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Immunohistochemical Expression of B7-H4 in Ovarian Tumors

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Abstract: In cancers, inflammation, and autoimmune illnesses, the coinhibitory molecule B7-H4, an important member of the B7 family, is inappropriately produced. By reducing T cell proliferation, cytokine release, and cell cycle, B7-H4 negatively controls T cell immune response and promotes immunological escape. Furthermore, B7-H4 plays a critical function in carcinogenesis and tumor growth, including cell proliferation, anti-apoptosis, tumor, invasion and metastasis development. As a result, B7-H4 has been identified as a potential therapeutic target for malignancies, inflammation, autoimmune disorders, and organ transplantation. So, the objective of the current study was to evaluate the immunohistochemical expression of B7-H4 in different ovarian tumor histological variants that would be used for target therapy in the future. This cross-sectional study was conducted at Ziauddin University and Hospital North campus, comprised of 71 cases. Consent and demographic details were obtained before the selection of cases. The obtained data were analyzed statistically via SPSS version 21. Positive IHC expression was seen in 48 of 71 ovarian cancers (67.6%). The majority of B7-H4 IHC positive patients (22/48; 45.8%) were malignant, with benign (20/48; 41.7%) and borderline (6/48; 12.5%) tumors following closely behind, indicating a statistically significant link between B7-H4 and ovarian cancers (p=0.004). B7-H4 protein expression was also found in some unusual ovarian tumor morphological forms, such as 5/5 dysgerminoma and 2/2 Brenner tumor of the ovaries. These uncommon variations are also statistically linked to B7-H4 protein expression (p-value: 0.011). Most positive samples had protein expression strength of 1 on a scale of 4 with a significant statistical estimate of p-value 0.015.

Keywords: B7-H4, ovarian tumors, serous tumor, mucinous tumor, Brenner tumor.

乙7-H4在卵巢腫瘤中的免疫組化表達

摘要：在癌症、炎症和自身免疫性疾病中，乙7 家族的重要成員—共抑制分子乙7-H4 的產生不當。通過減少吨細胞增殖、細胞因子釋放和細胞週期，乙7-H4 負向控制吨細胞免疫反應並促進免疫逃逸。此外，乙7-H4 在致癌作用和腫瘤生長中起著關鍵作用，包括細胞增殖、侵襲、轉移、抗細胞凋亡和腫瘤發展。因此，乙7-H4 已被確定為惡性腫瘤、炎症、自身免疫性疾病和器官移植的潛在治療靶點。因此，本研究的目的是評估乙7-H4 在未來用於靶向治療的不同卵巢腫瘤組織學變異中的免疫組織化學表達。這項橫斷面研究是在齊奧丁大學和醫院北校區進行的，包括 71 個病例。在選擇病例之前獲得同意和人口統計細節。獲得的數據通過 SPSS 21 版進行統計學分析。在 71 例卵巢癌中的 48 例（67.6%）中觀察到陽性国际健康委员会表達。大多數乙7-H4免疫組化陽性患者 (22/48; 45.8%) 是惡性的，良性 (20/48; 41.7%) 和交界性 (6/48; 12.5%) 腫瘤緊隨其後，表明具有統計學意義的聯繫乙7-H4 和卵巢癌之間的差異(磷=0.004)。在一些不尋常的卵巢腫瘤形態學形式中也發現了乙7-H4 蛋白表達，例如卵巢的 5/5 無性細胞瘤和 2/2布倫納腫瘤。這些不常見的變異也與乙7-H4 蛋白表達在統計上相關（磷值：0.011）。大多數陽性樣品的蛋白質表達強度為 1，等級為 4，磷值的顯著統計估計值為 0.015。

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关键词：乙7-H4，卵巢肿瘤，浆液性肿瘤，粘液性肿瘤，布伦纳肿瘤。

1. Introduction

Ovarian tumor is the 8th most frequent tumor worldwide, with an incidence rate comparable to cervical and endometrial tumors and having the highest mortality rate among the malignant gynecological tumors [1]. In 2018, 295414 new cases of ovarian tumor were reported globally, with 184799 fatalities [2].The highest incidence rates were observed in North America (8 per 100,000), Eastern and Central Europe (11.4 per 100,000 and 6.0 per 100,000, respectively). In contrast, the lowest rates were identified in Africa and Asia (less than 3 per 100,000) [3]. The annual incidence of ovarian tumors in Pakistan is 4.8 per 100,000, while 7.2 per 100,000 in India [4]. In Karachi, the annual incidence of ovarian tumors is 10.2 per 100,000 [5].

Women over the age of 55 are more likely to get an ovarian tumor, with the age range of 55 to 64 having the highest global occurrence with a 5-years survival rate ranging from 30% to 40% worldwide [6]. Nearly 90% of ovarian tumors are epithelial neoplasms. Germ cell and sex cord-stromal cell cancers are substantially less common, accounting for fewer than 10% of ovarian malignancies [7]. Germline mutations in BRCA1, BRCA2, or mismatch repair genes cause about 10% of the familial ovarian malignancies. First-degree relatives of the patient have a 3-to-7-fold higher risk of developing an ovarian tumor [8].

The total number of years exposed to estrogen and contraceptive use during reproductive age plays a role in pathogenesis because they stimulate and multiply cells by activating the mitogen-activated kinase (MAPK) [9]. Ovarian tumor symptoms are generic and imprecise, making early detection challenging. Histopathological examination is the gold standard approach for the diagnosis of ovarian tumors [10]. Currently, ovarian tumor screening procedures, such as transvaginal ultrasonography or biomarker tests such as CA125, have not improved the stage of diagnosis or prognosis. Furthermore, CA-125 is used exclusively to diagnose epithelial tumors. Other immunomarkers have been identified, including human kallikerin 10 (HK10), human kallikerin 6 (HK6), osteopontin (OPN), claudin 3, and a recently recognized novel biomarker with prognostic and therapeutic significance [11, 12].

B7-H4 is a 282-amino-acid immunoregulatory membrane-anchored protein located on chromosome 1 and belongs to the B7 superfamily [13]. B7-H4 is a highly glycosylated, glycosylphosphatidylinositol (GPI)-linked protein. A short intracellular region, a hydrophobic transmembrane domain, and an external immunoglobulin-like domain are required for ligand binding [14]. Although B7-H4 protein expression is severely restricted due to its tight translational regulation, mRNA is expressed at extremely low levels in typical healthy tissues such as the thymus, kidneys, liver, pancreas, and ovaries [15]. B7-H4 has been discovered in breast malignancies, ovarian tumors, hepatocellular carcinoma, osteosarcoma, and gastric tumor. According to the literature, B7-H4 interrelates to its receptors on activated CD4+ and CD8+ T lymphocytes, decreasing their effector activity by increasing the G0/G1 phase of the cell cycle, thus lowering proliferation and cytokine production [16]. Intracellular effects of B7-H4 expressing cells include decreased apoptosis, increased proliferation, and facilitated metastasis. As a result, the current study's goal was to look at B7-H4 expression in ovarian cancers of varied morphologies.

2. Methods

This cross-sectional study was conducted at Ziauddin University and Hospital North campus, comprised of 71 cases. Cases were recruited by using the purposive sampling technique.

2.1. Tissue Sampling and Histological Diagnosis

Paraffin-embedded, formalin-fixed tissue blocks of ovarian tumors received between 2017 and 2020 at the histopathology laboratory Ziauddin hospital North campus Karachi were selected for the study. The study comprised biopsy-proven ovarian tumor cases aged 18 and above with no chemotherapy or radiotherapy before an ovarian biopsy. Patients not giving consent and with malignancies other than ovarian tumors were excluded from the trial. WHO criteria were used to determine histologic diagnoses by a panel of histopathologists, including tumor grade, which were then validated by reviewing the original H&E stained tissue sections. The American Joint Committee on Cancer (AJCC) staging system was used to determine the clinical stage [17]. Histological grade for all cases of dysgerminoma could not be assessed due to the lack of a grading system available. Cases included serous tumors (Benign, borderline, and malignant), mucinous tumors (Benign, borderline, and malignant), and some rare histological variants like benign Brenner tumor and dysgerminoma. The study project was evaluated and reviewed by the ethical review committee of Ziauddin University, and approval was taken according to institutional guidelines (Ref # 1721219MRPAT).

2.2. Immunohistochemistry

For immunohistochemistry, the most representative

block with the largest tumor volume was chosen. The approach described by Signoretti et al. was employed for immunohistochemistry [18]. Briefly, formalin-fixed, paraffin-embedded tissue blocks were cut into 3mm slices, immersed in a hot water bath, and transferred to glass slides, followed by antigen retrieval treatment with 0.1mol/L citrate buffer in a microwave for 15 minutes. TAB 250, a monoclonal mouse anti-B7-H4 antibody, was made at a 1:50ml dilution. The preparation was then applied to the tissue segment for 45 minutes, followed by a buffer rinse and the development of enzyme activity separately. The sections were then dehydrated and counter-stained with hematoxylin for 1 minute before rinsing with distilled water and drying at room temperature. After that, the coverslip was applied with mounting media. The positive controls were known B7-H4 positive gallbladder slides, while the negative controls were tissue slides without primary antibodies. The same consultant histopathologist analyzed the IHC sections to assess the B7-H4 staining pattern. Dark brown cytoplasmic staining observed under light microscopy in tissue sections was taken as positive.

2.3. Evaluation of Immunohistochemical Staining of B7-H4

B7-H4 expression was detected in all instances that displayed at least mild focal staining. The fraction of B7-H4 positive cells was determined after a thorough examination of the complete histologic section, and a final score was provided (1+ 0–10% positive cells; 2 + >10–50% positive cells; 3+ >50–80% positive cells; 4+ >80–100% positive cells) [19].

2.4. Statistical Analysis

The statistical analysis was performed using SPSS Statistics 21. Ratios and percentages were used for qualitative variables, and for quantitative variables, mean and standard deviation were used. The relationship between B7-H4 expression and clinicopathological factors such as age, tumor grade, and the stage was investigated using the chi-square test. A p-value of 0.05 was established as the statistical significance criterion.

3. Results

3.1. Patient Characterization

In this research, we evaluated the 71 patients with their demographic and clinicopathological characteristics. Among these, 48/71 (97.6%) females were equal to or less than 50 years of age. Predominantly they were married 55/71 (77.4%), and multiparous females 44/71 (61.9%) had the tumor in the left ovary 6/71 (64.7%). None of these factors have a statistical correlation with the B7-H4 immunohistochemical expression.

3.2. Immunohistochemical Expressions

We were able to perform B7-H4 immunohistochemical analysis of 71 ovarian tumor biopsy blocks, which comprised of benign 39 (54.9%), borderline 6 (8.6%), and malignant 26 (36.6%) cases.

The positive IHC expression was seen in 48 cases (67.6%) out of 71 ovarian tumors. The majority of B7-H4 IHC positive cases (22/48; 45.8%) were malignant, followed by benign (20/48; 41.7%) and borderline (6/48; 12.5%) tumors, showing a significant statistical relationship between B7-H4 and ovarian tumors (p = 0.004) (Table 1).

Table 1 Clinicopathological and demographic characteristics of ovarian tumors and statistical estimates

Demographic and Clinicopathological Characters	n=71	B7-H4		P-value
		Positive	Negative	
Tumor				
Benign	39	20	19	
Borderline	06	06	00	0.004
Malignant	26	22	04	
Marital Status				
Single	16	11	05	1.00
Married	55	37	18	
Site				
Right	25	18	07	0.606
Left	46	30	16	
Age				
≤ 50	48	34	14	0.427
> 50	23	14	09	
Parity				
Nulliparous	27	19	08	0.797
Multiparous	44	29	15	
Grade				
Mildly Differentiated	07	06	01	
Moderately Differentiated	03	02	01	0.427
Well Differentiated	11	09	02	
Stage				
I A	09	09	00	
I C	02	01	01	
II A	08	06	02	0.152
III C	02	01	01	

B7-H4 protein expression was found in 27/40 serous ovarian tumors, including 13/40 serous cystadenocarcinomas, 2/40 serous tumors of low malignant potential, and 12/40 serous cystadenomas. Mucinous cystic tumors are the second most prevalent morphological variant identified, with positive B7-H4 protein expression in 14/22 tumor tissues, with 4/22 being positive mucinous cystadenocarcinomas, 4/22 being mucinous tumors with low malignant potential, and 6/22 being mucinous cystadenomas. They have an insignificant statistical association with B7-H4 expression with p-values of 0.051 and 0.195, respectively (Table 2).

Table 2 Association of B7-H4 expression with morphological variants of ovarian tumors

Histological Variants		B7-H4 +ve	B7-H4 -ve	P-value
Serous Tumors	Serous adenocarcinoma n = 15	13	02	0.051
	Serous borderline tumor n = 02	02	0	
	Serous cyst adenoma n = 23	12	11	
Mucinous Tumors	Mucinous adenocarcinoma n = 06	04	02	0.195
	Mucinous borderline tumor n = 04	04	0	
	Mucinous cystadenoma n = 12	06	06	
Other Rare variants	Dysgerminoma n = 05	05	0	0.011
	Brenner tumors n = 02	02	0	
	Seromucinous cyst adenoma n = 02	0	02	

B7-H4 protein expression was also observed in some rare morphological variants of ovarian tumors like in 5/5 dysgerminoma and 2/2 Brenner tumor of ovaries (Fig. 1).

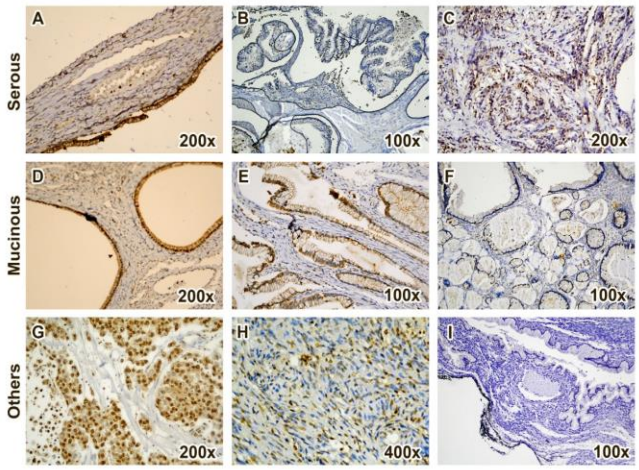

Fig. 1 Distribution of B7-H4 among (A) Serous cystadenoma (B) Borderline serous cystadenoma (C) Serous cystadenocarcinoma (D) Mucinous cystadenoma (E) Borderline mucinous cystadenoma (F) Mucinous cystadenocarcinoma (G) Dysgerminoma (H) Brenner tumor (I) Seromucinous cystadenoma displaying cytoplasmic immunoreactivity as; weak (B), Moderate (C and H), high (A, D and E) and strong (G) scores. In contrast, (F) and (I) are B7-H4 negative sections

These rare variants also have a significant statistical link with B7-H4 protein expression (p-value: 0.011). The strength of protein expression in the majority of the positive sample was observed 1 on the scale of 4 with a significant statistical estimate of a p-value of 0.015 (Table 3). Some studies showed the high intensity of B7-H4 tissue immunohistochemical expressions that may be encountered due to intra observers and inter observers bias and expertise. B7-H4 expression in ovarian tumor was found to be unrelated to clinical or pathologic parameters such as tumor size, stage, or grade.

Table 3 Immunohistochemical staining intensity of B7-H4 in ovarian tumors with statistical estimates

Positive Histological Variant	Cases Number	IHC Scoring and the Proportion of B7-H4 Positive Cells				P- value
		1+ >0-10%	2+ >10-50%	3+ >50-80%	4+ >80-100%	
Serous adenocarcinoma	13	01	06	05	01	0.015
Serous borderline tumor	02	02	00	00	00	
Serous cyst adenoma	12	08	04	00	00	
Mucinous adenocarcinoma	04	02	02	00	00	
Mucinous borderline tumor	04	03	00	01	00	
Mucinous cystadenoma	06	02	03	01	00	
Brenner tumors	02	00	00	02	00	
Dysgerminoma	05	01	01	01	02	

4. Discussion

Ovarian tumor has been more common in recent years, and patients with the disease are presenting at younger ages, with a mortality rate that ranks top among gynecologic tumors. Early detection and active treatment of ovarian tumors can dramatically improve a patients' prognosis and survival rate; therefore, finding effective and accurate diagnostic techniques has become a priority in ovarian tumor research [20]. Multiple markers on the surface of tumor stem cells have been discovered in the last few years. B7-H4, a novel member of the B7 family, suppressed T-cell immunologic responses by decreasing cell proliferation and preventing cytokine production and cell cycle progression [21]. B7-H4 is widely expressed in various tumor tissues, while B7-H4 protein expression is limited. Recent research has linked high levels of B7-H4 expression in numerous malignant tumors to tumor occurrence, progression, and prognoses like cervical cancer, lung tumors, and other epithelial tumors.

Overexpression of B7-H4 in tumor tissues may one day serve as a new tumor diagnostic or therapeutic target. [15, 22].

The current investigation found that the B7-H4 protein was highly expressed in ovarian tumors, more commonly in serous tumors and mucinous tumors. Some studies have comparable results with ours using similar monoclonal antibodies; Choi and colleagues discovered that 85 percent (22/26) of ovarian tumors expressed B7-H4 [23]. Tringeler and colleagues discovered that 100 percent (32/32) of serous ovarian carcinomas, 78 percent (18/23) of borderline serous tumors, and 77 percent (20/26) of serous cystadenomas expressed B7-H4 using the monoclonal antibody A57.1 [24]. Another research done by Barbara Tringler et al. concluded that in all primary serous (n = 32), endometrioid (n = 12), and clear cell carcinomas (n = 15), as well as all metastatic serous (n = 23) and endometrioid (n = 7) ovarian carcinomas, B7-H4 cytoplasmic and membranous expression, was found [24]. These findings comply with our results and imply that B7-H4 may have a role in ovarian tumor developing, and it could be used as a candidate marker for epithelial tumors.

Simon et al., in their research, compared the tissue expression and serum levels of B7-H4 in ovarian tumors found that B7-H4 expression in ovarian tumor tissue was consistent with expression in patients' serum, suggesting that B7-H4 expression in tissues could represent the patients' preoperative serum levels. [25]. These results imply that measuring serum B7-H4 levels could aid in the early ovarian tumor identification – one of our limitations is that we could not compare the serum levels with the tissue levels.

Furthermore, B7-H4 was highly expressed in two Brenner cell tumors and five dysgerminomas, while no other variant was discovered in the current investigation. Tringler et al., on the other hand, found that B7-H4 was expressed 100% in primary ovarian serous carcinomas (32 cases), endometrioid carcinomas (12 cases), clear cell carcinomas (15 cases), and all metastatic serous carcinomas (23 cases) and metastatic endometrioid carcinomas (7 cases), but only 1 out of 11 cases with mucinous carcinomas. The discrepancy between their findings and the findings of this study in terms of B7-H4 expression specificity on a larger scale must be clarified. Different rare variants found in our area may be due to risk exposure, genetic makeup, and customs.

5. Conclusion

In conclusion, B7-H4 expression is linked to the onset and ovarian tumor progression, and it is predicted to become a new molecular marker for assessing the biological behavior of these tumors. However, research with bigger sample sizes is needed to confirm the effects of B7-H4 on ovarian tumor development and its clinical utility. Furthermore, studying other B7 family members (i.e., B7.1, B7.2, inducible co-stimulatory ligand, B7-H3, B7-H5, B7-H6, B7-H7) in ovarian and other malignancies could help us learn more about tumor immune evasion and identify new immunotherapeutic targets.

According to our extensive investigation, no study has been conducted to assess the relationship between B7-H4 expression and the various histological variants in our population.

Because CA125 is typically increased in women with benign conditions like endometriosis, its value as a diagnostic screening tool is restricted. As a result, new serum markers that are more sensitive and specific for identifying ovarian cancer, whether employed alone or in combination with CA125, are urgently needed. Additional research with well-characterized samples on B7-H4 is needed to confirm and broaden our findings. Furthermore, the utility of B7-H4 as a prognostic marker in tissue biopsies and for patient monitoring following surgery should be investigated. Finally, B7-H4's cell surface location and tissue selectivity make it an attractive antibody-based treatment target. Immunotherapeutic medicines that target B7-H4 are currently being developed. Anti–B7-H4 medication could be tried in the future in patient-derived xenograft models and/or clinical trials. B7-H4 appears to be a promising immunotherapeutic target in ovarian cancer, according to our findings and those of other authors. [26].

6. Limitations

The study was cross-sectional due to ethical issues to get the biopsy samples from normal controls, although it was supposed to be a case-control study. As this was a cross-sectional study, we could not follow the patients and obtain data on their survival or cancer recurrence. In the current study, we only used immunohistochemistry due to financial constrain; otherwise, we would have compared the expressions with serum levels. We also could not get the CA-125 levels to compare them with expressions.

Ethical Approval

IRB: Approved by Ethical Review Committee, Ziauddin Medical University, Ref # 1721219MRPAT, dated February 04, 2020.

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