ERBB3 Methylation and Immune Infiltration in Tumor Microenvironment of Cervical Cancer

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Research Article

Keywords: ERBB3, Methylation, m6A methlation, Immune infiltration, CESC

Posted Date: January 10th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1156359/v1

Abstract

ERBB3, a member of the ERBB family of receptor tyrosine kinases, plays an important role in cancer, despite its lack of intrinsic Carcinogenic mechanism of CESC. Research on bioinformatics methods through multi-omics, this work proves that ERBB3 gene mutation, methylation modification have extensive regulatory mechanisms on the CESC microenvironment. We found that ERBB3 is involved in carcinogenesis of cervical cancer and is not associated with its prognosis. The carcinogenic mechanism is mainly related to the suppression of the immune system between TILS and the methylation of the RNA level. Our study indicated ERBB3 is more likely to be a carcinogenic factor than a key prognostic factor for cervical cancer. Methylation of ERBB3 may work as a chekpoint immunotherapy target in CESC, DNA methylation modification of the 4480 base pair downstream of ERBB3 transcription initiation site was the highest.

Simple Summary

We first explore the carcinogenic effects of ERBB Family based on TCGA (Cancer Genome Atlas) and GEO (Gene Expression Comprehensive) datasets in cervical cancer. ERBB family is highly expressed in most cancers. The expression of ERBB3 is significantly associated with the prognosis of cancer patients. The relationship between the ERBB3 gene mutation and its clinical significance was studied, focusing on the functional mechanism of the protein nucleic acid methylation and tumor immune microenvironment in cervical cancer.

1. Introduction

A comprehensive genomic and molecular biology study of cervical cancer in the 2017 Cancer Genome Atlas (TCGA) project[1] first proposed ERBB3 as a new significant mutation gene (SMG) in cervical cancer and ERBB3 (HER3) as a therapeutic target. ERBB receptor tyrosine kinase family[2] regulates a variety of biological processes, including cell proliferation, migration, invasion and survival [3][4]. The family comprises four members: ERBB-1, also known as epidermal growth factor receptor (EGFR) HER-1, ERBB-2 (HER-2), ERBB-3 (HER-3) and ERBB-4 (HER-4). High expression or aberrant activation of epidermal growth factor receptor (EGFR) is related to tumor progression and therapy resistance[5] across cancer types[6][7], including CESC (cervical and endocervical cancer)[8]. ERBB3 plays a key role in driving the proliferation and survival of cancer-causing cells in cervical tumors[9][10]. ERBB3 is the least studied member of the ERBB family. However, recent evidence supports the key role of ERBB3 in cell transformation and tumor malignancy[11] [12].

Recent studies have focused on the role of RNA m6A modification in tumorigenesis and development [13] [14]. Abnormal DNA methylation is an early event in tumorigenesis. The study of disease-specific methylation markers can provide new ideas for cancer screening, diagnosis and treatment [15]. In vitro laboratory tests showed that in the evolution of cervical cancer, the degree of methylation was related to the degree of cervical lesions [16]. The overall methylation level increased with the carcinogenesis process, and the increase of methylation level would increase the severity of cervical diseases [17].

However, it is unclear whether methylation modification also plays a potential role in tumor microenvironment (TME) formation and immune regulation. DNA methylation is an early event of cervical cancer. At present, there is no unified understanding about which gene methylation detection can effectively predict or early detect precancerous lesions. The research on molecular markers of cervical cancer methylation is still in the early stage. The existing research shows us the prospect of clinical application of methylation, New methylation markers will become a useful tool for accurate diagnosis of cervical cancer.

Based on 22 m6A regulators, this study comprehensively evaluated the methylation modification patterns of 607 cervical cancer samples, systematically studied the correlation between methylation modification patterns and immune cell infiltration in tumor microenvironment, and sought more effective and accurate immunotherapy strategies.

2. Materials And Methods

2.1.Data

The TCGA data involved in this study are downloaded from UCSC cancer browser (http://xena.ucsc.edu) with 10 GTEx normal samples; 3 TCGA adjacent samples ; TCGA tumor samples. Methylation analysis data mainly include : (1) Illumina Infinium Human Methylation450K BeadChip methylation data. We take the basic beta value for analysis ; (2) Illumina HiSeq 2000 RNA sequencing data. We download the level3data in TCGA, which is standardized by log2 (FRKM + 1).

2.2. Gene expression analysis

Gepia2 [18] (http://gepia2.cancer-pku.cn) was used to compare the expression of ERBB Family and m6A regulators [19]. UALCAN(http://ualcan.path.uab.edu) was used to To study the correlation between target gene and clinical data in CESC (Chandrashekar et al., 2017) [20].

2.3. RNA m6A methylation analysis data

The literature related to m6A RNA methylation was searched in the NCBI-pubmed literature database (http://www.ncbi.nlm.nih.gov/ncbisearch/). After extensive reading of the literature, 22 m6A regulators that have been clearly identified in our study, including m6A methyltransferase (writer) : METTL14, METTL3, METTL16, WTAP and VIRMA ; m6A demethylase (eraser) : ALKBH5, FTO; and m6A binding proteins (reader) : YTHDF3, HNRNPA2B1, HNRNPC, YTHDF2, YTHDF1, YTHDC1, YTHDC2, IGF2BP2, IGF2BP1, RBM15B, CBLL1, ZC3H13, ZCCHC4. CBioPortal (http://www.cbioportal.org/) is used to analyse Genomic multiomics data of m6A regulators in CESC.

2.4. Genetic alteration analysis

After logging into the cBioPortal web (https://www.cbioportal.org/) [21][22], in the ‘Quick Selection’ section, we selected 'TCGA Pan Cancer Atlas Studies' and entered 'ERBB3' to query the gene change characteristics of ERBB3. The frequency, mutation type and CNA (copy number change) results of CESC in TCGA were observed in the 'Cancer Types Summary' module. The information of ERBB3 mutation sites can be displayed

in the protein structure diagram and three-dimensional structure diagram through the ‘Mutations’ module. Kaplan-Meier diagram with logarithmic rank P value was also generated by the ‘Comparison‘ module.

2.5. Immune infiltration analysis

Tumor development and treatment are closely related to the immune system in tumor microenvironment. In order to promote the comprehensive study of tumor immune interaction, through TISIDB (http://cis.hku.hk/TISIDB) to analysis the association between methylation of ERBB3 and immune characteristics of TCGA cancer types, such as lymphocytes, immunomodulators and chemokines, was calculated in advance. In TISIDB, we cross-validated the role of interest genes in tumor immune interaction through literature mining and high-throughput data analysis [23].

2.6. The Human Protein Atlas Gene set enrichment analysis database

The "Human Proteome" chapters provide a knowledge-based analysis and entry into the Human Protein Atlas (https://www.proteinatlas.org/) from different defined transections of the human tissue proteome. Analysis of Four Proteins Expressed in the ERBB Family in CESC Patients Using Tissue Microarray (TMAs) Immunohistochemistry (IHC).

2.7. GO and KEGG Analysis

Using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) [24] (https://david.ncifcrf.gov/), Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis based on co-expressed genes (https://www.kegg.jp/), $P < 0.05$.

2.8. Survival prognosis analysis

We used the "survival map" module of gepia2 to obtain the OS (overall survival) and DFS (disease-free survival) significance map data of four genes in ERBB family of CESC in all TCGA. The high and low groups expression thresholds is set as the high cut-off (50%) and low cut-off (50%) values. The hypothesis was tested by log rank test, and the survival map was obtained through the "survival analysis" module of gepia2. The correlation between overalls survival time and m6A regulators expression are measured using Kaplan-Meier Plotter Database (https://kmplot.com/analysis/) [25].

2.9. Statistical Data

Statistical analysis on the TCGA data were performed with R software (version 3.6.0). In the previous analysis, except specifically mentioned, P value less than 0.05 was considered statistically significant.

3. Results

3.1.Gene differential expression and clinical significance of ERBB3 in CESC

We first analyzed the expression pattern of EGFR,ERBB2,ERBB3,ERBB4 in CESC tumor and normal tissues. ERBB3 obtain a significant difference in CESC(Fig. 1A).At the same time, GEPIA2 provided the structural map of the isomeric protein domain [26] based on Pfam prediction to show the structural differences among the isomers (Fig. 1B). Fig. 1C,D,F analyze the clinical significance of the ERBB family. The disease-free survival Prognostic (Fig. 1C) and overall survival rate(Fig. 1D) of EGFR, ERBB2, ERBB3 and ERBB4 have no significance(P>0.05). Fig. 1F shows the accordion diagram of each gene in cervical cancer staging. We found that the difference in the expression of the four genes between different stages of cervical cancer were not significant.

Figure 1.**E** and **Table.1** list the Genotype-Tissue Expression (GTEx) project collects and analyzes human cervical tissues. RNA-seq data of EGFR, ERBB2, ERBB3, and ERBB4 in cervical and ectocervix tissue type was mapped based on RSEMv1.2.22 (v7) and the TPM values have been included in the human protein map for four genes in the ERBB gene family in this study. All transcriptomics data have been retrieved from the cancer genome map. Immunohistochemistry **(IHC**) based on tissue microarray (**TMAs**) was used to analyze the expression levels of four proteins in the tissues of cervical cancer patients in this study, showing the corresponding protein-coding genes. We showed the mutation site (V104ML) (case ID list as TCGA-C5-A1MI-01, TCGA-C5-A7CO-01, TCGA-EA-A5FO-01) in receptor domain with the highest change frequency in the three-dimensional structure of ERBB3 (Fig. 2B,C). The potential correlation between mutation status and progression-free, overall, disease-specific and disease-free survival of CESC not significant (Fig. 2D).

Table 1. This is a table shows the RNA-seq data of ErbB Family in GTEx

Gene	GTEx sample Id	Sample description	pTPM
EGFR	GTEX-S341-1126-SM-4AD6T	40-49 years, female	38.1
	GTEX-T5JW-0726-SM-4DM6D	20-29 years, female	37.8
	GTEX-U3ZN-1626-SM-4DXTZ	30-39 years, female	35.2
	GTEX-S4UY-1426-SM-4AD6Y	40-49 years, female	21.8
	GTEX-TSE9-2826-SM-4DXTF	60-69 years, female	17.7
	GTEX-S32W-1526-SM-4AD6Z	50-59 years, female	15
	GTEX-S341-1326-SM-4AD72	40-49 years, female	41.8
	GTEX-T6MO-1426-SM-4DM73	40-49 years, female	24.7
	GTEX-TSE9-2726-SM-4DXSQ	60-69 years, female	20.1
	GTEX-S32W-1626-SM-4AD6G	50-59 years, female	17
	GTEX-TML8-0726-SM-4DXTT	40-49 years, female	12.4
ERBB2	GTEX-U3ZN-1626-SM-4DXTZ	30-39 years, female	78.3
	GTEX-T5JW-0726-SM-4DM6D	20-29 years, female	76.8
	GTEX-S341-1126-SM-4AD6T	40-49 years, female	48.9
	GTEX-S4UY-1426-SM-4AD6Y	40-49 years, female	44.2
	GTEX-TSE9-2826-SM-4DXTF	60-69 years, female	32.9
	GTEX-S32W-1526-SM-4AD6Z	50-59 years, female	25.3
	GTEX-S341-1326-SM-4AD72	40-49 years, female	66.8
	GTEX-T6MO-1426-SM-4DM73	40-49 years, female	56.5
	GTEX-TML8-0726-SM-4DXTT	40-49 years, female	42
	GTEX-TSE9-2726-SM-4DXSQ	60-69 years, female	32.8
	GTEX-S32W-1626-SM-4AD6G	50-59 years, female	32.7
ERBB3	GTEX-U3ZN-1626-SM-4DXTZ	30-39 years, female	77.5
	GTEX-T5JW-0726-SM-4DM6D	20-29 years, female	61.9
	GTEX-S341-1126-SM-4AD6T	40-49 years, female	14.1
	GTEX-S4UY-1426-SM-4AD6Y	40-49 years, female	10.4
	GTEX-TSE9-2826-SM-4DXTF	60-69 years, female	6.9
	GTEX-S32W-1526-SM-4AD6Z	50-59 years, female	4.1
	GTEX-T6MO-1426-SM-4DM73	40-49 years, female	33.2

	GTEX-S341-1326-SM-4AD72	40-49 years, female	27.1
	GTEX-TML8-0726-SM-4DXTT	40-49 years, female	25.3
	GTEX-S32W-1626-SM-4AD6G	50-59 years, female	8.9
	GTEX-TSE9-2726-SM-4DXSQ	60-69 years, female	4.5
ERBB4	GTEX-TSE9-2826-SM-4DXTF	60-69 years, female	0.4
	GTEX-S32W-1526-SM-4AD6Z	50-59 years, female	0.3
	GTEX-S341-1126-SM-4AD6T	40-49 years, female	0.3
	GTEX-S4UY-1426-SM-4AD6Y	40-49 years, female	0.1
	GTEX-T5JW-0726-SM-4DM6D	20-29 years, female	0
	GTEX-U3ZN-1626-SM-4DXTZ	30-39 years, female	0
	GTEX-S341-1326-SM-4AD72	40-49 years, female	5.8
	GTEX-T6MO-1426-SM-4DM73	40-49 years, female	2.5
	GTEX-TML8-0726-SM-4DXTT	40-49 years, female	0.2
	GTEX-S32W-1626-SM-4AD6G	50-59 years, female	0.1
	GTEX-TSE9-2726-SM-4DXSQ	60-69 years, female	0

3.2. m6A Regulators Is Differently Expressed in CESC Cancer

We analyzed the expression of 22 major m6A RNA methylation modulators in 607 CESC patients from the TCGA dataset.This study showed that mutations in the m6A regulator of the human CESCs genome were associated with pathogenesis. There were 9 m6A regulatory factors significantly increased in CESCs : **HNRNPC**, **YTHDF1**, **HNRNPA2B1**, **IGF2BP1**, **VIRMA**, **IGF2BP2**, **YTHDF2**, **RBM15**, and **IGF2BP3**. There were 7 m6A regulators with significantly reduced expression : **ZCCHC4**, **METTL3**, **ZC3H13**, **YTHDC1**, **YTHDC2**, **METTL16**, and **FTO**.

This study focuses on the role of m6A in CESC. Detection of genetic variation of m6A regulators in 607 patients using cBioPortal database (Fig. 3), among which **IGF2BP2** displayed the highest incidence rate (17%). Tumor tissue is higher than normal tissue are: HNRNPC (Fig. 4C), YTHDF1 (Fig. 4D), HNRNPA2B1 (Fig. 4F), IGF2BP1 (Fig. 4G), VIRMA(Fig. 4K), IGF2BP2 (Fig. 4P), YTHDF2 (Fig. 4Q), RBM15 (Fig. 4R), IGF2BP3 (Fig. 4V);Tumor tissue is lower than normal tissue are : ZCCH4 (Fig. 4A), METTL3 (Fig. 4B), ZC3H13 (Fig. 4H), YTHDC1 (Fig. 4J), YTHDC2 (Fig. 4M), METTL16 (Fig. 4O), FTO (Fig. 4U);Among them, the most significant (***p <0.0001) is HNRNPC, YTHDF1, IGF2BP1, YTHDF2, RBM15, IGF2BP3 (Yellow background with increased expression in tumor tissues) and YTHDC1, YTHDC2, METTL16, FTO (Blue background with decreased expression in tumor tissues).

In Fig. 5, we obtain the prognostic value of 22 m6A regulators in CESC through the Kaplan-Meier plot database. We revealed higher levels of ZC3H13, WTAP, HNRNPC, YTHDF3, and VIRMA were significantly

associated with worse outcomes in CESC(HR>1,blue background), while YTHDC1, YTHDF1 were protective factors of cervical cancer (HR<1,orange background). It indicates these m6A regulators had key roles in CESC prognosis.

3.3. Relationship between ERBB3 and m6A regulators.

The upper part of Fig. 6 is the expression of ERBB3 gene, the lower part is expression of m6A regulator genes after Z-score transformation.The thermal maps show ERBB3 expression has the most significant relation with YTHDC1, METTL14, RBM15, RBM15B, CBLL1, ZC3H13, METTL3, YTHDC2, ZCCHC4.

For the sake of investigating the downstream pathways of hub m6A regulators in CESC, we performed GO and KEGG analysis using co-expression genes of 6 m6A regulators. The results showed that YTHDC1 (Fig. 7A) and YTHDC2 (Fig. 7B) belong to RNA binding protein families. Both YTHDC1 and YTHDC2 can recognize and bind RNA containing N6 methyladenosine (m6A); The YTH domain mediates this binding [27–29]. M6A is a modifier existing in mRNA and some non-coding RNA internal sites, and plays a role in mRNA splicing, processing and stability regulation. YTHDC1 (also known as splicing factor yt521) can be used as a key regulator of exon addition or exon skipping to regulate selective splicing. YTHDC1 can promote exon increase by recruiting srsf3 into the region containing m6A, and inhibit exon skipping by blocking srsf10 binding to these same regions [13]. RBM15(Fig. 7C,D)

Figure 8 shows ERBB3 DNA methylation site.The methylation probe cg26929894 (Fig. 8A), cg11835619 (Fig. 8B), cg00907267 (Fig. 8C), cg10869879 (Fig. 8D), cg04794420 (Fig. 8E), cg22514674 (Fig. 8F), cg19258882 (Fig. 8G), cg26344379 (Fig. 8H) are significant (P<0.001). The probe of cg11835619(TSS+4480)has the highest relation with ERBB3 DNA methylation(r=0.62).

3.4. ERBB3 methylation and TIME (Tumor Immunity in the MicroEnvironment) classification

There are interactive regulatory mechanisms in the local immune microenvironment of cervical cancer tumors.Cells, related cytokines, and immune effector cells have different distribution patterns and infiltration densities. We studied TME cell infiltration characteristics in distinct DNA-Methylation modification of ERBB3 with association of TILS, Immunomodulator, Chemokine.

3.4.1. TILs

Correlation between ERBB3 methylation and three different kinds of CD8 cells in CESC : Effector memory CD8 T cell(Tem_CD8) r=0.625(p<0.01),Activated CD8 T cell(Act_CD8) r=0.538(p<0.01), Central memory CD8 T cell(Tcm_CD8) r=0.391(p<0.01) (Low correlation) ; Correlation between ERBB3 methylation and three different kinds of B cells in CESC : Activated B cell (Act_B) r=0.658(p<0.01), Immature B cell(Imm_B) r=0.684(p<0.01), Memory B cell(Mem_B) r=0.121(p<0.01)(Low correlation); Correlation between ERBB3 methylation and Type 1 T helper cell(Th1),Myeloid derived suppressor cell(MDSC),Macrophage, Regulatory T cell(Treg) in CESC, respectively.Th1 r=0.683(p<0.01),MDSC r=0.647(p<0.01),Macrophage r=0.634(p<0.01),Treg r=0.611(p<0.01). ERBB3 DNA methylation is closely related to a variety of immune cells and factors in the tumor microenvironment, especially tumor-infiltrating lymphocytes. It can be seen from

Table 2 that the TILS with the highest correlation with ERBB3 DNA methylation is TH1 .It helps cells are mainly used to fight the immune response of intracellular bacteria and protozoa. They are mainly induced by interleukin 12 (IL-12). The main cytokine for execution is interferon gamma (IFN-γ).

Table 2. Spearman correlations between Methylation (met) of ErbB3 and TILs, immunomodulators, chemokines (or receptors) across CESC.

	Factor	Rho (P□0.01)		Factor	Rho (P□0.01)		Factor	Rho (P□0.01)
Immuno-I	TIGIT	0.656	**Immuno-S**	ICOS	0.702	**MHC**	HLA-E	0.514
	CTLA4	0.642		CD48	0.659		*HLA-QA2*	*0.471*
	CD96	0.605		IL2RA	0.648		*TAP1*	*0.471*
	BTLA	0.599		CD27	0.626		*HLA-DPB1*	*0.464*
	HAVCR2	0.57		CD28	0.607		*HLA-DRA*	*0.455*
	CD244	0.558		CD86	0.604		*HLA-PA1*	*0.454*
	PDCD1	0.557		KLRK1	0.591			
				TNFRSF8	0.585			
				TNFSF13B	0.576			
				LTA	0.571			
TILS	Tem_CD8	0.625	**CK**	CXCL9	0.602	**R**	CXCR5	0.671
	Act_CD8	0.538		CXCL5	0.596		CCR5	0.656
	Tcm_CD8	*0.391*[a]		CXCL13	0.594		CCR7	0.62
	Act_B	0.658		CXCL11	0.56		CXCR6	0.618
	Imm_B	0.684		*CCL19*	*0.476*[a]		CXCR3	0.609
	Mem_B	*0.121*[a]		*CCL18*	*0.467*[a]		CCR2	0.589
	Th1	0.683		*CCL13*	*0.45*[a]			
	MDSC	0.647		*CCL21*	*0.425*[a]			
	Macrophage	0.634		*CCL22*	*0.4*[a]			
	Treg	0.611						

[a] Spearman correlations r□0.5

Immuno-I: Immunoinhibitor; **Immuno-S:** Immunostimulator; **MHC**: MHC molecule; **TILs**: tumor-infiltrating lymphocytes; **CK**: Chemokinnes; **R**: Receptors; **Tem_CD8**: Effector memory CD8 T cell; **Act_CD8**: Activated CD8 T cell; **Tcm_CD8***:* Central memory CD8 T cell*;* **Imm_B**: Immature B cell; **Mem_B***:* Memory B cell*;* **MDSC**: Myeloid-derived suppressor cells; **Treg**: Regulatory T cells.

3.4.2. Immunoinhibitor, Immunostimulator, MHC

Spearman correlations between methylation (met) of ERBB3 and Immunoinhibitors across CESC. From (**1**)-(**9**) follows as:TIGIT r=0.656(p<0.01) ,CTLA4 r=0.642(p<0.01),CD96 r=0.605(p<0.01),BTLA r=0.599(p<0.01), HAVCR2 r=0.57(p<0.01),CD244 r=0.558(p<0.01), PDCD1 r=0.557(p<0.01),PDCD1LG2 r=0.548(p<0.01), LAG3 r=0.519(p<0.01).(**E**) Spearman correlations between methylation (met) of ERBB3 and Immunostimulators across CESC. From (**1**)-(**10**) follows as: ICOS r=0.702(p<0.01),CD48 r=0.659(p<0.01), IL2RA r=0.648(p<0.01),CD27 r=0.626(p<0.01),CD28 r=0.607(p<0.01), CD86 r=0.604(p<0.01), KLRK1 r=0.591(p<0.01),TNFRSF8 r=0.585(p<0.01), TNFSF13B r=0.576(p<0.01),LTA r=0.571(p<0.01); (**F**) Spearman correlations between methylation (met) of ERBB3 and MHCs across CESC. From (**1**)-(**6**) follows as: HLA-E r=0.514(p<0.01),HLA-DQA2 r=0.471(p<0.01), TAP1 r=0.471(p<0.01), HLA-DPB1 r=0.464(p<0.01), HLA-DRA r=0.455(p<0.01), HLA-DPA1 r=0.454(p<0.01).

3.4.3. Chemokine and receptors

In CESC, the order of chemokines associated with ERBB3 methylation from high to low is as follows: CXCL9 (r=0.602, P < 0.01), CXCL5 (r=0.596, P < 0.01), CXCL13 (r=0.594, P < 0.01), CXCL11 (r=0.56, P < 0.01), CCL19 (r=0.476, P < 0.01), CCL18 (r=0.467, P < 0.01), CCL13 (r=0.45, P < 0.01), CCL21 (r=0.425, P < 0.01), CCL22 (r=0.4, P < 0.01) ; The order of Receptor associated with ERBB3 methylation from high to low is as follows: CXCR5 (r=0.671, P < 0.01), CCR5 (r=0.656, P < 0.01), CCR7 (r=0.62, P < 0.01), CXCR6 (r=0.618, P < 0.01), CXCR3 (r=0.609, P < 0.01), CCR2 (r=0.589, P < 0.01).(External Links include HGNC, NCBI, Ensembl, Uniprot, GeneCards data base).

4. Discussion

ERBB receptor is a typical cell membrane receptor tyrosine kinase, which is activated by dimerization after binding to ligands. ERBB receptor tyrosine kinase family contains four cell surface receptors : ERBB1/EGFR/HER1, ERBB2/HER2, ERBB3/HER3 and ERBB4/HER4. HER3/ERBB3 is a member of the ERBB receptor protein tyrosine kinase family, but lacks tyrosine kinase activity. The tyrosine phosphorylation of ERBB3 depends on its binding to other ERBB tyrosine kinases. When binding to ligands, heterodimers were formed between ERBB3 and other ERBB proteins, and ERBB3 was phosphorylated by activated ERBB kinase on tyrosine residues [30] [31]. There are at least nine potential tyrosine phosphorylation sites in the tail region of carboxyl end of ERBB3. These sites are the common binding sites of signal transduction proteins (including Src family members, Grb2 and PI3 kinase p85 subunit), which can mediate downstream signal transduction of ERBB [32]. The Tyr1222 and Tyr1289 sites of ERBB3 are located in the YXXM motif and participate in PI3K signal transduction [33]. Researchers have found that ERBB3 is highly expressed in many cancer cells [34].

In this study, it was found that in ERBB family, the expression of ERBB3 in cervical cancer tissues was higher than that in normal tissues (Fig. 1A). The pTPM values of EGFR, ERBB2 and ERBB3 in cancer tissue sections were significantly higher than those of ERBB4 (Fig. 1E). However, ERBB3 had no statistical significance in clinical disease staging and disease prognosis (Fig. 1C,D,F **and** Fig. 2D). Therefore, we speculate that ERBB3 is a pathogenic factor of cervical cancer rather than a prognostic factor. The main mutation site of ERBB3 in cervical cancer is base pair 104 (Fig. 2A,B).

Abnormal DNA methylation is a prominent feature of cancer. It is unclear how DNA methylation affects immune surveillance and tumor metastasis. N6-methyladenosine (m6A) is one of the most common and representative chemical modifications in eukaryotic RNA. It is a kind of dynamic and heritable information, which is widely present in a variety of organisms. M6A is mainly divided into its important roles in regulating gene expression, splicing, RNA editing, regulating RNA stability, and controlling mRNA degradation. It is a reversible epigenetic modification. Mainly divided into three categories: m6A methyltransferase (writer), m6A demethylase (eraser), m6A binding protein (reader).

Among them, we found higher expression of HNRNPC, YTHDF1, IGF2BP1, YTHDF2, RBM15, and IGF2BP3, lower expression of YTHDC1, YTHDC2, METTL16, and FTO in CESC (Fig. 4.). YTHDC1 and YTHDF1 have anti-cancer effects in cervical cancer, while ZC3H13, WTAP, HNRNPC, YTHDF3 and VIRMA have tumor-promoting effects in cervical cancer (Fig. 5.).We compared the methylation regulatory factors with the highest correlation in Figure 5 and Figure 6, and obtained three factors in Figure 7. Through further GO and KEGG clustering analysis of the functions of these three factors (YTHDC1, YTHDC2 and RBM15), We infer that ERBB3 RNA methylation plays an important role in the occurrence and progression of cervical cancer.

According to the analysis of ERBB3 DNA methylation in Figure 8, in cervical cancer, the correlation of DNA methylation modification of the 4480 base pair downstream of ERBB3 transcription initiation site was the highest, but whether the gene was finally regulated still needs further experiments to determine the causal relationship.

GO and KEGG analysis confirm ERBB3 Methylation were involved in regulating RNA splicing, RNA stability, and cell proliferation create Tumor and immune system interaction, which integrates multiple heterogeneous data types (Figure 7).

We researched the relationship between ERBB3 methylation and immune cell infiltration in cervical cancer microenvironment, finding that (Table 1) the abundance of TH1, MDSC, Macrophage, effector memory CD8 T cell, activated CD8 T cell, immature B cell and regulatory T cell have the significant association with methylation of ERBB3 in cervical tumor immune microenvironment (R>0.6). Relations betwwenn three kinds of immunomodulators and methylation of ERBB3 as follows: TIGIT(R=0.656), ICOS(R=a0.702), and HLA-E (R=0.514). **TIGIT** (T cell Ig and ITIM domain) is a member of the poliovirus receptor (PVR)/Nectin family [35]. It is expressed in lymphocytes, especially effector and regulatory $CD4^+$ T cells, follicular auxiliary $CD4^+$ T cells, effector $CD8^+$ T cells and natural killer (NK) cells. TIGIT plays an inhibitory role in multiple steps of tumor immune cycle [36]. The immune regulation of **ICOS** is manifested in the following aspects : enhancing the pattern recognition receptor signal of dendritic cells, inducing $CD4^+$ T cells to produce **IL-10** and producing high affinity antibodies against specific antigens..

CXCR5 [37] (R=0.671) is a G protein-coupled seven transmembrane receptor, belonging to the CXC chemokine receptor family, and its ligand is the chemokine **CXCL13** (R=0.594). **CCR5** [38] (R=0.656) is the receptor of intracellular β-chemokines (RANTES, MIP1α and MIP1β), which has the function of regulating the migration, proliferation and immunity of T cells and monocytes/macrophage cell lines. It is mainly expressed in the memory quiescent T Lymphocytes, monocytes, immature dendritic cells, etc. on the cell membrane. When cancer cells spread in the body, secondary tumors called metastases can form. These secondary tumors cause approximately 90% of cancer patients' deaths. An important way to spread cancer cells is through the lymphatic system, which runs through the entire body like the vascular system and connects the lymph nodes to each other. When white blood cells migrate through the lymphatic system to coordinate defenses against pathogens, as a specific membrane protein, chemokine receptor 7 (**CCR7**) [39] (R=0.62) plays an important role. It is located in the cell membrane, and it can receive external signals and transmit these signals to the inside of the cell. The receptor for **CXCL9** (R=0.602) (also known as IFN-r) is **CXCR3** (R=0.609) of the CXC subfamily [40].

5. Conclusions

In summary, this work proves that ERBB3 gene mutation, methylation modification have extensive regulatory mechanisms on the CESC microenvironment. ERBB3 is more likely to be an important carcinogenic factor of cervical cancer, but it has no significant effect on the clinical stage and prognosis of the disease. The differences of methylation modification patterns lead to the heterogeneity and complexity of CESC tumor immune microenvironment. Our study found that m6A regulator was an important biomarker of CESC and was closely related to tumor immune infiltration in cervical cancer. A comprehensive assessment of the ERBB3 multi-omics will help to enhance our understanding of the characteristics of cell infiltration in CESC tumor microenvironment and guide more effective immunotherapy strategies. DNA methylation modification of the 4480 base pair downstream of ERBB3 transcription initiation site was the highest.Further experiments will verify the carcinogenic mechanism of this methylation site on ERBB3 in cervical cancer.

Declarations

Author Contributions: Conceptualization, **Xiaoyue Yang**. and **Weipei Zhu**.; methodology, **Weipei Zhu**.; software, **Mei Li** and **Ying Chen**.; investigation, **Xiaoyue Yang**.; resources, **Xing Hu**.; data curation, **Xiaoyue Yang**.; writing—original draft preparation, **Xiaoyue Yang**.; writing—review and editing, **Xiaoyue Yang**.; visualization, **Xiaoyue Yang**.; supervisions, **Xiaoyue Yang**.; project administration , **Weipei Zhu**.; funding acquisition, **Xiaoyue Yang** and **Weipei Zhu**. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by “Research on early diagnosis of cervical cancer based on terahertz technology” in Zhenjiang social development project, SH2020051 ; “Observation and study on the clinical efficacy of a vaginal gel for the treatment of female genital HPV infection and related diseases” 2019 Key Laboratory of Pharmacodynamics and Safety Evaluation of Traditional Chinese Medicine in Jiangsu Province, JKLPSE201906; “Study on the Innovation of Distance Teaching Mode of Gynecological Laparoscopic Surgery under the Background of New Medicine” Project of Cooperative Education between

Industry and Education in 2020, Project number : 202002144004.Open Project of State Key Laboratory of Radiology and Radiation Protection (GZK1202106).

Data Availability Statement: The raw data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

Acknowledgments: Professor Zhang Hong's theoretical guidance of the Second Affiliated Hospital of Suzhou University, and Professor Su Zhaoliang's theoretical guidance of Jiangsu University Medical College**.**

Conflicts of Interest: The authors declare no conflict of interest

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Figures

Figure 1

(**A**) The expression of EGFR、ERBB2、ERBB3、ERBB4 in different tumors or specific tumor subtypes in TCGA and GTEx databases analyzed by TIMER2. (**B**) Isoform structure of EGFR, ErbB2, ErbB3, ErbB4. PFAM domains are indicated in color. (**C)** The disease free survival analyses based on the cancer type and cancer subtype showed the significant prognostic impact of four genes in CESC : EGFR (p=0.29), ErbB2 (p=0.057), ErbB3 (p=0.21), ErbB4 (P=0.14). (**D**) The overal survival analyses based on the cancer type and cancer subtype showed the significant prognostic impact of four genes in CESC: EGFR (p=0.055), ErbB2 (p=0.94), ErbB3 (p=0.28), ErbB4 (P=0.88). (**E**) EGFR, ErbB2-4 RNA-Seq data generated by the Genotype-Tissue Expression (GTEx) project from normal cervical tissues is reported as average pTPM. (**F**) Analyze expression of four genes in CESC by clinical stages.

Figure 2

Mutation characteristics of ERBB3 in different tumors. The cBioPortal tool was used to analyze the mutation characteristics of ERBB3 in TCGA tumors. Shows the frequency of mutation type (**A**) and mutation site (**B**). The graphical view shows the Pfam protein domains(**C**) and the positions of specific mutations. The length of the line connecting the mutation annotation to the protein is indicative of the number of samples that have the mutation. The most recurrent mutations are labeled in the graphical view. The cBioPortal tool was used to analyze the potential correlation between mutation status and progression-free, overall, disease-specific and disease-free survival of CESC (**D**).

Figure 3

Amplification, Deletion, Genetic variations and mutation of m6A regulators in in 607 CESC cases using the cBioPortal database.

Figure 4

Expression profile of m6A regulators in CESC. (**A–V**) The expression levels of m6A regulators showed expression in CESC samples, including (**A**) ZCCHC4,(**B**) METTL3, (**C**) HNRNPC, (**D**) YTHDF1, (**E**) RBM15B,(**F**) HNRNPA2B1,(**G**) IGF2BP1,(**H**) ZC3H13, (**I**) ALKBH5, (**J**) YTHDC1,(**K**) VIRMA ,(**L**) WTAP,(**M**) YTHDC2,(**N**) CBLL1, (**O**) METTL16,(**P**) IGF2BP2,(**Q**) YTHDF2,(**R**) RBM15,(**S**) YTHDF3,(**T**) METTL14,(**U**) FTO,(**V**) IGF2BP3. *P < 0.05; **p < 0.01; ***p < 0.0001 compared with normal tissues.

Figure 5

Relationship between different expression of the 22 m6A regulators and ERBB3 with OS of cervical cancer.

Figure 6

The correlation between the high and low expression of ERBB3 gene and m6A regulators in cervical cancer (ns , $p \geq 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Figure 7

GO and KEGG enrichments. (**A–D**) Bioinformatics analysis of YTHDC1, YTHDC2, RBM15 in CESC.

Figure 8

TPM values of ERBB3 in RNAseq data and Beta value corresponding to different methylation probe cg26929894 (**A**), cg11835619 (**B**), cg00907267 (**C**), cg10869879 (**D**), cg04794420 (**E**), cg22514674 (**F**), cg19258882 (**G**), cg26344379 (**H**).The beta value is the ratio of the methylated probe intensity to the overall intensity (the sum of the methylated and unmethylated probe intensities).