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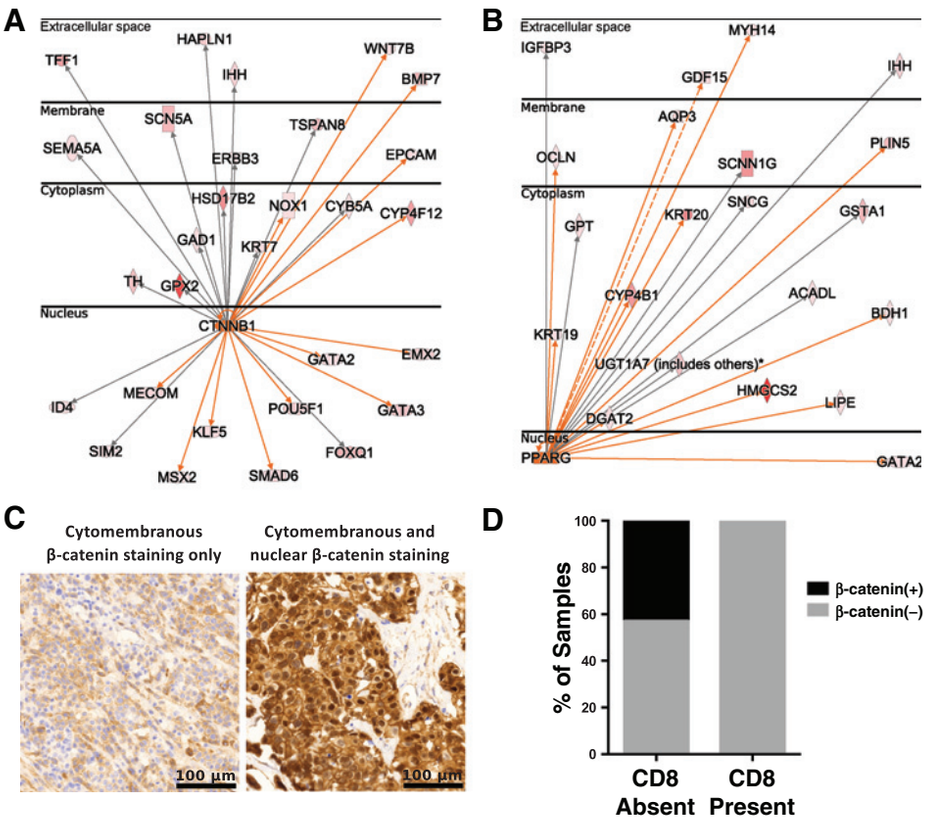

Figure 3.
A and B, cellular localization of molecules upregulated in the non–T-cell-inflamed group and their relationship with (A) β-catenin and (B) PPARG. Arrows point from the regulator toward a molecule being regulated. Intensity of red color is proportional to level of upregulation. Dotted lines indicate indirect relationships. Lines highlighted in orange show predicted activation. C, all bladder tumors showed cytomembranous staining for β-catenin (left); however, a fraction of non–T-cell-inflamed tumors showed nuclear staining for β-catenin (right). D, nuclear β-catenin staining was only found in tumors with an absence of $CD8^+$ T cells ($P = 0.036$, Fisher exact test, $n = 19$).

inflamed phenotype, we compared gene expression patterns between T-cell–inflamed and non–T-cell-inflamed bladder cancers. We found 3,112 genes differentially expressed between the two groups ($Q < 0.01$; Supplementary Table S1), with 730 being preferentially expressed in the non–T-cell-inflamed tumors. We focused on the latter set of genes and evaluated for pathway enrichment using Ingenuity Pathways Analysis. The top activated upstream regulators identified were β-catenin and PPAR-γ (both $P = 0.003$). Thirty genes related to β-catenin signaling and 22 genes related to PPARG-γ were overexpressed in the non–T-cell-inflamed tumors (Fig. 3A and B).

We further explored the two pathways by evaluating gene expression of PPARG and of WNT7B, the primary ligand associated with β-catenin signaling in melanoma (14). Both genes showed an inverse correlation with expression of CD8A ($P < 0.0001$), further suggesting a link between activation of those pathways and the absence of intratumoral T cells. To confirm this pattern at the protein level, we used IHC to examine expression of stabilized β-catenin versus extent of $CD8^+$ T-cell infiltration. Indeed, an inverse relationship was observed between nuclear staining of β-catenin and the presence of intratumoral $CD8^+$ T cells ($P = 0.036$; Fig. 3C and D). Nuclear staining was only found in samples without intratumoral $CD8^+$ T cells, although it only accounted for 43% of the non–T-cell-inflamed tumors. These results are consistent with what was observed in melanoma, in which 48% of the non–T-cell-inflamed tumors showed β-catenin pathway activation (14).

To assess a potential underlying mechanism of T-cell exclusion in this tumor type, we evaluated expression of the chemokine CCL4 and the transcription factor BATF3, which are associated with recruitment of the critical population of dendritic cells necessary for T-cell activation in the melanoma context (14). CD8 transcripts were correlated with CCL4 and BATF3 expression within the tumor microenvironment (Supplementary Fig. S1A). We confirmed the expected inverse correlation with WNT7B expression, but also found that PPARG showed the same relative expression pattern (Supplementary Fig. S1B). Thus, the recruitment of BATF3-lineage dendritic cells necessary for cross presentation of tumor antigens might also be negatively influenced by PPARG. Lastly, of the 29 β-catenin target genes overexpressed in our dataset, only IHH and GATA2 overlapped with the 22 target genes of PPARG. Despite this lack of overlap, target gene expression for both pathways was strongly correlated ($P < 0.0001$), indicating a potential synergistic mechanism of immune exclusion.

Mutated FGFR3 and coactivators of PPAR-γ in non–T-cell-inflamed cancers

We next interrogated somatic mutations between T-cell–inflamed and non–T-cell-inflamed subtypes to identify mutations in pathways that might be exclusive to the non–T-cell-inflamed subset. Analysis of exome sequencing data revealed no difference in the overall number of nonsynonymous mutations per patient for each group ($P = 0.80$). Thus, a lower mutational density alone could not explain the absence of T cells in a major subset of tumors. We therefore reviewed differential mutations at the individual gene level. We found 891 genes mutated in two or more samples exclusive to

Table 1. FGFR3 mutations found in non–T-cell-inflamed bladder cancers

TCGA sample ID	Mutation	Amino acid substitution
TCGA-DK-A3IS-01	chr4:1803377:G:A	E216K
TCGA-DK-A3IS-01	chr4:1803395:G:A	D222N
TCGA-DK-A3IS-01	chr4:1803435:G:A	G235D
TCGA-FJ-A3ZF-01	chr4:1803738:G:A	V306I
TCGA-GU-A42R-01	chr4:1805533:C:T	H349Y
TCGA-UY-A78N-01	chr4:1805561:C:T	P358L
TCGA-CF-A5U8-01		
TCGA-E7-A3Y1-01	chr4:1806089:G:T	G370C
TCGA-E7-A7DU-01		
TCGA-BT-A42C-01		
TCGA-CF-A47W-01		
TCGA-DK-A6B0-01	chr4:1806099:A:G	Y373C
TCGA-E7-A5KF-01		
TCGA-DK-A3IS-01	chr4:1808937:C:-:	A790fs
	Fusions	
TCGA-CF-A3MF-01		
TCGA-CF-A3MG-01	FGFR3-TACC3	
TCGA-CF-A3MH-01		

noninflamed tumors (Supplementary Table S2). FGFR3 was the most common mutation exclusive to noninflamed tumors ($P < 0.0001$). Fourteen separate FGFR3 mutations were detected in 11 of the non–T-cell-inflamed samples, with no mutations detected in the T-cell–inflamed samples. Nine unique mutations were identified (Table 1), and two were recurrent (G370C in three samples and Y373C in four samples). The two most common FGFR3 mutations, Y373C and G370C, have been reported previously in bladder cancer and lead to activation of the receptor through increased ligand-independent dimerization and phosphorylation (18). In addition, recurrent in-frame activating FGFR3–TACC3 fusions were found exclusively in non–T-cell-inflamed tumors (three samples). We also analyzed FGFR3 gene expression and found an inverse correlation with expression of CD8A ($P < 0.0001$). Non–T-cell-inflamed tumors showed higher expression of FGFR3 as compared with the T-cell–inflamed tumors.

Finally, we also discovered that PPARGC1A and PPARGC1B were exclusively mutated in non–T-cell-inflamed tumors. Both are coactivators of PPAR-γ, which was one of the top activated regulators in our gene expression pathway analysis. PPARGC1A mutations included A728D, C726Y, K663*, R657Q, R485W, and R408T. PPARGC1B mutations included P607L, V703I, E787K, and S994L. These mutations were evaluated by a prediction algorithm, SIFT, and 70% were predicted to affect protein function (17). Together, these data identify the β-catenin pathway, the PPAR-γ pathway, and active FGFR3 as three potential cancer-intrinsic pathways that could contribute to T-cell exclusion in bladder cancer.

Discussion

Using our immune gene signature, we found that urothelial bladder cancer segregated into T-cell–inflamed and non–T-cell-inflamed subtypes, and the latter phenotype correlates with an absence of $CD8^+$ T cells by IHC. Unsupervised clustering analyses of muscle-invasive bladder cancer based on global gene expression segregate tumors into two to four intrinsic subtypes (19–21), but previous studies did not evaluate segregation based on T-cell infiltration in bladder cancer, as we have done. Our strategy was based on the presence of immune cells in the tumor microenvironment, rather than clustering by tumor-derived genomic pathways. Although the latter approach might better identify chemotherapeutic resistance pathways, the former is more likely to yield insight into immunotherapy resistance. PPAR-γ pathway activation and FGFR3 mutations are associated with the "luminal" intrinsic bladder subtype, and we found both were also linked with the non–T-cell-inflamed phenotype. Further bioinformatic and experimental analyses are needed to determine the relationship of the non–T-cell-inflamed subtype and previously described bladder cancer–intrinsic subtypes.

Our use of immune gene expression profiling identified three molecular pathways linked with the non–T-cell-inflamed subtype. Our methodology selected for genes overexpressed in tumors with an absence of immune-infiltrating cells, which by inference were highly likely to arise from tumor-intrinsic activated molecular pathways. Modulation of these targets could prove useful to overcome primary resistance to immune checkpoint blockade such as PD-1/PD-L1–targeted therapies. One limitation to this analysis is its correlative nature. However, similar methods have led to the discovery of a causal link between activated β-catenin and intratumoral T-cell restriction via ATF3-mediated CCL4 repression in melanoma, which was, indeed, confirmed through careful experimental studies in genetically engineered mouse models (14). Our study independently validated this pathway as active in non–T-cell-inflamed bladder cancers. We also identified two other molecular pathways not previously found in melanoma. The effect of modulation of these pathways on the induction of a T-cell–inflamed phenotype will be an attractive area of research to pursue in future studies. The availability of FGFR3 inhibitors for clinical testing provides a rationale and feasibility for combination with monoclonal antibodies to PD-1 or PD-L1.

An understanding of the non–T-cell-inflamed phenotype in urothelial bladder, as well as other cancers, is critical to improving response rates for future immunotherapeutic development (22, 23). Three immune checkpoint inhibitors have been approved by the FDA since 2011 for melanoma, renal, and non–small cell lung cancers (23–25). It is critical to continue advancing our biologic understanding of tumors with primary resistance to expand the proportion of patients responding to these immunotherapies. A better understanding of resistance will catalyze the development of new clinical trial strategies with combination therapies that overcome resistance in urothelial bladder cancer and, ultimately, other tumor types.

Disclosure of Potential Conflicts of Interest

W.M. Stadler reports receiving commercial research support from Johnson & Johnson and is a consultant/advisory board member for AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R.F. Sweis, G.P. Paner, G. Steinberg
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Study supervision: R.F. Sweis, G. Steinberg, T.F. Gajewski

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