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Expression Profiling of EMT Transcription Factors in Epithelial Cancer vs. Colon Cancer from a Clinical Dataset (Snail, Slug, Zeb-1, Twist)

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Abstract: ***Colorectal cancer (CRC) is the second most common cancer and a leading cause of cancer-related deaths worldwide. Epithelial-mesenchymal transition (EMT) is a biological process that enables a polarized epithelial cell to go through a variety of biochemical changes and adopt the phenotype of a mesenchymal cell, which includes increased migratory ability and invasiveness. Here, we aim to explore the role of a comprehensive analysis of the dynamic regulation of EMT transcription factors Slug, Snail, Twist, and Zeb1 in CRC. By examining expression patterns across different tumor stages, age groups, and genders, the research elucidates the stage-dependent and demographic-specific modulation of EMT drivers. Findings reveal that key EMT regulators such as Slug and Snail are predominantly active during early CRC stages, contributing to tumor initiation and local invasion. At the same time, Twist and Zeb1 become more prominent during advanced stages, facilitating metastasis and tumor progression. Notably, gender differences influence the expression of these factors, with females exhibiting higher levels of Slug and Twist. The study underscores the potential of these transcription factors as stage- and patient-specific biomarkers and therapeutic targets, emphasizing the importance of personalized strategies in CRC management. Future investigations aimed at functional validation and clinical trials could pave the way for stage-specific EMT-targeted interventions, ultimately improving prognosis and treatment efficacy in CRC.***

Keywords: Colorectal cancer, Epithelial-mesenchymal transition, Epithelial-mesenchymal transition TFs, **Slug, Snail.**

I. INTRODUCTION

Epithelial-mesenchymal transition (EMT) is a cellular mechanism that plays a vital role in embryonic development, tissue repair, and disease. When cells are losing their epithelial characteristics and gain mesenchymal properties, allowing them to increase their motility and develop an invasive phenotype [1]. Epithelial cells are usually closely arranged with tight junctions, exhibiting distinct apical and basal features [2]. When EMT is initiated, the epithelial cells lose their tight connections, leading to a breakdown of their organized structures and shifting towards a more flexible and mobile mesenchymal traits that resemble the shapes of scattered stones [3]. This transformation enables the cells to move to new locations within the body [4]. Hence, EMT plays an important role in spreading primary tumor cells to the distant site and thereby developing secondary tumor or metastasis [5]. In the last ten years, increasing research has shown strong evidence of the critical role that EMT plays in the development and spread of various cancers, including CRC [6]. EMT includes the loss of tight junctions (TJs), adherent junctions (AJ), desmosomes, and gap junctions. A family of intracellular adhesion molecules build the adhesion junctions, which include transmembrane proteins like nectin and E-cadherin, related cytoplasmic proteins like catenin that are directly connected to the actin cytoskeleton. These proteins are crucial for forming and preserving connections between cells [7]. Tight junctions (TJs) are complex structures that form barriers between neighboring epithelial cells near the surface, blocking the passage of substances between cells. TJ proteins are comprised of two parts: I) cytoplasmic scaffold proteins, which are topologically present on the intracellular side of the plasma membrane, and II) transmembrane proteins, the extracellular domains of which, cross the plasma membrane and connect with other proteins from the adjacent cells. The transmembrane TJ proteins, including, Occludin (OCLN), claudins (CLDNs), and junctional adhesion molecules (JAMs), form a linear barrier at the apical-lateral membrane of the cell. Cytoplasmic scaffold proteins, including cingulin, and zonula occludens (ZOs) proteins, connect the transmembrane TJ proteins with the intracellular cytoskeletons. Desmosomes are patch-like intercellular junctions connecting adjacent cells with desmoglein and desmocollins to stabilize tissue under significant mechanical stress by linking as anchor points for intermediate filaments. They are located at the lateral sides of plasma membranes [7]. When a mesenchymal cell moves out of its original epithelial layer by breaking down the basement membrane below, it signals the start of EMT [8]. The downregulation of epithelial-specific markers, along with transcription factors that differentiate between epithelial

and mesenchymal states, occurs during the initiation of EMT. As EMT begins, there is a reduction in the expression of epithelial markers such as cytokeratin, E-cadherin, ZO-1, and occludin. In contrast, there is an increase in mesenchymal markers like β-catenin, N-cadherin, and vimentin (VIM) [9], and fibronectin (FN) [10]. EMT is quite complex and requires the activation of special transcription factors and signaling pathways in epithelial cells. Expression of EMT-related transcription factors such as Snail, Twist, Zeb, and Slug are regulated at multiple post-transcriptional and post-translational levels. They promote the mesenchymal state and inhibit the transcription of genes that help in maintaining the epithelial state[11]. This adaptability for tumor cell plasticity is further observed in partially EMT-associated phenotypes as well [12].

A. Epithelial-mesenchymal transition (EMT) transcription factors

The process of EMT, which involves a different type of transcription factors (TFs), including Snail1/Snail, Snail2/Slug, Twist, and Zeb-1, is involved in the orchestration of EMT [13]. This transition is tightly regulated by a network of transcription factors (TFs), which orchestrate gene expression changes to drive the phenotypic switch. The primary EMT-inducing transcription factors, depending on the specific biological context, include Snail (SNAI1), Slug (SNAI2), Twist (TWIST1), and Zinc Finger E-Box Binding Homeobox (ZEB1). It is important to note that EMT regulators are controlled at both mRNA and protein levels through post-transcriptional regulation [14].

B. Key EMT Transcription Factors:

Snail Family SNAI1: Snail is a member of a well-conserved family of C2H2 zinc finger proteins that have been widely studied for their roles in development, cell morphogenesis, and tumor metastasis [15]. It was first identified in Drosophila, where mutant embryos exhibit defects in mesoderm formation during gastrulation (Simpson 1983). It mediates transcriptional repression by recruiting two corepressors: the C-terminal-binding protein (dCtBP) and Ebi, which recruits histone deacetylase 3 (HDAC3). The functionality of Snail as a repressor is compromised when any of the corepressor interaction motifs in its N terminus are mutated. This impairment affects not only its repressor function but, particularly for the dCtBP interaction motifs, its ability to coordinate mesoderm development. An analysis of the repressive effects of Snail across various enhancers showed that its function is dependent on distance. Snail is classified as a short-range repressor that inhibits activators located within 100 base pairs (bp) of the same enhancer or core promoter [15], [16]. The Snail family, which includes Snail, consists of zinc-finger transcriptional repressors that bind to E-box sequences in the promoter regions of epithelial markers, such as E-cadherin. This binding leads to the repression of E-cadherin. The loss of E-cadherin is a hallmark of EMT, which promotes a mesenchymal phenotype. Snail1 is recognized as the master regulator of EMT and is induced by signaling pathways, including TGF-β (Transforming Growth factor beta), Wnt, and Notch. It represses genes associated with epithelial traits while activating genes linked to the mesenchymal phenotype, such as N-cadherin and Vimentin.

C. ZEB Family (Zeb-1)

Zeb1 was first identified as a repressor of the d1-crystallin enhancer in chicken embryos in the early 1990s. At that time, it was thought to play a role in embryogenesis due to its specific expression in mesodermal tissues, the nervous system, and the lens of the chicken embryo. A small group of pleiotropic transcription factors regulates the EMT program. One of the most significant activators of EMT is Zeb1, a transcription factor characterized by double zinc finger and homeodomain motifs. Zeb1 is associated with aggressive behavior, metastasis, treatment resistance, and poor prognosis across various cell types. Also known as TCF8 or DeltaEF1, Zeb1 represses the expression of epithelial genes, a critical feature in the context of metastasis. The expression of Zeb1 is regulated by several signaling pathways, including WNT, NF-kB, TGF-β, COX2, HIF signaling, and various microRNAs. Zeb1 belongs to a family of transcription factors distinguished by two clusters of zinc fingers that facilitate DNA binding, as well as a centrally located homeodomain [17]. Additionally, Zeb1 contains several protein-binding domains, including the Smad Interaction Domain (SID), the CtBP Interaction Domain (CID), and the p300-P/CAF Binding Domain (CBD). Through its zinc finger clusters, Zeb1 can bind to specific DNA sequences known as E-boxes [18]. By recruiting co-suppressors or co-activators via the CID, SID, or CBD, Zeb1 can modulate the expression of its target genes. For instance, Zeb1 directly binds to the E-box in the promoter region of the CDH1 gene, which encodes E-cadherin. This binding recruits the CtBP transcriptional co-repressors and/or the SWI/SNF chromatin-remodeling protein BRG1, leading to the repression of CDH1 transcription and the induction of EMT [17].

Zeb1 can also recruit p300-P/CAF and Smad proteins, allowing it to activate the transcription of genes responsive to TGF-β and promote osteoblastic differentiation [19].

D. TWIST Family (TWIST1)

Twist1, a basic helix-loop-helix (bHLH) domain-containing transcription factor, was originally identified in Drosophila as an essential regulator during embryogenesis, particularly in mesoderm formation, specification, and differentiation [20]. Drosophila embryos harboring Twist1 mutations fail to invaginate properly, resulting in embryos devoid of internal organs with a "Twisted" appearance [20], [21]. In humans, the Twist1 gene is located on 7q21.2, containing two exons and one intron. Mutation of Twist1 in humans leads to Saethre-Chotzen syndrome, a disease of autosomal dominant inheritance characterized by manifestations such as craniosynostosis, ptosis, and hypertelorism [21] . Twist2 is another member of the Twist subfamily of bHLH proteins in humans, which shares great structural similarity with Twist1. Both Twist1 and Twist2 are key regulators in embryonic development and organogenesis. While a great number of studies have extensively demonstrated that Twist1 is implicated in tumor initiation, stemness, angiogenesis, dissemination, and chemoresistance in various carcinomas, sarcomas, and haematological malignancies, the biological functions of Twist2 in tumors are still highly controversial or unexplored [22], [23].

E. EMT TFs' role in Different Types of Cancer

Snail1 is a transcription factor that plays a crucial role in promoting cell movement, which is essential for cancer progression and metastasis. In patients with colon cancer, elevated levels of Snail1 are often linked to poor clinical outcomes, likely due to the downregulation of E-cadherin expression [24], [25]. The high mortality, accounting for over 90% of colorectal cancer (CRC) related deaths, is linked to the ability of cancer cells to spread beyond the large intestine to distant locations [26]. These metastatic abilities of colon cancer cells are closely associated with EMT and transcription factors, which are responsible for their activation. Therefore, it is a discussion of the role of Snail1 in CRC progression and metastasis. The first stage of CRC is adenoma. During cancerogenesis, this lesion progresses to adenocarcinoma and then to metastatic cancer. Adenoma is also the first stage of CRC carcinogenesis, exhibiting Snail1 expression, which is inversely correlated with expression of E-cadherin [26]. Regarding Snail1 expression in colorectal mucosa with no specific pathological changes, the result suggests that Snail1 is not connected with malignant transformation only, but, in normal tissue, this factor may be involved in the maintenance of homeostasis by regulation of cellular proliferation, differentiation, and apoptosis [27]. Snail1 expression was detectable in most of the CRCs but showed no significant association with E-cadherin loss, clinical pathological characteristics, or overall survival. Snail-overexpressing CRC cells were more chemoresistant to oxaliplatin than control cells. Increased Snail expression induces EMT and the CSC-like phenotype in CRC cells, which enhances cancer cell invasion and chemoresistance. In melanoma, cancer cells reside in a heterogeneous tumour microenvironment that acts as a crucial regulator of its progression. Snail1 is an EMT TF that is expressed during development and reactivated in pathological situations, including fibrosis and cancer [28], [29]. Research has shown that Snail1 is activated in the melanoma microenvironment, particularly in fibroblasts. Analysis of mouse models that allow stromal Snail1 depletion and therapeutic Snail1 blockade indicates that targeting Snail1 in the tumour microenvironment decreases melanoma growth and lung metastatic burden, extending mice's survival. Another transcriptomic analysis of melanoma-associated fibroblasts and analysis of the tumours indicate that stromal Snail1 induces melanoma growth by promoting an immunosuppressive microenvironment and a decrease in anti-tumour immunity [30]. Slug and Snail, two closely related genes, both of which promote cell motility, appear to be differentially regulated in melanocytes and melanoma cells [31].It plays an important role in the invasive characteristics of lung carcinoma, influencing the survival of the patients [32]. When Snail is knocked down, it might thus be one option for targeted molecular therapy in lung cancer. Another study showed that Snail knockdown influenced the expression of claudins individually in a cell-line dependent manner but did not influence matrix metalloproteinase (MMP) expressions or activations to any significant degree [32], [33]. Expression of snail is associated with poor prognosis in patients with Lung cancer. Snail has an important role in invasion and metastasis, and silencing the gene may be a potential therapeutic target in Renal cell carcinoma (RCC) [34]. Slug and Snail could be useful immunohistochemical markers for staging and prognosis in patients suffering from various RCCs, representing potential targets for further therapy strategies of renal cancer. Slug statistical analysis indicated that elevated Snail, MMP2, and MMP9 protein expression are significantly worse predictors of disease-free and disease-specific survival of the patients with RCC [34]. In conclusion, these data suggest that Snail has an important role in invasion and metastasis, and that silencing the gene may be a potential therapeutic target in RCCs. Slug is more frequently expressed in tumors with a sarcomatoid component, which confirms its role in the EMT process. The survival period is significantly longer in patients whose tumors do not express Slug. Our results show the correlation of Snail expression, especially nuclear expression, with higher stages of kidney tumors, a higher degree of invasiveness of the tumors themselves, and shorter survival [35]. Our results suggest that Slug and Snail may be useful immunohistochemical markers for staging and

prognosis in patients suffering from various RCCs, representing potential targets for further therapy strategies of renal cancer. In the present study, we have provided the evidence that Snail is immunolocalized to carcinoma cells in high-grade RCCs with correlations to pathological tumor stage and the presence of sarcomatoid carcinoma, and that all metastatic RCCs show strong Snail expression with decreased E-cadherin expression [36], [37]. In lung cancer, Slug has been extensively studied for its role in tumor progression [38]. Research indicates that Slug expression is associated with increased invasion and resistance to targeted therapies. Specifically, Slug can repress E-cadherin expression, leading to enhanced EMT and metastatic potential. Additionally, Slug has been linked to resistance against therapies targeting the epidermal growth factor receptor (EGFR), suggesting its role in mediating treatment resistance in lung cancer [39]. The role of Slug as a mediator of EMT in human colon cancer cells. We demonstrated that the relative level of Slug expression in a panel of human colon cancer cell lines is correlated with critical EMT properties, including loss of E-cadherin as well as increased migration and invasion. To examine this correlation experimentally, we examined the impact of Slug expression in DLD-1 cells., Slug expression in the DLD-1 colon cancer cells leads to morphologic and phenotypic changes consistent with EMT, including increased spindle shape and pseudopodia formation and decreased E-cadherin expression. In our orthotopic model, Slug-expressing DLD-1 cells developed significantly more tumor growth than both the parental DLD-1 cell line as well as the empty vector (control) DLD-1 cells [40], [41]. Slug is a bona fide transcriptional repressor of E-cadherin as well as a regulator of P-cadherin in melanoma cells, and its knockdown attenuated invasive behavior and blocked SPARC-enhanced cell migration [42]. High expression of Slug in gastric cancer tissue was associated with lymph node metastasis and poor survival. Evaluation of Slug would be useful for discriminating patients at high risk of lymph node metastasis in early gastric cancer [43]. Researchers have demonstrated that knocking down Twist1 inhibits proliferation and increases the percentage of apoptotic cells in CRC cell lines. All the findings suggested that Twist1 enhances the growth and resistance to chemotherapy of CRC cells [44]. Twist1 may be a potential prognostic marker and a molecular target for therapies. Taken together, these studies highlight the mechanistic involvement of Twist1 in the deregulation of factors that maintain genome stability during EMT in CRC cells. Twist1 overexpression enhances genome instability in the context of EMT, which further contributes to cellular heterogeneity. In addition, these studies imply that Twist1 downmodulates nuclear lamins that further alter the spatiotemporal organization of the cancer genome and epigenome. In genetic background, CRC cells nevertheless maintain their overall ploidy, while the downstream effects of Twist1 enhance CIN and DNA damage, enriching for sub-populations of aggressive cancer cells [45]. It was found a negative correlation in between the Twist level and p53 level, probably due to transcriptional regulation. Our results have identified Twist as a critical regulator of gastric cancer cell proliferation and migration, suggesting a potential therapeutic approach to inhibit the growth and metastasis of gastric cancer through inactivation of Twist [46]. Twist2 promotes kidney cancer cell proliferation and invasion by regulating ITGA6 and CD44 expression in the ECM-receptor interaction pathway [47]. Furthermore, in comparison to control cells, the lung cancer cells with ectopic expression of twist showed a significant phenotype alteration through EMT and an increasing ability to migrate in vitro, in part, due to a tenfold increase in matrix metalloproteinases activity and almost a 60% increase in modulation of focal adhesion kinase activity, although a contribution of microRNA appeared unlikely in our study [48]. Our present analysis of twist expression in lung cancer provides clues to a comprehensive understanding of the mechanisms by which metastasis often develops in lung cancer. Another TF Zeb1, enhances LOXL2 transcription and expression through direct binding to its promoter. Gain-of-function assays indicate that LOXL2 expression is involved in increased cell migration and invasion, but does not affect cell proliferation. IHC evaluation of Zeb1/LOXL2 provides important prognostic information for CRC patients [49]. Furthermore, Zeb1 knockdown initiated a chemosensitization effect, induced G1/S arrest, and increased apoptosis, which functionally validated the three Zeb1 downstream targets. In summary, the present study identified three DDR-associated genes as Zeb1 downstream targets, and demonstrated that their suppression by Zeb1 contributes to Zeb1-mediated chemoresistance [50]. In addition, Zeb1 expression in the early-stage IB primary NSCLC correlated with the tumor-node-metastasis stage. These findings indicate that Zeb1-induced EMT and associated molecular changes in ESRP1 and CD44 contribute to early pathogenesis and metastatic potential in established lung cancer [51]. Despite its growth-inhibiting effect, EGFR inhibitor-induced Zeb1 strongly promotes EMT-dependent resistance to EGFR inhibitors partially through NOTCH1, suggesting a multifunctional role for NOTCH1 in EGFR-mutated cells [52]. These results support a previously unrecognized genetic cell context-dependent role for Zeb1 and suggest that NOTCH1 may be a useful target for treating resistance to EGFR inhibitors, especially EMT-driven resistance. The results showed that the relative expression levels of Zeb1 were significantly higher in CRC tissues compared to the normal adjacent mucosa, and higher expression of Zeb1 correlated with liver metastasis [53].

Kaplan-Meier analysis indicated that patients with high Zeb1 had a poor overall survival. Moreover, the multivariate analysis showed that high expression of Zeb1 was an independent predictor of overall survival. The results also demonstrated that Zeb1-AS1 positively regulates E2F2 expression by competitively binding to miR-365a-3p. It was further revealed to enhance liver cancer

cell proliferation. Thus, these results indicate that Zeb1-AS1 is required for liver cancer progression in a ceRNA-dependent manner [54]. In this study, we applied quantitative real-time polymerase chain reaction (RT-qPCR) and found that LINC01559 expression was significantly enhanced in GC cells [55].

In return, LINC01559 recruited insulin-like growth factor 2 mRNA binding protein 2 (IGF2BP2) to stabilize Zeb1 mRNA to upregulate Zeb1 in GC cells [54], [55]. In short, the findings in this research might provide a novel target for GC treatment. Upregulation of Zeb1 and Zeb2 mRNA in KIRC correlated with Anoikis, cytotoxic immune cell infiltration, and patient survival outcomes. Zeb1 and Zeb2 are regulated by microRNAs. Here, we demonstrated that Zeb1 directly binds to the CD274 promoter, induces PD-L1 mRNA transcription, and increases its expression at the cell membrane [56].

II. MATERIALS AND METHODS

1) Download of expression data from the clinical tissue dataset.

To investigate the expression patterns of specific proteins within clinical tissues, a crucial step involves downloading relevant data from the Human Protein Atlas (HPA) website (https://www.proteinatlas.org). The HPA offers a comprehensive resource for exploring protein expression across various human tissues, including valuable clinical data [57]. Navigating to the appropriate section of the HPA, search for our EMT transcription factors Snail, Slug, Zeb-1, and Twist protein, and access the available data sheets. Users can typically search for specific genes or proteins of interest and then access associated expression data. This often involves selecting the "Tissue" tab or a similar categorization, where they can then find options to download expression profiles, usually in a user-friendly format like Excel. These downloaded datasets provide raw quantitative information on protein abundance within different clinical tissue samples, forming the foundation for subsequent bioinformatic analysis and biological interpretation.

2) Downloading of normal and cancerous datasets.

Further analysis of protein expression patterns in both normal and cancerous tissues using the HPA website, the initial and crucial step involves systematic downloading of relevant datasets. Navigating to the HPA website (https://www.proteinatlas.org) provides access to its comprehensive database [58]. Users can then influence the site's spontaneous search functionalities and data visualization tools to identify and select datasets about specific normal tissues and their corresponding cancerous counterparts. Download the extensive clinical transcriptome data to compare colon cancer to other epithelial malignancies to assess the expression of EMT transcription factors, which include Twist, Zeb-1, Slug, and Snail. Strong baseline comparisons were provided by using datasets from normal tissue.

3) Screening expression level data from a cancer stage-wise dataset.

The ability to effectively screen expression level data from a cancer stage-wise dataset holds immense significance for advancing at various points in the disease progression.

The primary goal is to identify significant differences in expression patterns that correlate with specific cancer stages, from early-stage initiation to advanced metastatic disease. For comparing the expression of EMT-related transcription factors (Snail, Slug, Zeb-1, Twist) between normal tissue samples and other epithelial-derived cancers across tumor stages. Furthermore, these stage-specific expression signatures can serve as powerful prognostic indicators, helping clinicians stratify patients and predict disease aggressiveness [59]. Ultimately, this screening process acts as a critical first step in pinpointing molecular targets that are actively contributing to disease progression at particular stages, paving the way for the development of more precise and personalized treatment strategies.

4) Screening expression level data from a cancer gender-wise dataset.

To screen expression level data from a CRC gender-wise dataset, specifically focusing on EMT-related transcription factors. We aimed to compare the expression patterns of key EMT regulators – Snail, Slug, Zeb-1, and Twist between CRC patient samples. By leveraging the comprehensive and standardized clinical and transcriptomic datasets available through the expression levels were meticulously normalized and then stratified by gender. This gender-wise stratification allowed us to identify potential gender-specific trends or differences in the expression of these critical EMT factors, which may contribute to distinct tumor behaviors or responses to therapy in male versus female patient populations.

5) Analysis of combined expression data for screening of the most potentially vital transcription factors for CRC.

Analysing combined expression data offers a powerful strategy for identifying potentially vital TFs. By integrating transcriptomic profiles from diverse patient cohorts and experimental models, we can leverage the collective insights to pinpoint TFs that exhibit consistent dysregulation across various CRC subtypes and stages. This meta-analysis allows for the identification of TFs whose expression patterns correlate significantly with disease progression, patient outcomes, or specific molecular features, such as mutational status or pathway activation. Furthermore, combining data from different sources enhances statistical power and reduces the impact of batch effects or noise inherent in individual studies, thereby increasing the confidence in the identified TF candidates [58], [60]. Ultimately, the systematic analysis of combined expression data serves as a crucial first step in pinpointing the most influential TF regulators that could serve as therapeutic targets or biomarkers for effective CRC management.

6) Statistical analysis.

Statistical analysis formed the cornerstone of our investigation into the differential involvement of key EMT regulators. By meticulously integrating multiple datasets, which crucially included comprehensive information on cancer stages and fragment per kilobase of transcript per million mapped reads (FPKM) values, we embarked on a rigorous analytical journey. The FPKM values, serving as a quantitative measure of gene expression, were carefully normalized to account for variations in sequencing depth and RNA composition across different samples [57]. Subsequently, we employed a suite of robust statistical tests to identify significant differences in the expression levels of EMT regulators.

III. OBSERVATION

The study observes that EMT transcription factors in CRC exhibit distinct, stage-dependent expression patterns influenced by demographic factors such as age and gender. Specifically, Slug and Snail are more actively expressed during early stages, particularly in younger and female patients, while Twist and Zeb1 become increasingly prominent in later stages, correlating with metastasis. Additionally, gender differences suggest a higher EMT activity in females, potentially contributing to differences in disease progression and prognosis. These findings highlight the complex, dynamic regulation of EMT in CRC and underscore the importance of considering patient-specific factors for targeted therapeutic strategies.

IV. RESULTS AND DISCUSSIONS

A. Stage-Specific Dynamics of EMT Transcription Factors in Colorectal Cancer: A Comparative Cancer-Type Analysis.

EMT is a key process driving cancer progression, invasion, and metastasis. To understand the role of EMT in CRC, we analysed the expression patterns of four core EMT transcription factors, Slug, Snail, Twist, and Zeb1, across different clinical stages of various epithelial cancer data. This analysis provides insight into the temporal regulation of EMT during CRC progression. The stage-wise comparison highlights CRC's unique EMT profile relative to other cancer types. Slug TF expression demonstrates distinct tissue- and stage-specific patterns across normal and cancer samples. In normal tissues, the highest Slug expression is observed in the lung, followed by the CRC, while the liver, Renal, stomach, and melanoma show relatively lower levels, indicating baseline tissue-specific expression. During cancer progression, Slug expression becomes particularly prominent in stomach and renal cancers starting from Stage I, with consistently high levels maintained through Stages II to IV, suggesting a significant role in early tumorigenesis and later metastatic progression in these tissues. Moderate expression is seen in CRC and melanoma, while liver consistently shows low Slug levels across all stages, implying a limited role or compensation by other EMT regulators in hepatic cancers. Notably, lung cancer shows declining Slug expression in later stages, hinting at its reduced relevance in advanced disease. Overall, Slug appears to play a critical and sustained role in EMT-related processes, especially in stomach and renal cancers.

Snail TF expression displays a dynamic yet tissue-specific pattern across normal and cancer stages. In normal tissues, the lung exhibits the highest Snail levels, while colon and skin show lower expression, and liver, kidney, and stomach maintain moderate levels, reflecting tissue-specific baseline regulation. During cancer progression, Snail expression is highest in stomach cancers starting from Stage I and remains elevated through Stages II to IV, suggesting a strong and persistent involvement in EMT-driven processes. CRC and renal cancers also show notable Snail expression in early stages, while melanoma becomes increasingly expressive by Stage III, indicating its emerging role in advanced disease. In Stage IV, Snail remains prominently expressed in renal and stomach cancers, while other cancer types display moderate levels. This pattern highlights Snail's key role in EMT and tumor progression, especially in stomach, renal, and late-stage melanoma. Twist TF expression reveals a highly selective and stage-specific pattern, with notably high expression in normal skin tissue, suggesting a physiological role in skin development or structure. In cancer stag-

es, Twist shows a dramatic spike in expression in melanoma during Stage I, reaching around 30 units, indicating a strong involvement in early EMT and tumor initiation. This elevated expression continues in Stage II, particularly in melanoma and to a lesser extent in renal cancers, while other cancers like CRC and liver remain low, emphasizing Twist's specificity to certain tumor types. However, from Stage III onward, Twist expression significantly drops across most cancers, with only modest levels in lung and CRC, implying that its role may be restricted to early tumor transformation. In Stage IV, Twist expression peaks again in lung cancer but remains low elsewhere, supporting the idea that Twist functions predominantly in early or intermediate tumor progression rather than during metastasis in most cancer types. Zeb1 TF expression exhibits consistently high levels in specific tissues and cancer types, highlighting its sustained role in tumor progression. In normal tissues, the highest Zeb1 expression is seen in the colon, followed by the lung and skin, indicating a baseline functional role in these epithelia. During cancer development, Zeb1 expression is markedly elevated in stomach and renal cancers from Stage I onward and remains persistently high through Stages II, III, and IV, suggesting a continuous involvement in driving EMT and promoting tumor aggressiveness in these tissues. CRC, lung, and melanoma show moderate expression in early stages, with melanoma levels increasing during progression. In advanced stages, particularly Stage IV, Zeb1 remains elevated in stomach and renal cancers, while CRC, lung, and liver maintain stable but comparatively lower levels. This pattern underscores Zeb1's pivotal and sustained contribution to EMT and metastatic potential, especially in gastric and renal tumor contexts. An integrated analysis of the four key EMT transcription factors Slug, Snail, Twist, and Zeb1 demonstrates that CRC maintains a moderate but consistent expression profile across all stages, indicating a balanced engagement with EMT-related pathways (Fig. 1), (Table 1**)**. In Stage I, CRC ranks mid-range (3rd–4th), showing moderate expression of Snail and Slug and lower levels of Twist and Zeb1. This pattern continues in Stage II, where CRC falls slightly to the 4th–5th position due to relatively low Twist expression. In Stage III, CRC shows elevated levels of Slug, Snail, and Twist, moving up to a higher EMT activity rank (2nd–3rd), while Zeb1 remains stable. By Stage IV, CRC returns to a mid-range position (3rd–4th), with consistent expression of Slug, Snail, and Zeb1, and a slight increase in Twist. Overall, CRC demonstrates a stable EMT transcriptional footprint, without extreme fluctuations, reflecting a sustained but controlled role of EMT in its tumor progression, with the most notable activity observed during Stage III. In summary, CRC displays a moderate and stage-specific EMT transcriptional profile, characterized by the expression of Slug, Snail, Twist, and Zeb1. In early stages (I–II), partial EMT is indicated by moderate Slug and Snail expression, while Twist and Zeb1 remain low. EMT activity peaks in Stage III with increased expression of Slug, Snail, and Twist, supporting invasion and progression. In Stage IV, CRC maintains moderate expression of all factors, suggesting a hybrid epithelial/mesenchymal state for metastatic adaptability. Overall, EMT in CRC is strategically activated, with its highest impact during intermediate progression.

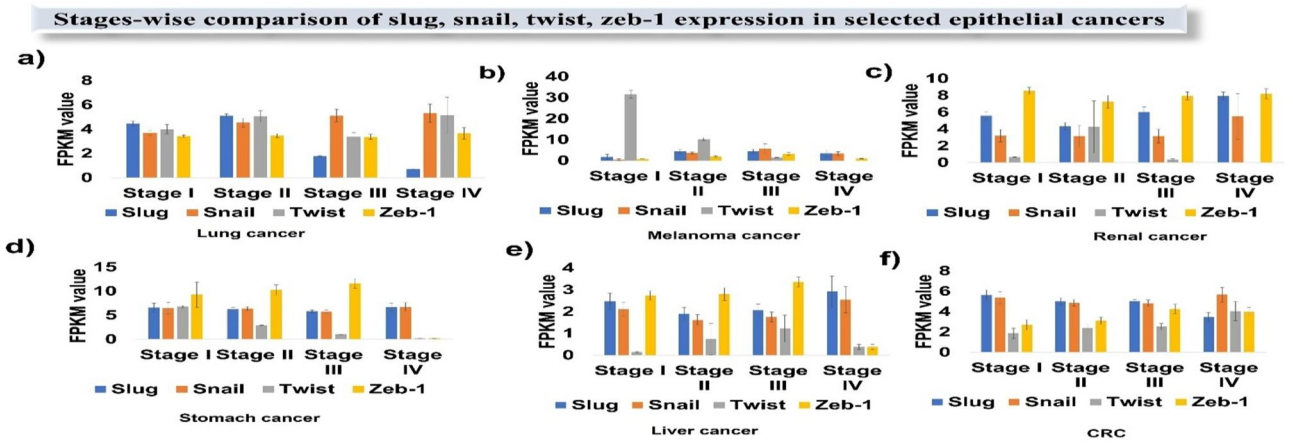


Fig 1: Stage-wise Comparison of EMT Transcription Factor Expression Across Epithelial Cancers. a. Lung cancer: Snail and Twist expression increase slightly from stage I to III, with a drop in stage IV. b. Melanoma: Twist shows high expression at stage I, decreasing in later stages; other TFs remain low. c. Renal cancer: Zeb-1 is consistently high across all stages; other TFs show minor stage-wise variations. d. Stomach cancer: High Zeb-1 expression in early stages, dropping sharply at stage IV. e. Liver cancer: Moderate and steady expression of all TFs across stages. f. CRC: All four TFs show relatively stable expression, with minor differences across stages.

Table 1. Clinical stage-wise patient sample of six epithelial cancers.

Transcription factors (TFs)	Stages	Epithelial cancers					
		LUNG	LIVER	STOMACH	RENAL	MENALOMA	CRC
SLUG	STAGE I	506	69	45	444	2	49
	STAGE II	274	44	110	110	62	123
	STAGE III	163	81	143	187	27	104
	STAGE IV	32	4	34	187	4	47
SNAIL	STAGE I	267	69	35	19	2	39
	STAGE II	117	44	113	25	62	98
	STAGE III	81	81	151	14	27	72
	STAGE IV	25	4	33	6	4	35

Transcription factors (TFs)	Stages	Epithelial cancers					
		LUNG	LIVER	STOMACH	RENAL	MENALOMA	CRC
TWIST	STAGE I	506	69	45	444	2	49
	STAGE II	274	44	110	101	62	123
	STAGE III	163	81	143	187	27	104
	STAGE IV	32	4	34	102	4	47
ZEB-1	STAGE I	507	69	45	444	2	49
	STAGE II	273	44	110	101	62	123
	STAGE III	163	81	143	187	27	104
	STAGE IV	32	4	34	102	4	47

B. Comparative Analysis of EMT-Related TFs Across Age Groups in CRC Patients.

The age-wise comparison of EMT transcription factors in CRC reveals distinct expression patterns for each factor, though overall age-related differences are relatively subtle. Slug expression remains stable in normal colon tissues but shows a gradual increase in CRC patients, particularly in the 50–79 age group, suggesting enhanced EMT activity with advancing age. In contrast, Snail is slightly elevated in younger CRC patients (30–49), indicating a possible role in early-onset CRC, while remaining comparable to normal levels in older patients. Twist also follows an age-dependent trend, with the highest expression in younger CRC patients (30–39) and a steady decline in older age groups, implying its involvement in aggressive early-stage tumors. Zeb1 exhibits a markedly different profile, with high expression in normal tissues across all ages but significantly reduced levels in CRC patients, regardless of age, indicating a general downregulation possibly due to tumor-related suppression. While each factor shows some age-associated differences, the limited variability across the 30–79 range suggests that age alone may not be the primary determinant of EMT factor regulation in CRC, prompting further exploration through gender-wise comparisons. Despite these observations, the overall differences in TF expression between age groups remain subtle. Since the age range of 30–79 is common to most patients (Fig. 2), (Table 2) and doesn't reveal strong divergence in EMT profiles, we extended the analysis to a gender-wise comparison to explore whether sex-based biological differences might better explain variations in EMT factor regulation and CRC progression. In summary, Slug and Twist are more elevated in younger CRC patients, implying early EMT activation. Snail plays a variable role, while Zeb1 is suppressed in CRC compared to normal tissue, regardless of age**.**

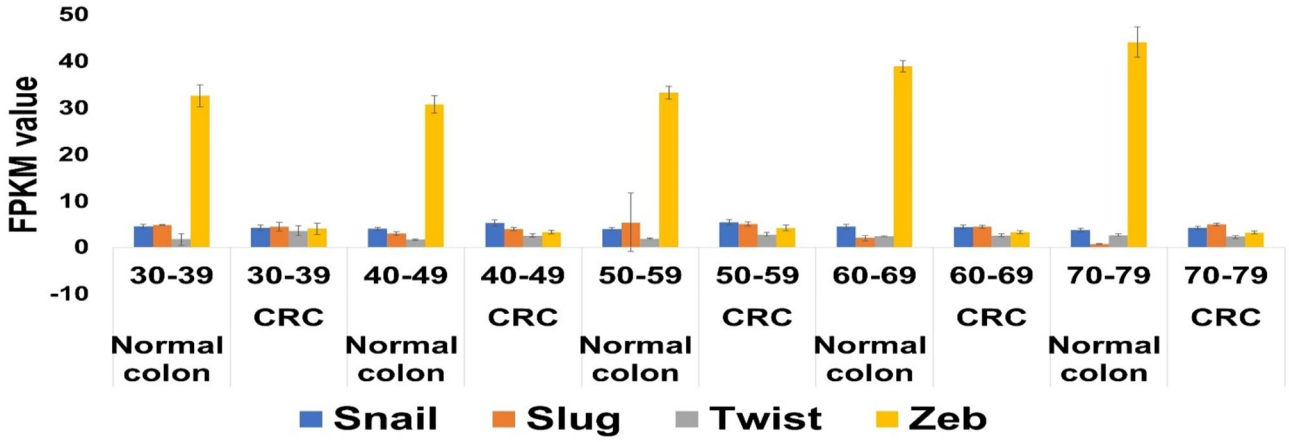


Fig. 2: Age- and Stage-specific Expression of EMT Transcription Factors in CRC. A. FPKM values of EMT transcription factors (Snail, Slug, Twist, Zeb-1) in normal colon and CRC tissues across different age groups (30–39 to 70–79 years). Zeb-1 shows markedly higher expression in CRC tissues across all age groups compared to normal colon, while other TFs show moderate variations.

Table 2. Clinical patient sample for Normal Colon vs CRC.

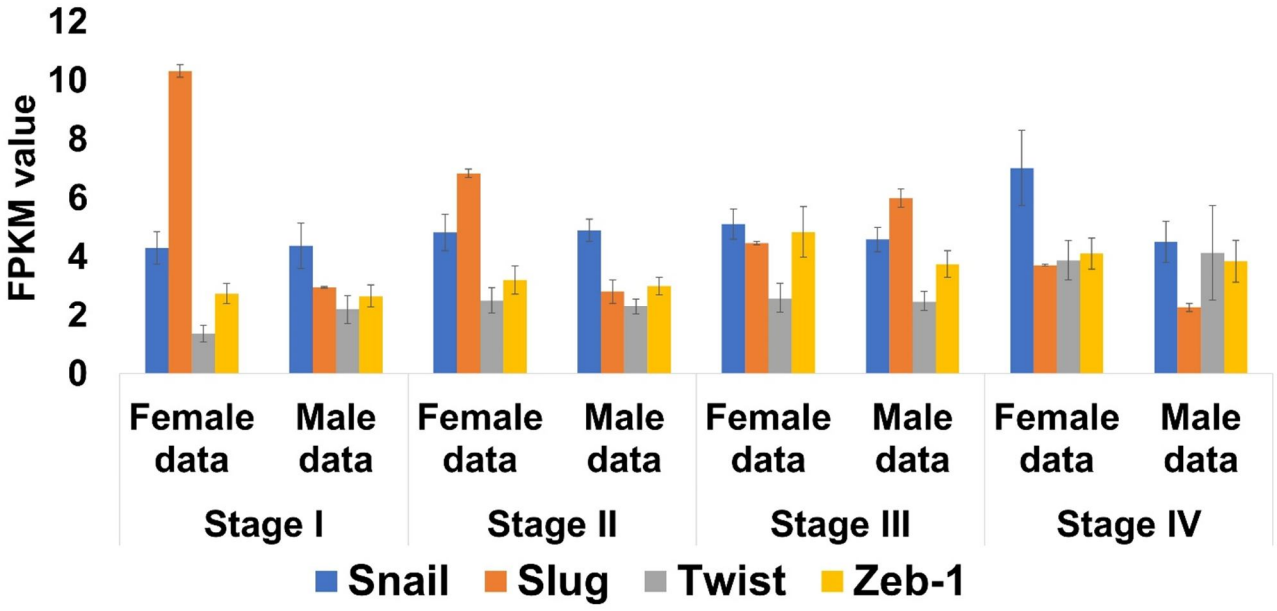
Cancer	**Age**	**Epithelial-mesenchymal transition TFs**							
CRC	**30-39 age**	Normal colon data	CRC	Normal colon data	CRC	Normal colon data	CRC	Normal colon data	CRC
		86	12	166	9	86	12	86	12

Cancer	Age	Epithelial-mesenchymal transition TFs							
	40-49 age	Normal colon data	CRC	Normal colon data	CRC	Normal colon data	CRC	Normal colon data	CRC
		129	38	129	27	136	38	129	38
	50-59 age	Normal colon data	CRC	Normal colon data	CRC	Normal colon data	CRC	Normal colon data	CRC
		245	68	245	48	245	68	245	68
	60-69age	Normal colon data	CRC	Normal colon data	CRC	Normal colon data	CRC	Normal colon data	CRC
		217	92	217	71	217	92	217	92
	70-79 age	Normal colon data	CRC	Normal colon data	CRC	Normal colon data	CRC	Normal colon data	CRC
		23	95	21	64	22	95	22	95

C. Gender-wise Differences in EMT Transcription Factor Expression in Colorectal Cancer.

The gender-wise comparison of EMT transcription factors in CRC indicates that female patients may exhibit stronger EMT-associated molecular signatures, particularly during early and advanced stages (Fig. 3). Slug expression is markedly higher in females at Stage I (~10 units) and declines with progression, while males consistently show lower levels, suggesting that Slug may play a more dominant role in initiating EMT and early tumor development in females. Snail expression remains relatively stable

across both sexes (~4–5 units), with slightly more variability in females, indicating a context-dependent but not sex-specific role. Twist expression increases progressively in females, peaking at Stage IV, whereas males show lower and more variable expression, highlighting a more sustained involvement of Twist in metastasis and late-stage EMT in females. Zeb1 levels are uniformly low in both sexes, with a slight increase in females at Stage III, suggesting limited but stable activity. These patterns collectively suggest that females may exhibit a higher EMT transcriptional load, potentially contributing to increased CRC susceptibility, progression, or aggressive phenotypes compared to males. Females exhibit higher early Slug expression and a progressive rise in Twist, indicating



potential sex-specific EMT regulation. Snail and Zeb1 show minimal gender-dependent variation.

Fig. 3: Stage-wise and sex-specific expression levels of Snail, Slug, Twist, and Zeb-1 in CRC samples. Zeb-1 expression remains relatively stable across stages, with a noticeable increase in Snail and Slug expression in later stages, particularly among females.

V. CONCLUSION

The comprehensive evaluation of EMT transcription factor expression in CRC across stages, age groups, and genders highlights a coordinated and dynamic regulation of EMT throughout disease progression. In the early stages (Stage I and II) of CRC, Slug and Snail emerge as key drivers of EMT initiation.

Their elevated expression, particularly in younger patients and female individuals, suggests that these factors are critical in establishing early epithelial-to-mesenchymal plasticity, facilitating tumor cell detachment, local invasion, and early stromal interactions. This phase represents the transition from benign epithelial behavior to an invasive phenotype, setting the foundation for further tumor progression. As the disease advances into Stage III and IV, there is a noticeable shift in the EMT landscape, with Twist and Zeb1 taking on more dominant roles. Twist expression becomes increasingly prominent in later stages, especially in female patients, indicating its contribution to sustained EMT, migration, and possibly distant metastasis. Zeb1, although consistently lower in CRC compared to normal tissues, shows a relative increase in advanced stages, suggesting a reactivation or compensation mechanism that supports prolonged mesenchymal traits and survival advantages in hostile microenvironments. Notably, gender-based differences are evident throughout: females consistently show higher expression of Slug and Twist, pointing to a sex-specific modulation of EMT that could influence disease aggressiveness, therapeutic response, or prognosis. Age-wise, the variation in expression is subtle but informative younger CRC patients tend to express higher levels of Snail and Twist, hinting at a more aggressive EMT phenotype in early-onset CRC cases. In contrast, Slug becomes more prominent with increasing age, peaking in older individuals, potentially reflecting age-associated epigenetic or inflammatory influences on EMT regulation. Taken together, these findings underscore a stage-dependent transition in EMT drivers with Slug and Snail initiating EMT in early CRC and Twist and Zeb1 contributing to progression and metastasis in later stages. The interplay of age and gender further refines this regulatory pattern, offering insight into how demographic factors may influence EMT dynamics and CRC behavior. These observations not only enhance our under-

standing of EMT in CRC but also suggest that targeted interventions could be more effective if tailored to stage- and patient-specific EMT profiles.

VI. FUTURE DIRECTIONS

Based on the observed stage-, age-, and gender-specific expression patterns of EMT transcription factors in CRC, several avenues for future research emerge. First, functional validation of the roles of Slug, Snail, Twist, and Zeb1 in stage-specific EMT progression is essential, particularly through in vitro and in vivo models that mimic early- versus late-stage CRC. These models could help decipher the molecular mechanisms through which each factor contributes to invasion, metastasis, and therapy resistance. Second, the gender-based differences, especially the heightened EMT activity in female patients, warrant further investigation to understand the underlying hormonal, genetic, or epigenetic factors that may regulate EMT differently in males and females. Additionally, longitudinal patient studies integrating gene expression with clinical outcomes such as recurrence, metastasis, and survival will be vital to determine the prognostic significance of EMT factor expression across CRC stages. The potential for EMT-targeted therapies should also be explored, with the possibility of stage-specific interventions, such as inhibiting Slug or Snail in early-stage CRC to delay progression, or targeting Twist and Zeb1 in late-stage disease to suppress metastasis. Lastly, developing personalized treatment strategies based on a patient's EMT profile taking into account their age, sex, and tumor stage, could pave the way for more precise and effective CRC management.

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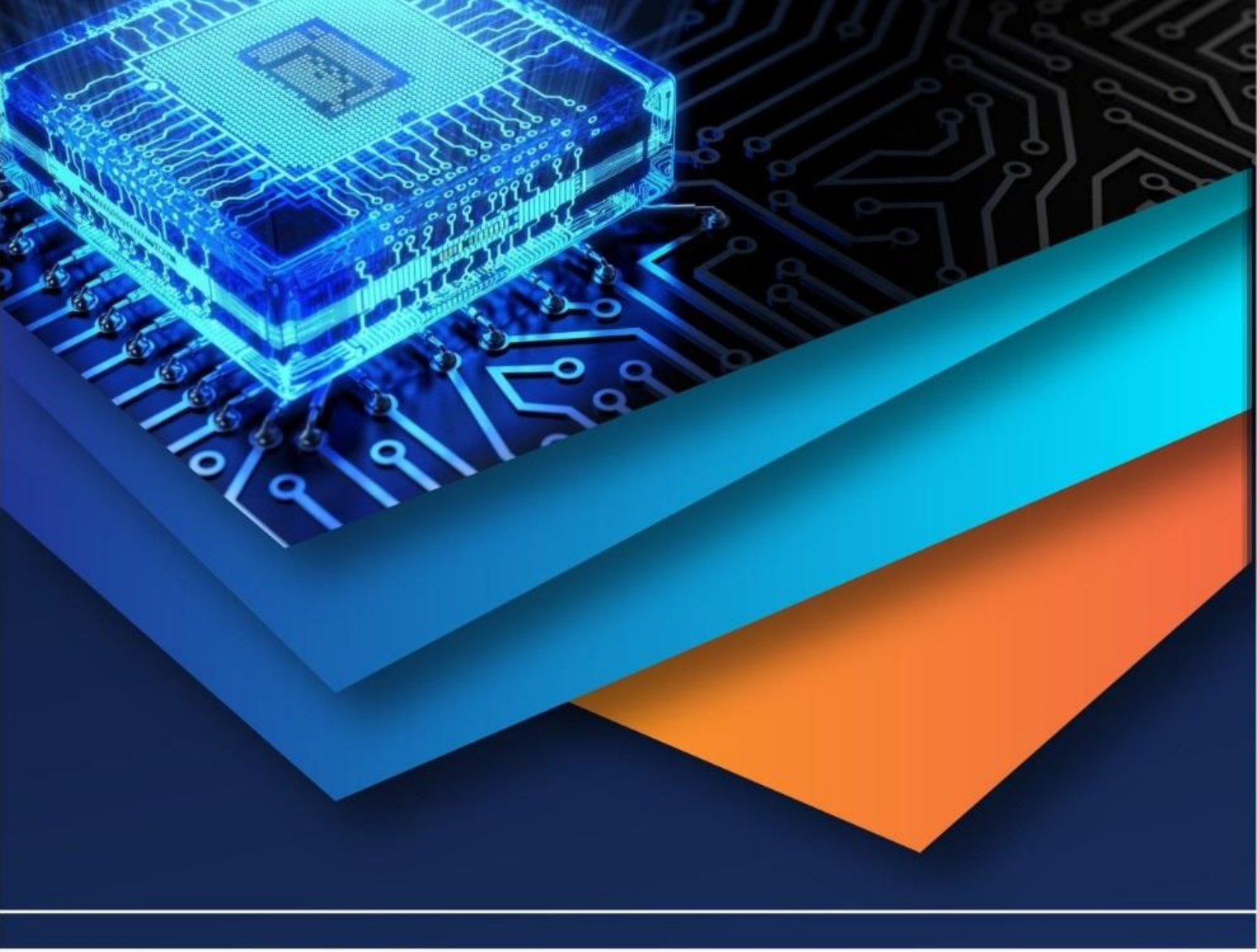

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