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Surrogates of immunologic cell death (ICD) and chemoradiotherapy outcomes in head and neck squamous cell carcinoma (HNSCC)

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ABSTRACT

Objectives: Chemoradiation can induce immunogenic (ICD) or tolerogenic cell death. ICD relies on the generation of damage-associated molecular patterns which can stimulate toll-like receptors (TLRs). We sought to determine whether we can predict responses to chemoradiation by measuring surrogate biomarkers of ICD in a cohort of patients with locally advanced (LA) head and neck squamous cell carcinoma (HNSCC).

Materials and Methods: In a cohort of 113 LA HNSCC pts we evaluated expression of TLR4, TLR7 and TLR9 in the EpCAM + circulating tumor cell (CTC) fraction at baseline and after cisplatin chemoradiation. We also quantified changes in chemokines CXCL10, CXCL16 and IL-2R in the serum.

Results: Seventy three patients had evaluable specimens. Among cases with biomarker assessment at baseline and post treatment, 36.8% had an increase in CXCL10 levels ($p = 0.022$), 73.7% had an increase in CXCL16 levels ($p = 0.002$) and 63.8% had an increase in IL2Ra levels ($p = 0.032$) with treatment. 52.0% of evaluable cases at baseline and post-treatment had an increase in TLR4 levels ($p = 0.996$), 42.9% had an increase in TLR7 levels ($p = 0.042$) and 27.7% had increase in TLR9 levels ($p = 0.011$) with treatment. CXCL10 levels at baseline were significantly associated with PFS and OS ($p = 0.010$ and $p = 0.032$, respectively).

Conclusions: Our results suggest that chemoradiation leads to quantifiable effects in surrogate markers of ICD. These effects may inform trials combining chemoradiation with immune checkpoint inhibitors. In addition, CXCL10 has prognostic effect in pts treated with chemoradiation.

Introduction

Optimal management of locally advanced (LA) head and neck squamous cell carcinoma (HNSCC) requires a multidisciplinary approach and it is well known that combined chemotherapy and radiation has been shown to improve tumor control, preserve organ integrity and prolong survival in this patient population [1,2]. There is accumulating evidence that chemoradiation (CRT) mediates its cytotoxic effects by directly inducing different forms of cell death [3]. Depending on the irradiation dose, the type of chemotherapy, the genetic background of

Abbreviations: APC, antigen presenting cell; CRT, chemoradiation; CTC, circulating tumor cell; CTL, cytotoxic T cell; CTLA-4, cytotoxic T lymphocyte antigen 4 (CTLA-4); CXCL10, chemokine ligand 10; DAMP, damage-associated molecular patterns; DC, dendritic cell; HNSCC, head amd neck squamous cell carcinoma; ICD, immunologic cell death; ICIs, immune checkpoint inhibitors; NF-κB, nuclear factor-κB; OS, overall survival; PD-L1, programmed cell death ligand-1; PFS, progression free survival; TLR, toll like receptor

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the host and the cellular origin, diverse phenotypes of apoptosis, necrosis, mitotic catastrophe, and/or senescence can occur, which clearly vary in immunogenic potential [4,5]. The concept of damage-associated molecular patterns (DAMPs), has been recently introduced to explain this potential immunogenicity of stressed or dying uninfected cells. DAMPs are released from dying cells to be recognized by antigen presenting cells (APCs) followed by formation of T-cell-mediated adaptive immunity [6]. Therefore, it has been suggested that for cell death to be immunogenic (ICD), it must release DAMPs to stimulate immune cells. Several parameters, including the intrinsic antigenicity of the cells, the nature of the cell death inducer, the precise cell death pathway that is triggered and the availability of the immune cells capable of response, determine whether cell death is immunogenic or not [7].

Comprehension of ICD gradually increases its significance, particularly in the field of cancer immunotherapy. Mechanistically, DAMPs induced by exposure to CRT are sensed by APCs through immune sensors, such as Toll-like receptors (TLRs), which are cell surface or intracellular transmembrane proteins that have a crucial role in early host defense against invading pathogens [8]. Stimulation of TLRs leads to inflammation and activation of transcription factor nuclear factor-κB (NF-κB), which induces expression of genes encoding inflammatory cytokines, type I interferons (IFN) and IFN-inducible genes, such as the gene encoding CXC-chemokine ligand 10 (CXCL10), therefore leading to an increase in the level of cytokine production (IL-2, IL10, IL-6), migration of immune cells due to the increased production of chemokines and immune activation [9–11].

Although it has been shown that CRT may induce ICD, there is still limited information to support this theory directly in a clinical setting, probably due to the lack of accurate assay systems to evaluate antigen-specific T-cell responses in patients with cancer receiving CRT. We hypothesized that ICD is induced by CRT in patients with LA HNSCC, which serves as a paradigm of malignancy characterized by intrinsic immunogenicity [12–14]. Furthermore, it is well known that Circulating Tumor Cells (CTCs) represent a valuable tool for real-time monitoring of tumor status in HNSCC and other solid tumors [15]. Based on these considerations, we sought to determine whether we can predict clinical responses to CRT by evaluating T-cell responses based on expression of *TLRs* 4,7 and 9 and other surrogate biomarkers of ICD on CTCs at baseline and after completion of CRT in a cohort of patients with LA HNSCC treated with curative intent. To achieve this, we first developed a highly sensitive, specific and reproducible RT-qPCR assay for the quantification of *TLRs* 4,7 and 9 mRNA expression in CTCs. We also quantified changes in chemokines CXCL10, CXCL16 and IL-2 receptor (IL-2R) at baseline and after chemoradiation using ELISA. We believe that systemic anti-tumor immune responses induced by ICD might be the underlying driving forces of a potential synergism between immunotherapy and CRT in this patient population.

Patients and methods

Study design

One hundred and thirteen patients with LA HNSCC were included in this analysis. Written informed consent was obtained from all patients before participating in the study. The present study was approved by the Medical Ethical Committee of Attikon University hospital (Athens, Greece) and complies with the principles laid down in the Declaration of Helsinki.

Patients with newly diagnosed HNSCC were eligible if they had histologically confirmed squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx or larynx deemed inoperable due to technical or organ preservation reasons. Major exclusion criteria were prior treatment for LA HNSCC, poor performance status (ECOG > 2) and other concomitant neoplasms. Stage was determined according to the TNM classification. Staging examinations included computed tomography (CT) scan of the head and neck and CT scan of the thorax and abdomen. Patients were enrolled before initiation of treatment. All patients were treated with cisplatin CRT with curative intent. Samples were obtained at baseline and at the end of CRT. For all patients the "end of treatment" samples were obtained a week post completion of concurrent CRT. Assessment of response was performed 12 weeks after completion ofcisplatin CRT. During follow-up, patients were investigated (ENT evaluation and CT of neck/chest) every 3 months for the first 2 years and every 6 months after the period of two years.

We evaluated expression of *TLR4*, *TLR7* and *TLR9* in the EpCAM + CTC fraction at baseline and after cisplatin-based CRT.

Isolation of EpCAM(+) CTCs

For the isolation of EpCAM(+) CTCs from peripheral blood (30 mL) we followed our previously described protocols [16,17].

RNA extraction

Total RNA from the EpCAM(+)CTC fraction was isolated using the miRNeasy micro kit (QIAGEN, Germany), according to manufacturer's instructions.

cDNA synthesis

cDNA synthesis was performed using the SuperScript ™ First-Strand Synthesis System for RT-PCR (Life technologies, USA) according to manufacturer's protocol, using 7 μL of isolated total RNA as starting template.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) assay for the quantification of the TLR4, TLR7 and TLR9 mRNA

Primer and Probe Design. We designed in-silico the primers and hydrolysis probes (Taqman) for *TL4*, *TLR7*, *TLR9* using Primer Premier 5.0 software (Premier Biosoft, CA, USA). Our primers and probes were carefully designed to completely avoid primer–dimer formation, false priming sites, formation of hairpin structures, and hybridization to genomic DNA, while amplify specifically only *TLR4*, *TLR7* and *TLR9* target genes according to our search in the BLAST Sequence Similarity Search tool (NCBI, NIH). *B2M* (β2-microglobulin) was used as a reference gene, as we have previously described [18]. The sequences of primers and probes are available upon request.

qPCR. qPCR reaction was performed in the Rotor Gene Q (QIAGEN). Detailed optimization experiments were performed (results not shown). The amplification reaction mixture for *TLR4* contained 2 μL of the PCR Synthesis Buffer (5X), 1.2 μL MgCl2 (25 mM), 0.15 μL dNTPs (10 mM), 0.8 μL BSA (10 μg/μL), 0.1 μL Taq DNA polymerase (5 U/μL), 0.3 μL of forward and reverse primer (10μM), 0.67 μL Taqman probe (3 μM) and H2O to a final volume of 10 μL; for *TLR7* it contained 2 μL of the PCR Synthesis Buffer (5X), 1.2 μL MgCl2 (25 mM), 0.15 μL dNTPs (10 mM), 0.8 μL BSA (10 μg/μL), 0.1 μL TaqDNA polymerase (5 U/μL), 0.3 μL of forward and reverse primer (10μM), 1 μL Taqman probe (3 μM) and H2O to a final volume of 10 μL; for *TLR9* it contained 3 μL of PCR Synthesis Buffer (5X), 1.2 μL MgCl2 (25 mM), 0.2 μL dNTPs (10 mM), 0.8 μL BSA (10 μg/μL), 0.1 μL TaqDNA polymerase 5 U/μL, 0.3 μL of forward and reverse primer(10μM), 1 μL Taqman probe (3 μM) and H2O to a final volume of 10μLand for B2M it contained 1 μL of PCR Synthesis Buffer (5X), 1.2 μL MgCl2 (25 mM), 0.15 μL dNTPs (10 mM), 0.3 μL BSA (10 μg/μL), 0.1 μL Taq DNA polymerase 5 U/μL, 0.25 μL of forward and reverse primer (10μM), 0.83 μL Taqman probe (3 μM) and H2O to a final volume of 10 μL. PCR reaction cycling conditions for all *TLRs* and *B2M* were: 95 °C/2min; 45 cycles of 95 °C/10 s, annealing at 58 °C/20 s and extension at 72 °C/20 s.

Normalization of qPCR data in clinical samples. qPCR data for *TLR4*, *TLR7* and *TLR9* expression were normalized in respect to *B2M* expression in the same cDNAs, using the $2^{-\Delta\Delta Cq}$ approach [19]. CTCs isolated through positive immune-magnetic enrichment are partly contaminated; since "contamination" of PBMC in the EpCAM(+)CTC

fraction could affect TLR assay specificity, we assessed this contamination by analyzing peripheral blood samples from 20 healthy individuals in exactly the same way as patients. We estimated a cut-off based on *TLR4*, *TLR7* or *TLR9* normalized expression in respect to B2M expression in this control group. Using this approach, we defined a sample as *TLR4*, *TLR7* or *TLR9* overexpressed based on the fold change of *TLR4*, *TLR7* or *TLR9* expression in the EpCAM(+)CTC fraction in respect to the corresponding EpCAM(+) fraction in the group of 20 healthy individuals.

Enzyme-linked immunosorbent assay (ELISA) for quantification of CXCL10, CXCL16 and IL2R

We also quantified changes in chemokines CXCL10, CXCL16 and IL-2 receptor (IL-2R) at baseline and after chemoradiation. The protein levels of CXCL10, CXCL16, and IL-2R in plasma samples collected were measured using the corresponding ELISA kits (Human Elisa kit, R&D Systems, Inc.) according to the manufacturer's instructions.

Statistical analysis

The statistical software SPSS version 22.0 was used for all analyses. Baseline biomarker levels were compared with post-treatment levels by Wilcoxon test. Median baseline biomarker levels were compared across different patient subgroups by Kruskal-Wallis test. Independent associations between baseline biomarker levels and tumor characteristics were assessed by linear regression. The association between biomarker levels and tumor response to treatment was examined by logistic regression.

The prognostic significance of baseline biomarker levels for progression-free (PFS) and overall survival (OS) was assessed by Cox proportional hazard models. PFS was defined as the time from registration to the date of tumor progression or death from other causes or censored at the time of last contact. OS was defined as the time from registration to the study to death from anycause or censored at the time of last contact. Survival curves were generated by Kaplan-Meier analysis and tested for significance by Mantel-Cox log rank test. All statistical tests were two-sided with a level of significance p-value < 0.05.

Results

Patient population

In our cohort, samples from 113 patients with LA HNSCC were obtained at baseline and after completion of CRT. Baseline patient demographic and clinical characteristics are shown in Table 1. Median age was 64.1 years; 75.3% were male, 71.2% were heavy smokers and 19.2% reported heavy alcohol use. Oral cavity was the primary site in the majority of patients (42.4%).Forty-six patients (63%) had stage IVA disease.

ICD biomarker levels at baseline and post treatment

In our cohort, 73 patients had evaluable specimens for CTC gene expression analysis. Among cases with biomarker assessment at baseline and post treatment, 52.0% had an increase in *TLR4* levels with treatment, 42.9% had an increase in *TLR7* levels and 27.7% had increase in *TLR9* levels. Post treatment levels compared to baseline were significantly higher for *TLR7* (p = 0.042) and *TLR9* (p = 0.011), while they did not differ significantly for *TLR4* (p = 0.996). With regards to chemokine levels in corresponding serum samples, CXCL10 levels were statistically significantly decreased with treatment (p = 0.022), while CXCL16 (p = 0.002) and IL-2R (p = 0.032) levels were significantly increased post chemoradiation as compared to baseline. More specifically, 36.8% of evaluable cases at baseline and post-treatment had an increase in CXCL10 levels with treatment, 73.7% had an increase in CXCL16 levels and 63.8% had an increase in IL2Ra levels. Line charts demonstrating biomarker levels before and post chemoradiation are shown in Fig. 1.

Table 1
Basic patient and disease characteristics.

Characteristic	N	(%)
Age		
Median (years)	64.1	(range, 18–91)
< 65	38	(52.1)
≥65	35	(47.9)
Sex		
Male	55	(75.3)
Female	18	(24.7)
Smoking		
Heavy (≥20 pack-years)	52	(71.2)
Light (< 20 pack-years)	7	(9.6)
Never	14	(19.2)
ETOH		
Heavy	14	(19.2)
Social	35	(47.9)
No	24	(32.9)
Primary site		
Oral cavity	31	(42.4)
Oropharynx	17	(23.3)
Hypopharynx	8	(11.0)
Larynx	17	(23.3)
AJCC Stage		
I	1	(1.4)
II	11	(15.1)
III	9	(12.3)
IVA	46	(63.0)
IVB	6	(8.2)
Histological grade		
Low	10	(13.7)
Intermediate	36	(49.3)
High	23	(31.5)
Unknown	4	(5.5)

Association of biomarker levels with tumor characteristics

Correlation of baseline biomarker levels with tumor characteristics are illustrated in Table 2. Patients with oral or oropharyngeal compared to those with laryngeal or hypopharyngeal primary site, as well as those with more advanced compared to those with earlier tumor stage, had significantly higher baseline CXCL10 levels (Kruskal-Wallis, $p = 0.017$ and 0.006, respectively). No other correlation was observed. Furthermore, no baseline characteristic or biomarker levels were associated with response to CRT (data not shown).

Association with clinical outcome

The present cohort had a median follow-up of 39.4 months (range, 3.4–72.2), during which 24 patients progressed and 34 died. In our cohort, all biomarker levels were evaluated for association with PFS and OS. Among them, only CXCL10 levels at baseline were significantly associated with PFS and OS (p = 0.010 and p = 0.032, respectively), in univariate Cox regression analysis (Table 3). Importantly, these were shown to be of prognostic significance independently of other established covariates, as shown in Table 3. Survival curves according to CXCL10 levels are demonstrated in Fig. 2. On the other hand, we found no association of post-treatment CXCL10 levels with clinical outcome. In addition, increase in CXCL10 levels with treatment did not correlate with PFS (HR 0.52, 95%CI 0.17–1.64, p = 0.263) or OS (HR 0.65, 95%CI 0.27–1.58, p = 0.338).

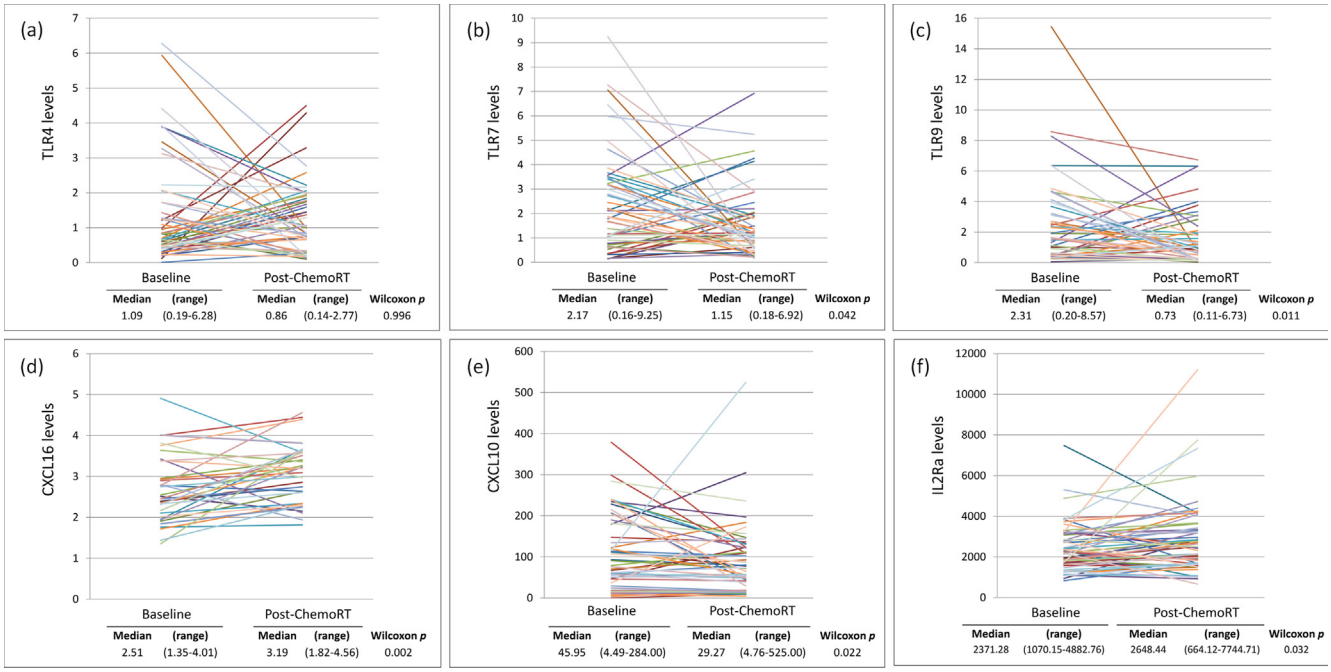

Fig. 1. Line charts demonstrating biomarker levels before and post chemoradiation. (a) TLR4, (b) TLR7, (c) TLR9, (d) CXCL16, (e) CXCL10, (f) IL2Ra.

Table 2
Association of baseline biomarker levels with tumor stage, histological grade and primary site.

Characteristic	TLR4		*p*-value	TLR7		*p*-value	TLR9		*p*-value
	Median	(range)		Median	(range)		Median	(range)	
Stage			0.557			0.790			0.730
I-II	0.65	(0.19–3.92)		1.78	(0.16–6.45)		1.78	(0.21–8.28)	
III	1.27	(0.12–7.89)		2.10	(0.16–4.63)		1.32	(0.20–4.69)	
IVA	0.97	(0.01–6.28)		2.13	(0.14–9.25)		1.53	(0.06–15.45)	
IVB	1.50	(0.30–3.12)		1.88	(1.35–7.26)		0.70	(0.21–4.63)	
Grade			0.593			0.545			0.525
Low	0.81	(0.41–5.94)		2.17	(0.14–7.06)		2.31	(0.38–15.45)	
Intermediate	0.87	(0.01–7.89)		2.01	(0.16–9.25)		1.78	(0.20–8.28)	
High	1.25	(0.26–3.92)		1.77	(0.14–7.26)		1.14	(0.06–4.63)	
Not reported	0.58	(0.19–1.72)		0.93	(0.16–2.79)		1.87	(0.45–8.57)	
Primary site			0.368			0.365			0.973
Oral cavity	0.78	(0.01–6.28)		1.92	(0.16–9.25)		1.13	(0.21–15.45)	
Oropharynx	0.99	(0.40–3.89)		2.45	(0.36–7.26)		1.64	(0.35–4.86)	
Hypopharynx	1.83	(0.41–7.89)		1.90	(0.34–3.16)		1.36	(0.30–4.63)	
Larynx	0.72	(0.19–5.94)		1.34	(0.14–3.48)		2.36	(0.06–4.11)	
	CXCL10		*p*-value	CXCL16		*p*-value	IL-2R		*p*-value
	Median	(range)		Median	(range)		Median	(range)	
Stage			0.017			0.561			0.864
I-II	31.92	(8.08–298.47)		2.45	(1.76–3.37)		1843.25	(1271.57–3898.07)	
III	36.98	(4.49–116.40)		2.34	(1.44–4.01)		2377.30	(943.28–3821.08)	
IVA	77.36	(10.04–239-95)		2.78	(1.35–4.90)		2140.38	(830.84–7480.82)	
IVB	226.95	(134.19–378.59)		2.31	(2.16–4.01)		2330.92	(2185.74–3237.36)	
Grade									
Low	61.71	(10.04–147.53)	0.873	2.59	(1.90–3.42)	0.890	1921.65	(830.84–3284.10)	0.596
Intermediate	90.64	(10.25–378.59)		2.75	(1.76–4.01)		2306.32	(943.28–7480.82)	
High	57.72	(4.49–284.00)		2.24	(1.35–4.90)		2140.38	(1140.98–3618.20)	
Not reported	69.47	(8.08–190.01)		2.45	(2.10–2.54)		2765.44	(1786.14–4882.76)	
Primary site			0.009			0.407			0.172
Oral cavity	109.07	(4.49–378.59)		2.54	(1.76–3.81)		1878.87	(830.84–5306.09)	
Oropharynx	92.88	(15.22–239.95)		2.79	(1.94–4.90)		2265.96	(1099.97–7480.82)	
Hypopharynx	19.34	(10.04–204.27)		2.12	(1.35–3.63)		2633.99	(2140.38–3284.10)	
Larynx	27.76	(8.08–228.43)		2.45	(1.44–4.01)		1964.52	(1070.15–4882.76)	

Primary site was the only tumor characteristic independently associated with baseline CXCL10 levels (linear regression, $p = 0.006$).
No baseline characteristic or biomarker levels were associated with response to chemoRT.

Table 3
Prognostic significance of baseline CXCL10 levels compared to other covariates.

Covariates	Values	Progression-free survival					
		Univariate			Multivariate		
		HR	95% CI	*p*-value	HR	95% CI	*p*-value
CXCL10	Above vs. below median	2.87	1.29–6.36	0.010	2.42	1.08–5.45	0.033
Age (years)	≥65 vs. < 65	1.27	0.68–2.39	0.454			
Sex	Male vs. female	1.08	0.51–2.28	0.843			
Smoke	Heavy vs. light/never	0.70	0.36–1.36	0.291			
ETOH	Heavy vs. social/no	1.38	0.65–2.93	0.401			
Stage	IV vs. I-III	1.56	0.74–3.28	0.247			
Primary	Oral/Oropharyngeal vs. Laryngeal/Hypopharyngeal	1.96	0.95–4.02	0.068			
Grade	High vs. low/intermediate	0.97	0.49–1.93	0.929			
Covariates	Values	Overall survival					
		Univariate			Multivariate		
		HR	95% CI	*p*-value	HR	95% CI	*p*-value
CXCL10	Above vs. below median	2.65	1.09–6.48	0.032	2.50	1.02–6.16	0.046
Age (years)	≥65 vs. < 65	1.31	0.66–2.60	0.437			
Sex	Male vs. female	1.45	0.63–3.34	0.386			
Smoke	Heavy vs. light/never	0.86	0.42–1.74	0.666			
ETOH	Heavy vs. social/no	1.32	0.57–3.06	0.519			
Stage	IV vs. I-III	1.46	0.66–3.22	0.355			
Primary	Oral/Oropharyngeal vs. Laryngeal/Hypopharyngeal	2.62	1.14–6.03	0.023			
Grade	High vs. low/intermediate	0.78	0.37–1.64	0.507			

Discussion

Depending on the initiating stimulus, cancer cell death can be immunogenic or non-immunogenic. ICD is characterized by the exposure of “eat me” signals on the cell surface through the release of DAMPs. Such signals stimulate a series of receptors expressed by dendritic cells (DCs) to trigger the presentation of tumor antigens to T cells[20]. Hence, ICD involves the recruitment of the host's immune system as a contributor of response to chemotherapy and radiation, there by resulting in immune memory and advantageous systemic effects [21]. Indeed, it constitutes a significant pathway for the activation of the immune system against cancer, which could define the long-term success of anticancer therapies; therefore, a treatment regimen that fails to induce ICD might result in therapeutic failure. The present study contains novel findings to support the concept of ICD induced by CRT in patients with HNSCC. First, we demonstrated that in a cohort of patients with LA HNSCC, serum levels of TLRs and chemokines were significantly altered following CRT. Second, CXCL10 was found to be a poor prognostic indicator independently of other established covariates.

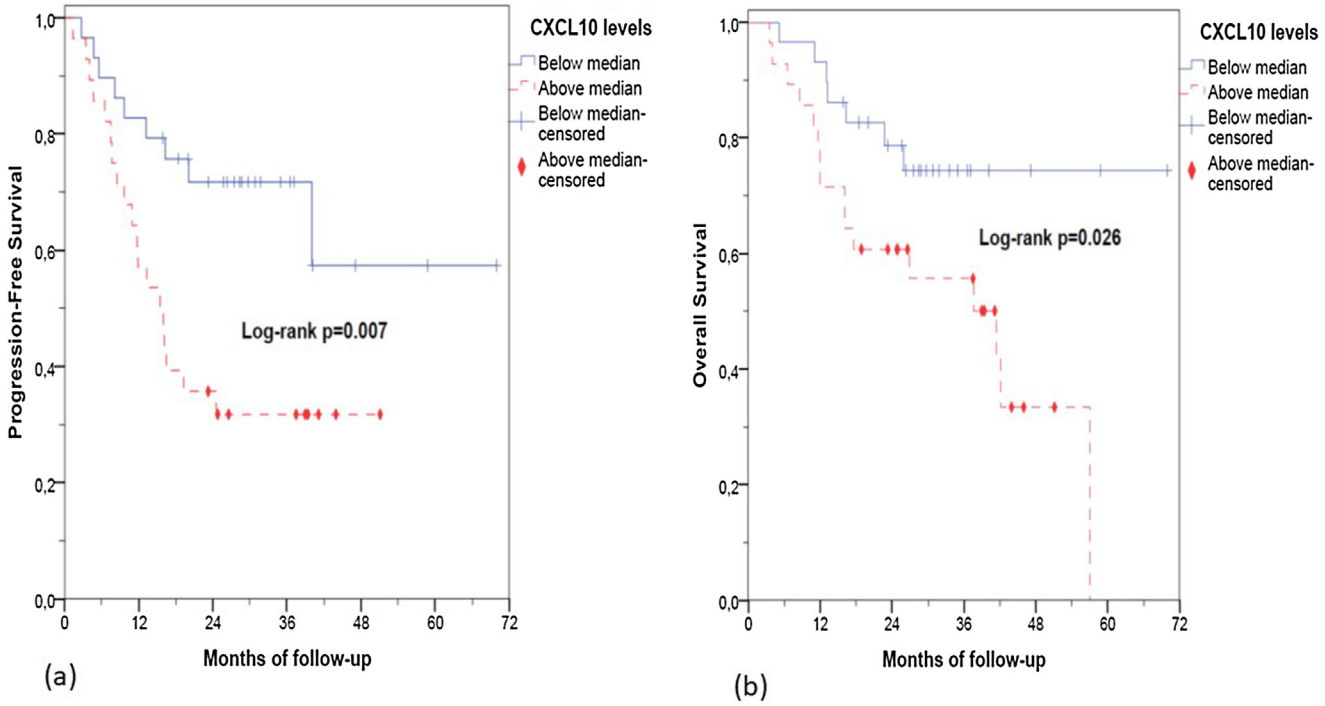

Fig. 2. Kaplan–Meier (a) Progression Free Survival (p = 0.043) and (b) Overall Survival (p = 0.021) curves for *PDL-1* and *IDO1* expression levels after treatment.

In this study, we noted decreased serum CXCL10 following treatment, which is consistent with prior studies [22,23]. Most importantly, we demonstrated that CXCL10 levels at baseline were significantly associated with PFS and OS ($p = 0.010$ and $p = 0.032$, respectively), in univariate Cox regression analysis. This was shown to be of prognostic significance independently of other established covariates. CXCL10 and its receptor CXCR3 are over-expressed in many solid tumors and have been correlated with poor prognosis and metastasis in several cancers, including colon cancer, multiple myeloma and basal cell carcinoma [24–26]. In breast cancer, Hormone Receptor-independent elevation of CXCL10 has been linked to progression and metastasis [27]. In line with our findings, Rentoft et al. assessed the expression of CXC-chemokine ligands and their receptors in a group of patients with oral squamous cell carcinoma treated with radiotherapy and surgery and found that high CXCL10 expression post treatment was predictive for poor response to radiotherapy [22]. Based on these considerations, decreases in CXCL10 possibly indicate tumor death and a favorable response to therapy. This has therapeutic implications, as CXCL10 inhibitors are in early development [28].

On the other hand, we found increased serum CXCL16 and IL2R concentrations following CRT. CXCL16 is known to be expressed by immune cells such as dendritic cells and macrophages and to be upregulated during inflammation in different tissues and organs [29–31]. In addition, CXCL16 is known to attract tumour-infiltrating T cells or NK cells in lung and colorectal cancers [32,33]. In a prospective study conducted in patients with HNSCC treated with RT or CRT, CXCL16 serum levels were found to increase following RT [23]. Irradiation of human and mouse breast tissue *in vitro* and in mouse models *in vivo* has been shown to result in upregulation of CXCL16 secretion and recruitment of effector T cells [34,35]. Indeed, it is hypothesized that RT could stimulate dendritic cells to produce CXCL16 [36]. The molecular mechanism responsible for up-regulation of CXCL16 following CRT is currently unknown. On the other hand, it is known that binding of IL-2 leads to proliferation of immune cells and biosynthesis of IL-2R. Therefore, expression of IL2Rα is considered a landmark of T cell activation [37]. However, the soluble form of IL2Rα that can be found in the serum is also expressed by cancer cells and can negatively modulate immune response [38,39]. Thus, increase of IL2Ra levels following treatment might be an inhibitory signal secreted from the tumor, as shown in other studies [23].

Traditionally, it is postulated that conventional treatments such as chemotherapy and radiation induce an immunosuppressive state in both T-cell and natural killer-cell immunity and directly kill tumor cells through their cytostatic/cytotoxic effects by triggering apoptotic cell death that may be immunologically silent [40]. However, accumulating evidence suggests that a decisive contribution to the long-term successful elimination of cancer by radiation and some traditional chemotherapeutic agents is made by alerting the immune system to the presence of dying cancer cells, therefore inducing TLR-dependent antigen specific T cell immunity [41]. Interestingly, distinct chemotherapeutic agents are not equivalent in their ability to trigger ICD. Indeed, cisplatin, a platinum agent most commonly used concurrently with CRT in HNSCC, does not induce ICD, and this is attributed to the lack of calreticulin exposure, which serves as a signal for dendritic cells to engulf the dying cell [40,42,43]. In our cohort, the clinical efficacy of cisplatin could be potentiated by ICD as it is co-administered with radiotherapy, a potent inducer of calreticulin exposure. Existing evidence indicate that combining cisplatin with compounds that induce calreticulin exposure leads to full-blown ICD [44]. Indeed, combinations of radiotherapy and chemotherapy are more effective in inducing immune mediated local and abscopal effects [45]. Golden et al. showed that radiation improves carboplatin and paclitaxel-induced ICD when given simultaneously in breast cancer cells [46]. Of note, chemotherapeutic agents that are considered potent ICD inducers include anthracyclines (doxorubicin, idarubicin, epirubicin), platinum derivatives (oxaliplatin), alkylating agents (cyclophosphamide) and proteasome inhibitors (bortezomib). Their ability to trigger ICD is mainly dependent on the generation of Endoplasmic Reticulum (ER) stress and Reactive Oxygen Species, that play an important role in DAMP function [47,48]. Although the ability of taxanes in triggering ICD is not equivalent, it has been shown that docetaxel might enhance the immunogenicity of tumor cells and anitumor acitivity [49,50].

An important question is how to further exploit the immunogenic potential of CRT to increase its anticancer efficacy. One novel approach to achieving this goalis combination with immune checkpoint inhibitors (ICIs). Immune checkpoints modulate signaling and either limit (co-inhibitory) or enhance (co-stimulatory) T-cell response. Cytotoxic T lymphocyte Antigen 4 (CTLA-4) and Programmed Cell Death protein 1 (PD-1) are distinct examples of co-inhibitory molecules [51]. Anti-PD-1 monoclonal antibodies pembrolizumab and nivolumab have received FDAapproval for use in recurrent/metastatic HNSCC [52,53]. Preclinical studies that combined RT with PD-1 blockade have found synergistic antitumor responses, thought in part to be due to the enhanced diversity of the antitumor T-cells that result from RT [54]. There is a good rationale to combine CRTwith ICIs: we showed that cisplatin-based CRT results in elevation of TLR and chemokine levels; hence, it canprovide ICD. Furthermore, CRT can induce tumor cell sensitization to cytotoxic T cell (CTL) lysis and downregulation of PD-L1, while the checkpoint blockade unleashes the killing potential of activated T cells [43,55]. In addition, ICD-inducing agents, including RT, have been shown to upregulate type I IFN response genes within the tumor cell. Recently, induction of a type I IFN response via radiation therapy was shown to overcome tumor resistance to anti-PD1 therapy. Indeed, CRT may increase tumor responsiveness to anti-PD-1 therapy by enhancing antigen availability and disrupting immune-regulatory networks. In the clinical setting, a phase I study in LA HNSCC has shown that pembrolizumab in combination with weekly cisplatin-based CRT is safe and does not significantly impair radiation or chemotherapy dosing [56]. Efficacy is currently being assessed in phase II/III trials.

On the other hand, we showed a statistically significant elevation of *TLR7* and *TLR9* levels post CRT in patients with LA HNSCC. This analysis was performed on CTCs and it is the first time that these markers are checked in serial samples. This has therapeutic implications given the promising clinical activity of drugs that interfere with the NF-κB and TLR signaling pathways. TLR7 and TLR9 agonists trigger the development of a tumor-specific immune response by inducing apoptosis, necrosis, and activating immune and dendritic cells [57]. Topical imiquimod, a synthetic imidazoquinoline that targets *TLR7*, is indicated for the treatment of superficial basal cell carcinoma and genital/perianal warts [10] and has been shown to induce temporary regression of treatment-refractory breast cancer chest wall metastases in combination with nab-paclitaxel in a phase II trial [58]. In oral cancer, it has been shown to reduce the degree of dysplasia in a mouse model [59] and has demonstrated anecdotal clinical activity in recurrent disease [60]. Indeed, local TLR7 activation by imiquimod has been shown to alter the tumor microenvironment and create an inflammatory environment suitable for antigen cross-presentation and infiltration by effector T cells and DCs with cytotoxic potential [61]. These are particularly desirable features for cancer immunotherapeutics and could lead to augmentation of tumor immunogenicity and increase the efficacy of ICIs in combination with CRT. On the other hand, CMP-001, a TLR9 agonist, when combined with pembrolizumab, has recently shown objective, durable tumor responses with tolerable toxicities in subjects with advanced melanoma resistant to prior anti-PD-1 therapy [62].

A major limitation of our study is that it is a single institution cohort and our results need to be validated in large cohorts with longer follow-up.

In conclusion, research published over the last decade has established that emission of danger signals or DAMPs as a part of an elaborate danger signaling module that can help in elevating the immunogenicity of dying, stressed or dead cancer cells; this cell death

pathway has been termed ICD. ICD can convert dying or dead cancer cells into a “vaccine” capable of inducing anticancer immunity. Our study strongly suggests that CRT can trigger ICD and induce tumor antigen-specific T-cell responses in patients with HNSCC. Furthermore, it demonstrates the significance of chemokine CXCL10 as a valuable poor prognostic biomarker. Despite still being in early stages of investigation, interests in therapeutic application of ICD are constantly increasing, particularly since the clinical implementation of immunotherapeutic approaches. Indeed, there is an urgent need to characterize the impact of ICD in the clinic and to identify ICD-related biomarkers capable of predicting the likelihood of a patient to respond to anticancer therapies that engage the host’s immune system.

Declaration of Competing Interest

None declared.

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