Tumor-infiltrating lymphocytes in colorectal tumors display a diversity of T cell receptor sequences that differ from the T cells in adjacent mucosal tissue

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Abstract

Tumors from colorectal cancer (CRC) are generally immunogenic and commonly infiltrated with T-lymphocytes. However, the details of the adaptive immune reaction to these tumors are poorly understood. We have accrued both colon tumor samples and adjacent healthy mucosal samples from 15 CRC patients to study lymphocytes infiltrating these tissues. We apply a method for detailed sequencing of T-cell receptor (TCR) sequences from tumor infiltrating lymphocytes (TILs) in CRC tumors at high throughput to probe T-cell clones in comparison to the TCRs from adjacent healthy mucosal tissue. In parallel, we captured TIL counts using standard immunohistochemistry. The variation in diversity of the TIL repertoire was far wider than the

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variation of T-cell clones in the healthy mucosa, and the oligoclonality was higher on average in the tumors. However, the diversity of the T-cell repertoire in both CRC tumors and healthy mucosa was on average 100-fold lower than in peripheral blood. Using the TCR sequences to identify and track clones between mucosal and tumor samples, we determined that the immune response in the tumor is different than in the adjacent mucosal tissue, and the number of shared clones is not dependent on distance between the samples. Together, these data imply that CRC tumors induce a specific adaptive immune response, but that this response differs widely in strength and breadth between patients.

Keywords

tumor infiltrating lymphocytes; immune repertoire sequencing; T-cell receptor β; colorectal cancer

INTRODUCTION

Tumor immunogenicity is emerging as an important but incompletely understood contributor to cancer progression. A tumor's ability to both evade and suppress the host's T lymphocyte response likely affects survival, progression, and metastasis [1]. The presence of tumor infiltrating lymphocytes (TILs) is often interpreted as evidence that the host's immune system, at some level, is responding and attempting to eliminate the tumor [2]. This interpretation is supported by multiple observations that for several known immunogenic cancers, including colorectal cancer (CRC), the presence, density, and phenotype of TILs is correlated with outcomes including Disease-Free Survival (DFS) and Overall Survival (OS) [3,4,5]. However, T cell count is a very gross view of a T cell population. Underneath the total number of infiltrating T cells is a complex population that may be characterized by a diverse or restricted T cell receptor (TCR) repertoire. Capturing not just the total cell count, but the clonal lineages of T cells allows a new way to analyze and understand TIL populations. This project uses high-throughput sequencing of the TCR Beta (TCRB) repertoire to quantify the immune repertoire of infiltrating lymphocytes in CRC tumors and paired mucosal tissue. These sequencing data complement previously-collected CD3+ immunohistochemistry data. These data allow us, as a vital first step, to characterize the variation in diversity and clonality of T cells infiltrating both normal mucosa and colon tumor.

While many cancers are considered immunogenic, data on CRC gives strong evidence that anti-tumor immune response may be a clinically reliable prognostic indicator [3]. Multiple independent studies have indicated that the presence and density of TILs in CRC tumors are strongly correlated with DFS and OS. The methods and the type of T cells that were quantified varied by study; overall, presence and high density of CD3+ (total), CD8+ (cytotoxic), CD4+ (helper), and memory TILs were associated with better outcomes, while the presence and high density of Tregs were associated with poorer outcomes in CRC patients [1,4]. In addition, higher expression of genes involved in the cytotoxic T cell response (e.g. interferon gamma) is also correlated with lower risk of relapse. Pages et al. have reported that immune markers alone predicted outcome better than TNM staging [5].

Thus, CRC is an ideal cancer in which to study the abundance, phenotype and clonal structure of tumor-infiltrating T lymphocytes.

While data on the prognostic significance of total count and phenotype of TILs is strong, less is known of the significance of the antigen receptor repertoire. Quantifying the antigen receptor repertoire would allow researchers to elucidate, if the population of T cells that infiltrates tumor cells is measurably different than the T cells that are associated with adjacent tissue. We apply this approach to investigate whether TIL populations harbor a specialized repertoire of T cells (presumably targeting specific tumor antigens), or whether the intratumoral T cell repertoire is closely related to the repertoire of T cells infiltrating healthy adjacent tissue. Given the heterogeneity of patients' immune response to tumors, the answer to this question could easily vary between patients.

However, quantifying the clonal structure of the TCRB repertoire is technically difficult as the cellular adaptive immune system generates a remarkable breadth of diversity in antigen-specific TCRs by combinatoric shuffling of gene segments in somatic cells. The TCR is composed of two peptide chains, encoded by the *TCRA* and *TCRB* genes, respectively. The antigenic specificity of αβ T lymphocytes is in large part determined by the amino acid sequence in the hypervariable complementarity-determining region 3 (CDR3) regions of TCRs [6]. The existence of multiple V_β, D_β, and J_β gene segments in the TCRB locus permits a large combinatorial diversity in receptor composition, and template-independent deletion or insertion of nucleotides at the V_β-D_β, and D_β-J_β junctions further adds to the potential diversity of receptors that can be encoded.

Previous technologies permitted quantification of one component of the CDR3 chain diversity at a time (either V gene usage with Betamark or CDR3 length distribution with spectratyping). While betamark spectratyping combines these two measures, it is still an indirect measure of the TCRB repertoire diversity, both because betamark technology only assays some V gene segments and because V gene usage and CDR3 length together are still insufficient to generate a unique molecular tag for each T cell clone. Herein, we apply a multiplex PCR method that amplifies rearranged TCRB CDR3 sequences [7] and exploits the capacity of high-throughput sequencing technology to sequence tens of thousands of TCRB CDR3 chains simultaneously. By directly sequencing the TCRB CDR3 chains, the assay captures the true diversity of the TCRB repertoire since the CDR3 region contains enough information for its full sequence to act as a unique molecular tag identifying each T cell clone. We apply the multiplex PCR assay to amplify TCRB CDR3 chains from T cells infiltrating both colorectal tumor and matched mucosal tissue from 15 CRC patients.

With these data we can describe the TCRB repertoire of colorectal tumor and adjacent mucosal tissue. Specifically, with these data we can assess 1) if T cells infiltrating colorectal tumors are a different population than mucosal tissue-localized T cells, 2) if the population-level characteristics of the intratumoral TCRB repertoire in colorectal tumors are different than those of mucosal tissue, and 3) if such variations in T cell repertoire are consistent across individuals.

METHODS

Study population

This study is based on CRC patients who participate in the ColoCare study, Heidelberg, Germany. The ColoCare Consortium is a multicenter initiative establishing an international cohort of CRC patients for interdisciplinary studies of CRC prognosis and outcomes, with sites at the Fred Hutchinson Cancer Research Center, Seattle (Washington, USA), H. Lee Moffitt Cancer Center and Research Institute, Tampa (Florida, USA), and the National Center for Tumor Diseases, Heidelberg (Germany). This pilot study involves n = 15 CRC patients, recruited between October 2010 and November 2011 at the Division of Preventive Oncology, in Heidelberg, Germany (Table 1). Eligibility criteria for this study included: at least 18 years of age, first diagnosis of colon or rectal cancer (stages I–IV) and availability of both tumor and healthy mucosal tissue. The study was approved by the Institutional Review Board and all participants provided written informed consent.

Sample Collection

Colorectal tumor tissue and healthy mucosal tissue were collected during surgery in collaboration with the University Clinic Section for Surgical Oncology, Heidelberg. Specimens were reviewed by pathologists to determine TNM (tumor, nodes and metastases) stage. Tissue samples were collected, processed and frozen within 45 minutes of excision (cold ischemia) and placed into a storage cryovial pre-labeled with a unique specimen ID, immersed in liquid nitrogen and stored at −80°C until analysis. Additional sections of tumor were formalin-fixed and paraffin-embedded. DNA was extracted from ~50mg each of frozen tumor and healthy mucosal tissue using the QIAGEN AllPrep DNA/RNA Mini Kit.

Microsatellite instability analysis

Tumors were classified based on microsatellite instability phenotype. Microsatellite instability (MSI) typing was performed using the marker panel CAT25, BAT25, and BAT26 [8]. Tumors showing microsatellite instability at two or more markers were scored as MSI-H; tumors were classified as microsatellite stable (MSS) if no instabilities were detected. The marker combination encompassing the quasi-monomorphic mononucleotide markers CAT25, BAT25, BAT26 equals the sensitivity and specificity of the Bethesda marker panel and other five-nucleotide marker panels in colorectal cancer [8,9].

TIL Quantification

Tumor infiltration by CD3+ cells was analyzed immunohistochemically. Tumor sections were sliced into four μm thick sections from FFPE tissue. Sections were deparaffinized, rehydrated, and placed on slides than transferred to a fully automated staining facility (Leica BOND-MAX™, Leica Microsystems). To retrieve antigens, slides were boiled in 10 mM citrate buffer (pH 6) for 20 min then endogenous peroxidase activity was quenched by 10 min incubation with 0.6% H_2O_2 in methanol and blocked with 10% normal goat serum (Vectastain® Elite ABC kit, Vector). Mouse monoclonal antibodies recognizing human CD3ε (1:50 dilution, clone PS1, Acris) were applied as primary antibody at room temperature (RT) for 30 min. To amplify the response, slides were then incubated with

secondary antibody (rabbit-anti-mouse IgG, Bond Refine Detection Kit, Leica) for 8 min at RT followed by incubation with a third antibody, conjugated with horseradish peroxidase and coupled to dextrane molecules, for 8 min at RT (mouse-anti-rabbit IgG, Bond Refine Detection Kit, Leica). Revelation was performed by a colorimetric reaction with 3,3-di-amino-benzidine (DAB+ chromogen, Leica). Sections were counterstained with hematoxylin (Leica) and mounted with Aquatex (Merck).

Positively stained immune cells were counted using a computerized image analysis system consisting of a NDP Nanozoomer (Hamamatsu Photonics) and a personal computer. The whole center of the tumor was analyzed – so in this analysis, the average CD3+ cell density across the measured region as well as the individual density of cells for each mm^2 of tissue analyzed was used. All results are expressed as the mean of positively stained cells per mm^2. Full sections were analyzed, with all evaluable tissue on the slide being used for quantification. Cell counts were performed with a dedicated software (VIS software suite, Visiopharm) to measure cell densities across a given region of interest as previously reported [10,12,13]. All evaluations were visually checked for consistency.

High-throughput sequencing

Sequencing CDR3 regions—The *TCRB* CDR3 regions were amplified and sequenced from 600 ng of total extracted genomic DNA. Amplification and sequencing of *TCRB* CDR3 regions was carried out on the ImmunoSEQ platform (Adaptive Biotechnologies, Seattle, WA) at Adaptive Biotechnologies as previously described [7]. The sequences for both the *TCRB* CDR3 regions were delineated according to the definition established by the International ImMunoGeneTics collaboration[14]. Sequences that did not match CDR3 sequences were removed from the analysis. A standard algorithm was used to identify which V, D, and J segments contributed to each *TCRB* CDR3 sequence[14].

Sequence analysis & statistical analysis

In order to control for the types of CRC tumors, MSI-H and MSS, TCR data was analyzed using the entire data set (15 samples) and excluding the two MSI-H samples (13 samples). To ascertain if the sequencing data was consistent with IHC data, the correlation co-efficient was calculated between log-transformed values of CD3+ cells/mm^2 and unique TCRB clones sequenced.

In order to test whether tumor and mucosal tissue samples had different variances with respect to the number of unique TCR clones observed, we used a nonparametric Fligner-Killeen [15] test for homogeneity of variances. To address the diverse vs. oligoclonal nature of each sample's TCR repertoire, we calculated the Shannon entropy [16] of the observed frequency distribution of TCR clones, then normalized this value by log_2 (# of unique TCR clones observed), which produces a metric that represents the clonal nature of a sample while being insensitive to total sampling depth. To compare this metric between tumor and mucosal samples, we used a nonparametric two-tailed Wilcoxon signed rank test. For Figure 4, the proportion of TCR overlap between tumor and matched mucosal tissue samples was calculated as (# of TCR sequencing reads from clones found in both samples) / (# of total TCR sequencing reads from both samples).

RESULTS

Our study cohort consisted of 15 patients diagnosed with colorectal cancer. Table 1 summarizes the characteristics of the study population, including stage, patient metadata, and tumor type. All tumor samples were CIMP negative. The paired primary tumor and nearby healthy mucosal tissue were analyzed using the ImmunoSEQ assay to generate a total of approximately 14 million sequencing reads distributed among about 32,000 unique TCRB rearrangements, summarized in Table 2. Using these data we have attempted to characterize the intratumoral T cell repertoire in colorectal tumors and compared it to nearby mucosal tissue.

TIL density

The average density of TILs varied by tumor, with the median at 120.1 cells/mm^2. Seven tumors' average TIL count was < 100 CD3+ cells/mm^2, six were between >100 and <500 CD3+ cells/mm^2, and two samples had > 1000 CD3+ cells/mm^2. While the sample with the highest TIL density (3940 cells/mm^2) was classified as MSI-H, the other sample classified as MSI-H was the median for TIL density (120.1 cells/mm^2). Average TIL density was moderately correlated with unique TIL TCR sequences. (r = 0.5, p = 0.04 by one-tailed normal approximation).

Distribution of T cell clones infiltrating colorectal tumor and adjacent normal mucosal tissue

We used the junctional diversity that is present in CDR3 regions to identify T cell clones and thus observe the number of unique clones as well as the distribution of clonal frequencies in each sample. We observed that the number of unique T cell clones in each sample varied largely, ranging from a minimum of 26 T cell clones to a maximum of 2,781 clones. Despite such a large variance across all samples, the number of T cell clones we sequenced in tumors was similar to the number observed in healthy mucosal tissue samples (a median of 914 clones were observed in mucosal tissue v. 1195 in tumor samples, p = N/S). We observed no relationship between the number of T cell clones detected in matched tumor and mucosal tissue samples, indicating the disparate nature of the T cell repertoire in each tissue type.

Diversity of TILs in colorectal cancer is far more variable than in mucosa

While we observed similar numbers of T cell clones in tumor and mucosal samples overall, there was a considerable discrepancy in the variance observed in the two tissue types: mucosal tissue samples had a standard deviation of 394 T cell clones, versus 778 for tumor samples (p = 0.04 using a Fligner-Killeen test, Figure 1; the same pattern is observed [p = 0.07; Supplemental Table 1] when ignoring the two MSI samples). The higher variance observed in tumor samples is consistent with substantial diversity in the breadth of the intratumoral T cell repertoire among patients.

CRC TILs are more oligoclonal than T cells in mucosa

To quantitatively assess the distribution of T cell clonal frequencies, we calculated the Shannon entropy of each tumor or mucosal tissue sample, and normalized each sample's entropy based on the number of T cell clones observed (Figure 2). This measure reflects the

oligoclonal vs. polyclonal nature of each sample's T cell repertoire independent of the total number of T cells that we sequenced. By this measure, the tumor samples tend toward oligoclonality compared to mucosal tissue samples (mean normalized entropy 0.88 for mucosal samples vs. 0.84 for tumor samples; p = 0.01 by two-tailed Wilcoxon signed-rank test; the same pattern is observed [p = 0.006, Supplemental Table 1] when ignoring the two MSI samples). We hypothesize that the more restricted T cell repertoire observed in tumor samples may be indicative of a specific and oligoclonal T cell response to antigens that are present in and restricted to the tumor environment.

Repertoire-level characteristics are similar in CRC vs. adjacent mucosal tissue

To determine if this variance in distribution of T cell clones was matched by variation in the T cell clones themselves, we characterized the high-level repertoire features of T cells sequenced from the fifteen tumor and matched healthy mucosal tissue samples by computing the following: usage frequency of the various *TCRBV* and *TCRBJ* gene segments; distribution of CDR3 length; number of unique T cell clones detected by sequencing; and distribution of T cell clonal frequencies. Figure 3 presents a summary of our results. As expected, V and J segment usage frequencies and CDR3 length distribution are quite similar among tumor and mucosal tissue samples, consistent with an intratumoral immune environment dominated by many specific interactions between TCRs, peptide antigens and HLA proteins without a broad-based bias toward particular lengths or gene segments. Crude measurements of the T cell repertoire such as antibody- or electrophoresis-based assays would be unlikely to identify any differences between tumor and healthy tissue samples.

Overlap in cellular adaptive immune response in CRC vs. adjacent mucosal tissue

In order to investigate the relationship between the immune environment in tumor compared to healthy mucosal tissue, we assessed the degree to which tumor samples and matched mucosal tissue samples shared common TCRB rearrangements, indicating the presence of clonally-related T cells in both tissues (Figure 4). By counting the number of TCRB CDR3 region sequencing reads (considering both the tumor and mucosal tissue samples) that originated from T cell clones which were observed in both samples, we calculated a TCRB overlap index that ranges from 0 (if no clones are detected in both samples) to 1 (if an identical set of clones were detected in both samples). A wide range of overlap values are observed among the fifteen patients in this study: results ranged from 0.002, indicating that 0.2 percent of T cells we sequenced were observed in both samples, to 0.38, indicating that 38% of sequenced T cells were present in both the tumor and mucosal tissue samples. A median of 5% overlap was observed – far higher than would be expected when comparing unrelated individuals, but low enough to strongly suggest that tumors have T cell repertoires quite distinct from those of surrounding healthy tissue. Using these data and distance between mucosal and tumor tissue, we found no evidence for a relationship between the proportion of T cell overlap and the distance between tumor samples and matched mucosal tissue samples at the time of collection (Fig.4).

Shared or public intertumoral immune response between CRC patients

To investigate if there were public TCRs that respond to colorectal tumors, we investigated the presence of T cell receptor sequences that were observed in multiple patients

independently. Table 2 compiles all the TCR sequences seen in at least 2 separate tumor samples, and provides the V and J gene segments used as well as the CDR3 sequence. To avoid any possibility of contamination from the sequencing process, we only counted a TCR protein sequence as having been observed twice if the amino acid sequence was coded by different DNA sequences in the two samples. In total, we observed 29 TCR sequences in two separate tumor samples, and a further 3 TCR sequences that were present in two patients and had been independently rearranged twice in one of those patients (for a total of 3 AA-identical TCRs). While potential public T cell responses to antigens specific to colorectal cancers are expected to be HLA-restricted and will require a much larger sample size to study thoroughly, the ability to sequence TCRs opens up the possibility of such studies and the presence of many TCR sequences observed in multiple patients suggests that such public responses may exist.

DISCUSSION

There is no measurable difference between the V gene usage and CDR3 length of the TCRB chains sequenced from colorectal tumor and mucosal tissue. Previously used technologies, specifically Betamark and spectratyping, would be unable to differentiate the two tissues' TCRB repertoire[17]. However, by using the TCRB CDR3 sequences it is apparent that the TCRB repertoire of the two tissues is markedly different. Tumor tissue and mucosal tissue diverged in two ways: the TCRB repertoires of the tumor tissues are more oligoclonal than their matched tumor tissues and the infiltrating T cell clones differed between matched tumor and mucosal tissue based on TCRB CDR3 sequences – independent of the physical distance between the two tissues (Fig. 4).

Although the gastrointestinal system has a large population of T cells, like peripheral blood, the mucosal T cell population is estimated to have a different TCRB repertoire with the mucosal tissue estimated to have a more oligoclonal population of T cells[18,19,20]. In our dataset we find that measures of TCRB diversity in T cells from mucosal tissue and its distribution are similar across individuals. In contrast, the TCRB repertoire diversity and distribution of tumor infiltrating lymphocytes is not similar across individuals (Fig. 3). Within individuals, the TCRB repertoire, as measured by unique TCRB CDR3 sequences, has a different distribution between mucosal and tumor tissue. Individuals' tumor samples have a measurably more clonal TCRB repertoire (Fig. 1). This matches observations that a targeted immune response to a tumor is dominated by a few clones [21,22,23]. While clinical outcome data is not yet available for this dataset, the variation we see between individuals could be representative of the variation in the immune response to the tumor. While abundance of TILs is correlated with better prognosis, it is currently not understood how TIL TCR diversity impacts prognosis. When clinical data is available, we will test this hypothesis.

Given the variation in the TCRB repertoire diversity between mucosal tissue and matched tumor tissue, we tested to see if other measures of TCRB repertoire were divergent between the two tissue types. Like Ochsenreither *et al.* [24] we find that the V gene usage and CDR3 length are conserved between these two tissue types (Fig. 3). However, when we used TCRB sequence data to test if the same T cell clones infiltrated the two tissues, we find that the two

tissue types are divergent. Based on shared CDR3 sequences, mucosal and tumor tissue have very different T cell populations. This highlights that the immune response to the tumor is likely unique and separate from the standard immune repertoire of the adjacent mucosal tissue. In addition, these data highlight how repertoire sequencing permits greater granularity of tracking the immune response in the colorectal tumor.

Given that the tumor tissue has a unique repertoire relative to the mucosal tissue, we asked if there were public, i.e. TCRB CDR3 chains shared by individuals, colorectal tumor T cell clones. Previous TCRB repertoire sequencing data found that the peripheral repertoires of unrelated individuals had an unexpectedly high number of shared TCRB CDR3 chains, >10,000 shared TCRB amino-acid sequences in the CD8+ memory compartment [25]. Later TCRB sequencing studies identified that a known EBV specific clone is public. If colorectal tumor cells present similar antigens, it is possible that we could find public T cell receptors. We searched for shared TCRB CDR3 amino-acid chains between individuals and found that several individuals have a few shared TCRB chains, including a chain with the same amino-acid sequence but many different underlying DNA sequences (Table 3). While the size of this study precludes the ability to identify true public CRC tumor clones, further experiments with a larger sample size would permit this search.

This is the first study to sequence at high-throughput the TCRB repertoire of tumor tissue. While other technologies exist to survey the immune repertoire of tissue, like immunohistochemistry, spectratyping, and V beta spectratyping (Betamark), this study demonstrates the limitations of these tools. Both TCRB sequencing and V beta spectratyping find that the TCRB repertoire of colon tissue is oligoclonal [18,19,20]. However, we find that mucosal tissue and CRC tumor tissue have substantially different populations of infiltrating T cells based on their TCRB sequences (Fig. 1& 2). Yet, the CDR3 length (assessed by spectratyping) and V gene usage (Betamark) was indistinguishable between the two tissue types (Fig. 3). V beta spectratyping would be unable to detect the variation between these two tissue types. While we recognize the limitations of the present study, namely the small sample size for this first investigation, the need for clinical outcomes that have not yet been ascertained, and to test for tumor heterogeneity, we identified characteristics of TIL populations that were not measurable using previous tools.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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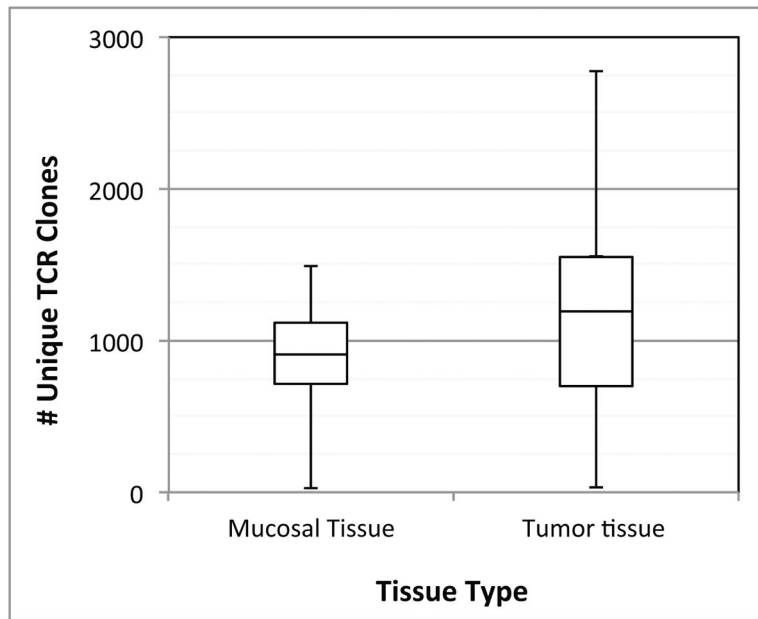
Figure 1. Comparison of TCR diversity, colorectal tumor vs. mucosal tissue
The range of TCR diversity (as measured by the number of unique TCR clones identified) is presented for 15 paired mucosal and tumor tissue samples. Values are given in quartiles, from min to max. There is no significant difference between the number of unique TCR clones identified in the two tissue hypes; however, the variance in TCR diversity is significantly higher in tumor than in mucosal tissue ($p = 0.04$ by a Fligner-Killeen test).
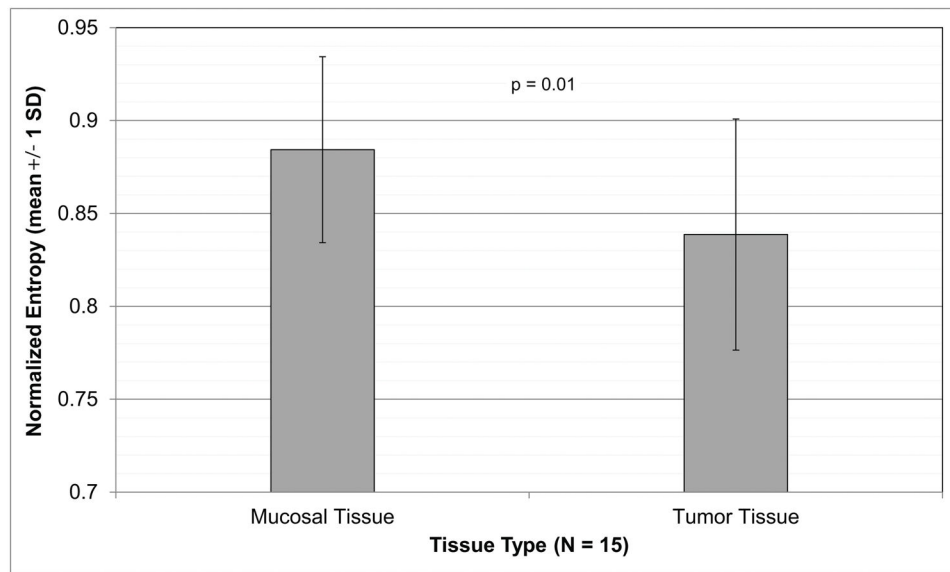
Figure 2. Comparison of TCR distribution entropy, colorectal tumor vs. mucosal tissue
Above is a comparison of Shannon entropy, normalized based on the number of unique TCR clones detected, between 15 mucosal and 15 tumor samples. The mean normalized entropy value is reported for each tissue type, with error bars representing one standard deviation. A two-tailed Wilcoxon signed-rank test (a nonparametric equivalent of the paired t-test) demonstrates a significant trend toward lower entropy values (i.e, a more clonal TCR frequency distribution) in tumor samples (p = 0.01).
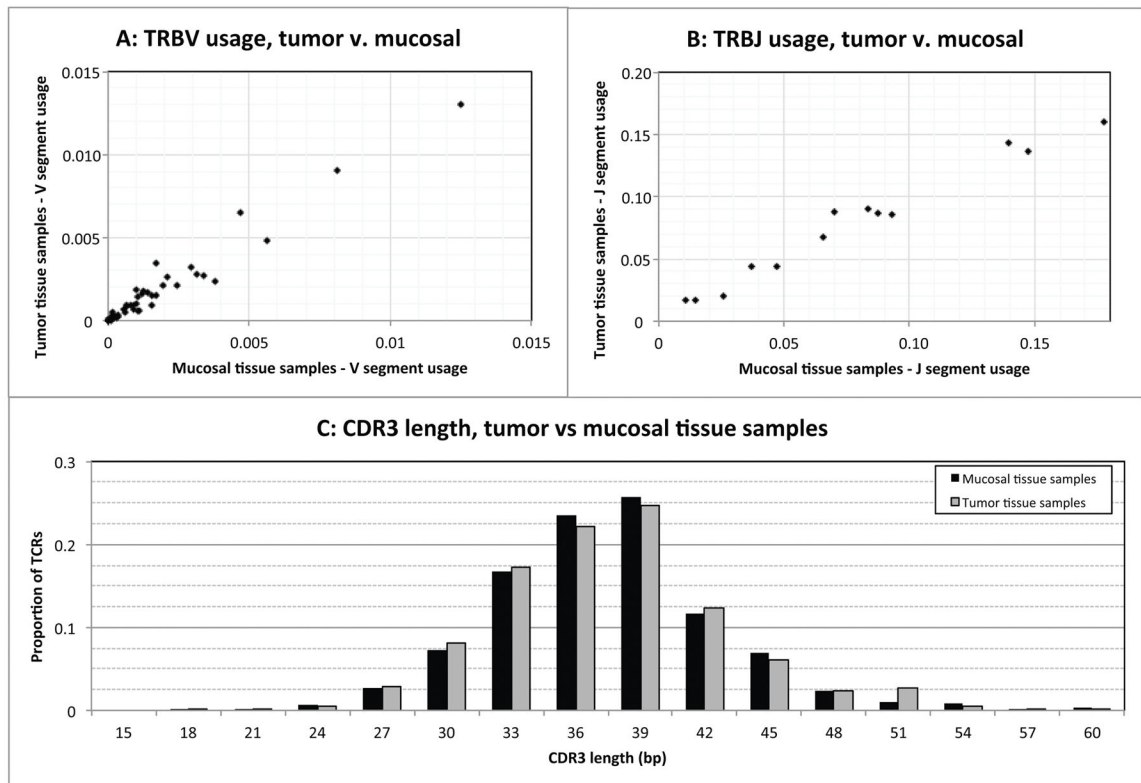
Figure 3. TCRβ sequence properties in tumor and mucosal tissue samples

A: A comparison of TRBV gene segment usage in tumor and mucosal tissues (N = 15 samples of each tissue type). Each *TRBV* gene is graphed according to its frequency of usage in tumor and mucosal samples. *TRBV* gene usage is quite similar in the two tissue types.

B: A comparison of TRBJ gene segment usage in tumor and mucosal tissues. Each *TRBV* gene is graphed according to its frequency of usage in tumor and mucosal samples. *TRBJ* gene usage is very similar in both tissues.

C: A comparison of CDR3 region lengths between tumor and mucosal tissue samples. The proportion of TCR sequences with CDR3 regions of each length are presented in a histogram for each tissue type. CDR3 region length is similar in tumor and mucosal tissue samples.
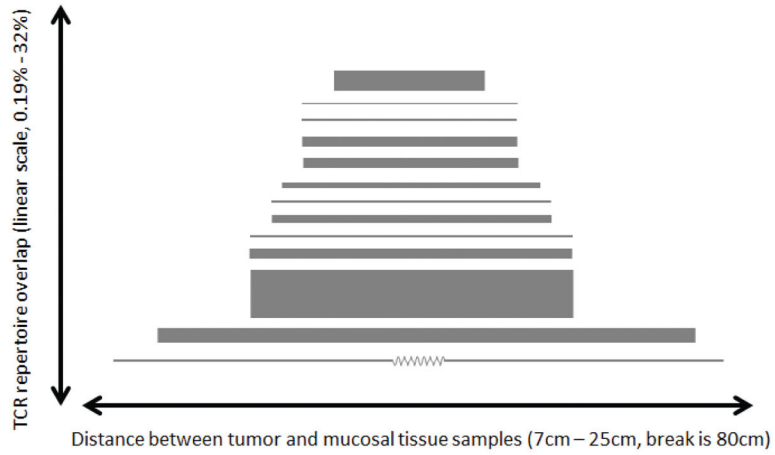
Figure 4. Relationship between spatial distance and TCR repertoire overlap
Above, we present each of 13 samples for which paired tumor and mucosal tissue samples were available along with information about the distance between the tumor site and the healthy mucosal tissue sample. The line connecting each pair of samples has a length proportional to the spatial distance between the two samples, and a width proportional to the proportion of TCR sequencing reads belonging to TCR clones shared in both samples. Samples are sorted by (distance / TCR overlaps). There is no significant evidence of a relationship between the distance between tumor and nearby healthy tissue and the degree of TCR repertoire overlap (Pearson's r = 0.16).

Table 1

Study population

Variable	NT
Sex	
Female	7 (47%)
Male	8 (53%)
Age	62 (27–78)
Tumor Stage	
I	3 (20%)
II	5 (33%)
III	5 (33%)
IV	2 (13%)
Tumor location	
Colon	12 (80%)
Rectum	3 (20%)
BMI	
25 kg/m^2	7 (47%)
>25 kg/m^2	8 (53%)
MSI	
MSI-H	2 (13%)
MSS	13 (87%)

Table 2

ColoCare_ID	MSI-Status	CD3+Cells/mm2	%stroma	TCRB CDR3 Total	TCRB CDR3 Unique	TCRB CDR3 Total	TCRB CDR3 Unique
		Tumor				**Mucosal**	
400464	MSS	319.25	20	60,876	1,836	321,096	1,466
400480	MSS	52.01	15	130,921	2,432	15,556	1,773
400488	MSI-H	3944.79	15	180,152	2,090	19,324	699
400600	MSS	59.09	0	19,366	862	15,573	984
400712	MSS	77.67	15	6,536	203	167,141	667
400728	MSS	18.00	15	1,115	41	29,676	1,110
401144	MSS	1509.00	20	384,228	1,390	13,052	1,040
401176	MSS	261.44	15	41,096	723	65,571	883
401248	MSS	42.98	15	8,104	391	551,093	1,844
401256	MSS	262.61	20	37,093	1,711	13,572	1,068
401264	MSS	422.97	15	5,483,101	3,849	36,639	910
401304	MSS	244.68	20	1,962,748	1,659	21,987	1,612
401320	MSS	28.49	15	6,312,534	2,933	27,601	1,667
401336	MSI-H	120.88	15	257,195	988	18,932	1,228

Table 3

TCR sequences observed in multiple tumor samples

CDR3 Sequence	# Patients	# Rearrangements	TRBV	TRBJ
CASSPYQETQYF	2	3	TRBV18	TRBJ2-5
CASSLGGRDGYTF	2	3	TRBV5-4	TRBJ1-2
CASSLGPIQETQYF	2	3	TRBV7-9	TRBJ2-5
CASSPGGYEQYF	2	2	TRBV18	TRBJ2-7
CASSPSPNTEAFF	2	2	TRBV18	TRBJ1-1
CASRGQGNQPQHF	2	2	TRBV19	TRBJ1-5
CASRLAGETQYF	2	2	TRBV19	TRBJ2-5
CASSLGTGNNEKLFF	2	2	TRBV19	TRBJ1-4
CASSRTGGNTEAFF	2	2	TRBV19	TRBJ1-1
CASSTGQGALSGANVLTF	2	2	TRBV19	TRBJ2-6
CSAELQETQYF	2	2	TRBV20-1	TRBJ2-5
CSARDLEAGEFNEQFF	2	2	TRBV20-1	TRBJ2-1
CSARDRVYEQYF	2	2	TRBV20-1	TRBJ2-7
CAWSPGLNTEAFF	2	2	TRBV30	TRBJ1-1
CASSEEPSSGNTIYF	2	2	TRBV6-1	TRBJ1-3
CASSELAGGPNEQFF	2	2	TRBV6-1	TRBJ2-1
CASSELAGGQETQYF	2	2	TRBV6-1	TRBJ2-5
CASSELAGGYNEQFF	2	2	TRBV6-1	TRBJ2-1
CASRISYEQYF	2	2	TRBV6-2/TRBV6-3	TRBJ2-7
CASSSGRDTGELFF	2	2	TRBV6-2/TRBV6-3	TRBJ2-2
CASSYSSGGAETQYF	2	2	TRBV6-2/TRBV6-3	TRBJ2-5
CASSDSTSGGADTQYF	2	2	TRBV6-4	TRBJ2-3
CASSLAGGPTDTQYF	2	2	TRBV7-2	TRBJ2-3
CASSLEAEGTQYF	2	2	TRBV7-2	TRBJ2-3
CASSSSGTWGETQYF	2	2	TRBV7-3	TRBJ2-5
CASSADRGNTGELFF	2	2	TRBV7-8	TRBJ2-2
CASSLGREAQETQYF	2	2	TRBV7-8	TRBJ2-5
CASSLVPGASYNEQFF	2	2	TRBV7-8	TRBJ2-1
CASSLDDSPLHF	2	2	TRBV7-9	TRBJ1-6
CASSLDGTGANEQFF	2	2	TRBV7-9	TRBJ2-1
CASSLGAEQFF	2	2	TRBV7-9	TRBJ2-1
CASSLGGSSYEQYF	2	2	TRBV7-9	TRBJ2-7