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RESEARCH ARTICLE

High Expression of C-C Chemokine Receptor Type 5 in Urothelial Carcinoma When Compared to Other Common Malignancies Based on a Novel, Quantitative Immunohistochemistry Assay and Scoring System from FFPE Tissue not Requiring Antigen Retrieval

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ABSTRACT

Purpose: To compare C-C chemokine receptor type 5 (CCR5) expression in urothelial carcinoma tissue to other common malignancies using a specific CCR5 inhibitor-based assay.

Methods: We used an immunohistochemistry (IHC) assay to compare the expression of CCR5 in urothelial carcinoma to different types of common cancer. The IHC assay was based on labeled PRO140 (PRO140 CCR5 Hu IgG4a), a CCR5 inhibitor with potential therapeutic uses. The expression levels were compared using Percent Scores and H-Score methods.

Results: Quantification of CCR5 expression utilized a panel of 63 evaluable samples from 9 cancer indications as follows: 6 UC, 7 non-small cell lung (NSCL) adenocarcinoma, 9 NSCL squamous cell carcinoma, 5 triple negative breast cancer, 5 breast cancer, 7 pancreatic cancer, 9 colorectal cancer, 7 head, and neck cancer, and 8 sarcoma. Overall, most of the 63 cases evaluated tended to have either H-Scores >175 (highly reactive) (24/63) or <100 (low or non-reactive) (32/63). Urothelial cancer had the highest expression (H score=183.3), while sarcoma exhibited the most moderate expression of CCR5 (H score =23.6).

Conclusions: We show, for the first time, a high expression of CCR5 in the tissue of urothelial carcinoma using an assay based on a safe and effective receptor inhibitor. Our findings may have therapeutic implications if validated in more extensive studies.

Introduction

Systemic inflammation is associated with reduced survival in patients with urothelial carcinoma (UC)[1]. It is not clear how an inflammatory response would impact survival from cancer, but it is known that the release of proinflammatory cytokines and growth factors, not only stimulates tumor growth but also produces profound catabolic effects on host metabolism[1]. For example, interleukin 6, produced by the tumor or infiltrating inflammatory cells, is a growth promoter in UC[2]. Interleukin-6 also stimulates liver production of C-reactive protein, which increases the demand for certain amino acids in the body, which if not available in diet, is extracted from muscles leading to a functional decline and ultimately worse overall survival[3]. Patients with metastatic UC and high baseline inflammation had reduced responses to immune checkpoint inhibitors and chemotherapy. Yuen et al. analyzed circulating pIL-8 and IL8 gene expression in peripheral blood mononuclear cells and tumors of patients treated with atezolizumab (anti-PD-L1 mono-clonal antibody) from multiple randomized trials representing 1,445 patients with metastatic UC and metastatic renal cell carcinoma. High levels of IL-8 in plasma, peripheral blood mononuclear cells, and tumors were associated with decreased efficacy of atezolizumab in patients with UC, even in tumors that were classically CD8(+) T cell inflamed. Low baseline pIL-8 in patients with UC was associated with increased response to atezolizumab and chemotherapy. Yuen et al. then concluded that therapies that can reverse the impacts of IL-8-mediated myeloid inflammation will be essential for improving outcomes of patients treated with immune checkpoint inhibitors[4].

Several chemokines and their receptors induce the inflammatory response and immune-cell infiltration of malignant tumors[5]. Chemokines are a family of low molecular weight proteins (8–11 kDa) playing a significant role in regulating leukocyte adherence to endothelial cells, leukocyte migration through transendothelial membranes, tissue invasion, and metastasis[6,7]. Many tumor cell types can express chemokines and chemokine receptors, and high levels of C-C chemokine 5 and C-C chemokine receptor 5 (CCR5) expression have been demonstrated in human cancer tissues[8]. For various cancers, such as hepatocellular carcinoma, the CCR5/CCL5 interaction promoted migration and metastasis and data demonstrating the biological and clinical significance of the CCR5/CCL5 axis suggested that targeting of this axis is a promising avenue for the treatment of this disease[9].

In this study, we examined the expression of CCR5 in samples of UC labeled PRO140 (PRO140 CCR5 Hu IgG4a), a CCR5 inhibitor with potential therapeutic uses. We compared it to the levels of expression of this receptor in other common malignancies using a quantitative immunohistochemistry assay and scoring system that can be performed on formalin-fixed paraffin-embedded tissue (FFPE) without the use of antigen retrieval.

Methods

STAINING PROCEDURE

Immunohistochemical (IHC) staining for human CCR5 used the primary antibody PRO140 CCR5 Hu IgG4a to detect CCR5 in FFPE human tissues (Table 1). In brief, FFPE tissue blocks were cut at 4-5 µm thickness, and sections were mounted onto positively-charged, capillary gap glass slides. Slides were baked (60°C, dry heat) for at least 1 hour and 12 hours before use. Tissue sections were then de-waxed using organic solvents (xylene, 100%, four changes) and an alcohol series (100%, 70%, 30% ethanol) descending to distilled water to sufficiently hydrate the tissues and allow proper binding of the primary antibody and other detection reagents. Tissue sections were subsequently pre-treated with steam heat-induced epitope recovery (SHIER) or enzymatic digestion for antigen retrieval. No tissue pre-treatment conditions were referred to as NO SHIER. Samples were then tested by IHC using the TechMate instrumentation platform. Briefly, the detection of the primary antibody utilized biotinylated-mouse anti-human IgG4 Fc secondary antibody labeled with alkaline phosphatase (AP) and using ImmPRESS® Alkaline Phosphatase detection with HIGHDEF® blue chromogen, nuclei were counterstained using hematoxylin (blue stain) to assess cell and tissue morphology.

Table 1: Primary antibody used for Immunohistochemical (IHC) staining for Human CCR5 in different cancer tissue samples.

Antibody	CCR5
Source	INCELLDX (proprietary)
Discovery Lot No.	NT3141
Species/Isotype	Human IgG4a
Clonality	Monoclonal
CCR5 antagonist drug	Leronlimab
Suggested dilution	10.0 ug/ml
Incubation time	1 hour
Pre-treatment	None (NO SHIER)
Detection system	ImmPRESS®- Alkaline Phosphatase
Chromogen	HIGHDEF® Blue Chromogen

IHC SCORING

Paraffin-embedded tissue samples from lung, breast, colon, urothelial, pancreatic, head and neck carcinoma, and sarcoma were retrieved for this study from commercial resources. An independent, board-certified pathologist performed all pathology analysis (histology) and scoring for CCR5 expression. The evaluation was quantitative, with numeric scoring data as a measure of reactivity. For scoring, H&E (hematoxylin and eosin)-stained slides of all specimens were evaluated for the presence of tumor cells. Only tumor cells were scored for CCR5 using an IHC assay labeled PRO140 (PRO140 CCR5 Hu IgG4a). The scoring excluded any surrounding staining in the stroma, areas of non-tumor, areas of ischemia, areas of necrosis, and adjacent healthy tissue. In tumor cells, CCR5 was found to be reactive throughout the cytoplasm, associated with the plasma membrane, beneath the plasma membrane (submembranous), at the nuclear membrane (diffuse or fine granular), and facing the lumen of tubules (adluminal). All localizations were evaluated together. If present, true membrane staining could not be distinguished from membrane-associated staining (the signal density obscures separation) and, therefore, was included in the cytoplasmic score. The main components of scoring for CCR5 had percentages at differential intensities in determining Percent Scores and H-Scores (described below). Using this method, Percent Scores ≥1+ are equivalent to the overall percentage of tumor cells (0-100%) with CCR5 staining (% positive tumor cells). Percent Scores and H-Scores required recording the percentage of cells with CCR5 staining at a corresponding differential intensity on a four-point semi-quantitative scale (0, 1+, 2+, 3+). The following levels were assigned on the scoring scale: 0 = null, negative or non-specific staining, 1+ = low or weak staining, 2+ = medium or moderate staining, and 3+ = high or strong staining. The percentage of cells staining at each intensity was also estimated directly and typically reported as follows, with other percent increments used at the pathologist's discretion: 0, 1, 2, 3, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 97, 98, 99, or 100%.

PRECISION AND REPRODUCIBILITY

Four samples were selected for validation testing that included a range of high, moderate, low, and negative expression across the colorectal carcinoma (CRC) and Lung Squamous Cell Carcinoma (SCC) indications. Using the IHC assay, these samples were run in triplicate within one run for precision testing. In a separate series, a second operator ran the same samples in triplicate for reproducibility testing (Table 2 and Figure 1). Replicate slides were prepared as serial sections (one tissue section per slide with minimal material loss between preparations). In other words, intra-assay (precision) and inter-assay (reproducibility) were determined using a 2-run series with three replicate sections (per run) of each of the four selected samples for the CCR5 biomarker. Two trained operators ran the CCR5 assay using different TechMate instruments (Operator 1, Run 1; Operator 2, Run 2), and appropriate positive and negative controls were included in each staining run. All CCR5 replicates stained during validation were reviewed and scored by an independent Board-certified pathologist using the H-Score approach for cytoplasmic staining in tumor cells to determine precision and reproducibility. Full validation scoring, including statistical analysis for H-Score replicate sets, are shown in Tables 2 &3. Similar cellular patterns of CCR5 reactivity were observed in all replicates in the pattern, percent, and intensity of cytoplasmic staining (figure 1, supplemental figures 1 & 2). The staining specificity is demonstrated on control slides for all samples lacking primary antibody (supplemental figure 3). Any minor or graded changes observed in staining between replicates were attributable to slight changes in the amount of tumor in each serial section. Images of corresponding rabbit IgG negative controls are included in the bottom row of these figures.

Table 2: Inter-operator reproducibility and H-Score analysis in different cancer tissue samples of CCR5 IHC

Biomarker	Tissue	Q/MTB	Operator 1			Operator 2			H- score (Mean)	H-score (standard deviation)	H-Score SEM	H-score CV
			Rep1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3				
CCR5 tumor staining	Colorectal cancer	B426-3	300	300	300	300	300	300	300	0	0	0%
	Lung SCC	B3982	215	210	205	215	205	205	209.2	4.9	2	2%
	Colorectal cancer	B426-6	80	90	90	80	75	75	81.7	6.8	2.8	8%
	Lung SCC	B398-6	0	0	0	0	0	0	0	0	0	0%

Figure 1: Precision and reproducibility of CCR5 in lung SCC. Top three rows: CCR5, bottom row: rabbit IgG negative control, 40X

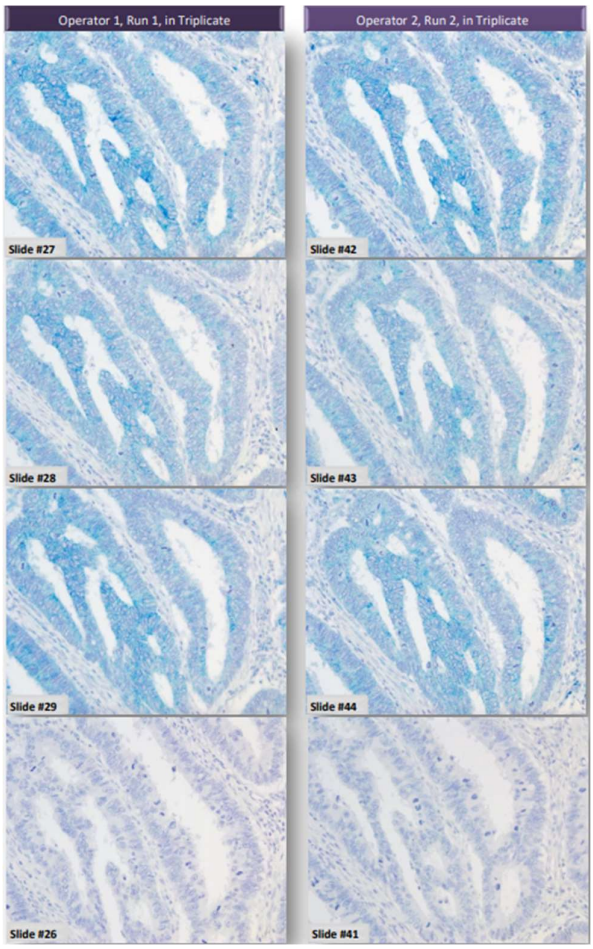


Table 3: CCR5 Reactivity in Tumor Types by Thresholds of ≥1+ staining

Breakdown of CCR5 Tumor Reactivity Data at Intensities ≥1+												
		≥1+ Staining in ≥1% Tumor Cells		≥1+ Staining in ≥10% Tumor Cells		≥1+ Staining in ≥25% Tumor Cells		≥1+ Staining in ≥50% Tumor Cells		≥1+ Staining in ≥75% Tumor Cells		≥1+ Staining in ≥90% Tumor Cells	
Tumor Type	Evaluable Cases	No. of Cases	% of Indication	No. of Cases	% of Indication	No. of Cases	% of Indication	No. of Cases	% of Indication	No. of Cases	% of Indication	No. of Cases	% of Indication
Lung Adeno	7	7	100%	7	100%	5	71%	4	57%	4	57%	4	57%
Lung SCC	9	8	89%	7	78%	7	78%	7	78%	6	67%	6	67%
TNBC	5	1	20%	1	20%	1	20%	1	20%	1	20%	1	20%
Breast Cancer	5	4	80%	4	80%	4	80%	4	80%	4	80%	4	80%
Pancreatic Cancer	7	4	57%	4	57%	4	57%	4	57%	3	43%	3	43%
Urothelial Cancer	6	6	100%	6	100%	5	83%	4	67%	4	67%	4	67%
Colorectal Cancer	9	8	89%	8	89%	8	89%	7	78%	5	56%	3	33%
H&N Cancer	7	4	57%	4	57%	3	43%	2	29%	2	29%	2	29%
Sarcoma	8	4	50%	3	38%	2	25%	1	13%	1	13%	1	13%

STATISTICAL ANALYSIS FOR THE PERCENT SCORE

Percent Scores were calculated by summing the percentages of intensities at either ≥1+, ≥2+ or ≥3+. Thus, scores range from 0 to 100. Percent Score ≥1+ = (% at 1+) + (% at 2+) + (% at 3+) , Percent Score ≥2+ = (% at 2+) + (% at 3+), and Percent Score ≥3+ = (% at 3+)

STATISTICAL ANALYSIS OF THE H-SCORE

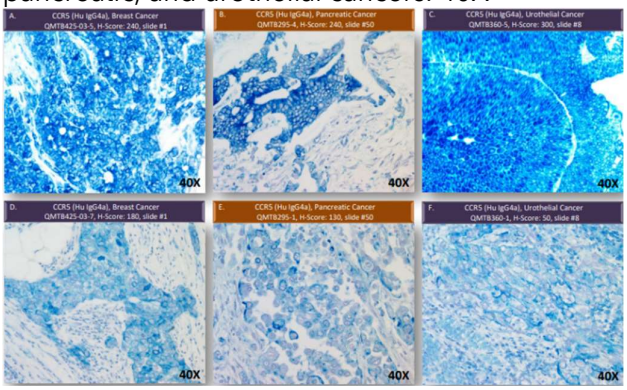
The H-Score was calculated by summing the percentage of cells with the intensity of expression (brown staining) multiplied by their corresponding differential intensity on a four-point semi-quantitative scale (0, 1+, 2+, 3+). Thus, scores range from 0 to 300.

H-Score = [(% at <1) x 0] + [(% at 1+) x 1] + [(% at 2+) x 2] + [(% at 3+) x 3]

Results

This study utilized a panel of 63 evaluable samples from 9 cancer indications as follows: 7 NSCL Adenocarcinoma, 9 NSCL squamous cell carcinoma, 5 TNBC, 5 non-TNBC, 7 pancreatic cancer, 6 UC, 9 colorectal cancer, 7 H&N cancer, and 8 sarcoma (Figures 2, and Supplemental Figures 1, 2, 3). Across the entire sample set, CCR5 displayed a full range of reactivity with H-Scores observed from 0–300. However, overall, most of the 63 cases evaluated tended to have either H-Scores >175 (highly reactive) (24/63) or <100 (low or non-reactive) (32/63). Very few samples (7/63) fell into a middle range with H-Scores from 100–175. Approximately half of the samples with H-Scores <100 lacked CCR5 expression (H-Score = 0). Within each cancer indication, the following average H-Scores were observed- UC: 183.3, breast cancer: 164.0, lung SCC: 162.8, colorectal cancer: 136.0, lung adeno: 125.1, pancreatic cancer: 112.9, H&N: 68.4, TNBC: 44.0, and sarcoma: 23.6. Thus UC had the highest expression of CCR5 based on our PRO140 labeled assay.

Figure 2. Representative CCR5 staining of breast, pancreatic, and urothelial cancers. 40X



Discussion

In the present study, we investigated the expression of CCR5 in samples from UC. We compared the expression levels in those samples to levels from tissue samples of other common tumors known to express high levels of CCR5 using an inhibitor-based IHC assay. To our knowledge, this is the first report of high levels of expression of CCR5 protein in UC in comparison to other tumors. With the maturation of CCR5 as a therapeutic target, our results may have clinical applications in treating this disease.

The CCR5 is a G protein-coupled receptor belonging to the beta chemokine receptors family of integral membrane proteins predominantly seen on T cells, macrophages, dendritic cells, eosinophils, microglia, and a subpopulation of breast or prostate cancer cells[10]. Recent studies indicate that CCR5 inhibitors blocked the migration and metastasis of breast and prostate cancer cells that expressed CCR5[11,12]. Sicoli et al. examined the molecular mechanisms governing prostate cancer bone metastasis by tranducing FVB murine prostate epithelium with oncogenic v-Src. These prostate cancer cell lines metastasized in FVB mice to brain and bone. Sicoli et al's - gene expression profiling of the tumors identified activation of a CCR5 signaling module when the prostate epithelial cells lines were grown in vivo vs. tissue cultures. The authors were then able to reduce the whole body, bone and brain metastatic prostate cancer burden by oral CCR5 antagonist[12]. Other studies suggested that CCR5 is expressed in some cancer stem cells, which are known to drive therapy resistance, and that CCR5 inhibitors may enhance the cancer response to chemotherapy[13].

There is very little literature investigating the association between UC and CCR5 variations. A few authors examined the effects of polymorphism in the genes encoding for CCR5 and the risk of UC in different populations. Kucukgergin et al. genotyped 142 histologically confirmed BC patients and 197 controls and demonstrated a positive association between CCR5Δ32 gene polymorphisms and BC risk. The frequency of the heterozygous wt/Δ32 genotype of CCR5 was more prevalent in BC patients than in controls[14]. Also, previously, genetic variants in CCR5 (CCR5wt/Δ32 andCCR5Δ32 alleles) were reported to confer a significantly high risk for early-onset smoking-related tumors. Srivastava et al. examined CCR5 Δ32 polymorphism in conferring genetic susceptibility to GBC. They found that such polymorphism was associated with gallbladder cancer, particularly in patients with early-onset and tobacco usage[15]. This a finding which bears significance since smoking is the number one risk factor for the development of BC.

Our results, if validated, may have therapeutic implications. CCR5 inhibitors have been shown to block metastasis of breast and prostate cancer. Velasco-Velázquez et al. conducted microarray analysis on 2,254 human breast cancer specimens and found increased expression of CCR5 in the basal and HER-2 genetic subtypes. The authors then showed that the CCR5 antagonists maraviroc or vicriviroc, developed to block CCR5 HIV coreceptor function, reduced in vitro invasion of basal breast cancer cells, and maraviroc decreased pulmonary metastasis in a preclinical mouse model of breast cancer. Velasco-Velázquez et al. then concluded that CCR5 antagonists might be used as an adjuvant therapy to reduce the risk of metastasis in patients with the basal breast cancer subtype[11]. The current study used an assay based on a diagnostic version of PRO140, a CCR5 humanized monoclonal antibody against CCR5 that, in pharmacologic form (leronlimab), showed proven efficacy and safety profile. A sub-Q injection form of PRO 140 was tested as monotherapy in HIV-1. It was generally well tolerated relative to placebo and demonstrated potent and prolonged CCR5 blocking activity when administered weekly or every other week[16]. Therefore, CCR5 inhibitors such as Leronlimab, Maraviroc, and Bristol Myers Squibb BMS-813160 may be potential therapeutic options for patients with UC. Patients

may also benefit from a combination of CCR5 inhibitors with currently approved systemic treatment for UC. Functional CCR5 was highly expressed by tumor infiltrating Treg, and CCR5 high Treg was previously shown to be more suppressive than their CCR5 low Treg counterparts[17]. Therefore, blockade of CCR5 on regulatory T cells (Treg) in combination with PD-L1 blockade could reduce the Treg suppression of CD8 cells and allow the anti-tumor activity of CD8 cells to endure in patients with BC treated with PD-L1 inhibitors such as pembrolizumab and Nivolumab. Also, since CCR5 is expressed on stem-like cancer cells, CCR5 inhibitors may potentiate the response to chemotherapy in the basal type of BC that is generally unresponsive to immunotherapy and thus resembles the basal type of breast cancer[11,18].

The limitations of this study include the lack of correlation between CCR5 expression and clinical outcomes. Another limitation is the small sample size which may affect the reproducibility of our results since protein expression shows wide geographical variations across populations and disease stages. Therefore, confirming the findings of this study in a large-scale cross-sectional prospective study may contribute to clarifying the role that CCR5 may play in the development, response to treatment, and outcome of UC.

Conclusion

We show, for the first time, a high expression of CCR5 in the tissue of urothelial carcinoma using an assay based on a safe and effective receptor inhibitor. Our findings need validation in bigger tissue samples and different disease stages, The high expression of CCR5 in UC may have therapeutic implications in this disease that is associated with high morbidity and mortality.

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Amruta Pise, Edgar Francisco, and Bruce K. Patterson MD are employees/stockholders in IncellDX Inc.

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Figure Legends

Figure 1. Precision and reproducibility of CCR5 in lung SCC. Top three rows: CCR5, bottom row: rabbit IgG negative control, 40X.

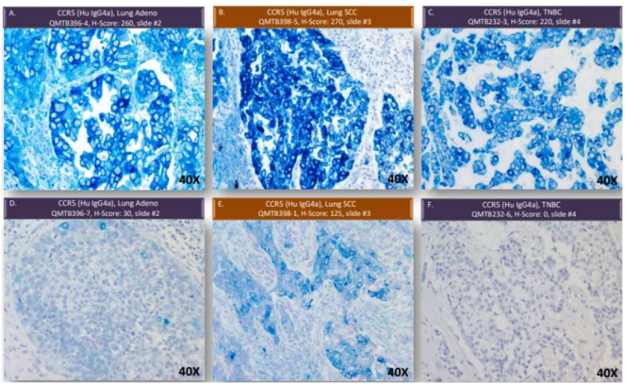
Figure 2. Representative CCR5 staining of breast, pancreatic, and urothelial cancers. 40X.

Table 1. A detailed description of the CCR5 diagnostic antibody PRO140.

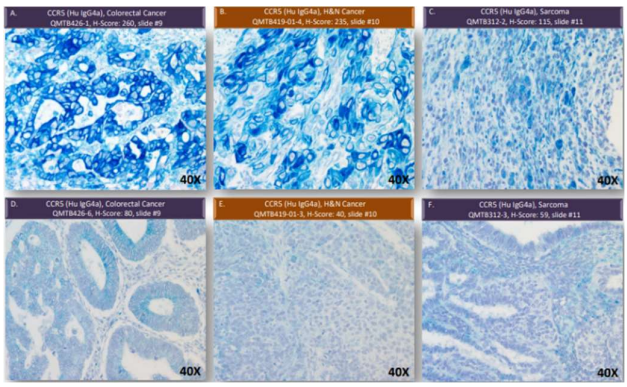
Table 2. Inter-operator reproducibility and H-Score analysis in different cancer tissue samples of CCR5 IHC.

Table 3. Testing precision and reproducibility for CCR5 staining by a separate operator in different cancer tissue samples.

Supplemental Figure 1. Representative CCR5 staining of lung adenocarcinoma, lung SCC, and triple-negative breast cancer (TNBC). 40X



Supplemental Figure 2. Representative CCR5 staining of colorectal cancer, head and neck cancer (H&N), and sarcoma. 40X



Supplemental Figure 3. Representative CCR5 staining control tissues lacking primary antibody. 40X