures (Table 1B). The association of hypoxia and immune score and the response to both EGFR inhibitors was evidenced in post- compared to the pre-treatment specimens (Fig. 1D reports the association between the decrease in Cl3 model and the increase in immune score). On the contrary, the predictive role of IFNγ was not confirmed in AFA pre- post-treatment samples.

Discussion

The collection of tissue specimens within a WOO study followed by GE profiling offers the possibility to evaluate the drug activity in unbiased conditions confirming if the drug targets the intended pathway. Herein, we exploited the availability of pre- and post-treatment tumor samples from two WOO studies conducted in HNSCC patients to assess whether already defined models/signatures characterizing hypoxia/immune score are associated with response to EGFR-inhibitor treatment. Due to the small sample size, it was not feasible: (i) to infer the contribution of specific immune subpopulations; (ii) to define the role of different mechanisms of action on EGFR receptor played by CET (monoclonal antibody) and AFA (tyrosine kinase inhibitor).

Despite these limitations, the data presented here provided a biological validation of the role of hypoxia assessed in pre-treatment biopsies to predict the clinical response to EGFR-inhibitors. Thanks to the results obtained from pre-post treatment, we provide novel evidence that the inverse correlation between hypoxia and immune score in pre-treatment tissues could be predictive of response to EGFR receptor inhibitors. The observation of a hypoxia score decrease in post-treatment tissues together with an immune score increase suggests an interplay between hypoxia and immunity in the context of EGFR inhibition that deserves further exploration.

Table 1A
Hypoxia and immune microenvironment models/signatures tested in CET and AFA pre-treatment tumor samples. Results of Spearman's correlation tests computed between ΔSUV versus hypoxia, immune microenvironment models/signatures in CET and AFA pre-treatment tumor samples. The rank correlation coefficients and p-values were calculated by Spearman and reported along with 95% confidence intervals. The significance level was set to 0.05.

CET			
Model/Signature Associated to:	**Model/Signature ID**	**Correlation (95% CI)**	**P-value**
Hypoxia	**Cl3**	**−0.505 (−0.786, 0.0494)**	**0.0327**
	Basal	**−0.606 (−0.836, −0.194)**	**0.0077**
	Winter	−0.364 (−0.71, 0.124)	0.1370
	Buffa	**−0.486 (−0.777, −0.0248)**	**0.0408**
	Eustace	−0.22 (−0.623, 0.275)	0.3810
Microenvironment	Immune_score	−0.0506 (−0.506, 0.426)	0.8420
	Microenvironment_score	−0.0423 (−0.499, 0.433)	0.8680
	IFNγ	−0.393 (−0.727, 0.0902)	0.1060
AFA			
Model/Signature Associated to:	**Model/Signature ID**	**Correlation (95% CI)**	**P-value**
Hypoxia	**Cl3**	**−0.682 (−0.868, −0.331)**	**0.0013**
	Basal	**−0.537 (−0.797, −0.109)**	**0.0178**
	Winter	**−0.475 (−0.765, −0.0271)**	**0.0397**
	Buffa	−0.323 (−0.678, 0.154)	0.1780
	Eustace	**−0.504 (−0.779, −0.0639)**	**0.0280**
Microenvironment	**Immune_score**	**0.496 (0.0546, 0.776)**	**0.0306**
	Microenvironment_score	0.395 (−0.0725, 0.72)	0.0944
	IFNγ	**0.551 (0.129, 0.804)**	**0.0145**

Table 1B
Results of paired t-tests for hypoxia and immune microenvironment models/signatures in pre-post neoadjuvant EGFR-inhibitor's treatment samples. The mean estimate represents the mean of differences in paired pre versus post scores (a positive or negative mean estimate for a signature corresponds to an increase or decrease in the post-treatment group, respectively) and was reported along with 95% confidence intervals. P-value was calculated by paired samples t-tests. The significance level was set to 0.05.

CET			
Model/Signature Associated to:	**Model/Signature ID**	**Mean estimate (95% CI)**	**P-value**
Hypoxia	**Cl3**	**−0.145 (−0.223, −0.068)**	**0.00135**
	Basal	**−0.288 (−0.451, −0.126)**	**0.00207**
	Winter	**−0.364 (−0.494, −0.235)**	**3.93E-05**
	Buffa	**−4.07 (−5.54, −2.6)**	**4.61E-05**
	Eustace	**−0.453 (−0.611, −0.295)**	**3.2E-05**
Microenvironment	**Immune_score**	**0.156 (0.0506, 0.261)**	**0.00698**
	Microenvironment_score	**0.201 (0.0791, 0.323)**	**0.00346**
	IFNγ	−0.402 (−1.11, 0.309)	0.243
AFA			
Model/Signature Associated to:	**Model/Signature ID**	**Mean estimate (95% CI)**	**P-value**
Hypoxia	**Cl3**	**−0.148 (−0.247, −0.0488)**	**0.0057**
	Basal	**−0.259 (−0.399, −0.119)**	**0.00109**
	Winter	**−0.177 (−0.338, −0.015)**	**0.0339**
	Buffa	**−2.81 (−4.35, −1.26)**	**0.00125**
	Eustace	**−0.344 (−0.542, −0.147)**	**0.00177**
Microenvironment	**Immune_score**	**0.128 (0.0244, 0.231)**	**0.0182**
	Microenvironment_score	**0.164 (0.0543, 0.274)**	**0.00569**
	IFNγ	0.0988 (−0.672, 0.87)	0.791

Conclusion

To our knowledge, the present work reports the first analysis of the inverse correlation between hypoxia and immune score with respect to the clinical activity of EGFR-inhibitors in HNSCC, suggesting a predictive role. These results seem to indicate that the use of anti-EGFR and immune checkpoint inhibitors (ICI), either in combination or in sequence, could sensitize to ICI the hypoxic cancers that are less sensitive to immunotherapy. Further studies are needed to confirm this hypothesis.

Declaration of competing interest

Lisa Francesca Licitra
Receipt of grants/research supports (Funds received by my institution for clinical studies and research activities in which I am involved):

Astrazeneca, BMS, Boehringer Ingelheim, Celgene International, Debiopharm International SA, Eisai, Exelixis inc, Hoffmann-La Roche ltd, IRX Therapeutics inc, Medpace inc, Merck–Serono, MSD, Novartis, Pfizer, Roche.

Receipt of honoraria or consultation fees (for public speaking/teaching in medical meetings and /or for expert opinion in advisory boards): Astrazeneca, Bayer, BMS, Eisai, MSD, Merck–Serono, Boehringer Ingelheim, Novartis, Roche, Debiopharm International SA, Sobi, Ipsen, Incyte Biosciences Italy srl, Doxa Pharma, Amgen, Nanobiotics Sa and GSK.

JP Machiels

Advisory board member or speaker with honoraria (managed by my Institution): Pfizer, Roche, Astra/Zeneca, Bayer, Innate, Merck Serono, Boerhinger, BMS, Novartis, Janssen, Incyte, Cue Biopharma, ALX Oncology, iTEOS, eTheRNA, NEKTARTravel expenses: Amgen, BMS, Pfizer, MSD. Data safety monitoring board with honoraria: Psioxus. Institutional conflict of interest (Funding to institution for research support): all companies.

Uncompensated advisory role: MSD.

Paolo Bossi

Honoraria: Bristol Myers Squibb, Merck, Regeneron, GlaxoSmithKline, MSD Oncology.

Consulting or Advisory Role: Merck Serono, Bristol Myers Squibb, Sanofi/Regeneron, Angelini Pharma, MSD Oncology, Sun Pharma, GlaxoSmithKline.

Research Funding: Amgen, Merck Serono, Roche, MSD Oncology, GlaxoSmithKline, Regeneron.

No other potential conflicts of interest were reported.

Acknowledgements

The research leading to these results has received funding from AIRC (IG 18519 and IG 23573 projects – P.I. Loris De Cecco).

We would like to thank Dr Silvana Canevari for critical reading of the manuscript and EORTC for sharing data and intellectual contribution.

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