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RESEARCH

The prognosis prediction value of CD69+ CD8+ tissue-resident memory T cell as a novel indicator of pathologic complete response heterogeneity following different neoadjuvant therapy regimen in esophageal squamous cell carcinoma

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Abstract

Background Improving pathological complete response (pCR) rate is currently the main goal of neoadjuvant therapy for locally advanced esophageal squamous cell carcinoma (LA-ESCC). However, improved pCR rates do not consistently translate into better prognosis, likely due to regimen-specific pCR heterogeneity. We investigated this heterogeneity and potential biomarkers between two common neoadjuvant regimens.

Methods We included 445 LA-ESCC patients from four centers, with 228 receiving neoadjuvant chemoradiotherapy (nCRT) and 217 undergoing neoadjuvant chemotherapy combined with immunotherapy (nICT). Propensity score matching ensured group comparability. We assessed pCR rates and their associations with overall survival (OS), disease-free survival (DFS), and recurrence patterns. Immune-related biomarkers were investigated through RNA sequencing and immune infiltration analysis, then validated via multiplex immunofluorescence staining.

Results Overall, pCR was associated with significantly higher DFS (HR = 0.3 [0.18–0.5], $P < 0.01$) and OS (HR = 0.19 [0.08–0.41], $P < 0.01$) compared to non-pCR. The nICT group had a lower pCR rate than the nCRT group (27.2% vs. 42.9%) but demonstrated comparable prognosis and reduced distant metastasis. Among pCR patients, DFS was significantly better in the nICT group (HR = 0.2 [0.05–0.86], $P = 0.031$), with a trend toward improved OS. Immune analysis revealed increased CD8 + T cell infiltration, particularly CD69 + CD8 + tissue-resident memory T cells (TRM), in the nICT pCR group. The proportion of CD69 + CD8 + TRM cells was significantly linked to improved DFS ($P = 0.016$) and OS ($P = 0.015$), suggesting they may be superior prognostic markers compared to pCR rates.

Conclusions The pCR obtained from different neoadjuvant treatments has distinct prognostic outcomes. The CD69 + CD8 + TRM, as a potential prognostic predictor, warrants further investigation.

Keywords Esophageal squamous cell carcinoma · Neoadjuvant · pCR · Immunotherapy · Tissue-resident memory T cell

Introduction

Esophageal carcinoma (EC) is the sixth leading cause of cancer-related deaths globally [1]. Approximately 90% of EC cases are esophageal squamous cell carcinoma (ESCC), primarily occurring in Asian nations [2]. Currently, neoadjuvant chemoradiotherapy (nCRT) combined with surgery is the standard treatment for locally advanced esophageal squamous cell carcinoma (LA-ESCC) [3]. Nevertheless, the advent of the immunotherapy era has significantly challenged the traditional nCRT approach. Neoadjuvant immunochemotherapy (nICT) has gained significant attention in clinical research due to its capacity to significantly increase the pathological complete response (pCR) rate with comparatively little side effects [4]. Multiple phase II single-arm clinical trials have been actively conducted, with reported pCR rates ranging from 20 to 50% [5–9]. Furthermore, a number of retrospective comparative studies have demonstrated that nICT is linked to improved perioperative safety, while also achieving comparable pCR rate to that of nCRT

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[10–12]. However, many of these studies are based on small-sample studies and are not supported by long-term survival data. The choice of neoadjuvant treatment regimen need more clinical data including prospective researches and real world studies.

Furthermore, present stage studies commonly use pCR as the primary endpoints and surrogate markers for survival outcomes. The utility of pCR in immunotherapy, however, is still debated. Several studies have demonstrated that achieving pCR does not closely correlate with improved survival outcomes [13, 14]. This outcome can be influenced by various factors such as tumor stage, lymph node status, and molecular subtype [15, 16]. A research comparing neoadjuvant chemotherapy to nCRT in esophageal adenocarcinoma showed that, in spite of a higher pCR rate with neoadjuvant chemoradiotherapy, patients achieving pCR with neoadjuvant chemotherapy had better Disease-free survival (DFS) [17]. This finding suggests that prognosis might vary depending on the treatment modality. We hypothesize that a similar phenomenon might also occur in ESCC populations and there is a need for more precise prognostic and predictive markers of therapeutic efficacy.

In recent years, the function of tissue-resident memory T cells (TRMs) in terms of anti-tumor immunotherapy has garnered significant attention. TRMs are a particular group of T cells that predominantly reside in tissues rather than circulating in the bloodstream. They can directly kill tumor cells, secrete cytokines to enhance immune responses and constitute the local anti-tumor immune microenvironment [18]. Additionally, TRMs stored in the tissue establish a long-term surveillance and rapid-response immune network that contribute to prevent tumor recurrence and metastasis [19]. Among them, CD8 + TRMs are considered to play a crucial role in antitumor activity [20]. Recent researches demonstrated a significant correlation between CD8 + TRM cells and improved outcomes from anti-PD-1 therapy in a various of tumors, including melanoma [21] and non-small cell lung cancer (NSCLC) [22]. TRMs are primarily characterized by high expression levels of CD103 and/or CD69, coupled with low expression levels of CCR7 and CD62L [23]. Balaji et al. [24] demonstrated that CD69 + CD103 + TRMs mediate antitumor immune responses in triple-negative breast cancer mouse model and are the key effector cells responsible for preventing tumor recurrence. Additionally, a study by Han et al. [25] reported that CD103 is a biomarker for tumor-reactive CD8 + tumor-infiltrating lymphocytes (TILs) and revealed that CD103 + CD8 + TILs can elicit potent anti-tumor immunity after anti-PD-1 blockade, independent of neoadjuvant chemotherapy. However, the function of CD8 + TRM cells in immunotherapy within human ESCC tissue need further exploration.

This multicenter retrospective research addresses a critical clinical question by directly comparing nCRT with nICT in LA-ESCC, and provides a comprehensive report on long-term survival results as well as recurrence patterns. Our team is the first to propose and elucidate pCR heterogeneity following neoadjuvant therapy of ESCC, revealing its potential underlying mechanisms. This study subsequently proposes novel immune-related biomarkers which showed relatively better prognosis prediction value with consideration to pCR heterogeneity.

Methods

Study design and population

Between March 2018 and July 2022, ESCC patients who underwent neoadjuvant treatment plus surgery were enrolled from four institutions in China. The study protocol was approved by the ethics committees of all involved institutions, with the approval number SDTHEC202410067. Since this study is retrospective in nature, patient informed consent was waived. The Declaration of Helsinki was followed in the planning and execution of the study.

Patients were eligible if they met the following criteria: (1) age ≥ 18 years, (2) histologically confirmed AJCC 8th stage II–IVa ESCC without distant metastasis, (3) underwent complete neoadjuvant treatment (nCRT or nICT) and the subsequent surgery. Exclusion criteria included: (1) had aortic and tracheal invasion, (2) lost to follow-up or (3) had severe disorders of liver, kidney, heart, or other severe diseases that affect the outcome.

Biopsy specimens taken before neoadjuvant therapy and postoperative pathological specimens were collected and analyzed using RNA sequencing (RNA-Seq) and multiplex immunofluorescence staining (mIF). Follow-up was carried out until July 2024.

Interventions

nICT

Neoadjuvant immunotherapy typically involves 1–4 cycles of a single immune checkpoint inhibitor (ICI) combined with platinum-based doublet chemotherapy, administered every 21 days. ICIs, including pembrolizumab (100–200 mg), tislelizumab (200–300 mg), camrelizumab (200 mg), sintilimab (200 mg), toripalimab (240 mg), and nivolumab (360 mg), were given intravenously on day 1. Chemotherapy was categorized into taxane-platinum (TP) and fluorouracil-platinum (PF) regimens. TP was administered on day 2 with taxane (paclitaxel: 175 mg/m^2; nab-paclitaxel: 260 mg/m^2; docetaxel: 70 mg/m^2) and platinum (cisplatin/nedaplatin:

80 mg/m^2; carboplatin: AUC = 5). PF included platinum (75–80 mg/m^2) and 5-fluorouracil (800 mg/m^2).

For patients unable to tolerate standard chemotherapy regimens, a weekly dosing schedule might be used, while keeping the total dosage equal.

nCRT

The selection of chemotherapy regimens was based on clinical guidelines and physician expertise. The most common chemotherapy regimen consisted of cisplatin (25 mg/m^2) plus paclitaxel (50 mg/m^2) administered weekly for 4 cycles. The PF regimen served as an alternative, consisting of platinum-based drugs (75–80 mg/m^2) and 5-fluorouracil (800 mg/m^2). Concurrently, three-dimensional conformal radiotherapy was initiated on the first day of chemotherapy. The total radiotherapy dose was 40 Gy, delivered in 20 fractions over 4 weeks, or 41.4 Gy, delivered in 23 fractions with 1.8 Gy per fraction.

Surgery

Surgery was scheduled within 4 weeks after neoadjuvant therapy. All patients were planned to undergo thoracoscopic surgery combined with radical resection for esophageal cancer.

Follow-up and response assessment

The disease of each patient was staged according to the 8th edition of the AJCC TNM classification for esophageal cancer. Before starting treatment, complete baseline clinical information and examination results were collected for each enrolled patient, including data on gender, age, BMI, upper gastrointestinal X-ray, endoscopy, pathology, and contrast-enhanced computed tomography (CT) findings. Clinical response was classified according to the Response Evaluation Criteria in Solid Tumors 1.1 (RECIST 1.1) as complete remission (CR), partial remission (PR), stable disease (SD), or progressive disease (PD). pCR refers to the absence of viable tumor cells in the surgical specimens, including the primary tumor site and lymph nodes. Patients received regular follow-up after surgery. In the first 2 years, upper gastrointestinal radiography, endoscopy, enhanced CT scans, and associated blood tests were conducted every 3 months. After 2 years, these follow-ups were performed every 6 months. Overall survival (OS) was defined as the duration from the initiation of treatment to death from any cause or the time of loss to follow-up. Disease-free survival (DFS) was defined as the time from the radical surgery to the first occurrence of tumor recurrence/metastasis or death from any cause.

Multiplex immunofluorescence staining (mIF)

Preoperative biopsy samples and postoperative samples from 80 patients were collected. The Opal™ 3 Color Kit (Akoya Biosciences, NEL811001KT) was used for mIF. The 4 μm-thick slides cut from the FFPE blocks were dewaxed with eco-friendly dewaxing solution. Then the tumor sections were deparaffinized, rehydrated, and stained using the following antibodies: CD69 (Abcam, ab233396), CD103 (Abcam, ab129202), CD8 (ZSGB-BIO, ZA-0508). Ultimately, the slides were stained for ten minutes using 4′-6′-diamidino-2-phenylindole (DAPI). Vectra Polaris (Akoyo Biosciences) was used to capture the images, and the Vectra Polaris imaging system (inForm 2.4.6) was used to evaluate the results. Cell counts and the percentage of positive cells (positive CD8 + T cells/total CD8 + T cells) were calculated.

RNA sequencing and data analysis

In accordance with the manufacturer's instructions, total RNA was extracted and purified by Qiagen RNeasy FFPE Kit (CAT: 73,504). RNA quantity and purity were measured with the NanoDrop ND-1000, and integrity was assessed using the Bioanalyzer 2100 (DV200 > 30% or RIN > 4.0), confirmed by denaturing agarose gel electrophoresis. The RNA libraries were constructed using the SMARTer Stranded Total RNA-Seq Kit v2 (Takara Bio USA, Mountain View, CA, USA). The cDNA libraries were sequenced on the Illumina NovaSeq 6000. Differential expression analysis was performed using DESeq2 and edgeR, with significant genes defined as having an absolute fold change ≥ 2 and false discovery rate (FDR) < 0.05. GO and KEGG enrichment analyses were conducted using GSEA (v4.1.0) and MSigDB, with significant terms/pathways showing |NES| > 1, NOM p-val < 0.05, and FDR q-val < 0.25. Tumor-infiltrating lymphocytes score was calculated by ImmuCellAI[26, 27].

TCGA data and analysis

The pan-cancer analysis using the TCGA database was conducted through the GEPIA2 web tool (http://gepia2.cancer-pku.cn/), which included the data of 33 types of cancer, such as esophageal cancer, colorectal cancer, lung adenocarcinoma, and lung squamous cell carcinoma [28]. High and low expression levels of CD69 and CD103 (also known as ITGAE) were defined based on median values.

Statistical analysis

The clinicopathological features were shown as totals and percentages, and the chi-square test or Fisher's exact test were used to test the differences between the two treatment groups. A 1:1 propensity score matching (PSM) with a 0.05 caliper was performed to balance baseline characteristics (gender, age, BMI, tumor location, clinical stage, T stage, N stage, primary tumor length, chemotherapy regimen). The Kaplan–Meier technique was used to evaluate the OS and PFS, and the log-rank test was used to investigate any differences. Differences in cell counts were determined using the Mann–Whitney U test or a paired Student's t-test with Bonferroni correction. Statistical analyses were performed, and corresponding graphs generated, using software such as SPSS 27.0 (IBM Corporation, Armonk, NY, USA), GraphPad Prism 8.0.2, and the R programming language version 4.4.0. A two-sided p-value of less than 0.05 was deemed statistically significant.

Results

Patient characteristics

Between March 2018 and July 2022, 853 patients from four centers were screened, with 445 included in the PSM analysis (Fig. 1a). Among them, 374 were male (86.0%) and the median age at the time of initial treatment was 62 years (range 42–79). The majority of patients (69.4%) had lymph node metastasis at the time of diagnosis, and 322 (74.0%) had tumors over 4.5 cm. In the nCRT group, 177 (79.7%) received radiotherapy at 41.4 Gy in 23 fractions. Among patients who received nICT, the most commonly used immunotherapy drug was camrelizumab, accounting for 70.9%, followed by sintilimab at 16.4%. The baseline characteristics are summarized in Table 1. Before PSM, the nCRT group had a higher percentage of stage II/III patients (97.3% vs. 88.3%) and the chemotherapy regimens also showed obvious difference. After matching, 191 patients were included in each group, with well-balanced baseline clinical characteristics, showing no statistically significant differences ($P > 0.05$).

Efficacy of neoadjuvant treatment

According to Table 2, Grade 3 or higher lymphopenia occurred more frequently in the nCRT group during treatment, with an incidence of 68.6%, compared to 11.51% in the nICT group. The nCRT group also showed a higher pCR rate (42.9% vs. 27.2%), a greater proportion of patients reached the ypT0 stage (50.8% vs. 28.8%), and a lower rate of positive lymph node detection (25.7% vs. 41.4%) compared to the nICT group. This suggests that nCRT might lead to better tumor regression compared to nICT, albeit with a potentially higher risk of hematologic toxicity. Despite these findings, no significant differences in DFS or OS were

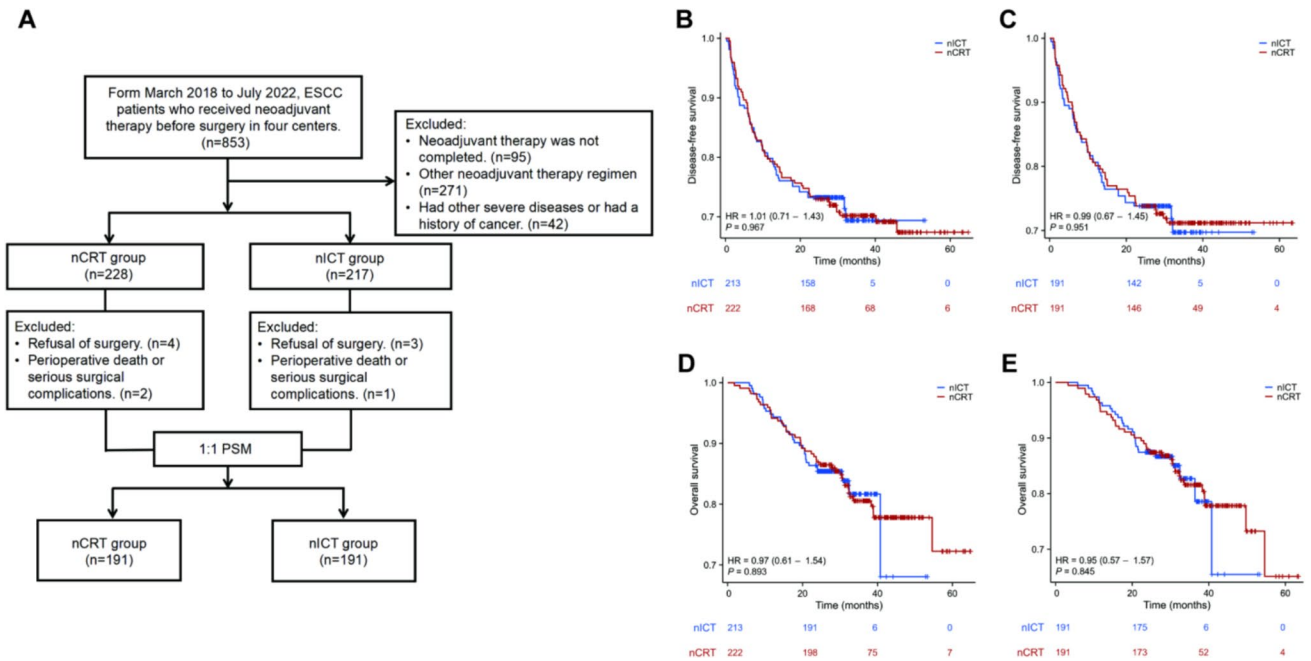

Fig. 1 The screening flowchart and the Kaplan–Meier analysis of all patients. **A** The screening flowchart; **B** disease-free survival before PSM; **C** disease-free survival after PSM; **D** overall survival before PSM; **E** overall survival after PSM

Table 1 Baseline demographic and clinical characteristics

	Before PSM				After PSM			
	nCRT (n = 222)	nICT (n = 213)	$\chi 2$	*P*	nCRT (n = 191)	nICT (n = 191)	$\chi 2$	*P*
Gender								
Male	189 (85.1)	185 (86.9)	0.267	0.606	166 (86.9)	167 (87.4)	0.023	0.878
Female	33 (14.9)	28 (13.1)			25 (13.1)	24 (12.6)		
Age (years)								
< 62	105 (47.3)	100 (46.9)	0.005	0.942	90 (47.1)	95 (49.7)	0.262	0.609
≥ 62	117 (52.7)	113 (54.0)			101 (52.9)	96 (50.3)		
BMI (kg/m^2)								
< 21	75 (33.8)	78 (36.6)	0.383	0.536	60 (31.4)	62 (32.5)	0.048	0.826
≥ 21	147 (66.2)	135 (63.4)			131 (68.6)	129 (67.5)		
Tumor location								
Up	31 (14.0)	35 (16.4)	1.871	0.392	28 (14.7)	34 (17.8)	3.820	0.148
Middle	65 (29.3)	71 (33.3)			50 (26.2)	63 (33.0)		
Down	126 (56.8)	107 (50.2)			113 (59.2)	94 (49.2)		
Clinical stage								
II	73 (32.9)	51 (23.9)	15.497	< 0.001	56 (29.3)	51 (26.7)	0.329	0.848
III	143 (64.4)	137 (64.3)			129 (67.5)	134 (70.2)		
IVA	6 (2.7)	25 (11.7)			6 (3.1)	6 (3.1)		
T stage								
2	10 (4.5)	12 (5.6)	0.450	0.799	7 (3.7)	12 (6.3)	3.149	0.220
3	203 (91.4)	194 (91.1)			175 (91.6)	175 (91.6)		
4	9 (4.1)	7 (3.3)			9 (4.7)	4 (2.1)		
N stage								
N0	77 (34.7)	56 (26.3)	3.608	0.058	60 (31.4)	55 (28.8)	0.311	0.577
N+	145 (65.3)	157 (73.7)			131 (68.6)	136 (71.2)		
Primary tumor length (cm)								
< 4.5	67 (30.2)	66 (31.0)	0.596	0.440	43 (22.5)	46 (24.1)	0.132	0.717
≥ 4.5	175 (78.8)	147 (69.0)			148 (77.5)	145 (75.9)		
Chemotherapy regimen								
TP	189 (85.1)	197 (92.5)	5.88	0.015	178 (93.2)	178 (93.2)	0.000	1.000
PF	33 (14.9)	16 (7.5)			13 (6.9)	13 (6.9)		
Radiotherapy regimen								
40 Gy/20 f	22 (9.9)	–	–	–	26 (13.6)	–		
41.4 Gy/23 f	177 (79.7)	–			151 (79.1)	–		
> 45 Gy	16 (7.2)	–			14 (7.3)	–		
Immunotherapy drugs								
Camrelizumab	–	151 (70.9)	–	–	–	139 (72.8)		
Pembrolizumab	–	11 (5.2)			–	8 (4.2)		
Toripalimab	–	6 (2.8)			–	6 (3.1)		
Sintilimab	–	35 (16.4)			–	30 (15.7)		
Tislelizumab	–	10 (4.7)			–	8 (4.2)		

nICT neoadjuvant immunochemotherapy; *nCRT* neoadjuvant chemoradiotherapy; *PSM* propensity score matching; *TP* taxanes combined with platinum-based drug regimen; *PF* fluorouracil combined with platinum-based drug regimen; *BMI* body mass index

observed between the groups, either before or after PSM (Fig. 1b–e), indicating that a higher pCR rate did not translate to survival benefits.

Differences were observed between the two groups in recurrence patterns, with a slightly higher 2-year recurrence rate (28.3% vs. 27.7%) and distant metastasis (11.5% vs. 5.2%) in the nCRT group (Supplementary Table 1).

Table 2 Patient demographic and clinical characteristics during and after neoadjuvant therapy

	Before PSM				After PSM			
	nCRT (n = 222)	nICT (n = 213)	χ2	*P*	nCRT (n = 191)	nICT (n = 191)	χ2	*P*
Grade of lymphopenia during neoadjuvant therapy								
0	5 (22.5)	65 (26.3)	157.730	< 0.001	2 (1.0)	58 (30.4)	178.535	< 0.001
1	21 (9.5)	70 (32.9)			18 (9.4)	67 (35.1)		
2	48 (21.6)	49 (23.0)			40 (20.9)	44 (23.0)		
3	115 (51.8)	23 (10.8)			99 (51.8)	18 (9.4)		
4	33 (14.9)	6 (2.8)			32 (16.8)	4 (2.1)		
ypT stage								
0	113 (50.9)	60 (28.2)	28.785	< 0.001	97 (50.8)	54 (28.3)	25.367	< 0.001
1	33 (14.9)	28 (13.1)			30 (15.7)	26 (13.6)		
2	25 (11.3)	38 (17.8)			20 (10.5)	32 (16.8)		
3	47 (21.2)	81 (38.0)			41 (21.5)	73 (38.2)		
4	4 (1.8)	6 (2.8)			3 (1.6)	6 (3.1)		
ypN stage								
0	167 (75.2)	122 (57.3)	20.529	< 0.001	142 (74.3)	112 (58.6)	14.605	0.002
1	41 (18.5)	51 (23.9)			36 (18.8)	44 (23.0)		
2	11 (5.0)	30 (14.1)			11 (5.8)	27 (14.1)		
3	3 (1.4)	10 (4.7)			2 (1.0)	8 (4.2)		
TRG								
0	113 (50.9)	59 (27.7)	37.028	< 0.001	97 (50.8)	55 (28.8)	28.914	< 0.001
1	43 (19.4)	34 (16.0)			35 (18.3)	30 (15.7)		
2	46 (46.7)	66 (31.0)			42 (22.0)	59 (30.9)		
3	20 (9.0)	54 (25.4)			17 (8.9)	47 (24.6)		
pCR								
yes	98 (44.1)	57 (26.8)	14.322	< 0.001	82 (42.9)	52 (27.2)	10.345	0.001
no	124 (55.9)	156 (73.2)			109 (57.1)	139 (72.8)		

nICT neoadjuvant immunochemotherapy; *nCRT* neoadjuvant chemoradiotherapy; *PSM* propensity score matching; *ypT* postoperative pathological stage T; *ypN* postoperative pathological stage N; *TRG* tumor regression grade; *pCR* pathological complete response

pCR is heterogeneous and patients who achieve pCR through nICT benefit more

A subgroup analysis was then conducted to explore the impact of various clinical characteristics on prognosis. As shown in Fig. 2a, none of these clinical parameters had a significant impact on OS. In the DFS analysis (Fig. 2b), the pCR subgroup had a significance (p for interaction = 0.034), indicating that nICT is more beneficial for prognosis when pCR is achieved (nICT vs. nCRT alone, HR = 0.20 [0.05–0.87], $P = 0.031$). Other characteristics showed no predictive value.

Details of the survival outcomes for the pCR group were calculated and are shown in Fig. 3. As shown in Fig. 3a–d, for the overall population, patients who achieved pCR had significantly higher DFS (HR = 0.3 [0.18–0.5], $P < 0.01$) and OS (HR = 0.19 [0.08–0.41], $P < 0.01$) compared to those who did not. For patients who achieved pCR, DFS in the nICT group was significantly better than that in the nCRT group (HR = 0.2 [0.05–0.86], $P = 0.031$; Fig. 3e, f). Although a trend toward improved OS was observed in the nICT group compared to the nCRT group, it did not reach statistical significance (HR = 0.36 [0.04–3.1], $P = 0.356$; Fig. 3g, h). However, for patients who did not achieve pCR, no differences in DFS or OS were observed between the two groups (Fig. 3i–l). This result suggests that pCR is heterogeneous, which we defined as superior DFS in patients with nICT-pCR over nCRT-pCR.

Recurrence patterns were also analyzed in patients with pCR and non-pCR. Both groups primarily exhibited locoregional recurrence or metastasis, with no significant differences observed between them (Supplementary Table 2).

CD8 + TRM-like cells might contribute to the heterogeneity of pCR

To investigate the mechanisms underlying gene changes associated with prognosis and treatment at the transcriptome level, as well as to explore the heterogeneity of pCR,

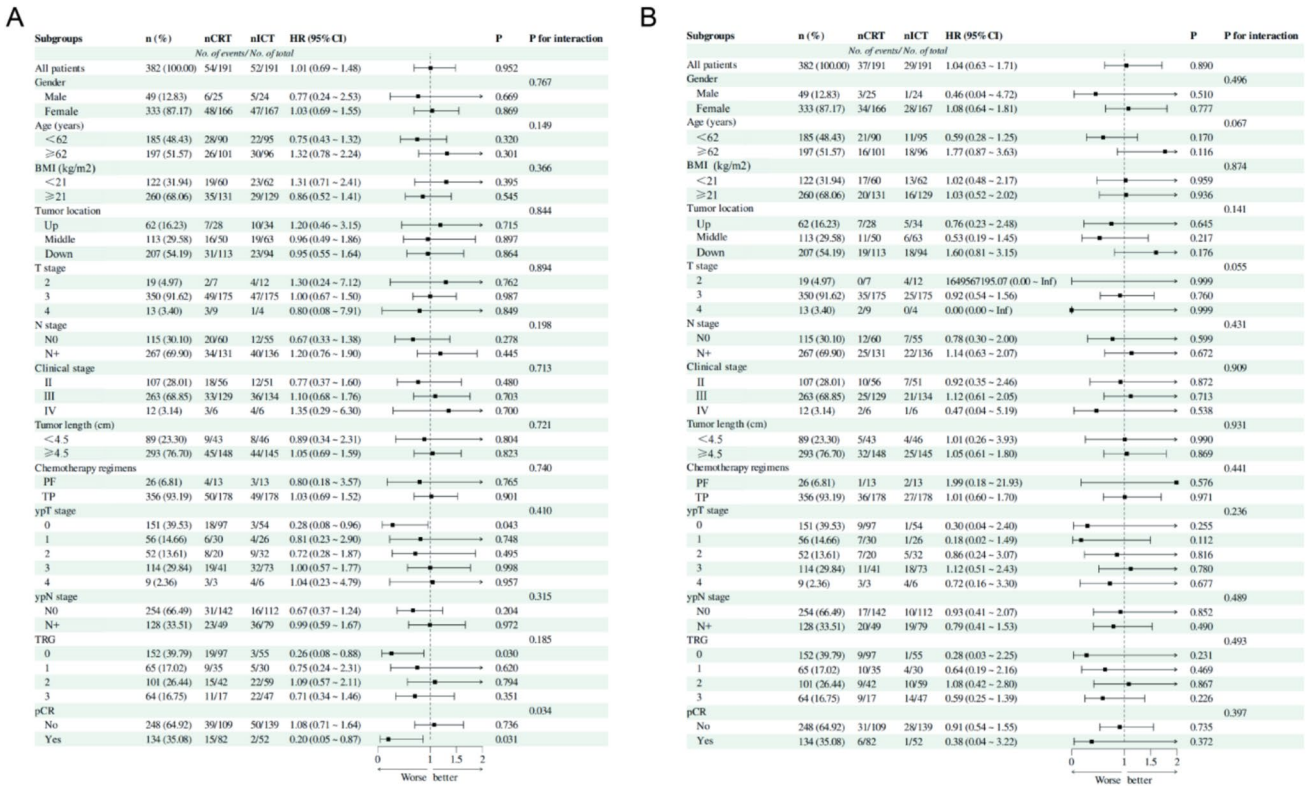

Fig. 2 Subgroup analysis of disease-free survival and overall survival. **A** Disease-free survival; **B** overall survival

we performed RNA sequencing on postoperative samples from 8 pCR patients in the nICT group and 8 pCR patients in the nCRT group. Given that no tumor cells were observed in the tissue of patients with pathological complete response after surgery, which was instead characterized by extensive infiltration of immune cells, we first conducted an immune cell infiltration analysis using ImmuCellAI. The results indicated a significant increase in the infiltration of CD8 + T cells, neutrophils, and cytotoxic T cells (Tc) in the nICT group (Fig. 4a). Subsequently,We compared gene expression between the nICT and nCRT groups, identifying 57 upregulated and 11 downregulated genes (Fig. 4b, c). The heatmap illustrates the elevated expression of the CD69 gene and several T cell co-inhibitory molecules like CD27 and CD28 in the nICT group (Fig. 4d). Additionally, both KEGG and GO enrichment analyses revealed the enrichment of multiple immune pathways, including those related to T cells (Fig. 4e, f). These results collectively indicate that the survival difference between pCR patients in the nICT group and the nCRT group is likely closely related to CD8 + T cells.

Given that CD69 is a classic marker for tissue-resident memory (TRM) cells, with CD103 (also known as ITGAE) being another well-established marker, we focused on a specific subset known as CD8 + TRM cells. To investigate the utility of CD69 and CD103 as biomarkers of anti-tumor T cell response in pan-cancer, we analyzed the expression of CD103 (ITGAE) and CD69 mRNA in The Cancer Genome Atlas (TCGA) pan-cancer dataset. The results showed that high expression of CD69 is associated with better DFS ($P < 0.001$), while CD103 did not reach statistical significance (Fig. 4g, h). The results from public databases further confirmed that upregulation of CD69 gene expression is beneficial for malignant tumor patients in achieving longer DFS.

The CD69 + CD8 + TRM cell subset was higher in nICT-pCR patients than nCRT-pCR pathents after surgery

To validate our hypothesis, we conducted mIF staining analysis on postoperative pathological sections from 80 patients. The results demonstrated that pCR patients exhibited greater CD8 + T cell infiltration (shown as the green fluorescence) postoperatively compared to non-pCR patients (Fig. 5a–d), regardless of treatment modality. The triple-positive cells of CD69, CD103, and CD8 indicated by the white arrows also show the same trend (Fig. 5a–d). Figure 5e–h showed the proportions of four CD8 + TRM cell subsets—CD69 + CD103+, CD69 + CD103−, CD69-CD103+, and CD69-CD103− —across different treatment groups in both pCR and non-pCR patients. The findings revealed a similar trend for the first two subsets, with a higher proportion observed in pCR patients compared to non-pCR patients of

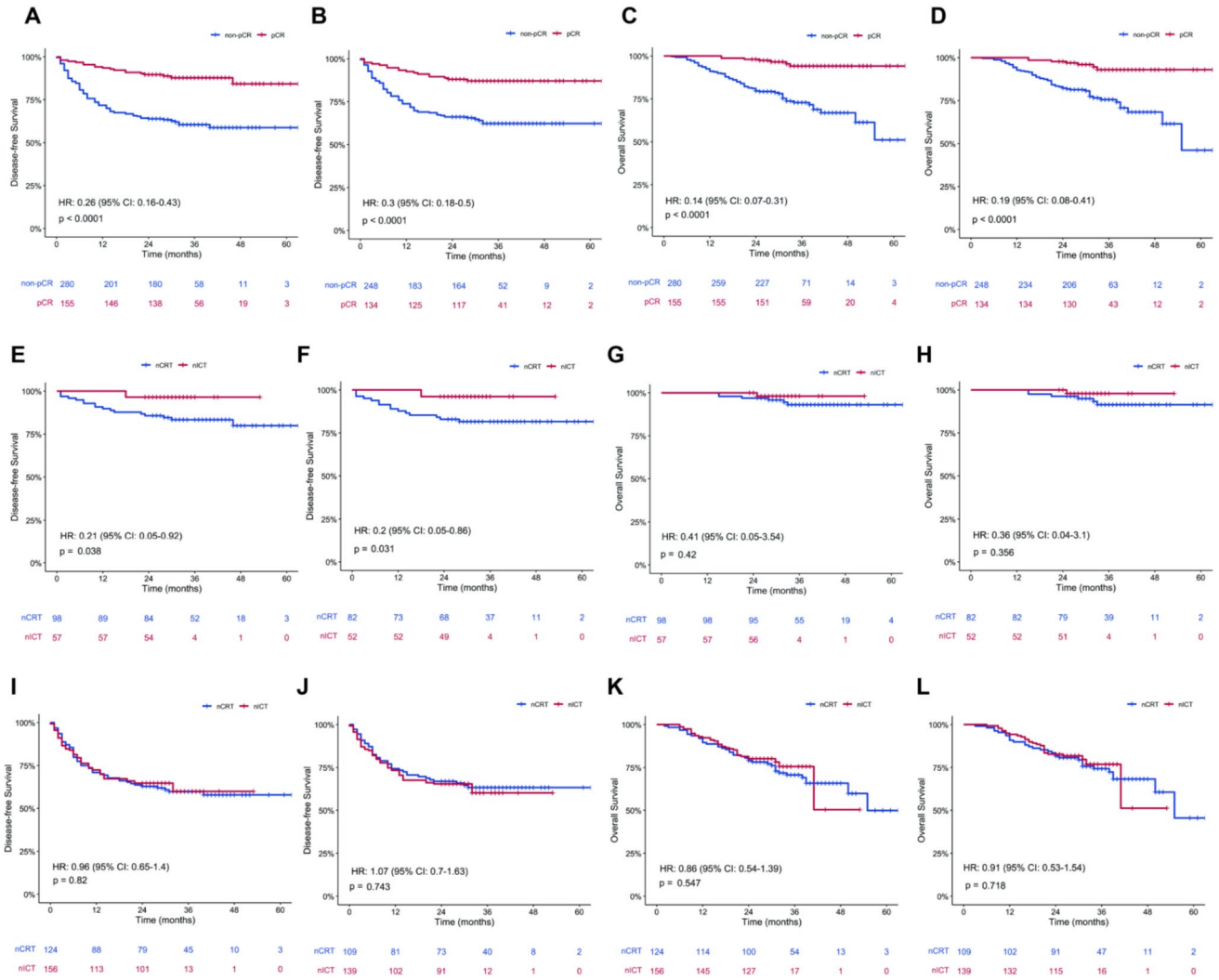

Fig. 3 The Kaplan–Meier analysis of disease-free survival and overall survival in different subgroups. **A** Disease-free survival of pCR and non-pCR patients before PSM; **B** disease-free survival of pCR and non-pCR patients after PSM; **C** overall survival of pCR and non-pCR patients before PSM; **D** overall survival of pCR and non-pCR patients after PSM; **E** disease-free survival between nICT and nCRT group in pCR patiens before PSM; **F** disease-free survival between nICT and nCRT group in pCR patiens after PSM; **G** overall survival between nICT and nCRT group in pCR patiens before PSM; **H** overall survival between nICT and nCRT group in pCR patiens after PSM; **I** disease-free survival between nICT and nCRT group in non-pCR patiens before PSM; **J** disease-free survival between nICT and nCRT group in non-pCR patiens after PSM; **K** overall survival between nICT and nCRT group in non-pCR patiens before PSM; **L** overall survival between nICT and nCRT group in non-pCR patiens after PSM

both the nICT and nCRT groups. Additionally, the proportion of the CD69 + CD103− CD8 + TRM subset was higher in pCR patients in the nICT group compared to those in the nCRT group. Furthermore, the combined analysis of CD69 + CD8 + T cells, regardless of CD103 expression, revealed a significantly higher infiltration levels in nICT-pCR patients than nCRT-pCR pathents (Fig. 5j).

The CD69 + CD8 + TRM cell subset might serve as a novel prognostic factor for patient survival

In the subsequent analysis, we examined preoperative paired samples from these patients. The results showed that in patients who achieved pCR, the proportions of the initial CD69 + CD103+, CD69 + CD103−, and CD69-CD103 + CD8 + TRM cell subsets in pretreatment biopsy samples were higher compared to those who did not achieve pCR (Fig. 6c–f). A combined analysis revealed that the CD69 + CD8 + TRM subset also exhibited a

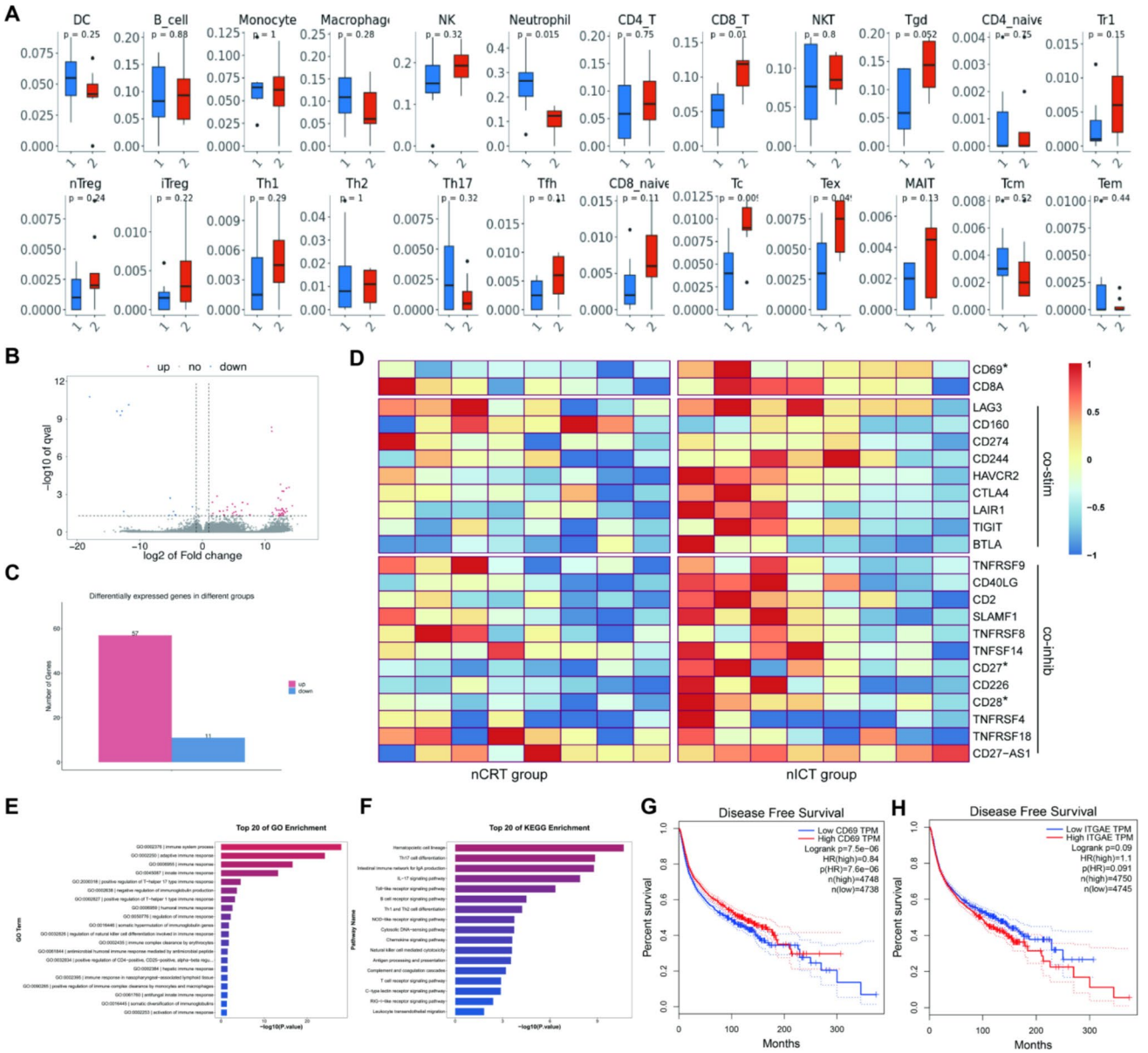

Fig. 4 RNA sequencing analysis between pCR patients in nICT and nCRT group. **A** Immunoinfiltration analysis: the blue box plot represents the nCRT group, and the red box represents the nICT group; **B**, **C** up-regulated and down-regulated differential genes; **D** heat map of co-stimulatory and co-inhibitory genes of T cell; **E** GO enrichment anlysis; **F** KEGG enrichment anlysis; **G** effect of CD69 on disease free survival in pan-cancer; **H** effect of CD103 on disease free survival in pan-cancer

similar trend, with a higher proportion in preoperative samples of patients who later achieved complete remission following treatment, while the CD103 + CD8 + TRM subset did not show significant differences (Fig. 6g–h). When comparing the patients who achieved pCR with different treatments, no statistical differences were observed in any of the subsets. A paired comparison between preoperative and postoperative samples was subsequently conducted. For patients who achieved pCR after surgery, the proportion of CD69 + CD8 + TRM cells increased significantly following nICT and nCRT (Fig. 6a–b, i–k). We used the median as the cutoff value to categorize pretreatment samples of CD69 + CD8 + TRM into "high" and "low" groups. Kaplan–Meier analysis showed that the group with a higher proportion of CD69 + CD8 + TRM

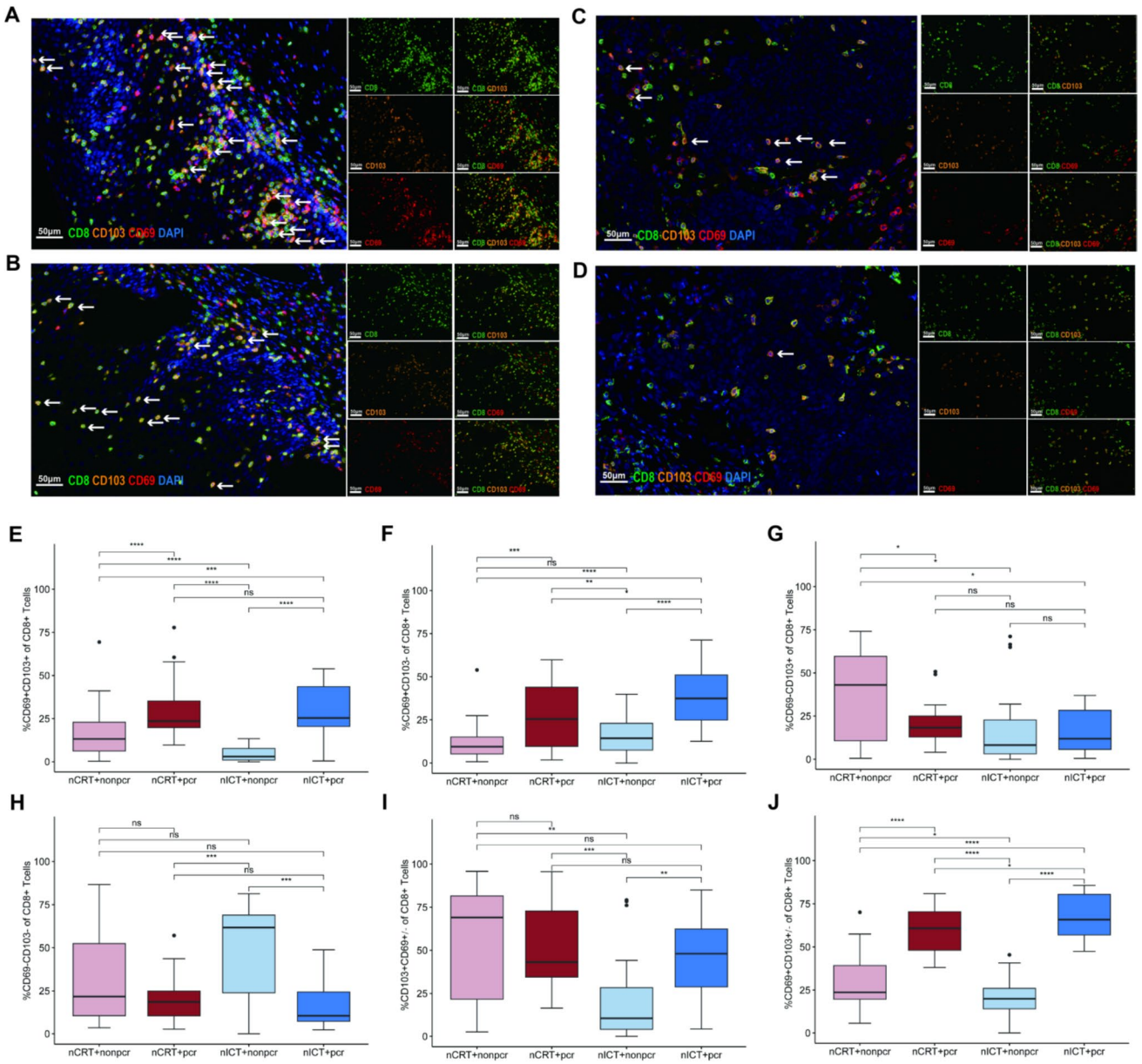

Fig. 5 mIF staining of postoperative biopsies. Cells indicated by white arrows are CD8, CD103, and CD69 triple-positive cells. **A** mIF staining of pCR patients in nICT group; **B** mIF staining of pCR patients in nCRT group; **C** mIF staining of non-pCR patients in nICT group; **D** mIF staining of non-pCR patients in nCRT group; an analysis of the proportions of four subsets of CD8+TRM cell: CD69+CD103+ (**E**), CD69+CD103− (**F**), CD69-CD103+ (**G**), CD69-CD103− (**H**), as well as their combined types CD103+CD8+ (**I**) and CD69+CD8+ (**J**) TRM cells

cells had better DFS ($p = 0.016$) and OS ($p = 0.015$) (Fig. 6l, m).

Discussion

Currently, nCRT plus surgery is the standard therapy of LA-ESCC. However, with the advent of the immunotherapy era, nCRT is increasingly challenged by immunotherapy, leading to ongoing debate on the optimal neoadjuvant treatment strategy. Currently, there is a lack of large-scale head-to-head clinical trials between nICT and nCRT to provide clear guidance. Our retrospective study, which is one of the largest known studies in this context, included 445 patients from four major clinical centers in China and directly compared two treatment modalities, nICT and nCRT. The results indicated that although nCRT showed a higher pCR rate compared to nICT (42.9% vs. 27.2%), there

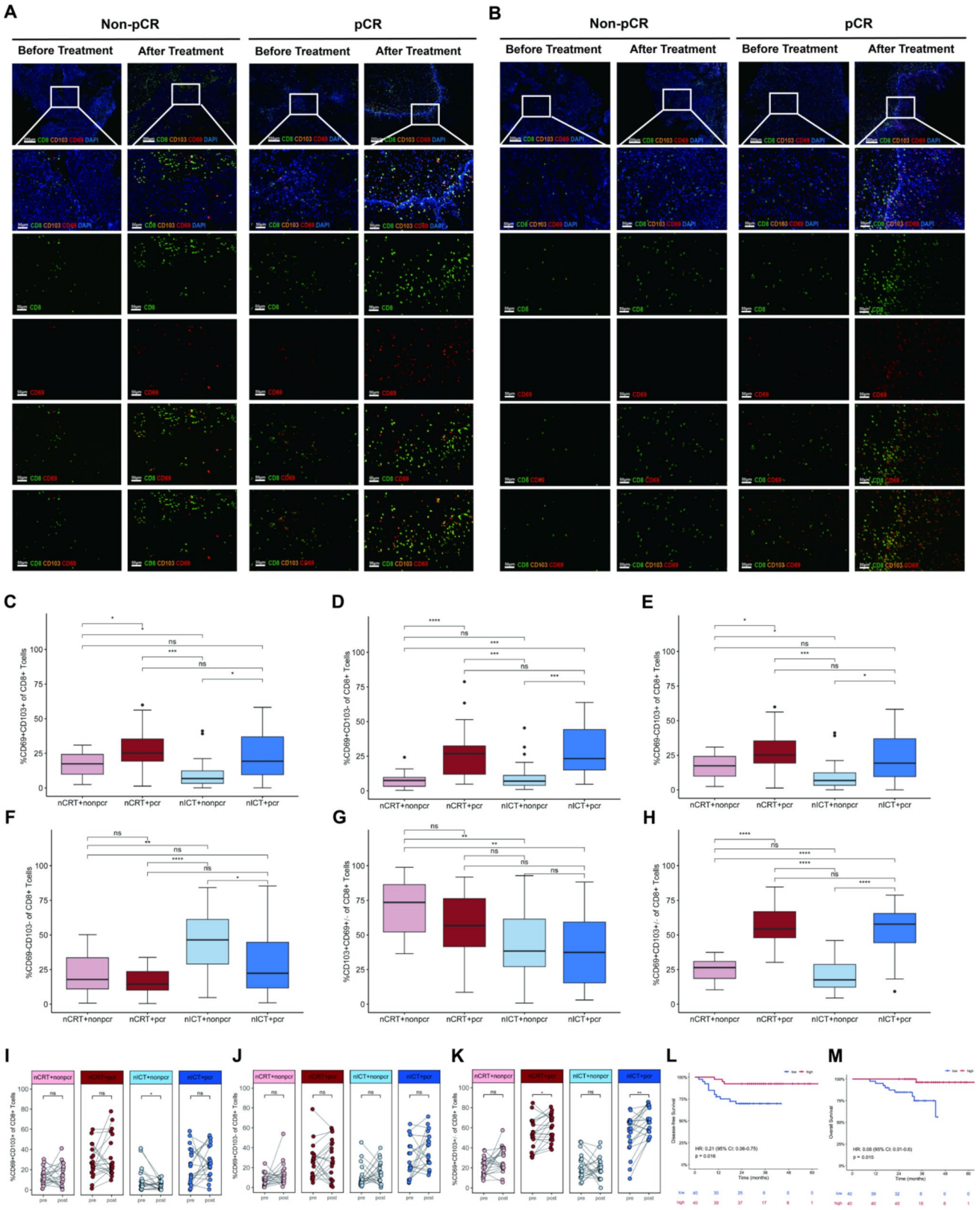

Fig. 6 mIF staining analysis of preoperative paired samples. mIF staining of biopsies in nICT group (**A**) and nCRT group (**B**); an analysis of four subsets of CD8+TRM cell: CD69+CD103+ (**C**), CD69+CD103− (**D**), CD69-CD103+ (**E**), CD69-CD103− (**F**), and their combined types CD103+CD8+ (**G**) and CD69+CD8+ (**H**) TRM cells in preoperative samples; changes of CD69+CD103+ (**I**), CD69+CD103− (**J**) and CD69+CD8+ (**K**) TRM before and after treatment; **K**–**M** curves of DFS (**L**) and OS (**M**)

were no significant differences in DFS and OS between the two approaches. nICT also showed better control of distant metastases without increasing locoregional recurrence within 2 years after surgery. Additionally, we observed heterogeneity in pCR outcomes, where patients in the nICT group who achieved pCR demonstrated better survival, highlighting potential advantages of immunotherapy in the current era. Finally, we proposed CD69 + CD8 + TRM as a prognostic predictor for neoadjuvant treatment in ESCC, and that this subpopulation may contribute to the survival difference of pCR patients between nICT and nCRT group.

Neoadjuvant chemoradiotherapy, as a standard treatment regimen, has demonstrated high complete response rates and sustained survival benefits in multiple clinical trials. The CROSS [29] study, a multicenter randomized controlled trial that established the standard of nCRT, reported a pCR rate of 49% in ESCC cases. The R0 resection rate increased from 69 to 92%, and the 5-year survival rate improved from 33 to 47%, with a 37% reduction in mortality risk. The multicenter NEOCRTEC5010 study [3] conducted in China replicated the findings of the CROSS study: the pCR reached 43%, and the R0 resection rate increased from 91.2 to 98.4%. The 5-year survival rate improved from 49 to 60%, with a 27% reduction in mortality risk. Although nICT has been in use for a shorter period, it has become a focus of clinical research due to its favorable pathological outcomes and high safety profile. Liu et al. conducted a multicenter, phase II research to assess the safety and effectiveness of camrelizumab plus chemotherapy as neoadjuvant treatment for LA-ESCC. Of the patients, 50 (98.0%) had R0 resection, and 20 (39.2%) had pCR [5]. Yang, W., et al. reported a 100% R0 resection rate, a 25% pCR rate, and a 50% MPR rate [30]. A meta-analysis with 24 phase II clinical trials included showed a 49.4% MPR rate and a pooled pCR rate of 32.4% [31]. Currently, head-to-head comparative studies between nICT and nCRT are relatively rare. A retrospective cohort research conducted by Yang et al. compared the postoperative outcomes and prognoses of these two treatment modalities, revealing that the pCR rates between the nICT and nCRT groups were similar (20.2% vs. 19.1%). The PFS was better in the nICT group, while there was no statistically significant difference in OS [32]. There are discrepancies between our study data and previously published retrospective findings, specifically in that the pCR rate of nICT is lower than that of nCRT (27.2% vs. 42.9%). Additionally, there is no difference in DFS and OS between the two groups.

Although our study did not observe an advantage of nICT over nCRT in terms of pCR rate and survival in the overall population, we identified its potential as a neoadjuvant treatment of LA-ESCC. First, our research indicates that nICT offers better control of systemic tumors, with a lower rate of distant metastasis within two years post-surgery. This finding is in line with the study by Xiao et al. [12] and Yang et al. [32] who reported that he nICT group had a lower recurrence rate at one year postoperatively compared to the nCRT group, with fewer patients developing distant metastasis. Second, our study shows that the incidence of grade three or higher lymphopenia during treatment was significantly lower in the nICT group at only 11.51%, compared to 68.6% in the nCRT group. Therefore, we hypothesize that immune checkpoint blockade therapy might prevent the formation of distant metastases by inducing both local and systemic immune responses and reducing damage to circulating immune cells. Additionally, it might result in fewer adverse effects compared to nCRT.

Given the general lack of long-term survival results, pCR is currently the primary surrogate endpoint and prognostic predictor in clinical research; however, there is still some controversy surrounding its use [16]. Our study indicates that an increased pCR rate does not necessarily translate into prolonged DFS and OS, as pCR exhibits heterogeneity depending on the therapeutic approach employed. Patients who achieved pCR with nICT had better DFS compared to those who achieved pCR with nCRT. Although the trend in OS did not reach statistical significance, this might be explained by limited follow-up time and a small number of endpoint events. Therefore, relying solely on the pCR rate as a prognostic marker is insufficiently accurate. More precise markers are needed to predict pathological outcomes and prognosis.

Multiple studies on human solid tumors have demonstrated the presence of TRMs within tumor immune infiltrates and elucidated their mechanisms of antitumor action. TRMs have been shown to directly mediate antitumor immunity by producing effector molecules and cytolytic mediators such as perforin and granzyme B (GzmB) [33, 34]. Additionally, TRMs possess immunostimulatory functions. Effector cytokines derived from TRMs activate local dendritic cells (DCs), natural killer (NK) cells, and T cells, thereby improving the antitumor immune response [35]. Moreover, compared to circulating memory T cells, TRMs exhibit a faster response upon antigen re-exposure [20]. Recent mouse infection models have suggested that pulmonary TRMs have the ability to move to draining lymph nodes (LNs), where they amplify immunological activity in neighboring LNs to improve local immune responses [36]. Collectively, these multifunctional capabilities make TRMs the pefect effector T cells for immunological responses against tumors. Moreover, in various tumors such as endometrial adenocarcinoma [37] and NSCLC [22], this subset of cells has been positively correlated with favorable outcomes following immunotherapy. Most studies have emphasized the close association between the CD103 + CD8 + TRM subset and patient survival, particularly in epithelial-derived cancers such as colorectal

cancer [38], ovarian cancer [39], and bladder cancer [40]. However, there are fewer reports on CD69 + CD8 + TRMs. A study by Balaji Virassamy et al. [24] demonstrated the presence of resident-like CD8 + T cells in the tumor microenvironment of both mice and patients with triple-negative breast cancer. These cells were categorized into two groups: CD69 + CD103− and CD69 + CD103 + . The CD69 + CD103− phenotype exhibited characteristics of exhaustion, whereas CD69 + CD103 + displayed a resident phenotype. Although both subsets possess antitumor responses, the latter demonstrated stronger antitumor activity, and was identified as the primary effector cell in suppressing in situ tumor recurrence. However, this is not static; under checkpoint inhibitor therapy or local inflammatory signals, including TGF-β, CD69 + CD103− T cells in the breast tumor microenvironment might also differentiate into CD69 + CD103 + T cells, thereby enhancing antitumor effects. Additionally, progenitor exhausted T cells (TPEX) have also been shown to proliferate in tumor-draining lymph nodes under PD-1 blockade [41]. This is consistent with our findings, where we observed that patients who achieved pCR exhibited higher proportions of both CD69 + CD103− and CD69 + CD103 + CD8 + TRM subpopulations, as well as their combined total, both preoperatively and postoperatively. However, the paired comparison only revealed an increase in the combined total, that is, the proportion of CD69 + CD8 + TRM cells postoperatively compared to preoperatively. We then stratified patients into high and low groups based on the median preoperative CD69 + CD8 + TRM proportion, and the Kaplan–Meier curves demonstrated that the high group had better DFS and OS outcomes. Based on the above, immunotherapy might sinificantly inhibit tumor recurrence and metastasis by increasing the infiltration of CD69 + CD8 + TRM cells in lesions and metastatic lymph nodes, thereby establishing sustained immune surveillance. This effect was often observed in patients who respond well to immunotherapy. This mechanism might underlie the better prognosis observed in patients who achieve pCR in the nICT group. Furthermore, the proportion of CD69 + CD8 + TRM cells might be a potential prognostic predictor.

Our study has several limitations. First, the inherent nature of a retrospective study might introduce unavoidable biases. To minimize these, we employed PSM to balance differences between the two groups as much as possible. Nevertheless, due to specific limitations, we used bulk RNA sequencing instead of single-cell sequencing to investigate pCR heterogeneity. However, this approach lacks precision. Future studies should utilize single-cell sequencing for more accurate interpretation. Second, our study did not provide a comprehensive comparison of the side effects associated with the two treatment modalities, which is also an important consideration in clinical decision-making. Future studies should aim to fill this gap. In addition, the follow-up duration was 24–63 months in this study. While a similar trend was noted between OS and DFS, the results did not reach statistical significance. This might be attributed to the relatively short follow-up period. Thus, we plan to extend the follow-up duration and conduct further studies. Lastly, our study primarily examined the prognostic differences among pCR patients between nCRT and nICT group. However, we did not investigate more specific prognostic predictors for pCR patients receiving the same treatment approach, which requires further exploration in future studies.

Conclusions

In summary, our multicenter retrospective study proposed the pCR heterogeneity in neoadjuvant therapy for ESCC: achieving a pCR with nICT provides greater survival benefits than pCR obtained through nCRT. Furthermore, the proportion of CD69 + CD8 + TRM cells might be a potential mechanism mediating pCR heterogeneity and holds promise as a novel prognostic predictor. The advent of the immunotherapy era has posed significant challenges to the traditional nCRT approach while offering new opportunities for patients with LA-ESCC.

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Author contributions Ao Liu and Defeng Liu contributed equally to this work, including study design, data collection, mIHC staining, statistical analysis, and manuscript writing. Xiuli Liu was involved in mIHC staining. Yuxiang Chi, Guiwen Zheng, Haiqun Lin , Qiuan Yang were responsible for data collection form different centers. Longxiang Guo, Dianxing Li, Qiankun Wang, Yuanlin Li, Yi Li, Yaru Tian performed proofreading. Minghuan Li and Jinming Yu made critical revisions to the manuscript for important intellectual content. All authors read and approved the final manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

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

Ethical approval and consent to participate The study protocol was approved by the ethics committees of all participating institutions, with the ethical approval number SDTHEC202410067. Informed consent

from patients was waived. The study was conducted according to the Declaration of Helsinki.

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