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ORIGINAL ARTICLE

Prognostic significance of PD-L1 expression on circulating tumor cells in patients with head and neck squamous cell carcinoma

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Background: Successful application of programmed death 1 (PD1) checkpoint inhibitors in the clinic may ultimately benefit from appropriate patient selection based upon predictive biomarkers. Molecular characterization of circulating tumor cells (CTC) is crucial for the investigation of molecular-targeted therapies while predictive biomarkers for response to PD1 checkpoint inhibitors are lacking. We sought to assess whether overexpression of *PD-L1* in CTCs could be detected at baseline and at different timepoints during treatment in a prospective cohort of head and neck squamous cell carcinoma (HNSCC) patients and used to predict clinical outcome after treatment with curative intent.

Patients and methods: We developed a highly sensitive, specific and robust RT-qPCR assay for *PD-L1* mRNA expression in EpCAM$^{(+)}$ CTCs. In a prospective cohort of 113 locally advanced HNSCC patients treated with curative intent we evaluated *PD-L1* expression in the EpCAM$^{(+)}$ CTC fraction at baseline, after 2 cycles of induction chemotherapy (week 6) and at the end of concurrent chemoradiotherapy (week 15).

Results: *PD-L1* overexpression was found in 24/94 (25.5%) patients at baseline, 8/34 (23.5%) after induction chemotherapy and 12/54 (22.2%) patients at the end of treatment. Patients with CTCs overexpressing *PD-L1* at end of treatment had shorter progression-free survival ($P = 0.001$) and overall survival ($P < 0.001$). Multivariate analysis revealed that *PD-L1* overexpression at end of treatment was independent prognostic factor for progression-free survival and overall survival. The absence of PD-L1 overexpression at the end of treatment was strongly associated with complete response with an odds ratio = 16.00 (95% CI = 2.76–92.72, $P = 0.002$).

Conclusions: We demonstrate that detection of CTCs overexpressing *PD-L1* is feasible and may provide important prognostic information in HNSCC. Our results suggest that adjuvant PD1 inhibitors deserve evaluation in HNSCC patients in whom *PD-L1*$^{(+)}$ CTCs are detected at the end of curative treatment.

Key words: circulating tumor cells, PD-L1, liquid biopsy, head and neck squamous cell carcinoma, cancer immunotherapy, checkpoint inhibitors

Introduction

Head and neck squamous cell carcinoma (HNSCC) represents the sixth most common malignancy, with 650 000 new cases and 350 000 HNSCC-related deaths reported annually worldwide [1, 2]. HNSCC are curable, if diagnosed at early stages (I and II); however, the majority of HNSCC present with locally advanced disease. The mortality rate of patients with locally advanced tobacco-induced HNSCC, especially heavy smokers with T4 tumor size or advanced nodal stage, remains high despite advances in treatment [3]. Certainly, there is a great need to improve outcomes of locally advanced HNSCC by identifying patients at high risk for relapse that may benefit from adjuvant therapy.

Circulating tumor cells (CTCs) are considered indicators of residual disease and thus are associated with an increased risk of metastasis [4, 5]. Moreover, hypoxic microenvironment, a major feature of HNSCC, plays a pivotal role in the emergence of CTCs and cancer stem cells (CSCs) [6]. The prognostic impact of CTCs and their central role in the metastatic cascade has been repeatedly demonstrated in many types of cancer [7–9] and their molecular characterization holds a very strong potential for novel approaches in the therapeutic management of cancer patients [10, 11]. Although rare and exposed to immune-mediated destruction, these cells manage to evade the immune system of the host. Therefore, a better understanding of the immunogenicity of these cells and their cross talk with immune cells may shed light to potential immunotherapy opportunities in HNSCC.

Tumor escape from immune-mediated destruction is due to immunosuppressive mechanisms that inhibit T cell activation. Programmed death 1 (PD1) is a negative immune-regulatory checkpoint, a negative signaling receptor expressed on activated T cells and $CD4^{(+)}$ $CD25^{(+)}$ Foxp3-expressing T regulatory (T-reg) cells [12]. Overexpression of PD-ligand 1 or 2 (*PD-L1* or *PD-L2*) by tumor cells activates the PD1 checkpoint pathway, by engaging to PD1 receptor, and attenuates the immune response [13]. Clinical trials in platinum-refractory recurrent/metastatic HNSCC have demonstrated safety and activity of PD1 checkpoint inhibitors in HNSCC [14–16].

Biomarkers are usually assessed only at baseline in different studies. However, it has been demonstrated that cancer treatments such as radiation may induce immune activation and cytokine production in the tumor microenvironment [17]. Therefore, the tumor immune phenotype may evolve in response to different treatments. Towards this direction, a recent study has shown overexpression of *PD-L1* in a subset of CTCs in breast cancer patients [18].

In the present study, we sought to prospectively determine *PD-L1* expression in CTCs at baseline, after induction chemotherapy (IC), after cisplatin chemo-radiation and at relapse in a cohort of locally advanced HNSCC patients treated with curative intend at our institution. To achieve this, we first developed and analytically validated a highly sensitive, specific and reproducible RT-qPCR assay for the quantification of *PD-L1*mRNA expression in CTCs. We demonstrate for the first time that detection of CTCs overexpressing *PD-L1* at the end of treatment is associated with shorter progression-free survival (PFS) and overall survival (OS) in locally advanced HNSCC.

Patients and methods

Study design

The 'liquid biopsy in HNSCC' project aims to detect and characterize CTCs in patients with HNSCC and examine their prognostic utility. 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. In the present report, we present the results obtained from the analysis of 113 HNSCC patients.

An additional cohort of 41 patients with recurrent/metastatic (R/M) HNSCC was included in the study as a test cohort since the likelihood to detect CTCs in R/M setting is higher compared with locally advanced HNSCC. The choice of chemotherapy in R/M patients was at the discretion of the treating physician. Specimens were obtained only at baseline in patients with R/M disease.

Patients with newly diagnosed inoperable HNSCC were eligible if they had histologically confirmed squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx, or larynx deemed unresectable due to technical or organ preservation reasons. Patients were excluded if they had received prior treatment of locally advanced HNSCC; patients with poor performance status (ECOG >2) and other concomitant neoplasms were also included. Tumor size, loco-regional spread and distant metastases were 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 chemo-radiotherapy with curative intent. IC with two cycles of TPF (docetaxel, cisplatin, 5-fluorouracil) preceded concurrent chemo-radiotherapy for patient selection when organ preservation approach was pursued or when a rapid response was deemed appropriate. Two cycles of induction TPF were followed by cisplatin chemo-radiotherapy in patients who attained at least a partial response to IC when an organ preservation strategy was pursued. The protocol called for specimen collection at baseline, at the end of IC (week 6), at completion of chemo-radiation (week 15) and at relapse. For all patients the 'end of treatment' samples were obtained 1 week post-completion of concurrent chemoradiotherapy. Assessment of response was carried out 14 days following the second cycle of IC and 12 weeks after completion of cisplatin chemo-radiotherapy. During follow-up, patients were investigated (ENT evaluation and CT of neck/chest) every 3 months for the first 2 years and after 24 months every 6 months. P16 protein status, assessed by immunohistochemistry, was used as a surrogate biomarker for human papillomavirus status. The study complied with the REMARK recommendations for tumor marker prognostic studies using biological material (available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2361579). A REMARK diagram is shown in supplementary Figure S1, available at *Annals of Oncology* online. Finally, a group of 20 healthy donors were used as controls.

Isolation of $EpCAM^{(+)}$ CTCs

For the isolation of $EpCAM^{(+)}$ CTCs from peripheral blood (30 ml) we followed our previously described protocols [19, 20].

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 carried out using the SuperScript™ First-Strand Synthesis System for RT-PCR (Life technologies) according to the manufacturer's protocol, using 7 μl of isolated total RNA as starting template.

RT-qPCR assay for *PD-L1*

Primer and probe design. We designed *in silico* the primers and hydrolysis probes for *PD-L1* and *HPRT* (used as a reference gene) using Primer Premier 5.0 software (Premier Biosoft, CA). 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 *PD-L1* and *HPRT* target genes according to our search in the BLAST Sequence Similarity Search tool (NCBI, NIH). The hydrolysis probe included a 5'-fluorescein (FAM) as a fluorophore covalently attached to the 5'-end of the oligonucleotide probe and a BlackBerry® Quencher as a quencher at the 3'-end, while the hybridization probe included a 3'-fluorescein (F) donor probe and a 5'-LC acceptor probe. The sequences of primers and probes are available upon request.

qPCR. qPCR was carried out in the LightCycler® 2.0 (Roche, Germany). Detailed optimization experiments were carried out (results not shown). The amplification reaction mixture for *PD-L1* contained 3 µl of the PCR synthesis buffer (5×), 1 µl $MgCl_2$ (25 mM), 0.2 µl dNTPs (10 mM), 0.6 µl BSA (10 µg/µl), 0.1 µl Hot-Start DNA polymerase (Promega), 0.3 µl of forward and reverse primer (10 µM), 0.83 µl hydrolysis probe (3 µM) and H_2O to a final volume of 10 µl, while the amplification reaction mixture for *HPRT* contained 2 µl of PCR synthesis buffer (5×), 1 µl $MgCl_2$ (25 mM), 0.2 µl dNTPs (10 mM), 0.3 µl BSA (10 µg/µl), 0.1 µl Hot Start DNA polymerase (Promega), 0.3 µl of forward and reverse primer (10 µM), 0.15 µl FRET probe (3 µM) and H_2O to a final volume of 10 µl. PCR cycling conditions for *PD-L1* were: 95 °C/2 min; 45 cycles of 95 °C/10 s, annealing at 58 °C/20 s and extension at 72 °C/20 s, while cycling conditions for *HPRT* were: 95 °C/2 min; 45 cycles of 95 °C/10 s, annealing at 59 °C/20 s and extension at 72 °C/20 s.

Preparation of PCR amplicons for evaluation of the analytical sensitivity of the assay. We generated individual PCR amplicons specifically for *PD-L1* and *HPRT* for the evaluation of the analytical sensitivity of the assay, using total RNA from the LNCAP and SKBR-3 cell lines as previously described [19, 20].

Normalization of qPCR data in clinical samples. qPCR data for *PD-L1* expression were normalized in respect to *HPRT* expression in the same cDNAs, using the $2^{-\Delta\Delta Ct}$ approach [21]. CTCs isolated through positive immune-magnetic enrichment are not 100% pure; since 'contamination' of PBMC in the $EpCAM^{(+)}$ CTC fraction could affect *PD-L1* assay specificity we evaluated this 'background noise' by analyzing peripheral blood samples from 20 healthy individuals in exactly the same way as patients. We estimated a cut-off based on *PD-L1* normalized expression in respect to *HPRT* expression in this control group. Using this approach we defined a sample as *PD-L1* overexpressed (*PD-L1* positive) based on the fold change of *PD-L1* expression in the $EpCAM^{(+)}$ CTC fraction in respect to the corresponding $EpCAM^{(+)}$ fraction in the group of 20 healthy individuals.

CellSearch™ analysis for PD-L1 expression on CTCs. We first carried out spiking experiments using a known number of SCC47 cells (0, 10, 50 and 100) spiked in 7.5 ml of whole blood, and analyzed them in the FDA-cleared CellSearch™ system according to a recently validated protocol [18]. *PD-L1* expression on CTCs was evaluated in peripheral blood of 16 patients with metastatic HNSCC disease using this protocol. For each patient, 30 ml of peripheral blood were collected in three CellSave tubes. Before CellSearch™ analysis, all samples were enriched for CTCs using the EpCAM independent RosetteSep System (Stem Cell Technology), following manufacturer instructions, so that the analysis could be carried out in 7.5 ml tubes, as required by the CellSearch™.

Statistical analysis

Objective response at the primary site was evaluated using the RECIST-modified criteria and was defined by either complete response or partial response. PFS was defined as the time from registration to the study to documented disease progression or death from other causes. Alive patients without documented events were censored at the time of the last disease evaluation. OS was defined as the time from registration to the study to death from any cause or censored at the time of last contact. The primary objectives of the study were the associations between *PD-L1* expression levels at baseline and at completion of treatment with PFS and OS.

Frequency and percentage were used to characterize patient demographics and disease characteristics and Fisher's exact test or χ^2 tests were used to make comparisons between groups. Event-time distributions were estimated by the Kaplan–Meier method and compared by the log-rank test. Univariable and multivariable Cox proportional hazards (PH) models were used to evaluate the relationship between *PD-L1* status (high versus low) and event-time distributions, with tumor stage, node's status, smoking, tumor site and age. Parametric and non-parametric tests were used to compare continuous variables between groups. All *P*-values are two-sided. A level of $P < 0.05$ is considered statistically significant unless specified otherwise.

Results

Development and analytical validation of the RT-qPCR assay for *PD-L1*

Limit of detection, linearity and analytical specificity. We evaluated the limit of detection (LOD) and limit of quantification (LOQ) for both *PD-L1* and *HPRT* as copies/µl in the reaction, using PCR amplicons, containing a known number of copies/µl, of each gene. Serial dilutions of these standards in triplicate, ranging from 10^5 to 10 copies/µl showed linearity, with correlation coefficients larger than 0.99 indicating a precise log–linear relationship (supplementary Figure S2, available at *Annals of Oncology* online). LOD for both assays was 3 copies/µl and LOQ was 9 copies/µl [22]. Ten different genomic DNAs (50 ng/µl) gave no amplification signal for any of the gene-target transcripts (results not shown). The characteristics of the calibration curves are given in supplementary Table S1, available at *Annals of Oncology* online.

Evaluation of intra- and inter-assay precision. Repeatability or intra-assay variance (within-run precision) of the *PD-L1* RT-qPCR, was evaluated by repeatedly analyzing three cDNA samples corresponding to low, medium and high *PD-L1* transcripts, while for *HPRT* by repeatedly analyzing 4 cDNA samples corresponding to 1, 10, 100 and 1000 SKBR-3 cells/µl in the same assay, in 3 parallel determinations. Intra-assay variance expressed as the CVs (%) of the Cq variance for *PD-L1*, ranged from 0.84% to 1.2%, while for *HPRT* ranged from 0.68% to 1.1% (Table 1). Intra-assay variance expressed as within-run CVs (%) of copies/µl ranged for *PD-L1*, from 16% to 20% and for *HPRT* from 20% to 24% (Table 1). Reproducibility or inter–assay variance (between-run precision) of the RT-qPCR assays, was evaluated by analyzing the same cDNA samples, for *HPRT* and *PD-L1* that were kept frozen in aliquots at −20 °C, over a period of 1 month on four separate assays carried out in 4 different days. Between-run CVs were 15.8% for *HPRT* and 15% for *PD-L1* (Table 1).

Table 1. RT-qPCR for PD-L1 and HPRT: evaluation of intra- and inter-assay precision

HPRT	Cq (SD)	CV%	Copies (SD)	CV%
SKBR-3	Intra-assay precision (n=3)			
1	30.64 (0.21)	0.68	0.84 (±1.6)×10	20
10	28.32 (0.25)	0.89	0.80 (±1.9)×10^2	24
100	25.59 (0.23)	0.90	1.1 (±0.3)×10^3	23
1000	21.97 (0.24)	1.1	3.7 (±0.86)×10^4	22
	Inter-assay precision (n=5)			
100	21.75 (0.065)	0.31	4.5 (±0.26)×10^4	5.8
PD-L1	Intra-assay precision (n=3)			
cDNA 1	35.63 (0.30)	0.84	6.5 (±1.3)	20
cDNA 2	31.48 (0.25)	0.79	0.97 (±0.14)×10^2	16
cDNA 3	24.45 (0.29)	1.2	1.0 (±0.18)×10^4	19
	Inter-assay precision (n=5)			
cDNA 4	29.57 (0.21)	0.71	5.32 (±0.82)×10^2	15

PD-L1 expression in the EpCAM$^{(+)}$ CTC fraction and corresponding EpCAM$^{(-)}$ PBMC fraction in healthy individuals and HNSCC patients

Before the analysis of *PD-L1* expression in all clinical samples we applied the RT-qPCR assay for *PD-L1* expression both in the EpCAM$^{(+)}$ CTC fraction and corresponding EpCAM$^{(-)}$ PBMC fraction in 10 healthy individuals and 54 HNSCC patients (Figure 1A). Median fold change of *PD-L1* expression in the EpCAM$^{(-)}$ PBMC fraction was 1.03 (range 0.07–3.40) in healthy individuals and 0.39 (range 0.01–1.82) in HNSCC patients (Mann–Whitney test, $=-1.692$, $P=0.091$). These distributions were not significantly different (Kolmogorov–Smirnov $Z=1.237$, $P=0.094$). Median fold change of *PD-L1* expression in the EpCAM$^{(+)}$ CTC fraction was 1.28 (range 0.64–2.43) in healthy individuals, 0.98 (range 0.03–1.64) in *PD-L1* negative HNSCC patients' samples (Mann–Whitney test, $=-2.120$, $P=0.034$) and 2.70 (range: 1.66–16.45) in *PD-L1* positive HNSCC patients' samples (Mann–Whitney test, $=-3.713$, $P<0.001$). The distribution between EpCAM$^{(+)}$ CTC fraction in healthy individuals and *PD-L1*$^{(+)}$ HNSCC patients' samples were significantly different (Kolmogorov–Smirnov: $Z=1.900$, $P=0.001$), but not in *PD-L1*$^{(-)}$ HNSCC patients' samples (Kolmogorov–Smirnov: $Z=1.137$, $P=0.151$).

PD-L1 expression in primary and recurrent/metastatic HNSCC

To investigate if *PD-L1* was overexpressed in the EpCAM$^{(+)}$ CTC fraction, we first checked a small cohort of 41 patients with recurrent and/or metastatic (R/M) disease, 25 of which had local recurrence and 16 distant metastases. Five out of 41 (12.2%) patients were found positive for *PD-L1* overexpression. Among those patients who were positive for *PD-L1* overexpression, 4/5 (80%) died, while among those patients who were negative at baseline, 20/36 (55.6%) deceased. Log-rank test showed no significant difference in OS between patients who were positive for *PD-L1* overexpression versus those who were not (log-rank $\chi^2=0.047$, $P=0.829$). Considering the diversity of the applied treatments in these patients with R/M disease, no definitive conclusion can be drawn about the prognostic significance of *PD-L1* overexpression.

We subsequently enrolled 113 patients with histologically confirmed primary HNSCC to test the prognostic importance of *PD-L1* overexpression in the EpCAM$^{(+)}$ CTC fraction at baseline and at the end of treatment. Among 8 oropharyngeal cancer patients with available tumor specimens for evaluation of p16 protein expression and known *PD-L1* overexpression status in the EpCAM$^{(+)}$ CTC fraction at baseline, 4 (50.0%) were p16 positive and 3/4 (75%) were positive for *PD-L1* overexpression at baseline, while none of the four p16 negative patients (0%) were positive for *PD-L1* overexpression (Fisher's exact test, $P=0.143$); 94/113 (83.2%) patients were evaluable for CTC analysis at baseline, 34/57 (54.9%) after IC and 54/106 (50.9%) at the end of chemoradiotherapy (supplementary Figure S1, available at *Annals of Oncology* online). Relative fold change ($2^{-\Delta\Delta Cq}$) of *PD-L1* in respect to *HPRT* in the EpCAM$^{(+)}$ CTC fraction for individual samples of healthy individuals ($n=20$) and all patient groups are shown in Figure 1B. Patient clinical characteristics in correlation to *PD-L1* status are shown in Table 2. At baseline 24/94 (25.5%) patients were found positive for *PD-L1* overexpression, at the post-IC setting 8/34 (23.5%) patients were positive and at the end of treatment 12/54 (22.2%) patients were positive for *PD-L1* overexpression. Patients with missing sample at the end of treatment did not differ from the ones included in the analysis with respect to patient gender, age, tobacco and alcohol use, TNM stage, histological grade and primary tumor site, as assessed by Fisher's exact test (Table 2). There was no correlation between *PD-L1* expression at any timepoint and basic patient characteristics (Table 2).

Clinical outcome

Data on response to treatment were available for 73/113 (64.6%) patients. Forty-three out if 73 (58.9%) patients achieved a complete response (CR) post-treatment, 9/73 (12.3%) a partial response, 3/73 (4.1%) stable disease, while 18/73 (24.7%) developed progressive disease during treatment or within the first 3 months post-treatment. Among 18 patients with overexpression of *PD-L1* at baseline, 7 (38.9%) achieved a CR post-treatment, whereas among 41 patients who were *PD-L1* negative at baseline, 27 (65.9%) achieved a CR post-treatment (Fisher's exact test, $P=0.085$). Among 10 patients with overexpression of *PD-L1* at the end of treatment, only 2 (20%) achieved a CR, whereas among 35 patients who were *PD-L1* negative at the end of treatment, 28 (80%) achieved a CR (Fisher's exact test, $P=0.001$). The absence of *PD-L1* overexpression at the end of treatment was strongly associated with complete response with an odds ratio = 16.00 (95% CI = 2.76–92.72, $P=0.002$).

Thirty-eight patients were assessed for *PD-L1* overexpression at both baseline and at the end of treatment. Among them, 13 (34.2%) overexpressed *PD-L1* at baseline and 8 (21.1%) overexpressed *PD-L1* at the end of treatment. This difference showed only a trend for statistical significance (McNemar test, uncorrected $\chi^2=2.778$, $P=0.096$), considering also the small sample size. Among 25 patients who were *PD-L1* negative at baseline, 23 (92.0%) remained negative at the end of treatment, while among 13 patients who overexpressed *PD-L1* at baseline, 6 (46.2%) remained positive at the end of treatment. For 16 patients, samples

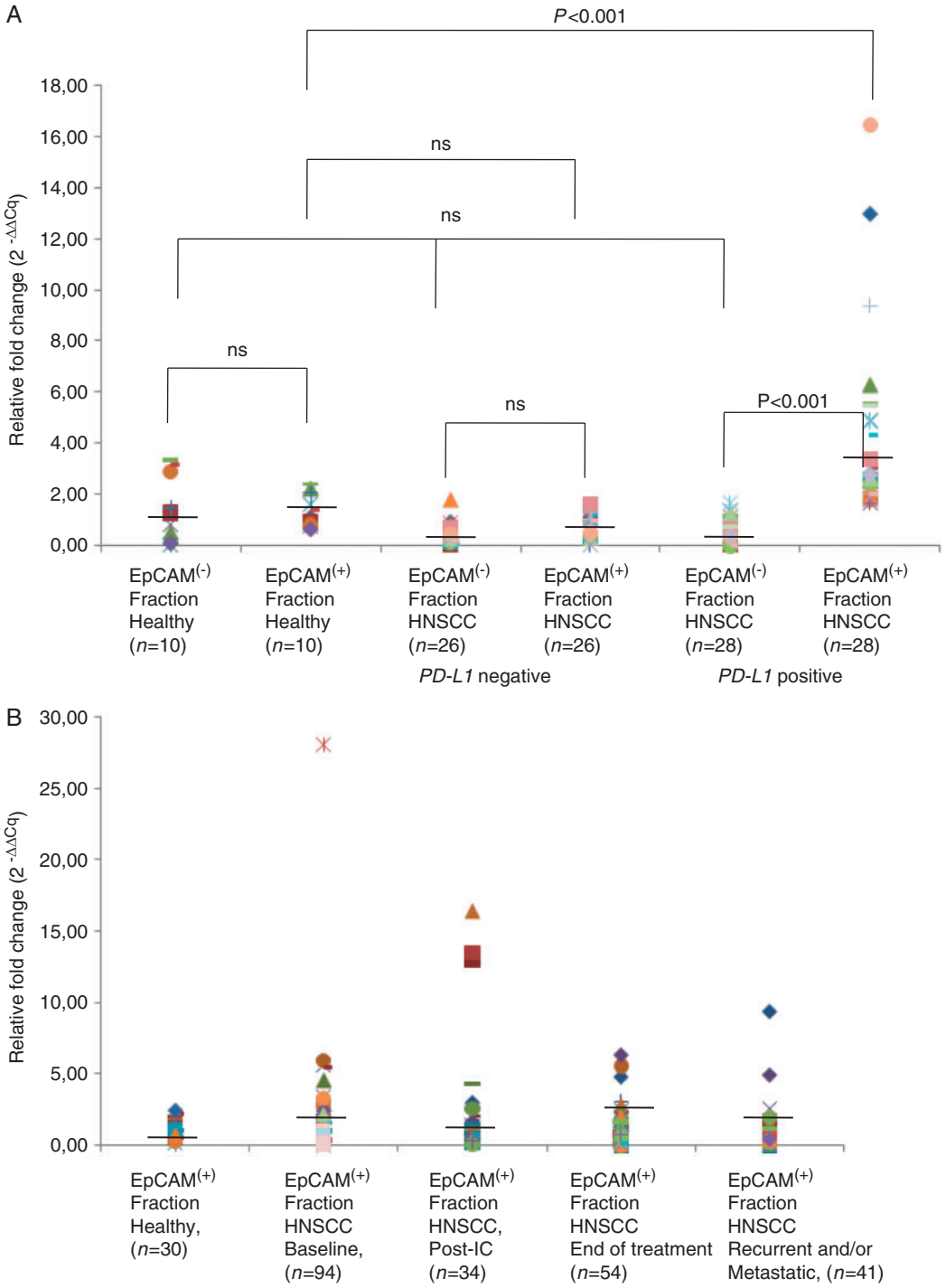


Figure 1. Relative fold change values ($2^{-\Delta\Delta Ct}$) between: (A) *PD-L1* and *HPRT* for individual samples in the EpCAM$^{(-)}$ and EpCAM$^{(+)}$ PBMC and CTC fractions, (B) *PD-L1* and *HPRT* for individual samples in EpCAM$^{(+)}$ CTC fractions for all groups.

at baseline, after 2 cycles of IC (week 6) and at the end of concurrent chemoradiotherapy (week 15) were available: serial longitudinal changes of *PD-L1* expression in CTC can be seen in supplementary Figure S3, available at *Annals of Oncology* online.

Survival

At a median follow-up of 18.9 months (range 0.2–54.9), 26/94 (27.7%) patients with baseline assessment developed progressive disease and 28/94 (29.8%) died, while 13/34 (38.2%) patients with post-IC assessment progressed and 14/34 (41.2%) deceased. Fourteen out of 54 (25.9%) patients with samples evaluable for *PD-L1* expression at the end of treatment progressed and 14/54 (25.9%) died.

Patients who had *PD-L1* overexpression in the EpCAM$^{(+)}$ CTC fraction at baseline did not have significantly different PFS (log-rank $\chi^2 = 0.09$, $P = 0.761$) and OS (log-rank $\chi^2 = 1.41$, $P = 0.235$) compared with those without *PD-L1* overexpression

Table 2. Clinical characteristics of the primary HNSCC patients (n = 94)

Patients enrolled	Baseline	*PD-L1* mRNA		Post-IC	*PD-L1*mRNA		End of treatment	*PD-L1*mRNA	
	All	Positive	Negative	All	Positive	Negative	All	Positive	Negative
	n (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)
	94 (100)	24 (25.5)	70 (74.5)	34 (100)	8 (23.5)	26 (76.5)	54 (100)	12 (22.2)	42 (77.8)
Sex	$P = 1.000$			$P = 0.645$			$P = 0.732$		
Male	75 (79.8)	19 (25.3)	56 (74.7)	26 (76.5)	7 (26.9)	19 (73.1)	37 (68.5)	9 (17.6)	28 (82.4)
Female	19 (20.2)	5 (26.3)	14 (73.7)	8 (23.5)	1 (12.5)	7 (87.5)	17 (31.5)	3 (24.3)	14 (75.7)
Age	$P = 0.812$			$P = 0.444$			$P = 0.513$		
≥65	40 (42.6)	11 (27.5)	29 (72.5)	13 (38.2)	2 (15.4)	11 (84.6)	25 (46.3)	7 (28.0)	18 (72.0)
<65	54 (57.4)	13 (24.1)	41 (75.9)	21 (61.8)	6 (28.6)	15 (78.4)	29 (53.7)	5 (17.2)	24 (82.8)
Smoke	$P = 0.622$			$P = 0.077$			$P = 1.000$		
No/light/Ex	33 (29.6)	7 (21.2)	26 (78.8)	9 (16.7)	0 (0)	9 (100)	17 (31.5)	4 (23.5)	13 (76.5)
Heavy	61 (70.4)	17 (27.9)	44 (72.1)	25 (83.3)	8 (32.0)	17 (68.0)	37 (68.5)	8 (21.6)	29 (78.4)
ETOH	$P = 0.123$			$P = 1.000$			$P = 0.714$		
No/light	65 (69.1)	20 (30.8)	45 (69.2)	27 (79.4)	7 (25.9)	20 (74.1)	42 (77.8)	10 (23.8)	32 (76.2)
MD/heavy	29 (30.9)	4 (13.8)	25 (86.2)	7 (20.6)	1 (14.3)	6 (85.7)	12 (22.2)	2 (16.7)	10 (83.3)
T stage	$P = 0.093$			$P = 0.170$			$P = 1.000$		
T_1/T_2	37 (39.4)	6 (16.2)	31 (83.8)	9 (26.5)	4 (44.4)	5 (55.6)	19 (35.2)	4 (21.1)	15 (78.9)
T_3/T_4	55 (58.5)	18 (32.7)	37 (67.3)	24 (70.6)	4 (16.7)	20 (83.3)	34 (63.0)	8 (23.5)	26 (76.5)
Unknown	2 (2.1)			1 (2.9)			1 (1.8)		
Lymph node status	$P = 0.452$			$P = 0.444$			$P = 1.000$		
N_0	31 (33.0)	6 (19.4)	25 (80.6)	13 (38.2)	2 (15.4)	11 (84.6)	18 (33.3)	4 (22.2)	14 (77.8)
N_1–N_3	63 (67.0)	18 (28.6)	45 (71.4)	21 (61.8)	6 (28.6)	15 (71.4)	36 (66.7)	8 (22.2)	28 (77.8)
TNM	$P = 0.340$			$P = 1.000$			$P = 0.328$		
I/II	15 (16.0)	2 (13.3)	13 (86.7)	1 (2.9)	0 (0)	1 (100)	7 (13.0)	0 (0)	7 (100)
III/IV	79 (84.0)	22 (27.8)	57 (72.2)	33 (97.1)	8 (24.2)	25 (75.8)	47 (87.0)	12 (25.5)	35 (74.5)
Grade	$P = 0.760$			$P = 0.673$			$P = 1.000$		
Low/intermediate	58 (61.7)	16 (27.6)	42 (72.4)	23 (67.7)	5 (21.7)	18 (78.3)	31 (57.4)	8 (25.8)	23 (74.2)
High	25 (26.6)	5 (20.0)	20 (80.0)	10 (29.4)	3 (30.0)	7 (70.0)	19 (35.2)	4 (21.1)	15 (78.9)
Unknown	11 (11.7)			1 (2.9)			4 (7.4)		
Primary site	$P = 0.779$			$P = 0.609$			$P = 0.708$		
Oropharynx	21 (22.3)	6 (28.6)	15 (71.4)	6 (17.6)	2 (33.3)	4 (66.7)	13 (24.1)	2 (15.4)	11 (84.6)
Other	73 (77.7)	18 (24.7)	55 (75.3)	28 (82.4)	6 (21.4)	22 (78.6)	41 (75.9)	10 (24.4)	31 (75.6)

(Figure 2A and B). Also, patients with *PD-L1* overexpression post-IC did not differ significantly in PFS (log-rank $\chi^2 = 0.835$, $P = 0.361$) and OS (log-rank $\chi^2 = 0.076$, $P = 0.783$) compared with those without *PD-L1* overexpression (data not shown). In contrast, patients with *PD-L1* overexpression at the end of treatment had poorer PFS (log-rank $\chi^2 = 11.11$, $P = 0.001$) and OS (log-rank $\chi^2 = 19.17$, $P < 0.001$) (Figure 2C and D).

Cox regression analysis, also revealed the significance of *PD-L1* overexpression in the EpCAM$^{(+)}$ CTC fraction of HNSCC patients after the completion of treatment. Univariate analysis showed a significantly higher risk of progression and death in the *PD-L1* positive compared with *PD-L1* negative patients. Finally, multivariate Cox regression model confirmed the prognostic value of *PD-L1* overexpression in the EpCAM$^{(+)}$ CTC fraction at the end of treatment of the prediction of PFS and OS, independently from patients' age and sex, smoking behavior, alcohol intake, tumor T stage, nodal status, primary site and histological grade. In Figure 3, the forest plots of univariate Cox-regression hazard models are shown.

Imaging of PD-L1$^{(+)}$ CTCs in HNSCC patients using the CellSearch^{TM} system

CTCs are highly heterogeneous, and it is expected that not all CTCs overexpress PD-L1. This has been shown clearly previously for breast cancer [18] using the CellSearch^{TM} system, where in the same breast cancer patients, PD-L1$^{(+)}$ and PD-L1$^{(-)}$ CTCs were detected. Based on these data, we wanted to evaluate the expression of PD-L1 in CTCs of HNSSC patients using the same methodology as previously described [18], for a limited number of HNSCC patients ($n = 16$). According to our results, in 8/16 (50%) patients CTCs were detected; however, only a limited number of CTCs (1–2) was detected in peripheral blood of these patients, and only a sub-fraction of these CTCs were PD-L1$^{(+)}$.There were also three cases, where CTCs were detected but these CTCs were negative for PD-L1 expression (Figure 4). Before the analysis of clinical samples in the CellSearch^{TM}, we also carried out spiking experiments. Our spiking experiments with the HNSCC SCC47 cell line have shown that when 100 SCC47 cells were spiked in 7.5 ml peripheral blood in a Cellsave

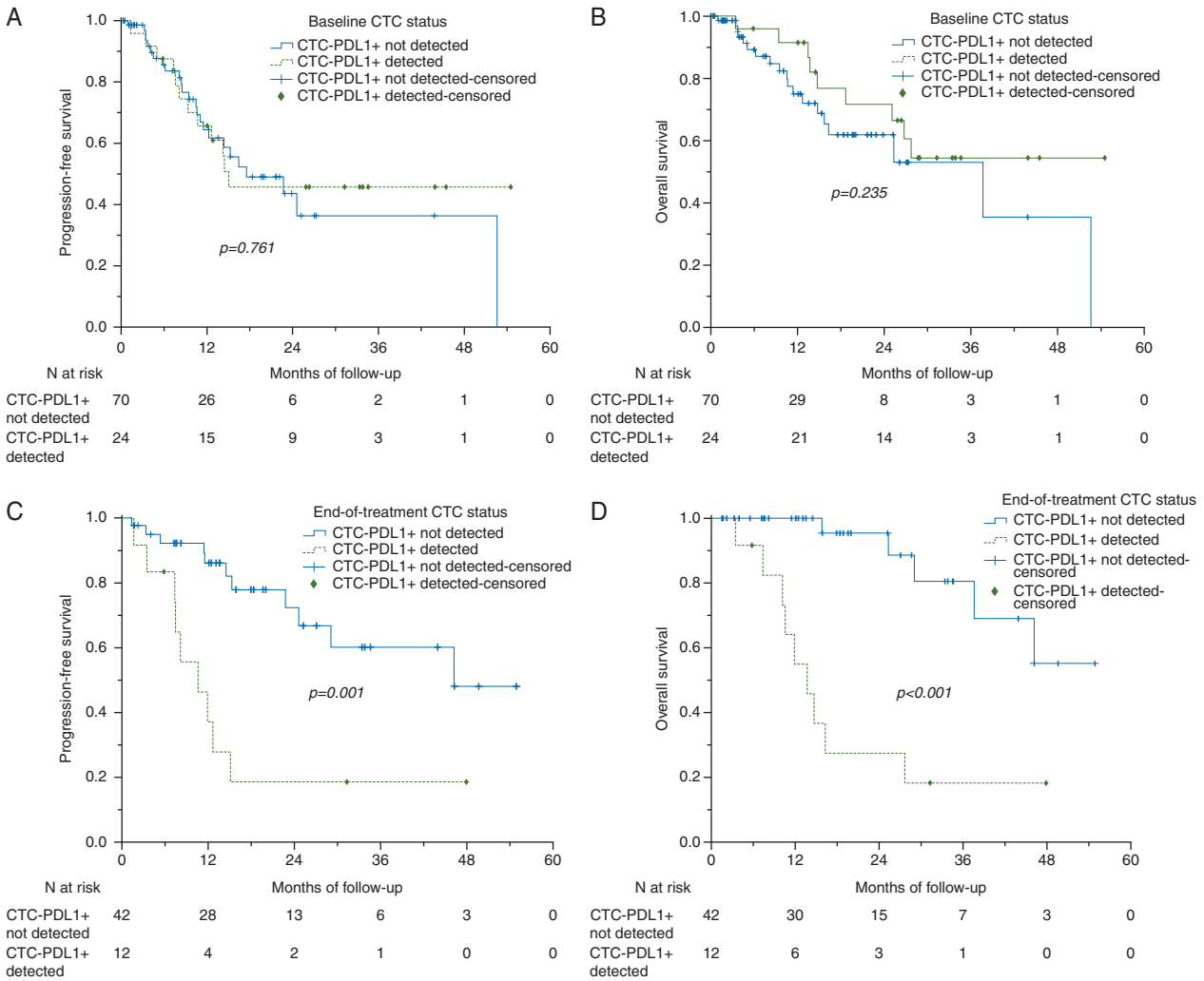


Figure 2. Kaplan–Meier estimates of (A) PFS for HNSCC patients at baseline with or without *PD-L1* overexpression ($P = 0.761$), (B) OS for HNSCC patients at baseline with or without *PD-L1* overexpression ($P = 0.235$), (C) PFS for HNSCC patients at the end of the treatment with or without *PD-L1* overexpression ($P = 0.011$) and (D) OS for HNSCC patients at the end of the treatment with or without *PD-L1* overexpression ($P = 0.004$).

tube, 98 tumor cells were detected in the CellSearch™ but only 12/98 (12.2%) of these cells were *PD-L1* positive. When 50 SCC47 cells were spiked, 59 tumor cells were enumerated and 13/59 (22.0%) out of these cells were *PD-L1* positive, while when 10 SCC47 cells were spiked, 11 tumor cells were counted and 2/11 (18.2%) cells were *PD-L1* positive. No tumor cells were detected in control blood samples (healthy individuals).

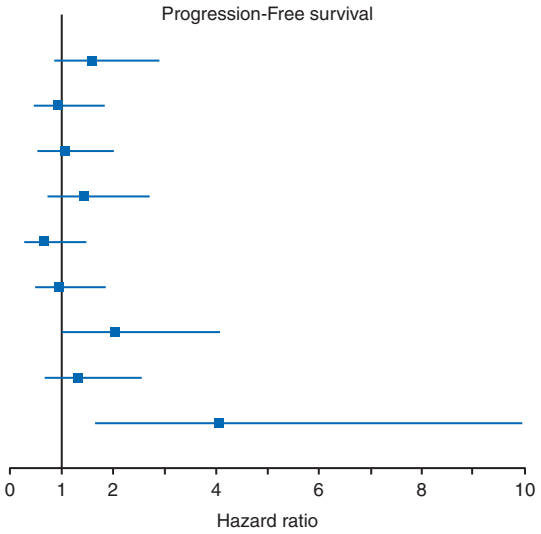
Discussion

We developed and evaluated an RT-qPCR assay for *PD-L1* mRNA transcripts and applied it to evaluate *PD-L1* overexpression in the EpCAM$^{(+)}$ CTC fraction, isolated from peripheral blood of HNSCC patients.

Many groups including ours have verified the importance of using molecular assays for CTC molecular characterization [19, 20, 23–33]. We have already shown that molecular assays based on real-time PCR carried out in nucleic acids material (RNA or genomic DNA) isolated from the EpCAM$^{(+)}$ CTC fraction can give valuable information for the molecular characterization of CTC at the gene expression [19, 20], DNA methylation [24–26] and DNA mutation level [27]. It is clear that in this approach we are not verifying the presence of CTCs by imaging through immunofluorescence, but through the genetic material isolated from the EpCAM$^{(+)}$fraction. This approach has been extensively and successfully used also for the molecular characterization of EpCAM$^{(+)}$ CTCs in breast and ovarian cancer patients by other research groups [28–30]. Direct comparison studies of our system with this commercially available test have shown satisfactory correlations when the same samples were analyzed by both methodologies [31]. In the present study, we sought to determine the prognostic significance of *PD-L1* overexpression in the EpCAM$^{(+)}$ CTC fraction at baseline and at the end of definitive treatment in locally advanced HNSCC. The use of chemotherapy as adjuvant/consolidation treatment has not yielded survival improvement in HNSCC [33–35].

A

Covariates	Covariate values	HR	95%CI
Age years	≥65 vs <65 years	1.59	0.88-2.88
Sex	Male vs Female	0.94	0.48-1.83
Smoke	Heavy vs Ex/Light/No	1.07	0.57-2.01
ETOH	Heavy vs Light/No	1.43	0.76-2.70
Primary site	Oropharynx vs other	0.66	0.29-1.48
Histology grade	High vs intermediate/Low	0.97	0.51-1.85
T stage	T3/T4 vs T1/T2	2.04	1.03-4.05
Lymph node status	N+ vs NO	1.33	0.70-2.54
PD-L1 expression	Yes vs No	4.07	1.67-9.91



B

Covariates	Covariate values	HR	95%CI
Age years	≥65 vs <65 years	1.68	0.85-3.31
Sex	Male vs Female	0.98	0.46-2.06
Smoke	Heavy vs Ex/Light/No	0.91	0.45-1.83
ETOH	Heavy vs Light/No	1.61	0.78-3.30
Primary site	Oropharynx vs other	0.56	0.27-1.18
Histology grade	High vs intermediate/Low	0.64	0.29-1.42
T stage	T3/T4 vs T1/T2	1.65	0.76-3.56
Lymph node status	N+ vs NO	1.96	0.85-4.50
PD-L1 expression	Yes vs No	7.96	2.65-23.89

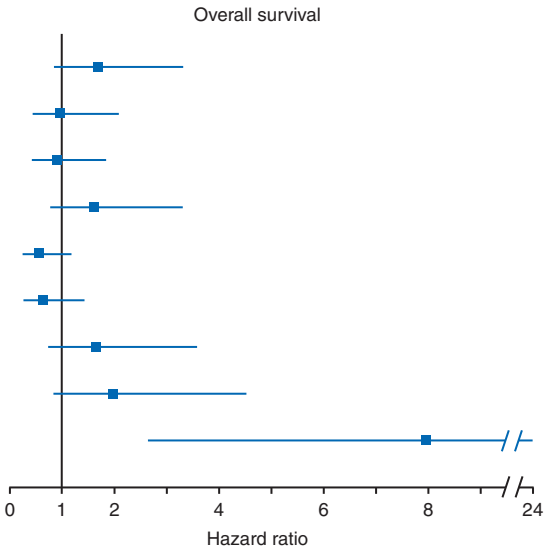


Figure 3. Forest plots of univariate Cox models for (A) OS and (B) PFS for HNSCC patients at the end of the treatment.

However, the more recent advent of targeted therapies urges re-examination of the question of adjuvant/consolidation systemic therapy following the completion of chemo-radiation. This concept has been successfully applied in breast cancer where 1 year of treatment with the HER2-targeted monoclonal antibody trastuzumab following adjuvant chemotherapy for early HER2$^{(+)}$ breast cancer represents currently the standard of care for these patients [36].The decision on adjuvant therapy in cancer is currently based on the characteristics of the primary tumor. However, the aim of administering adjuvant therapy in cancer is considered to be the eradication of minimal residual disease, which is present after primary surgery but undetectable by currently used conventional imaging approaches. It is therefore possible that decision on adjuvant therapy should be based on the detection and molecular characterization of CTCs [37].

Recent studies indicate that evaluation of PD-L1 protein status in tumor specimens could assist in patient selection for treatment with PD1 checkpoint inhibitors [38]. However, accurate measurement of PD-L1 protein levels in FFPE tumor samples, as discussed previously, is limited by the absence of reliable antibodies and interpretative uncertainties (i.e. positivity cutoff). Another limitation of *PD-L1* expression assessment on tumor biopsies is tumor heterogeneity. Several studies have demonstrated discordance in biomarker expression between primary tumor and CTCs [27, 29] indicating that heterogeneous clones exist that are often not represented in the biopsy material.

It appears, therefore, that liquid biopsies provide a non-invasive tool for molecular characterization of tumors which can be more informative than serial tumor biopsies. A very recent study has shown for the first time that PD-L1 protein status can be evaluated in CTCs of breast cancer patients with verified metastasis by using the FDA cleared CellSearch^{TM} system [18]. Other studies have also shown PD-L1 protein expression on CTCs in patients with bladder cancer [39] and NSCLC [40]. However, the evaluation of PD-L1 protein status using immunofluorescence is strongly affected by the specificity of antibodies used and interpretative uncertainties (i.e., positivity cutoff), unless a robust validation of the antibody is carried out [18]. Nicolazzo *et al.* recently evaluated the expression of PD-L1 in CTCs of non-small cell lung cancer patients treated with the PD1 inhibitor Nivolumab, using the CellSearch^{TM} system

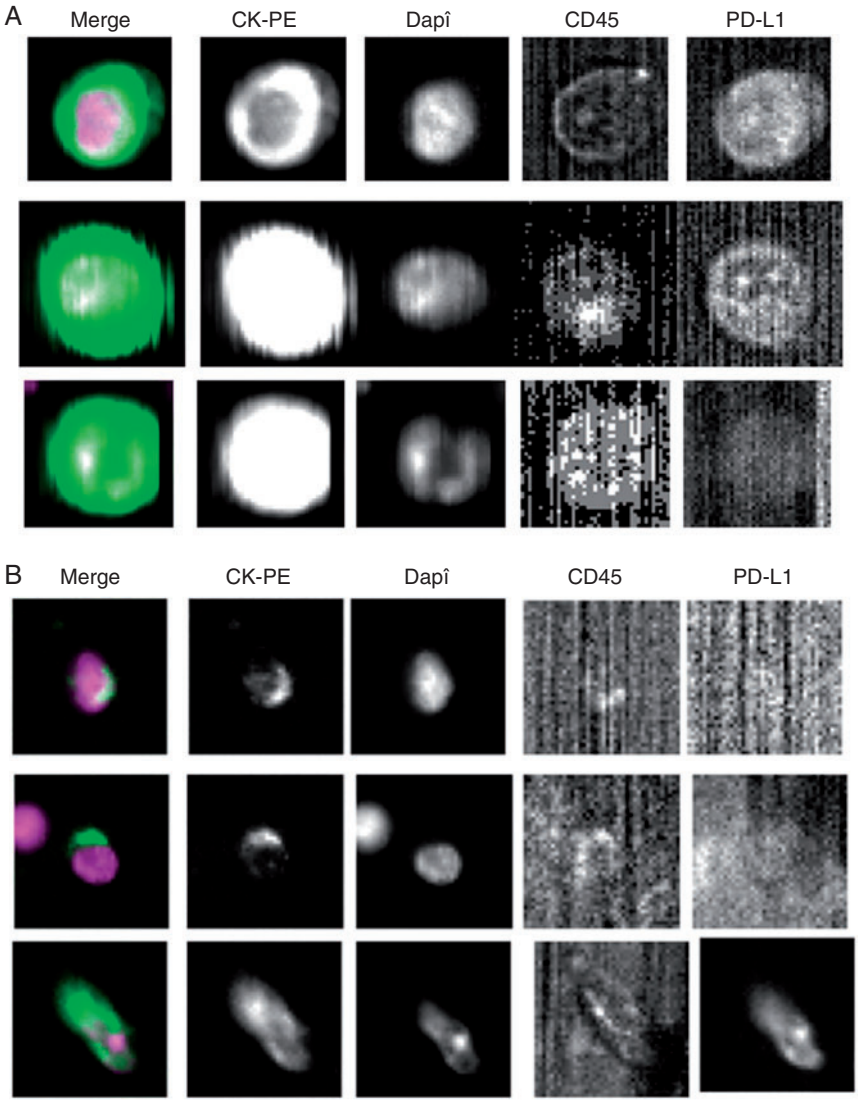


Figure 4. Imaging of PD-L1$^{(+)}$ CTCs using the CellSearch^{TM} system in (A) SCC47 cell line spiked in peripheral blood and (B) HNSCC patients peripheral blood samples.

[40]. According to this study, all patients with PD-L1$^{(-)}$ CTCs obtained a clinical benefit, while all patients with PD-L1$^{(+)}$ CTCs experienced progressive disease. Thus the persistence of PD-L1$^{(+)}$ CTCs is indicative of a mechanism of therapy escape. Therefore, liquid biopsy may allow real-time sampling of patients for PD-L1 through the course of the disease and provide information on mechanisms of tumor escape and response to immunotherapy.

By using this novel molecular assay, we demonstrate for the first time to our knowledge, in a cohort of locally advanced HNSCC treated with definitive non-surgical treatment including chemoradiation + IC, that detection of CTCs over-expressing *PD-L1* at the end of definitive treatment correlates with lower likelihood of attaining CR and higher risk for progression and death compared with *PD-L1* negative counterparts. Our results raise the question on whether PD1 targeted therapies can eliminate *PD-L1* over-expressing CTCs and increase cure rates in locally advanced HNSCC patients in whom *PD-L1* over-expressing CTCs are detected at the end of definitive treatment. Our results also demonstrate that serial *PD-L1* expression assessment in liquid biopsies is feasible and has potential to select and monitor patients for PD1 inhibitors. Because therapy with checkpoint inhibitors is cost intensive, cancer-bearing patients who may benefit from such treatments must be properly selected using appropriate biomarkers. This approach underscores the crucial need for predictive biomarkers that can be introduced in the clinical routine. Although studies of combination of chemotherapy and immunotherapy are being planned, the important question of how to integrate these novel therapies with the current clinical strategy still remains. This highlights the need to develop clear immune biological and clinical parameters that allow for rapid go/no-go decisions.

In the present study, we observed that *PD-L1* expression in the EpCAM$^{(+)}$ CTC fraction may evolve during treatment and this modulation may inform clinical trial design of the sequence of chemotherapy and/or radiation with immunotherapy.

A major limitation of our study is that it is a single institution cohort and our results need to be validated in larger cohorts. In addition, EpCAM-based isolation methods may miss CTCs

bearing mesenchymal rather than epithelial phenotype, a phenomenon that is not uncommon in HNSCC.

Since our RT-qPCR assay does not enumerate CTCs, a relevant question could be whether our findings are related to the extent of minimal residual disease (i.e. number of CTCs) at the end of treatment rather than the prognostic importance of *PD-L1* expression on CTCs as a biomarker. To answer this question, we evaluated PD-L1 expression on CTCs in peripheral blood samples for a limited number of HNSCC patients, using direct imaging through the CellSearch™ system. Previous experiments using the CellSearch™ system in breast cancer demonstrated that the percentage of CTCs expressing *PD-L1*is not directly correlated with the total number of CTCs since in the same breast cancer patients, there were PD-L1$^{(+)}$ and PD-L1$^{(-)}$ CTCs [18]. CTCs are indeed highly heterogeneous thus it is expected that not all CTCs overexpress PD-L1. Indeed our data verify this finding in HNSCC as well, since we have found that only a subset of CTCs detected were PD-L1$^{(+)}$ while there were also cases, where CTCs were detected but were negative for PD-L1 expression.

In summary, we demonstrate that serial determination of *PD-L1* expression in the EpCAM$^{(+)}$ CTC fraction is feasible and may inform clinical trial design.

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Disclosure

The authors have declared no conflicts of interest.

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