Representative Drivers for TB Development and p-EMT Induction

Subjects: Oncology

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Tumor budding (TB), a microscopic finding in the stroma ahead of the invasive fronts of tumors, has been well investigated and reported as a prognostic marker in head and neck squamous cell carcinoma (HNSCC). Epithelial–mesenchymal transition (EMT) is a crucial step in tumor progression and metastasis, and its status cannot be distinguished from TB. The current understanding of partial EMT (p-EMT), the so-called halfway step of EMT, focuses on the tumor microenvironment (TME). Although this evidence has been investigated, the clinicopathological and biological relationship between TB and p-EMT remains debatable. At the invasion front, previous research suggested that cancer-associated fibroblasts (CAFs) are important for tumor progression, metastasis, p-EMT, and TB formation in the TME.

head and neck squamous cell carcinoma tumor budding partial epithelial–mesenchymal transition tumor microenvironment

1. Hypoxia

Hypoxia in an advanced TME promotes TB development in various types of solid cancer. Hypoxia also promotes the recruitment of CAFs. Genes expressed by hypoxia-inducible factor (HIF)-1α promote EMTs [1]. A recent study has suggested that malic enzyme 1 (ME1) promotes the Warburg effect in cancer cells and induces EMTs in HNSCC cells. Yes-associated proteins (YAP) are activated in the hypoxic TME and abrogated by the knockdown of ME1 [2]. YAP activation promotes EMT and is involved in HNSCC progression [3]. During hypoxia, YAP activation is responsible for the upregulation of *GPRC5A* in combination with HIF-1α [4]. Reciprocally, activated YAP stabilizes HIF-1α and enhances its activity [5]. Inhibition of YAP activation by ME1 knockdown is associated with suppression of ME1 reprogramming of energy metabolism. Moreover, the levels of MMP9 and MMP7 are upregulated by HIF-1α activation; these markers are strong indicators of TB onset in HNSCC and CRC [6][7][8] and are local therapeutic candidates for the prevention of TB development. Therefore, the YAP activation cycle is a key pathway related to TB development induced in response to hypoxia and reprogramming of the energy metabolism (**Figure 1**).

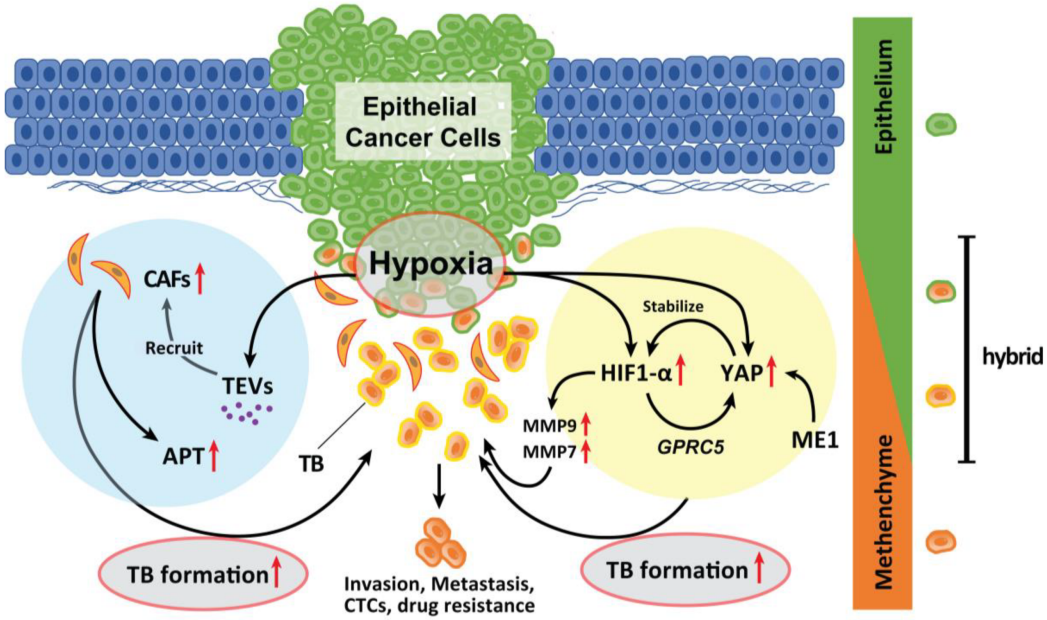

Figure 1. Role of hypoxia in the formation of TB. Hypoxia in advanced TME promotes TB development. Genes whose expression is induced by HIF-1α activation by hypoxia promote EMT. YAP is activated in a hypoxic TME and was abrogated by the knockdown of ME1 which promotes the Warburg effect in cancer cells and induces EMT. YAP activation is a significant factor that promotes the EMT phenotype and is deeply involved in the progression of the tumor. In a hypoxic situation, YAP activation is responsible for the upregulation of *GPRC5A* by binding to HIF-1α. Then, activated YAP also stabilizes HIF-1α and enhances its action. The expression of MMP9 and MMP7 is also upregulated by HIF-1α activation. Moreover, hypoxic TME can induce tumor cells to secrete enhanced amounts of TEVs. The dynamic intercellular crosstalk that is mediated by TEVs mobilizes oncogenic factors, relocalizes CAFs to tumor sites, p-EMT and APT development, and TB formation which sustains cancer progression and metastasis.

Moreover, hypoxic conditions within the TME can induce tumor cells to secrete enhanced amounts of tumor-derived extracellular vesicles (TEVs) [9]. TEVs play critical roles in tumor initiation, progression, and metastasis as vehicles of small molecules [10][11]. The dynamic intercellular crosstalk mediated by TEVs mobilizes oncogenic factors, relocalizes CAFs to tumor sites, and sustains metastasis [12] (**Figure 1**). Considering that TEVs contribute to the plasticity of cancer cells in multiple stages of cancer progression, TEV-mediated delivery of tumor suppressor cargo has broad possibilities for future clinical applications [13].

2. CAFs

CAFs are key factors in cancer cell invasion because they can remodel the extracellular matrix and provide mechanical propulsive forces that facilitate tumor invasion and metastasis. In vitro, Zhou et al. confirmed that CAFs induced more aggressive carcinoma cell proliferation and human umbilical vascular endothelial cells tube formation, whereas peritumoral fibroblasts strongly promoted the migration of tumor cells, both of which were isolated from patients with HNSCC [14]. Furthermore, it is well known that CAFs secrete molecules involved in

tumor invasion, EMTs, and metastasis, including TNF-α, IL-1α/β, IL-33, CCL7, SDF-1, MDNF, type 1 collagen, HGF, IGF2, BMP4, MMPs, PGE2, KGF, activin A, PDGF, and miRNAs [15].

In hepatocellular carcinoma, CAF-derived CCL5 (also known as RANTES) promotes metastasis by binding to a specific receptor, CCR5, and inhibiting the ubiquitination and degradation of HIF-1α, maintaining HIF-1α even under normoxia, thereby upregulating the downstream gene *ZEB1* and inducing EMT [16]. In CRC, CCL5 blockade reduced tumor xenograft growth, decreased the migration of tumor cells, reduced liver metastases, and decreased the infiltration of T_{regs} in the tumor [17]. The abnormal expression of CCL5 has been confirmed in many types of tumors, including CRC, breast, lung, ovarian, and prostate cancers [18][19]. Mielcarska et al. reported that CCL5 produced by macrophages can stabilize PD-L1 in CRC cells both in vitro and in vivo. CCL5 induces the formation of the nuclear factor kappa-B (NF-κB) p65/STAT3 complex, which upregulates the promoter of *COP9 signalosome 5* (CSN5). CSN5 stabilizes PD-L1 by regulating its deubiquitination at the cellular level, resulting in increased PD-L1 activity [20]. This may sensitize tumors to immune checkpoint inhibition therapy, even if the cancer cells are resistant to anti-EGFR therapy [21]. Thus, CCL5 blockade is a therapeutic candidate for inhibiting TB development and p-EMT induction in CRC.

CCR5 can modulate TGF-β activity, which subsequently promotes EMT and increases tumor cell migration via activation of the NF-κB pathway [22]. Considering that TB cells exhibit a particular gene expression signature, comprising factors involved in EMT and activated TGF-β signaling [23], the CCR5 contribution to drive p-EMT is promising. This further facilitates tumor angiogenesis and collagen synthesis and promotes tumor progression [24] (**Figure 2**).

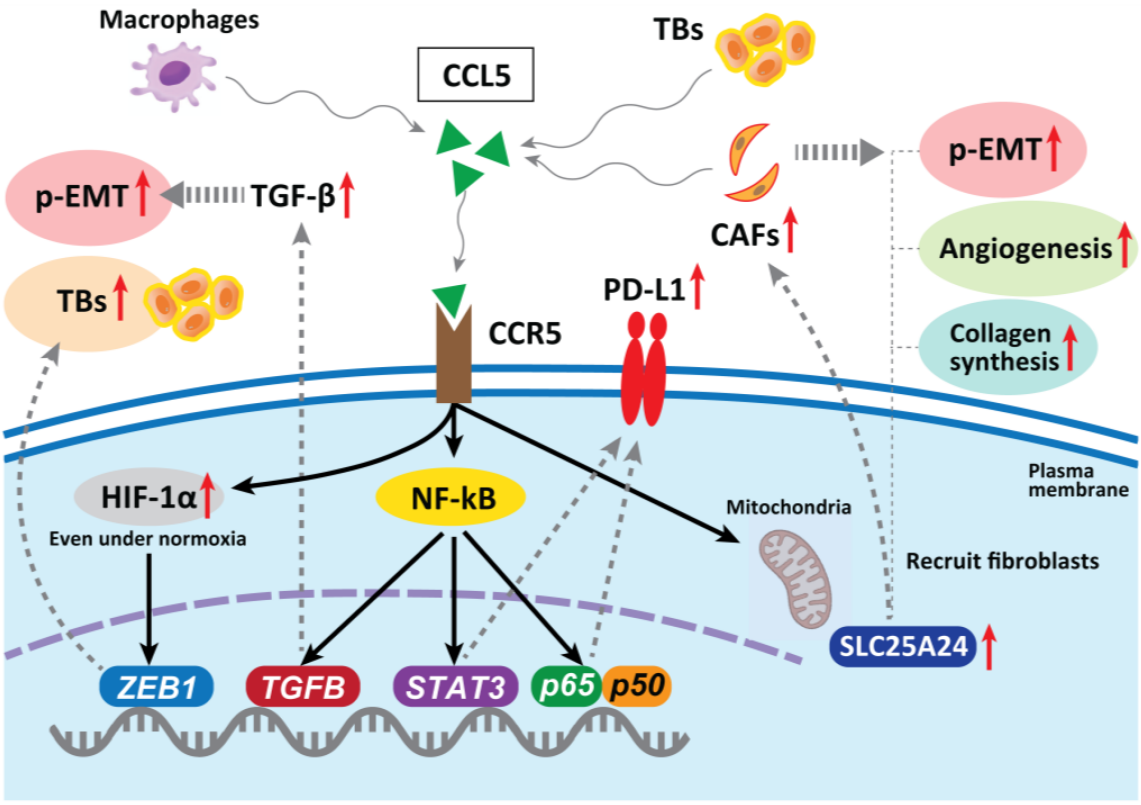

Figure 2. In hepatocellular carcinoma, CAF-derived CCL5 promotes metastasis by binding to specific receptors, CCR5, and stabilizing HIF-1α under normoxia, upregulating one of the EMT genes *ZEB1* and promoting TB development. In CRC, CCL5 blockade reduces tumor growth, decreases migration of tumor cells, reduces metastases, and decreases infiltration of T_{regs} in the tumor. CCL5 can stabilize PD-L1 in vitro and in vivo. CCR5 can also modulate TGF-β activity, which subsequently promotes an EMT. TB cells secrete high levels of CCL5, which recruits fibroblasts through CCR5–*SLC25A24* signaling and leads to the development of a characteristic fibroblast cluster around TB cells at the invasive front of CRC. This further facilitates tumor angiogenesis and collagen synthesis, recruitment of CAFs, and promotes malignant progression. CCR5 can also modulate TGF-β activity, which subsequently promotes an EMT and increases tumor cell migration via the activation of the NF-κB pathway.

In HNSCC, Chuang et al. reported that oral cancer cells with high invasiveness express CCR5 on their surface which regulates the increased migration of tumor cells and metastasis [25]. On the other hand, decreased expression of CCR5 in monocytes from HNSCC patients was reported [26]. Li et al. reported that patients with high CCR5 expression levels had worse overall survival (hazard ratio = 0.59, $p < 0.001$) and worse recurrence-free survival (hazard ratio = 3.27, $p < 0.001$). Moreover, they also observed the upregulation of CCR5 expression is associated with immunomodulators, chemokines, and infiltrating levels of CD4+ T cells, neutrophils, macrophages, and myeloid dendritic cells [27]. Moreover, González-Arriagada et al. reported that significant associations were detected in the relationship between high CCR5 expression and lymph node metastasis ($p = 0.03$), advanced clinical stage ($p = 0.003$), poor differentiation of tumors ($p = 0.05$), and tumor recurrence ($p = 0.01$) [28]. The evidence about the function and contribution of HNSCC CAFs in driving TB development and p-EMT induction is limited and further in-depth investigation will be needed as the above evidence indicates the potential relationship among CCR/CCL5 expression, development of TBs, and p-EMT demonstration.

3. Tumor-Associated Macrophages (TAMs)

TAMs have a leading position in the TME and are also key factors in EMT development. In CRC, Trumpi et al. reported that macrophages located around a tumor induce a loss of tight-junction proteins at the tumor cell–cell contacts and cause TBs in the colonosphere bulk [29]. This is because MMP7 is secreted by activation of the NF-κB pathway. Therefore, the macrophage-initiated NF-κB–MMP7 pathway functions as a central player in TB development.

Moreover, CAF-derived exosomes serve as chemoattractants that recruit various immune cells, including monocytes, thereby promoting CRC progression and the release of cancer-derived exosomes. CAF-derived exosomes containing granulocyte–macrophage colony-stimulating factor and IL-6 promote the differentiation of monocytes into M2 macrophages and activate M2 macrophages to release chemokines and macrophage-derived exosomes, which in turn drive angiogenesis, promoting TB development and metastasis [30] (**Figure 3**).

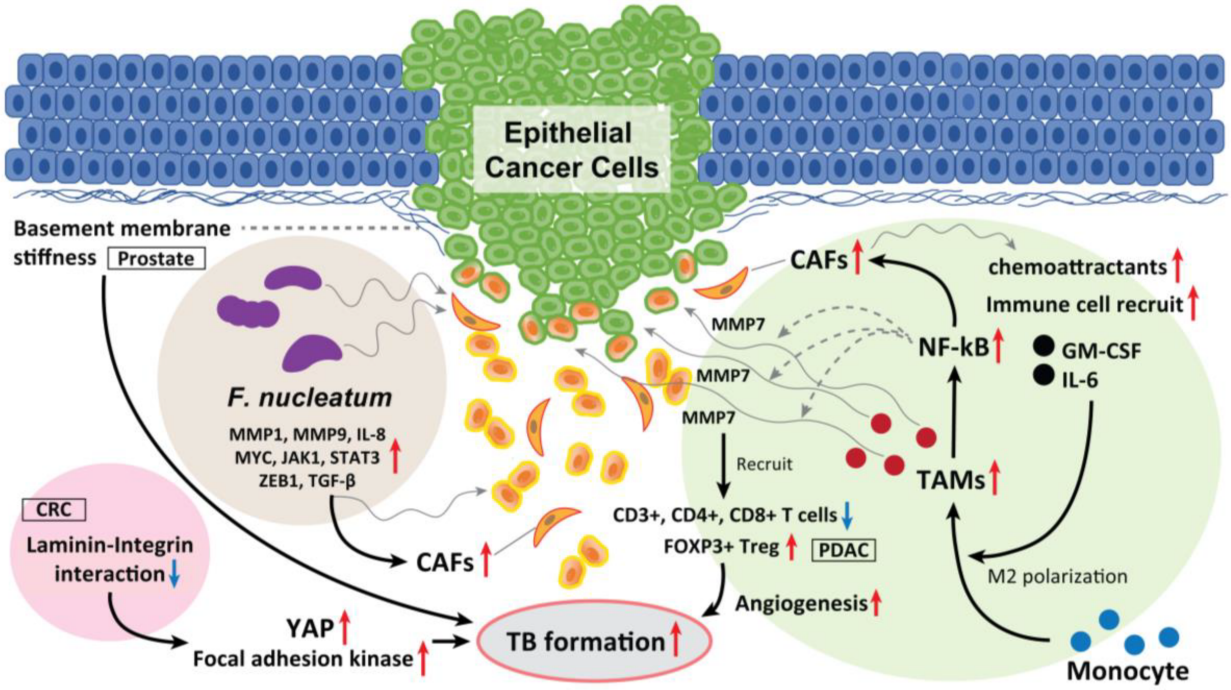

Figure 3. The role of TAM, laminin–integrin interaction, basement membrane stiffness, and *F. nucleatum* as a TB driver. TAMs located in the surrounding of the tumor mass induce loss of tight junction proteins at tumor cell–cell contacts, and cause TB from the colonosphere bulk of CRC. This is due to the MMP7 secretion by activating the NF-κB pathway. Moreover, CAF-derived exosomes serve as chemoattractants, which recruit various immune cells, including monocytes, promoting CRC progression and the release of cancer cell-derived exosomes. In PDAC, high-grade TB cases display lower M1 macrophages in the stroma and increased M2 macrophages in the tumor tissue, and displayed fewer CD3+, CD4+, and CD8+ T cells. Inversely, FOXP3+ T_{regs} were found to be elevated in high-grade TB cases. CAFs also recruit granulocyte-macrophage colony-stimulating factor and IL-6, promote the differentiation of monocytes into M2 macrophages and activate them to release chemokines and exosomes, which promote TB formation. Softening and enhanced remodeling of the basement membrane also promote TB development in stratified epidermis via the activation of focal adhesion kinase and YAP, while stiffening of the basement membrane promotes folding, and the laminin–integrin crosstalk in the basement membrane plays a key role to generate TBs. Moreover, *F. nucleatum* upregulated the expression of p-EMT-related genes in HNSCC cells with an epithelial phenotype. *F. nucleatum*-infected HNSCC cells had upregulated MMP1, MMP9, and IL-8. The expression of cell survival markers MYC, JAK1, and STAT3 and EMT markers ZEB1 and TGF-β were also significantly elevated and promoted TB development. These mediators also recruit CAFs in the TME and promote TB formation.

In oral SCC, the existence and roles of M1-like TAMs have been identified and revealed. High infiltration of M1-like TAMs has been identified to be associated with aggressive features of the disease. Xiao et al. demonstrated that exosome-transferred THBS1 polarized macrophages to the M1-like phenotype through p38, Akt, and SAPK/JNK signaling at the early-stage oral SCC [31]. Increased expression of THBS1 could significantly decrease the overall survival of HSNCC patients [32]. Moreover, their RNA sequencing analysis then revealed that M1-like TAMs tightly correlates with the EMT process and cancer-stem characteristics of oral SCC cells: M1-like TAMs could regulate

the EMT process of oral SCC cells through the IL6/Jak/Stat3 signaling pathway, which could subsequently promote the transcription and expression of THBS1 [32].

4. Laminin-5γ2 (LN-5γ2) and Integrin β1

The glycoprotein LN-5γ2 has recently become a focus of increased interest and investigation as a marker of invasion in gastrointestinal malignancies. In oral SCC, Peixoto da-Silva et al. suggested that heterogeneous LN-5γ2 chain expression in the invasive front of the tumor mediates the acquisition of the migrating and invading epithelial cell phenotype [33]. Among the interactions between a tumor and the surrounding stroma in oral SCC, Marangon Junior et al. reported that high-grade TB was associated with a higher expression of LN-5γ2, which is a cell–extracellular matrix adhesion molecule [34]. Zhou et al. reported that the interaction between LN-5γ2 and integrin β1 in CRC promoted TB development via focal adhesion kinase and YAP activation. This induces the nuclear translocation of YAP/TAZ, which consequently promotes tumor growth, EMTs, and TB development by regulating the transcription of downstream genes. Thus, high expression levels of LN-5γ2 and integrin β1 may indirectly improve the diagnostic sensitivity for occult TB development. They also found that a natural medicinal monomer, cucurbitacin B, inhibits the interaction between LN-5γ2 and integrin β1, inactivates YAP, and blocks TB development [35].

Fiore et al. reported that softening and enhanced remodeling of the basement membrane also promotes TB in the stratified epidermis while the stiffening of the basement membrane promotes folding [36]. Moreover, Wang et al. observed prominent TB formation in β-integrin–depleted prostate cancer spheroids in regions of a discontinuous laminin-332 layer, which was not observed in the controls. They concluded that the loss of integrin β4 expression generates laminin-332 continuity gaps, through which basal cells exit the spheroid, promoting the progression of high-grade prostatic intraepithelial neoplasia [37]. Thus, laminin–integrin interaction and basement membrane stiffness can also play a key role in promoting TBs, EMT/p-EMT, invasion, and metastasis (**Figure 3**). These physical properties of epithelial cancer tissue also affect the fate of tumor control due to TB development.

5. Fusobacterium nucleatum (F. nucleatum)

F. nucleatum has pathogenic effects on HNSCC and CRC [38]. *F. nucleatum* is detected more frequently in deeper areas of cancer tissues than in healthy subjects [39][40]. *F. nucleatum* is also frequently detected in CRC tissues and is directly involved in CRC development [41]. In addition, Harrandah et al. reported enhanced expression of three oncogenes (*STAT3*, *JAK1*, and *MYC*) and EMT markers in *F. nucleatum*-infected oral cancer cell lines. *F. nucleatum* can enhance MMP1, MMP9, and IL-8 expression and cancer cell invasiveness. They also upregulate the expression of p-EMT-related genes in oral SCC cells with an epithelial phenotype but not in those with a p-EMT or EMT phenotype.

F. nucleatum is one of the bacteria deeply involved in the development of oral cancer [42]. Harrandah et al. reported that *F. nucleatum*–polyinfected (c.f. *Porphyromonas gingivalis*) oral SCC cells showed synergistically upregulated

expression of MMP1, MMP9, and IL-8; the expression of cell survival markers MYC, JAK1, and STAT3, and the EMT markers ZEB1 and TGF-β were significantly elevated (**Figure 3**) [42][43]. Moreover, there is definite evidence of intratumoral microbiota that is highly organized in micro niches with immune and epithelial cell functions that support cancer progression. Epithelial cancer cells that are infected with *F. nucleatum* invade their surrounding TME as single cells and recruit myeloid cells to bacterial regions promoting transcriptional changes in epithelial cancer cells that facilitate invasion into the deeper [44]. Taken together, *F. nucleatum* drives TB development and EMT/p-EMT induction in the TME of oral cancer, which is promoted by polyinfection.

6. Human Papilloma Virus (HPV) Status

In HNSCC, HPV infection must be considered; however, there is no clear consensus that has yet been reached on the relationship between TB development and HPV infection. In vulvovaginal SCC, HPV-independent carcinomas are more likely to be moderately and poorly differentiated, with an intermediate-to-high TB status [45]. Since the expression of p16 correlates with high TWIST, SNAIL, and SLUG expression in oropharyngeal SCC [46], HPV infection itself may act as a TB driver. However, Prell et al. reported that the upregulation of $p16^{INK4a}$ may not be a strict requirement for TB development in CRC, suggesting that there is no correlation between the degree of p16 expression and TB development [47]. Further analyses of how HPV infection is related to TB development and p-EMT induction in HPV-associated HNSCCs (including the dynamics modulations of E5, E6, and E7 kinases) are required in the future.

7. Methylthioadenosine Phosphorylase (MTAP)

MTAP is a rate-limiting enzyme in the methionine salvage pathway, which recycles one carbon unit lost during polyamine synthesis into the methionine cycle. The *MTAP* gene, which is located at the chromosomal locus 9p21, is deleted in many human cancers because of its proximity to the tumor suppressor gene *cyclin-dependent kinase inhibitor 2A*. When the level of MTAP increases, it prevents the combination of cyclin D1 and CDK4, cell cycle arrest in the G1 phase, and the inhibition of cell proliferation. MTAP deficiency commonly occurs in hematological malignancies and various solid tumors, suggesting that MTAP may play a tumor-suppressing role in these types of cancer [48]. However, in CRC, MTAP expression is upregulated by LEF/TCF/β-catenin in parallel with tumor progression and cell dedifferentiation [49][50]. Amano et al. reported that concomitant nuclear and cytoplasmic expression of MTAP in OSCC is associated with a high TB score and might promote tumor aggressiveness through its activity in the methionine salvage pathway. In oral SCC, cytoplasmic MTAP expression is observed from an early stage of oncogenesis and persists throughout the progression of the disease. Both nuclear and cytoplasmic MTAP expressions are associated with an aggressive invasion pattern and EMT [51]. Thus, high MTAP expression levels in both may indicate the p-EMT/EMT process in oral SCC, and finding the relationship between and mechanism among MTAP, the LEF/TCF/β-catenin pathway, TB development, and their intermediates in the TME are required.

Representative evidence for TB drivers and p-EMT induction has been mentioned above; other well-described candidates for the TB driver are summarized in **Table 1** [52][53][54][55][56].

Table 1. Other evidence of TB drivers.

TB Driver	Mechanism	Cancer Type	Year	Reference
COL4A1/COL13A1	Activation of intracellular AKT pathway leads to an E/N-cadherin switch	Urothelial carcinoma of bladder	2017	[57]
SerpinE1 (known as plasminogen activator inhibitor type 1)	Regulation of the plasminogen activator system	HNSCC	2019	[58]
c-MET	Upregulation of MET transcription	CRC	2016	[52]
Tenascin-C	Tenascin-C induces cancer cell EMT-like change	CRC	2018	[53]
SREBP1	Upregulation of MMP7expression and NF-κB pathway activation	CRC	2019	[54]
Increased tumor stroma Percentage and LDH-5	Decrease CD3+ lymphocyte stromal density	CRC	2019	[55]
Thymosin β4/β10	Modulation of cytoskeleton organization	CRC	2021	[56]

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