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ORIGINAL ARTICLE

Predictive Value of CD8 Expression and FoxP3 Methylation in Nasopharyngeal Carcinoma Patients Treated with Chemoradiotherapy in a Non-endemic Area

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Abstract

Undifferentiated Nasopharyngeal Carcinoma (UNPC) is associated with Epstein-Barr Virus (EBV) and characterized by an abundant immune infiltrate potentially influencing the prognosis. Thus, we retrospectively assessed the significance of immunosuppression in the UNPC microenvironment as prognostic biomarker of treatment failure in a non-endemic area, and monitored the variation of systemic EBV-specific immunity before and after chemoradiotherapy (CRT). DNA and RNA were extracted from diagnostic biopsies obtained by tumor and adjacent mucosa from 63 consecutive EBV+ UNPC patients who underwent radical CRT. Among these patients 11 relapsed within 2 years. The expression of the EBV-derived UNPC-specific BARF1 gene and several immune-related genes was monitored through quantitative RT-PCR and methylation-specific PCR analyses. Peripheral T cell responses against EBV and BARF1 were measured in 14 patients (7 relapses) through IFN-γ ELISPOT assay. We found significantly higher expression levels of BARF1, CD8, IFN-γ, IDO, PD-L1, and PD-1 in UNPC samples compared to healthy tissues. CD8 expression was significantly reduced in both tumor and healthy tissues in UNPC patients who relapsed within two years. We observed a hypomethylated FOXP3 intron 1 exclusively in relapsed UNPC patients. Finally, we noticed a significant decrease in EBV- and BARF1-specific T-cells after CRT only in relapsing patients. Our data suggest that a high level of immunosuppression (low CD8, hypomethylated FoxP3) in UNPC microenvironment may predict treatment failure and may allow an early identification of patients who could benefit from the addition of immune modulating strategies to improve first line CRT.

Keywords Nasopharyngeal carcinoma · Immunosuppression · EBV-specific immunity · Chemoradiotherapy · CD8 · FoxP3

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Abbreviations

CRT	Chemoradiotherapy
EBV	Epstein-Barr Virus
IDO	Indoleamine 2,3-dioxygenase
IMRT	Intensity modulated radiotherapy
NP	Nasopharyngeal
PBMCs	Peripheral blood mononuclear cells
RT	Radiotherapy
Treg	Regulatory T cell
UNPC	Undifferentiated Nasopharyngeal Carcinoma

Introduction

Nasopharyngeal Carcinoma (NPC) has a peculiar geographic distribution since it is endemic in south-east Asia and China, but relatively rare in Europe and North America [1]. The World Health Organization (WHO) type 3 NPC (i.e. undifferentiated histotype of NPC, UNPC) encompasses over 95% of cases in the endemic areas. UNPC is strongly associated with Epstein-Barr Virus (EBV) infection [2, 3] and characterized by an early distant lymphatic and blood dissemination [3]. The standard of care for UNPC is radiotherapy (RT), in combination with chemotherapy (CRT) in advanced stages, with excellent response rates. Unfortunately, loco-regional recurrences occur in 10% to 20% of patients and represent a difficult task for clinicians, due to the complexity of surgical treatment, and the frequent onset of platinum resistance [2–4]. Additionally, metastatic UNPC patients still have an adverse prognosis [2, 3]. For these reasons, the early identification of prognostic biomarkers could favor the definition of risk classes, which in turn could contribute to ameliorate the management of these patients, possibly personalizing the therapeutic treatment.

Almost all UNPC cases show a retained EBV genome in cancer cells [5], characterized by type II latency, with the expression of a limited number of latent proteins, such as EBNA1, LMP-1, LMP-2, and BARF1 [6], an oncogenic protein expressed by the majority of these tumors [7, 8]. BARF1 mRNA is detected also in nasopharyngeal brushing of NPC patients, as recently demonstrated [9], and could thus represent a suitable NPC marker. Furthermore, this protein is immunogenic and able to induce the development of high numbers of circulating BARF1-specific T cells in UNPC patients, as we recently reported [10, 11]. On these grounds, we speculated that monitoring EBV-antigen expression by the tumor and host EBV-specific immunity may contribute to identify prognostic factors in UNPC management.

To date, tumor stage and quantitative analysis of EBV DNA in patients' serum are universally recognized as prognostic factors for UNPC [12, 13]. Morever, it is becoming increasingly clear that the abundant lymphocytic infiltrate that characterizes this malignancy supports an important contribution of host immune responses to UNPC evolution [2, 14]. Several subsets of infiltrating lymphocytes seem to differently correlate with UNPC progression [15], suggesting that, besides the presence of host anti-tumor immune effectors, a diffuse immune tolerance characterizes UNPC tumor microenvironment, particularly in advanced stages. In this regard, recent studies on oral squamous carcinoma described the presence of a regulatory T cell (Treg) population with a strong immunosuppressive activity and characterized by FoxP3 DNA hypomethylation in the tumor microenvironment [16]. Moreover, the anti-tumor immune response mediated by T lymphocytes through the production of IFN-γ is responsible for the induction of indoleamine 2,3-dioxygenase (IDO) [17], an enzyme with immunosuppressive activity, which also represents an independent prognostic factor of Disease-Free Survival in UNPC [18]. Finally, immune tolerance may also depend on the PD-1/PD-L1 immune checkpoint axis, responsible for the direct inhibition of T lymphocytes [19]. However, the prognostic value of PD-1/PD-L1 expression in primary UNPC is still debated [20–25]. PD-1 and PD-L1 are targets of the promising immune checkpoint inhibitors, whose efficacy has been positively correlated with the presence of an abundant lymphocytic infiltrate, in particular in virus-related tumors [26]. Moreover, recent studies demonstrated a synergistic activity between immune checkpoint inhibitors and RT [27], probably due to the immunogenic potential of ionizing radiations [28]. We thus hypothesized that also PD-1 and PD-L1 expression could influence the response to RT in UNPC.

On these grounds, we designed a retrospective study to evaluate the prognostic role of selected immune-related genes, and to verify their ability to predict local recurrence within 2 years in UNPC patients belonging to a non-endemic area. The early identification of patients at high risk of relapse after standard CRT may suggest which patients could benefit from the addition of further treatments, as for example immune checkpoint inhibitors.

Materials and Methods

Patients Assessments and Therapy

Between October 2002 and April 2015, 193 patients with nasopharyngeal cancer were consecutively observed in our center for CRT. 76 patients (39.4%) out of the 193 met the following inclusion criteria: caucasian patients, age $\geq$ 18 years, histological diagnosis of EBV-positive UNPC, II-IVB stage, CRT treatment between October 2002 and April 2015, with a minimum follow-up of 2 years, no evidence of metastatic disease. Patients received concurrent CRT whether affected by stage II disease and in presence of nodal involvement, and sequential therapy in presence of advanced stages (stage III-IVB). Neoadjuvant chemotherapy (CT) regimens employed were Cisplatin (CDDP), 5-Fluorouracil (5-FU) and Docetaxel (TPF scheme) or Bleomycin, Epirubicin, and

CDDP (BEC scheme). The former consisted of 3 cycles of Cisplatin (100 mg/m^2 IV on day 1) plus 5-FU (1000 mg/m^2 IV on days 1–5) plus Docetaxel (75 mg/m^2 IV on day 1), administered with a 3-weeks interval, while the latter included 3 cycles of Bleomycin (15 mg IV in push on day 1 and 12 mg/mq/24 h days 1–4), Epirubicin (70 mg/mq IV day 1), and CDDP (100 mg/mq IV day 1), administered with a 3-weeks interval. RT started within 3 weeks after the 3rd cycle of CT. RT treatment modalities consisted of accelerated hyperfractionated RT delivered with a co-planar multiple-field technique (HART) until 2005 and hypofractionated RT delivered with intensity modulated technique (IMRT) from 2005 (Supplemental Methods) [29, 30].

Patients underwent a weekly nasopharyngoscopy to assess response and toxicity. An MRI and an ^{18}F FDG-PET/CT were performed two and three months after the RT end, respectively, to confirm the clinical response. Follow-up examinations were planned every 3-months. Local recurrence within 24 months was the study endpoint.

The study was conducted with the approval of the local institutional review board and the Ethical Committee (Comitato Etico Unico Regionale CEUR-2017-Os-097-CRO). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Sample Collection

Biological samples collected from 76 UNPC patients were analyzed in the present study. The association with EBV was confirmed by in situ hybridization for EBERs for all UNPC tumor samples (data not shown). Thirteen out of 76 patients were excluded due to the scarceness of histological sample or for negative BARF-1 expression [10], thus leaving 63 patients eligible for the final analysis. Biopsies from UNPC tumor tissue and from normal adjacent nasopharyngeal mucosa ($n = 52$) were obtained at diagnosis, and divided into two parts: one half was immediately frozen at −80 °C and the second half was fixed in formalin for diagnostic purposes. Blood samples were collected from 14 UNPC patients at diagnosis and at the end of RT treatment. Peripheral blood mononuclear cells (PBMCs) were freshly isolated (within 5 h after blood drawing) from heparinised blood of patients (Supplemental Methods).

DNA and RNA Extraction and Molecular Analyses

Biopsies were homogenized through QIAGEN TissueLyser II as recommended. Extraction of nucleic acids was performed through All prep DNA/RNA Mini Kit, from QIAGEN, following manufacturer's recommendations.

One μg of RNA was retro-transcribed into cDNA for absolute quantification in qRT-PCR analysis, while bisulfite conversion was carried out on 500 ng genomic DNA for quantitative Methylation-Specific PCR (qMSP) analysis (Supplemental Methods).

Peptides Selection and Analysis of EBV- and BARF1-Specific T Cell Responses

A panel of 11 HLA class-I BARF1-derived 9-mers (Supplemental Table 2) was selected among a library of previously identified and validated peptides [10]. A further panel of 17 HLA class-II BARF1-derived 15-mers were selected on the basis of their predicted binding affinity to HLA class II alleles according to SYFPEITHI (www.syfpeithi.de) and NetMHC (http://www.cbs.dtu.dk/services/NetMHC/) prediction software available on the web. Peptides synthesis and storage are reported in Supplemental Methods.

Peripheral T cell responses against EBV and BARF1 protein were analysed by the interferon (IFN)-γ release enzyme-linked immunosorbent spot (ELIspot) assay using the commercial kit "Human IFN-γ Single-Color ELISPOT" (ImmunoSpot C.T.L.), according to manufacturer's instructions (Supplemental Methods).

Statistical Analysis

The Fisher exact test was employed for the comparison of UNPC patients by socio-demographic (sex and age) and clinical characteristic (TNM stage) according to recurrence within 24 months. BARF1 and immune-related biomarkers expression levels, and FoxP3 methylation percentages were expressed as median values, and the Kruskal-Wallis test was used to compare values observed in relapsed patients, with those detected in non-relapsing cases, according to cancer recurrence within 2 years. Gene expression levels measured in tumor samples and in the corresponding healthy samples were compared according to the Student's t test for paired data. Healthy/tumor tissue ratio was further calculated for each.

EBV- and BARF1-specific T cell responses were expressed as single dot plots for IFN-γ ELISPOT assay. The Student's t test for unpaired data was applied to compare T cell responses measured in relapsed versus non-relapsing patients both at diagnosis and after CRT, and the Student's t test for paired data was employed to highlight any significant difference within the single populations before and after CRT.

Results

Patients' Characteristics

The distribution of socio-demographic and clinico-pathologic characteristics of UNPC patients are presented in Table 1 and in Supplemental Table 3. Median age was 49 years (range:

18–71) and 46 patients (73%) were male. At diagnosis, TNM (7th edition – AJCC 2010) stage was II, III and IVa-IVb in 25 (39.7%), 23 (36.5%), and 15 (23.8%) patients, respectively. Neoadjuvant CT consisted of TPF and BEC in 48 (76.2%) and 15 (23.8%) patients. IMRT was employed in 50 pts. (79%) while HART in the remaining 13 (21%) pts. Among clinical characteristics only TNM stage was associated with recurrence (Table 1) even if the median value of EBV DNA plasma levels in recurrent cases was nearly 9 times higher to the same value measured in patients free from relapse within 2 years (analysis performed in a limited number of patients as reported in Supplemental Table 3).

The Expression Levels of Immune-Related Genes Varied between Tumor and Healthy Tissues

To compare tumor tissue with the adjacent non-tumor tissue usually represents the first step in search for biomarkers, in order to identify biological features unique to tumor tissue. To this end, BARF1 mRNA, and mRNA specific for several genes involved in the anti-tumor immune response (HLA-A, CD8, GATA3, IFN-γ), and in the induction of an immunosuppressed microenvironment (IDO, PD-L1, PD-1, FoxP3), were measured by quantitative RT-PCR analysis in UNPC samples ($n = 63$) compared with the matched adjacent tissue ($n = 52$) as control. Most of the investigated genes were differently expressed, with UNPC samples showing significantly higher expression levels of BARF1, CD8, IFN-γ, IDO, PD-L1, and PD-1 compared to the healthy tissues (Table 2). This was particularly evident for the samples of patients without recurrence ($n = 42$). In case of recurrence no differences were noticed for CD8, IFN-γ and PD-1. However, the number of patients undergoing a recurrence ($n = 11$) is probably too low to appreciate significant differences.

Table 1 Distribution of 63 patients with UNPC by socio-demographic and clinical characteristics according to recurrence within 24 months

	Recurrence within 24 months				Fisher exact test
	No		Yes		
	n	(%)	n	(%)	
Sex					
Man	36	(69.2)	10	(90.9)	
Woman	16	(30.8)	1	(9.1)	0.262
Age (years)					
<50	26	(50.0)	7	(63.6)	
≥50	26	(50.0)	4	(36.4)	0.515
TNM stage					
I-II	23	(44.2)	2	(18.2)	
III	20	(38.5)	3	(27.3)	
IVA-IVB	9	(17.3)	6	(54.6)	0.038

Table 2 Healthy/tumor tissue ratio (HTr) overall and according to cancer recurrence within 2 years

	All		Not recurred		Recurred	
	HTr	p	HTr	p	HTr	p
BARF1	0.00	$p < 0.001$	0.00	$p < 0.001$	0.00	$p = 0.002$
HLA-A	0.82	$p = 0.985$	0.76	$p = 0.688$	0.92	$p = 1.000$
CD8	0.29	$p = 0.043$	0.29	$p = 0.029$	0.32	$p = 0.770$
GATA3	0.76	$p = 0.671$	0.73	$p = 0.880$	1.02	$p = 1.000$
IFN-γ	0.11	$p < 0.001$	0.11	$p < 0.001$	0.16	$p = 0.082$
IDO	0.18	$p < 0.001$	0.23	$p < 0.001$	0.18	$p = 0.002$
PD-L1	0.48	$p = 0.026$	0.50	$p = 0.110$	0.46	$p = 0.084$
PD-1	0.17	$p < 0.001$	0.17	$p < 0.001$	0.24	$p = 0.160$
FoxP3	0.53	$p = 0.346$	0.64	$p = 0.467$	0.27	$p = 0.539$

The Impact of Immune-Related Genes and Proteins Expression on Outcome

To establish the putative prognostic value of these genes, we then performed an analysis to identify whether their differential expression was associated with UNPC recurrence. To this end, we compared UNPC patients who relapsed shortly after the end of the treatment (<2 year), and patients who did not relapse. Results reported in Table 3 demonstrated that, among all genes analyzed, only CD8 mRNA expression was significantly reduced in UNPC patients who relapsed within two years by the end of the treatment. Interestingly, a significant reduction in the CD8 mRNA levels was also observed in the corresponding uninvolved mucosa of relapsed patients (Table 3). On the contrary, no statistically significant differences in BARF1 expression were found in tumor tissue of UNPC patients who relapsed compared to those free from recurrence after 2 years (Table 3, Fig. 1). Since we noticed a slight increase in PD-L1 expression in relapsing patients already at diagnosis (Table 3, Fig. 1a), we better characterized PD-L1 expression by evaluating protein levels both in primary and in recurrent tumors through immunohistochemistry (Fig. 1b, Supplemental Fig. 1, and Supplemental Table 4), together with CD8 and FoxP3 expression (Supplemental Fig. 1). Interestingly, we observed that when expressed at diagnosis, PD-L1 protein expression was usually maintained also in the recurrent tumor (Fig. 1b), and we noticed that the majority of recurrent samples showed ≥5% PD-L1 expression (9/10 samples). Of note, FoxP3 gene expression was notably higher in tumor tissues of UNPC patients who relapsed within two years, even if statistical significance was not reached.

Table 3 Median values of selected biomarkers in tumor and healthy tissues according to cancer recurrence within 2 years

	Tumor tissue (/100,000)			Healthy tissue (/100,000)		
	Recurrence		Kruskal Wallis	Recurrence		Kruskal Wallis
	No	Yes		No	Yes	
BARF1	0.950	0.951	$p = 0.854$	0.000	0.000	$p = 0.127$
HLA-A	28.990	18.096	$p = 0.942$	25.320	25.27	$p = 0.758$
CD8	0.631	0.081	$p < 0.001$	0.189	0.030	$p < 0.001$
GATA3	0.170	0.126	$p = 0.486$	0.179	0.151	$p = 0.962$
IFN-γ	0.156	0.099	$p = 0.192$	0.020	0.019	$p = 0.662$
IDO	2.751	2.410	$p = 0.532$	0.617	0.425	$p = 0.530$
PD-L1	0.524	0.919	$p = 0.277$	0.289	0.559	$p = 0.343$
PD-1	0.041	0.040	$p = 0.730$	0.013	0.031	$p = 0.553$
FoxP3	0.040	0.127	$p = 0.094$	0.051	0.038	$p = 0.406$
FoxP3 methylation	94.68%	89.54%	$p = 0.020$	97.55%	95.52%	$p = 0.178$

Methylation Levels of FoxP3 Promoter Correlated with Recurrence

Next, we determined whether there was a relationship between the level of FoxP3 mRNA expression and the methylation status of the first intron of its gene, which usually shows complete demethylation in natural Tregs [31]. To this end, methylation of the FOXP3 intron 1 was evaluated by qMSP analysis in DNA obtained from 62 UNPC tissues and 52 nasopharyngeal normal tissues. Interestingly, FoxP3 intron 1 was hypomethylated in UNPC tissues of patients who relapsed compared to the tumor sample of patients free from relapse within 2 years (Table 3 and Fig. 2). The methylation percentage of FoxP3 promoter was comparable in the corresponding normal mucosa of the two cohorts of patients (Table 3).

Monitoring of T Cell Responses Against EBV and BARF1 Before and After IMRT

The presence of spontaneous T cell responses to EBV and BARF1 protein in particular was investigated by IFN-γ

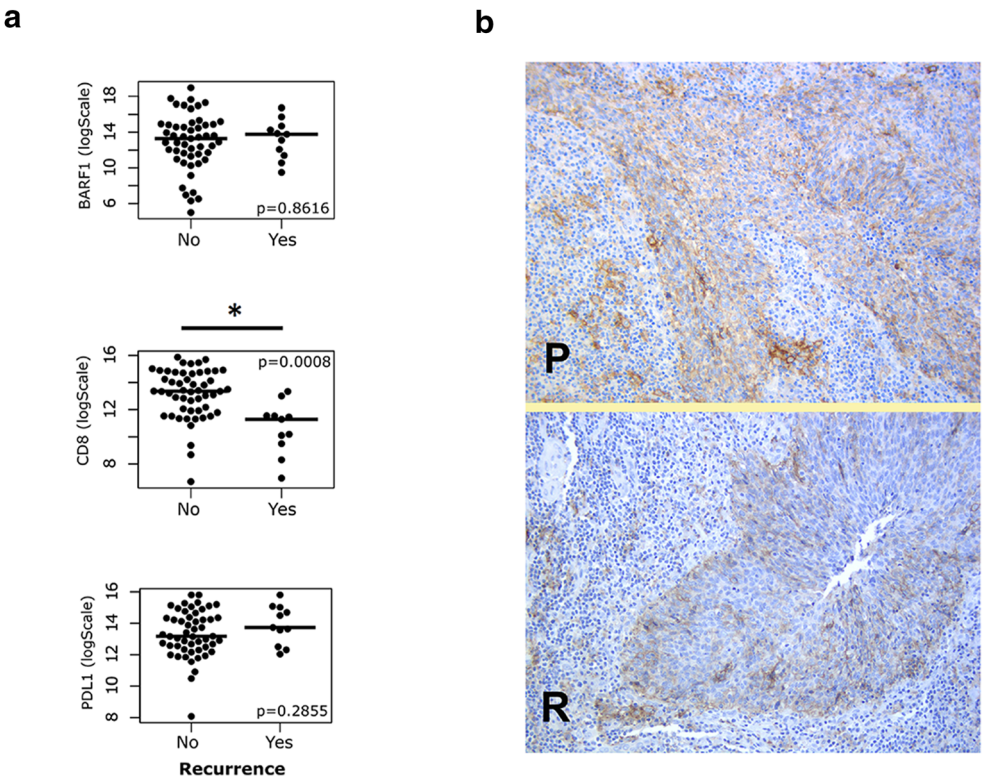

Fig. 1 BARF1, CD8, and PD-L1 mRNA expression levels and PD-L1 protein expression in UNPC patients. **a** Analysis of BARF1, CD8, and PD-L1 expression levels in patients who relapsed within 24 months from the end of treatment (yes) and in patients who did not (no). Black horizontal bars represent the median values of expression levels for each gene in both groups of patiens. *p* values were determined by the non-parametric Kruskal–Wallis test. **b** PD-L1 was expressed in both primary (P) and recurrent (R) tumors. FFPE immunohistochemistry. Hematoxylin counterstain, original magnification × 200

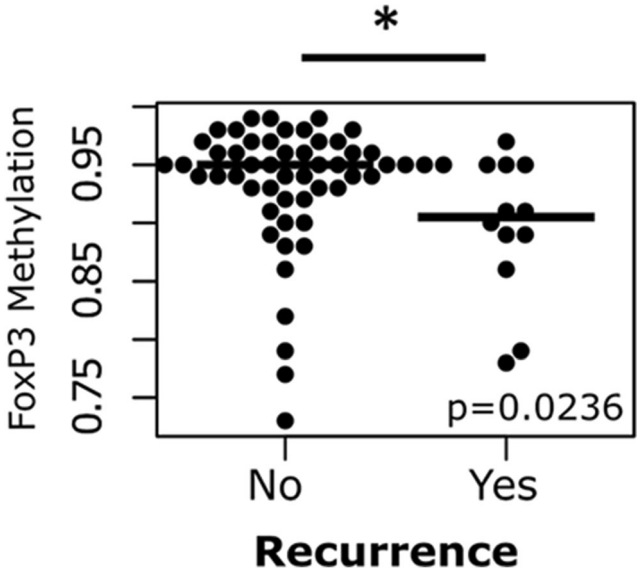

Fig. 2 FoxP3 intron 1 methylation in patients who relapsed within 24 months from the end of treatment (yes) and in patients who did not (no). Black horizontal bar represents the median values of FoxP3 intron 1 methylation for each group. p values were determined by the non-parametric Kruskal–Wallis test

ELISPOT assay, and by intracellular staining in flow cytometry, after stimulation of PBMCs through peptide mixes including validated peptides restricted for several HLA class I (EBV and BARF1 PepMixes) and class II alleles (only BARF1 PepMix) (Supplemental Table 2). The analysis was carried out in blood samples of 14 patients through ELISPOT assay, and in 13 of them also by intracellular staining, collected at diagnosis and after IMRT.

At diagnosis, ELISPOT assay detected the release of IFN-γ after stimulation with both, EBV and BARF1-PepMixes, in all patients, regardless of recurrence (Fig. 3). The amount of IFN-γ secreting T-cells after EBV and BARF1 stimulation significantly decreased after RT only in patients undergoing a relapse within 2 years (Fig. 3).

The qualitative analysis performed with intracellular staining seemed to reveal a prevalence of CD107a expression and IFN-γ production at diagnosis, and a majority of TNF-α production after RT, especially for BARF1 stimulation (Supplemental Fig. 2).

Discussion

Results from the present study revealed an association between a high level of immunosuppression in the tumor microenvironment at diagnosis and treatment failure after CRT. UNPC originates in a highly lymphoid rich organ and is characterized by a dense lymphoplasmacytic infiltrate [32]. On this assumption, the low level of CD8 expression that we observed in tumor tissues but also in the normal nasopharyngeal mucosa of relapsed patients could suggest a general deficiency in immune effectors already at diagnosis. A higher number of intratumor CD8+ T cells, but not of total TILs, was recently associated with increased Overall Survival [32], and a prognostic role for stromal TILs [33] was reported in 2 different cohorts of EBV+ NPC patients. Thus, the analysis of both intratumoral and stromal CD8 gene expression could be more

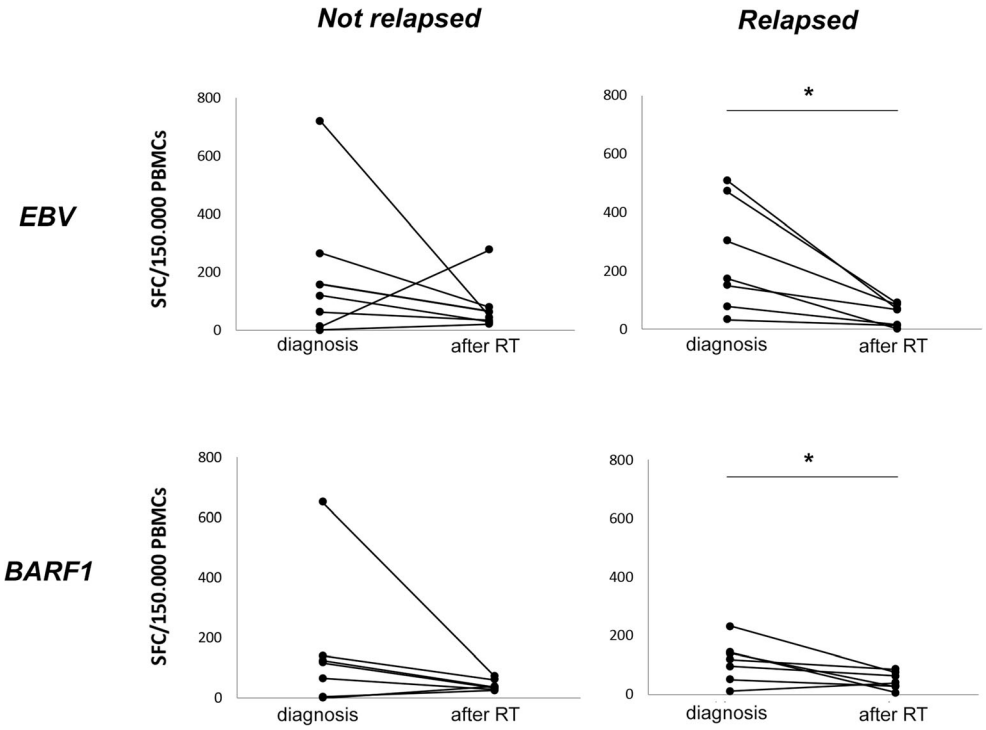

Fig. 3 Cellular immunity against EBV and BARF1 in UNPC patients at diagnosis and after CRT. Quantification of T cell responses against EBV- and BARF1-derived peptides was assessed in patients who relapsed within 24 months from the end of treatment (relapsed; $n = 7$) and in patients who did not (not relapsed; $n = 7$) by IFN-γ-ELISPOT. p values were determined by the Student's t test. SFC, spot forming cells; PBMCs, peripheral blood mononuclear cells. *, $p < 0.05$

accurate than the general quantification of intratumoral or stromal TILs to better define UNPC prognosis.

Several other immune cell populations have been consistently detected in NPC biopsies, including Tregs [34]. Treg functional activity can be monitored through the specific molecular marker FoxP3 [34], whose expression is regulated by intron 1 methylation [31]. Our data revealed that FoxP3 intron 1 was significantly hypomethylated in tumor tissues of relapsed patients, even in the presence of hypermethylated LINE-1 repetitive elements (a surrogate of the overall genomic DNA methylation content; Supplemental Table 5) [35], thus supporting this analysis as potential epigenetic prognostic marker.

Besides the hypermethylated genome [36], NPC carries also a heavily methylated EBV episome, expressing a limited number of viral transcripts including BARF1 [37, 38], hardly detectable as protein [39]. Consistently, we noticed an increased BARF1 expression in tumor samples compared to normal nasopharyngeal tissue in all UNPC patients. In these patients, serum antibodies against BARF1 are usually present at very low levels, because host immune responses against EBV are focused on EBNA1 [5]. By contrast, we measured circulating CD8 and CD4 T cell responses against BARF1-derived peptides in all patients at diagnosis, confirming that cellular immune responses against BARF1 are usually detected in NPC patients [10].

Despite the presence of BARF1-specific circulating T cells, tumor growth implies their inefficiency in the tumor microenvironment, probably induced by the activation of immune suppressive enzymes such as IDO [18]. High expression levels of IDO and low CD3+ cell infiltration have been associated with worse survival rates in a recent cohort of NPC patients [18]. In our study, we noticed an increased expression of IDO, IFN-γ, and PD-L1 in the tumor tissue compared to the normal nasopharyngeal mucosa, independently of recurrence. IDO could be induced by several factors, including IFN-γ [18, 40], which in turn could be also responsible for the overexpression of PD-L1 [41]. Although several studies have recently reported a high percentage (>70%) of NPCs expressing PD-L1 [20, 23, 42–44], its prognostic role is still debated [20, 23, 43, 44]. In our case study, we did not observe any association between PD-L1 expression and recurrence after CRT, but we found an increased expression of PD-1 in tumor tissues compared to normal mucosa. All T cells may express PD-1, but its expression on CD8+ T cells, in particular, was highlighted in NPC tissues [24, 42], and correlated with poorer prognosis [24]. The high expression of these immune checkpoints in NPC tumor microenvironment together with the high prevalence of CD8+ T cells make this tumor a good candidate for PD-1 and PD-L1 targeted therapies [23]. The expression of PD-L1 revealed in the majority of tumor tissues already at diagnosis, even if analyzed in a reduced number of patients, may support the potential efficacy of immune checkpoint inhibitors in this context. More importantly, we revealed a frequently high PD-L1 protein expression also in recurring tumor tissues (Fig. 1b, Supplemental Table 4), thus further supporting the eligibility of these patients for anti-PD-L1/PD-1 therapies. Furthermore, we noticed, even if only preliminarly due to the low number of patients investigated, significantly reduced EBV- and BARF1-specific immune responses especially in relapsed patients. This observation highlights the urgent clinical need to improve first line treatments in patients at high risk of relapse. In this respect, immunotherapeutic treatments could counteract the possible immunesuppressive activity of conventional therapy, thus exploiting the spontaneous anti-tumor immune responses already present in UNPC patients. Recent clinical trials employing immune checkpoint inhibitors reported a safety profile and a clinical efficacy of both pembrolizumab and nivolumab in metastatic or relapsed NPC patients previously treated with RT [42, 45].

In conclusion, our findings suggest that the evaluation of the immune cell markers CD8 and FoxP3 could be included in a prognostic panel together with activity markers (IFN-γ), and immune suppressive markers (IDO, PD-L1, PD-1) able to early identify UNPC patients at high risk of relapse and thus eligible for the addition of immunotherapy to convential CRT. Future prospective studies with larger sample populations need to be performed to confirm our observations.

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Authors' Contributions All authors contributed to the study conception and design. Elena Muraro conceived the study, performed part of the experiments and drafted the manuscript. Elisabetta Fratta, Damiana A Fae', Debora Martorelli, and Michela Cangemi performed the experiments and reviewed the manuscript. Jerry Polesel carried out statistical analysis and reviewed the manuscript. Elisa Comaro contributed to sample collection and data acquisition. Carlo Furlan, Giuseppe Fanetti, Federico Navarria, Carlo Gobitti collected and analyzed clinical data and reviewed the manuscript. Chiara Scaini, Chiara Pratesi, and Stefania Zanussi performed EBV DNA analysis. Valentina Lupato, Giuseppe Grando, Vittorio Giacomarra, Luigi Barzan collected and analyzed clinical data. Riccardo Dolcetti and Agostino Steffan reviewed the manuscript. Sandro Sulfaro and Vincenzo Canzonieri performed histopathological diagnosis and immunohistochemistry, and performed data analysis. Emanuela Vaccher and Giovanni Franchin collected and analyzed clinical data, conceived and design the study, reviewed and approved the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest

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