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Basic Study

Potential of six-transmembrane epithelial antigen of the prostate 4 as a prognostic marker for colorectal cancer

Fang ZX *et al*. STEAP4 in CRC

Abstract

BACKGROUND

Immune cells play a role in the regulation of tumor cell behavior, and accumulating evidence supports their significance in predicting outcomes and therapeutic efficacy in colorectal cancers (CRC). Human six-transmembrane epithelial antigen of the prostate (STEAP) proteins have been recognized and utilized as promising targets for cell- and antibody-based immunotherapy. One STEAP family member, STEAP4, is expected to be an attractive biomarker for the immunotherapy of prostate and breast cancer. However, an immunotherapeutic role of STEAP4 for colorectal carcinomas has not been demonstrated.

AIM

To explore the expression pattern of STEAPs in CRC and their relationship with immune infiltration, and investigate the potential utilization of STEAPs as novel prognostic indicators in colorectal carcinomas.

METHODS

The expression level of STEAPs in CRC was evaluated using various open-resource databases and online tools to explore the expression characteristics and prognostic significance of STEAPs, as well as their correlation with immune-related biomarkers, such as immune infiltration. Immunohistochemical (IHC) experiments were subsequently performed to verify the database conclusions.

RESULTS

The level of STEAPs in CRC was inconsistent. Expression of STEAPs 1-3 was not significantly different from normal tissues. However, STEAP4 mRNA levels were significantly lower than normal tissue and were positively correlated with immune-related biomarkers, such as immune cell infiltration, immune-stimulation, major histocompatibility complex levels and chemokines. Interestingly, the expression of

STEAP4 in microsatellite instability-high was higher than that in microsatellite stability in CRC subtypes. IHC staining was performed on colon cancer tissue samples and showed that high expression of STEAP4 in adjacent tissues positively correlated with immune-related biomarkers, including MLH1, MLH6 and PMS2, but negatively correlated with PDL1, to varying degrees.

CONCLUSION

Our results provide an analysis of the expression of STEAP family members in CRC. Among different STEAP family members, STEAP4 plays a different role in CRC than STEAPs 1-3. In CRC, STEAP4 expression is not only lower than that in normal tissues, but is also positively correlated with immune infiltration and immune-related biomarkers. These findings suggest that STEAP4 may be a potential biomarker for predicting CRC immune infiltration status.

Key Words: Six-transmembrane epithelial antigen of the prostate; Colorectal cancer; Immunotherapy; Target; Survival

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Core Tip: This study analyzes the expression level of six-transmembrane epithelial antigen of the prostate (STEAP) family members in colorectal cancer (CRC) and explores the potential biological function of STEAP4. It was found that expression of STEAP4 in CRC tissues has a positive correlation with immune infiltration and immune-related biomarkers, such as MLH1, MLH6, and PMS2, and a negative correlation with programmed death ligand 1. STEAP4 is expected to be a novel and potential prognostic biomarker for CRC.

INTRODUCTION

Colorectal cancer (CRC), ranks third in the world among malignant tumors, and ranks second in worldwide cancer mortality[1]. Currently, large changes in lifestyle and dietary habits are thought to have contributed to the increased incidence and mortality of CRC. For example, in China, the annual average increase of new CRC cases is estimated to be 4.2%[2]. Although gender and regional differences are considered as the prognostic factors for patients with CRC[3], the etiology of CRC oncogenesis and development is still complex and unclear.

With the development of standardized treatment for patients with CRC, the prognosis of CRC has greatly improved. However, the control of progression of metastatic disease is still intractable. Immune cells play an important role in the regulation of tumor cell behavior, and accumulating evidence supports their significance in predicting outcomes and therapeutic efficacy in CRC patients[4]. In this regard, attention is currently being paid to the immune microenvironment and immunotherapy of CRC, mainly focusing on T cells and therapeutic response as related to promising treatment strategies[5].

Immunotherapy serves as an alternative treatment for cancer patients, especially for those whose tumors overexpress antigens recognized by immune cells. The human six-transmembrane epithelial antigen of the prostate (STEAP) family of proteins belongs to a class of cellular transmembrane proteins and has been used to derive epitope peptides that stimulate T lymphocytes in patients with renal cell or bladder cancers[6]. Importantly, STEAPs are present at the intercellular junctions of the prostate secretory epithelium, and are overexpressed in prostate cancer, serving as attractive targets for prostate cancer immunotherapy[7].

Although STEAPs have been reported to be overexpressed in CRC[8-11], research on STEAPs in CRC remains limited and the immunotherapeutic role of STEAPs in colorectal carcinomas has not been shown. This research investigates the biological function of STEAPs in CRCs, in addition to the relationship between STEAPs and

immune infiltration, and demonstrates the potential of STEAPs to serve as novel and prognostic biomarkers for immunotherapy in colorectal carcinomas.

MATERIALS AND METHODS

Patient information and ethics statement

A tissue microarray with 87 matched primary CRC tissues and their corresponding adjacent normal colorectal tissue samples, and 6 extra samples of cancer cases without the corresponding paracancerous tissue were purchased from the Shanghai OUTDO Biotech Company, Shanghai, China (XT17-025, HColA180Su18). Pathological type was classified according to the prognostic degree of cancers. This study was approved by the Ethics Committee of Shantou University Medical college.

Comparison of STEAP expression in normal and cancerous tissues

TCGA datasets were used to evaluate the expression of STEAPs in normal and different cancerous tissues through the Tumor IMmune Estimation Resource (TIMER2.0) online source (http://timer.cistrome.org/)[12]. The UCSC Xena database (https://genome-cancer.ucsc.edu/)[13] was applied to analyze STEAP expression differences in colon adenocarcinomas (COAD) and rectal adenocarcinomas (READ) and related normal tissues. Regarding the different subtypes of CRC, namely microsatellite instability-high (MSI-H), microsatellite instability-low (MSI-L), and microsatellite stable (MSS)[14], MSI is a biomarker for response to immune checkpoint inhibitor (ICI), showing high disease control rates and good progression-free survival for with MSI-H CRC[15]. MSI-L tumors are phenotypically indistinguishable from MSS tumors, and the biological significance of MSI-L is unclear[16], so emphasis has been placed on MSI-H and MSS. The expression of STEAPs in MSI-H and MSS was also evaluated in the GEPIA2 database (http://gepia2.cancer-pku.cn/)[17].

Relationship between STEAPs and immune infiltration in CRC

Immune cells involved in CRC development were evaluated using the TIMER2.0 database to predict the association between the expression of STEAPs and the abundance of immune cells, including B cells, $CD4^+$ T cells, $CD8^+$ T cells, neutrophils, macrophages and dendritic cells, in the tumor microenvironment. In terms of immune characteristics of lymphocytes, immunostimulants, major histocompatibility complex (MHC) and chemokines, TISIDB, an integrated repository portal for tumor-immune system interactions (http://cis.hku.hk/TISIDB/)[18], was applied to explore the potential function of STEAPs in CRC immune infiltration. After being downloaded from the TISIDB, the dataset was analyzed and drawn with matrix2png (https://matrix2png.msl.ubc.ca/bin/matrix2png.cgi), an online mapping software[19].

Immunohistochemical staining

The immunohistochemical (IHC) staining of STEAP4 in tissue microarrays was conducted as described before[20]. The tissue microarray slide was dewaxed in xylene, hydrated in graded alcohols, and processed with 2% ethylenediamine tetraacetic acid antigen-repair solution (Fuzhou Maixin Biotechnology Development Co. LTD, Fuzhou, China) by microwave for epitope retrieval. After blocking endogenous peroxidase with 3% H_2O_2, the slide was incubated with anti-STEAP4 antibody (dilution: 1:1000, Proteintech 11944-AP) at 4 °C overnight. Stained tissues with DAB reagent were mounted using nuclear counterstaining with hematoxylin for visualization.

Sections were visualized under a bright-field microscope (Axio Imager A2, Zeiss, Germany) and evaluated independently by two investigators with no prior knowledge of the CRC patient information. For tissue expression of STEAP4, staining intensity was measured as 0, 1, 2 and 3 for colorless, light yellow, brown yellow and dark brown, respectively, while the percentage of positive cells, equaling 0%, 1%-25%, 26%-50%, 51%-75% and 76%-100%, was recorded as 0, 1, 2, 3 and 4 points, respectively. The final staining score for STEAP4 expression was calculated as the sum of staining intensity and the percentage of positive cells, and divided into low expression (scores 0-4) and high expression (scores 5-7) groups. The expression level of MLH1/2/6, PMS2 and

programmed death ligand 1 (PDL1) were included in the patient information and the cutoff were described before[21].

Statistical and survival analyses

SPSS 25.0 statistical software was used to analyze the results. Enumerated data were recorded as the number of cases (n = 93), and the relationship between STEAP4 and the clinicopathological parameters of CRC patients was analyzed by χ^2 and Fisher's exact probability tests. The relationship between expression of STEAP4 in CRC and adjacent normal tissues (n = 87 cases) was examined by the χ^2 test. Likewise, the correlation between highly expressed STEAP4 in CRC and the immune-related factors MLH1, MLH2, MLH6, PMS2 and PDL1 was determined by the χ^2 test. To investigate the prognostic value of STEAP4 in CRC patients, the Kaplan-Meier survival curve and log-rank test were used to evaluate the association of STEAP4 expression with CRC patient prognosis by SPSS 25.0 software. The difference was considered statistically significant at $P < 0.05$.

RESULTS

Expression of STEAPs in different types of malignant tumors

To determine the expression pattern of STEAPs in different types of malignant tumors, TIMER2.0 was used to analyze the difference between normal and cancerous tissues in TCGA. All STEAPs were found to be expressed at low levels in breast invasive carcinoma and kidney chromophobe compared to corresponding normal tissues, while in other types of malignancies, the expression patterns of STEAPs differed (Figure 1). Interestingly, in COAD, head and neck squamous cell carcinoma, lung adenocarcinoma, lung squamous cell carcinoma and READ, the expression of STEAP4 was lower than that in normal tissues. However, in such cancerous tissues, the level of STEAPs 1-3 was higher or not significantly different from normal, suggesting that STEAP4 may perform a different function from STEAPs 1-3 in patients with such cancers.

CRC tissues have lower STEAP4 Levels compared with normal tissues

To verify the expression pattern of STEAPs in CRC, another database, UCSC Xena, was analyzed to confirm the findings. Although expression of STEAPs 1 and 2 in COAD and READ were not different compared to their corresponding normal tissues, STEAP4 was lower, while STEAP3 was higher compared to corresponding normal tissues (Figure 2).

Low STEAP4 levels are associated with the MSS subtype of CRC

CRC is highly heterogeneous at the genetic and molecular level, affecting the efficacy of clinical therapy. A subset of CRCs exhibit MSI, indicating defective DNA mismatch repair and high mutational burden, different from the majority of MSS subtypes[22]. CRC patients, especially those with MSI-H tumors, are more sensitive to ICIs than those with MSS tumors, and MSI-H tumors have greater infiltration of immune cells, higher expression of immune-related genes and higher immunogenicity than MSS tumors[23]. Since the expression of STEAP4 is consistently low in CRC, different from the other three genes, we focused on STEAP4 for the remainder of this study. To explore STEAP4 expression levels in different subtypes of CRCs, the GEPIA2 database was used. Interestingly, the mRNA level of STEAP4 was highly expressed in MSI-H CRCs compared to MSI-L and MSS tumors (Figure 3).

STEAP4 is decreased in cancer tissues

As the expression pattern of STEAP4 seems to be different from the other STEAP members, paired normal and CRC tissues were used to evaluate the protein level of STEAP4. Representative images of STEAP4 expression are shown in Figure 4. The percentage of CRC tissues with high levels of STEAP4 was 51.7%, which was still lower than normal tissue (73.6%, $P = 0.003$) (Table 1).

Relationship between STEAP4 levels and clinicopathological parameters of patients with CRC

Clinicopathological analysis showed that STEAP4 expression was not associated with gender, primary tumor stage, lymph node status, American Joint Committee on Cancer (AJCC) stage or pathological type (Table 2). The expression of STEAP4 decreased with the increase of primary tumor stage, lymph node status, and AJCC stage.

STEAP4 protein levels are positively associated with MLH1, MLH6 and PMS2, and negatively associated with PDL1

Based on the IHC results, expression of STEAP4 and corresponding immune-related biomarkers in CRC were analyzed using χ^2 statistical analysis. In Table 3, a low level of STEAP4 was positively related to low levels of MLH1, MLH6 and PMS2, while negatively associated with PDL1 levels in CRC patients.

Expression level of STEAP4 is associated with the abundance of immune cell infiltration in CRC

Given the importance of antitumor immunity in the tumor microenvironment, the correlation between expression of STEAP family members and immune cells in CRC was analyzed. Based on the TIMER database, it was found that the expressions of STEAP1, STEAP2 and STEAP4 were negatively correlated with tumor purity and positively correlated with six types of immune cells, specifically B cells, $CD8^+$ T cells, $CD4^+$ T cells, neutrophils, macrophages and dendritic cells in COAD and READ (Figures 5A, 5B, and 5D). However, expression of STEAP3 was positively correlated with tumor purity, $CD4^+$ T cells, neutrophils, macrophages and dendritic cells, and negatively correlated with B cells and $CD8^+$ T cells in COAD and READ (Figure 5C).

STEAP4 is positively correlated with immune characteristics in CRC

According to the TISIDB database, Spearman correlation analysis showed that the expressions of STEAP1, STEAP2 and STEAP4 were positively correlated, but STEAP3 was negatively correlated with lymphocytes, immunostimulants, MHCs and

chemokines (Figures 6A-F and 6H), which is consistent with the results using the TIMER database (Figures 6C and 6G).

Low expression of STEAP4 in cancer tissues tends to predict poor overall survival in CRC patients

Based on the IHC results, protein expression of STEAP4 was analyzed by the Kaplan-Meier curve with log-rank test and demonstrated that STEAP4 expression was not significantly associated with the overall survival (OS) ($P > 0.05$, Figure 7). Although the difference did not meet the statistical criteria, it was found that high expression of STEAP4 tended to predict longer OS for CRC patients, suggesting the protein level of STEAP4 could be a predictor for the survival of CRC patients.

DISCUSSION

Members of the STEAP family were originally identified as metalloreductases *in vivo*, playing an important role in maintaining iron homeostasis[24]. Abnormal accumulation of iron caused by unbalanced iron metabolism has been reported to lead to the occurrence, progression and invasion of tumors[25]. Thus, the STEAP family bridges iron homeostasis and cancer[26]. As potential biomarkers and therapeutic targets of tumors, the STEAP family plays an important role in tumor therapy.

All four STEAP proteins are increased in prostate cancer and play important roles in the development and progression of prostate cancer[27]. Interestingly, although the structure of STEAP4 is similar to the three other STEAP family members, the function of STEAP4 may diverge in different types of cancers[24]. STEAP1 antibody can effectively activate $CD8^+$ T cells, natural killer cells and other immune-related factors against a broad spectrum of tumors[28]. In the process of this study, there was a great difference between STEA4 and STEAP1-3, and STEA4 promoted androgen-positive (PC) and inhibited (AR)- in PC, while it was very different from STEAP1-3 in CRC, so there might be different mechanisms. Interestingly, we found dual anti-STEAP1 antibody targeting T cells for cancer immunotherapy[29]. Combined with our study, it is

suggested that STEAP4 can be developed as a new therapeutic strategy. Therefore, this study explored the role of STEAP4 from mRNA and clinical tissue levels. And the STEAP4 is rarely studied in CRC. The current study used clinical tissues from CRC patients and characterized the mRNA and protein levels of STEAP4 to determine the expression of STEAP4 in CRC. Not surprisingly, low expression of STEAP4 was found in CRC tissues compared with adjacent tissues, which is consistent with the low mRNA expression of STEAP4 in CRC and a potential tumor suppressor role for STEAP4 in CRC patients.

To uncover the potential function of STEAP4 in CRC, clinicopathological parameters were analyzed. However, no statistical significance was found between the protein level of STEAP4 and primary tumor stage, lymph node metastasis or AJCC stage. Reduced STEAP4 expression tended to be associated with advanced CRC stage, increased lymph node and metastases, suggesting the suppression of STEAP4 could play a role in promoting the progression and metastasis of CRC.

Recently, Xue *et al*[11] investigated the molecular mechanism of STEAP4 involvement in the hypoxic metabolism of inflammatory bowel disease and the linkage with mitochondrial dysfunction in colon cancer. Increasingly high levels of STEAP4 result from increased levels of hypoxia and are associated with colitis in mouse models, and inflammatory bowel disease patients. Inflammatory factors were not examined in the current study, which may influence STEAP4 levels in different types of CRC. Based on the inflammatory environment, hypoxic conditions can be associated with mitochondrial iron dysfunction caused by increased STEAP4[30]. However, in the absence of inflammatory infiltration, the inhibition of STEAP4 was reversed to be a tumor suppressor through interactions with protein kinases[31].

Further analyses were performed to uncover the potential relationship of STEAP4 with immune infiltration. As expected, both the protein and mRNA levels of STEAP4 are associated with immune-related factors, predicting a potential role of STEAP4 in stimulating immune infiltration. MLH1 deficiency has been shown to be associated with cetuximab resistance in CRC[21]. The positive relationship between STEAP4 and MLH1

in CRC suggests an involvement of STEAP4 in the immune response of the tumor microenvironment. Recently, Ijsselsteijn *et al*[32] reported, for a cell-based model for Lynch syndrome, that DNA mismatch repair deficiency is related to the core DNA mismatch repair genes MSH2, MSH6, MLH1 and PMS2[32]. The protein level of STEAP4 was found to be positively correlated with such DNA mismatch repair genes in the current study. The above results support a role for STEAP4 in the immune response to CRC to prevent further development and metastasis.

CONCLUSION

In the current study, STEAP4 was found to be a protective factor in the intestinal tract and could be used as a prognostic indicator for patients with CRC. CRC patients with high STEAP4 tend to have longer survival.

ARTICLE HIGHLIGHTS

Research background

Six-transmembrane epithelial antigen of the prostate 4 (STEAP4) is an attractive biomarker for the immunotherapy of prostate and breast cancer. However, the immunotherapeutic role of STEAP4 for colorectal carcinomas has not been demonstrated.

Research motivation

Immunotherapy emerges with predicting outcomes and therapeutic efficacy in colorectal cancers (CRC).

Research objectives

To explore the expression pattern of STEAPs in CRCs and their relationship with immune infiltration, and investigate the potential utilization of STEAPs as novel prognostic indicators in colorectal carcinomas.

Research methods

CRC patients' tissues and online dataset were used to analyze the expression level of STEAP4 in different types of CRC and their relationship with immune characteristics.

Research results

The expression of STEAP4 is significantly decreased in CRC tissues compared with adjacent normal ones, related to immune-related biomarkers. Low STEAP4 level predicted poor overall survival of CRC patients.

Research conclusions

STEAP4 was found to be a protective factor in the intestinal tract and could be used as a prognostic indicator for patients with CRC.

Research perspectives

STEAP4 may be a potential biomarker for predicting CRC immune infiltration status.

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