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Lymphocyte Subset Distribution can Predict the Efficacy of First-line Treatment in Patients with Non-Hodgkin Lymphoma

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KEYWORDS

Non-Hodgkin's Lymphoma;
Diffuse Large B-Cell Lymphoma;
Nasal NK/T-Cell Lymphoma;
Lymphocyte Subsets;
Flow Cytometry;
Tumor-Specific Growth Factor (TSGF);
Lactate Dehydrogenase (LDH);
Ki67

ABSTRACT

Objective: To investigate the predictive value of pretreatment peripheral blood lymphocyte subset distribution and other laboratory indices for the efficacy of first-line chemotherapy in patients with diffuse large B-cell lymphoma (DLBCL) and nasal natural killer/T-cell lymphoma (ENKTL).

Methods: A retrospective analysis was conducted on 110 newly diagnosed lymphoma patients, comprising 63 cases of DLBCL and 47 cases of ENKTL. Demographic characteristics, disease stage, treatment regimens, and efficacy evaluations were collected, along with pretreatment levels of peripheral blood lymphocyte subsets (T cells, CD4+, CD8+, B cells, NK cells, CIK cells), tumor-specific growth factor (TSGF), lactate dehydrogenase (LDH), and Ki-67 expression. Univariate and multivariate logistic regression analyses were performed, with a significance threshold of $P < 0.05$.

Results: The DLBCL group predominantly presented with stage III–IV disease, while the ENKTL group mainly had stage I disease, demonstrating a statistically significant difference in stage distribution ($P < 0.001$). Multivariate analysis revealed that in DLBCL patients, disease stage (stage III: OR = 0.07, P = 0.043), elevated CD8+ T cell proportion (OR = 0.94, P = 0.047), and increased LDH levels (OR = 1.00, P = 0.021) were independent risk factors for poor treatment efficacy. In ENKTL patients, disease stage (stage II: OR = 0.03, P = 0.042; stage IV: OR = 0.00, P = 0.033) and elevated total T cell proportion (OR = 0.74, P = 0.049) emerged as independent predictors of unfavorable outcomes.

Conclusion: Pretreatment peripheral blood lymphocyte subset distribution is closely correlated with the efficacy of first-line chemotherapy in DLBCL and ENKTL patients. Elevated CD8+ T cell proportion indicates poorer outcomes in DLBCL, while increased total T cell proportion is associated with adverse treatment responses in ENKTL. Integrating lymphocyte subset testing with disease stage and LDH levels serves as a robust tool for individualized efficacy prediction, providing valuable insights for adjusting clinical treatment strategies.

Introduction

Lymphoma is the most common malignant tumor of the lymphohematopoietic system. The World Health Organization (WHO) classifies lymphoma into Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL), the latter originating from B cells or T cells/natural killer

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(NK)cells [1,2]. Diffuse large B-cell lymphoma (DLBCL) is the most common and aggressive type of B-cell lymphoma in adults, accounting for 30%–40% of NHL in China. DLBCL has great heterogeneity in clinical manifestations and prognosis [1]. Nasal NK/T-cell lymphoma (ENKTL) is a unique pathological subtype of non-Hodgkin lymphoma [3], which is highly aggressive, has poor response to treatment, and is prone to relapse [4]. ENKTL accounts for a relatively high proportion of head and neck lymphomas (about 22%), and occurs more frequently in the head and neck than lymphomas in other parts of the body [5]. Studies have found that nasal and sinus ENKTL and DLBCL have similar clinical symptoms, including nasal congestion, headache, and eye symptoms, which can easily lead to misdiagnosis. Clinically, it is necessary to combine other laboratory tests to improve the accuracy of diagnosis of the two [6]. Therefore, it is very meaningful to seek simple, readily avail-able, fast and accurate laboratory indicators to differentiate between the two.

Lymphocytes maintain dynamic balance in the body, thereby playing the functions of immune defense, immune surveillance and immune homeostasis. In the tumor state, the body's immune function is suppressed, which may lead to tumor proliferation, metastasis and spread, while peripheral blood lymphocyte subsets can reflect the immune function status of tumor patients and are related to the patient's prognosis [7]. Lactate dehydrogenase (LDH) has been included in the DLBCL international prognostic index scoring system, and is used in combination with age, Ann Arbor stage, performance status score and whether there is extranodal invasion to identify low, medium and high risk patients [1,2]. Tumor-specific growth factor (TSGF) is a protein produced by tumor cells during their formation. It is closely related to tumor growth and has relative specificity for tumors. Therefore, it has a strong ability to diagnose tumors before treatment and to evaluate the efficacy of treatment after recurrence [8]. Ki67 is a nuclear protein that has been widely used in clinical practice as a marker of proliferating cells. Its expression level is closely related to the degree of tumor cell proliferation, differentiation, malignancy (infiltration, metastasis) and patient prognosis [9]. Therefore, this study analyzed the distribution of lymphocyte subsets and the relationship between the expression of LDH, TSGF and Ki67 and the survival status and treatment effect of DLBCL and ENKTL, in order to find a more sensitive and reliable marker that can improve the diagnostic accuracy of DLBCL and ENKTL and predict the treatment effectiveness of both.

Materials and Methods

General Information

From August 2015 to December 2017, the patient sought medical treatment at Hunan Cancer Hospital and was diagnosed with DLBCL or ENKTL were included. Clinical data such as patient gender, age and time of diagnosis, disease stage (Ann Arbor staging), treatment regimen, and efficacy evaluation were collected. Patients were followed up to collect data on their survival status and survival time. Pre-treatment lymphocyte subsets (T, CD4, CD8, B, NK, CIK), TSGF, LDH, and Ki67 test results were retrieved and recorded.

Inclusion Criteria

1) Diagnosed as DLBCL or ENKTL (diagnostic classification 1 or 2);
2) Survival time is assessable (>1 month);
3) The results of the tests for T, CD4, CD8, B, NK, CIK, TSGF, LDH, and Ki67 are complete.

Peripheral Blood Lymphocyte Subset Analysis

EDTA-anticoagulated peripheral blood sample was collected from the patient. The flow cytometer used was a Beckman Coulter FC500 (USA); All flow cytometry reagents were manufactured by Beckman Coulter. Antibodies were as follows: a quadruple antibody of T lymphocyte subset: CD45-FITC/CD4-PE/CD8-ECD/CD3-PC5; a quadruple antibody of B lymphocyte subset: CD45- FITC/CD56-PE/ CD19-ECD/CD3-PC5 and standalone CD16-PE; quality control was IMMUNO-TROL ™ Cells whole blood quality control; red blood cell lysis buffer: Optilyse. The percentages of CD3+ T cells, CD4+ T cells, CD8+ T cells, CD19+ B cells, and CD3 - CD16+CD56+ NK cells, as well as the CD4+/CD8+ ratio, were analyzed using the FC500's built-in software.

Treatment Plan and Efficacy Evaluation

Treatment regimens included R-CHOP regimens (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone), GELOXD regimens (gemcitabine + asparaginase + oxaliplatin + dexamethasone), and others. Efficacy was evaluated according to the 2014 Lugano criteria, categorized as complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD). The overall response rate (ORR) was the sum of the CR and PR rates. In this study, response was defined as CR / PR; ineffectiveness as SD /PD.

Follow-Up

All patients were followed up through outpatient services and telephone at Hunan Cancer Hospital, with the follow-up deadline being June 30, 2023. Progression-free survival (PFS) refers to the time from diagnosis to disease recurrence/ progression or death from any cause, or the last follow-up.

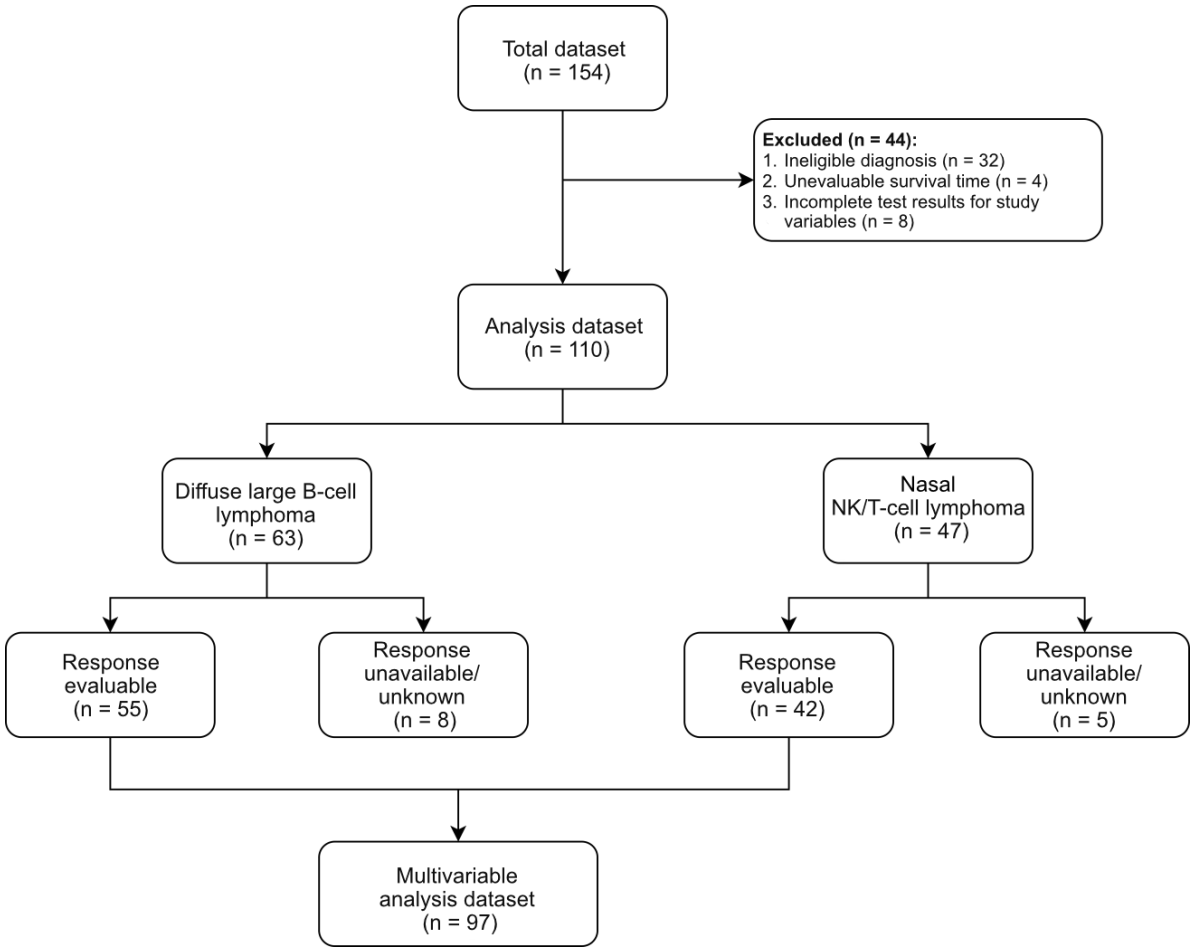


Figure 1 | Screening and group inclusion process flowchart

Flowchart of participant screening and group allocation in the study. The total initial dataset comprised 154 cases. From this, 44 cases were excluded for three reasons: ineligible diagnosis (n = 32), unavailable survival time (n = 4), or incomplete test results for study variables (n = 8). This yielded an analysis dataset of 110 cases, which was then stratified into two pathological subtypes: diffuse large B - cell lymphoma (n = 63) and nasal NK/T - cell lymphoma (n = 47). Within each subtype, cases were categorized by response assessability: "response evaluable" and "response unavailable/unknown". Specifically, for diffuse large B - cell lymphoma, 55 cases were response evaluable and 8 were response unavailable/unknown; for nasopharyngeal NK/T - cell lymphoma, 42 cases were response evaluable and 5 were response unavailable/unknown. Finally, a total of 97 cases across both subtypes were included in the multivariable analysis dataset.

Overall survival (OS) refers to the time from diagnosis to death from any cause, or the last follow-up.

Statistical Methods

Data analysis was performed using IBM SPSS 26.0 statistical software. Qualitative and ordinal data, including patient baseline characteristics (including gender, age, disease stage (Ann Arbor stage), treatment regimen, efficacy evaluation, survival time, and survival status), were compared using chi-square tests or Fisher's exact tests. Normality tests were performed on quantitative data. If the data followed a normal or approximately normal distribution, t-tests were used to compare the means; if the data did not follow a normal distribution, nonparametric methods were used to compare central tendency (median). Kaplan-Meier analysis was used for PFS and OS. Uni-variate and multivariate analyses were performed using Cox proportional hazards regression models. A p-value < 0.05 was considered statistically significant.

Results

Patient Baseline Information

Data Cleaning

Patients with evaluable survival time (>1 month) and complete pre-treatment lymphocyte subset (T, CD4, CD8, B, NK, CIK), TSGF, LDH, and Ki67 test results were included in the study. After data screening and filtering, 110 cases were included in the study; among them, 63 patients were diagnosed with DLBCL, and 47 patients were diagnosed with ENKTL. (The screening and enrollment process flowchart is shown in **Figure 1**).

Baseline Data Statistical Analysis

As shown in **Table 1**, the baseline distribution of DLBCL and ENKTL was as follows: there was no significant difference in gender between DLBCL and ENKTL patients; the majority of patients in both cases were under 60 years old

Table 1 | Population baseline data analysis (n%)

Factor		Diffuse large B-cell lymphoma n=63	Nasal NK/T-cell lymphoma n=47	Total population n=110	X^2 test, *P*
Gender	male	37 (58.73%)	26 (55.32%)	63 (57.27%)	X^2 = 0.128 , *P* = 0.721
	female	26 (41.27%)	21 (44.68%)	47 (42.73%)	
Age	<60	45 (71.43%)	44 (93.62%)	89 (80.91%)	X^2 = 8.580 , *P* = 0.003
	>=60	18 (28.57%)	3 (6.38%)	21 (19.09%)	
Disease staging	I	11 (17.46%)	26 (55.32%)	37 (33.64%)	X^2 = 22.928 , P = 0.000
	II	18 (28.57%)	13 (27.66%)	31 (28.18%)	
	III	9 (14.29%)	1 (2.13%)	10 (9.09%)	
	IV	22 (34.92%)	4 (8.51%)	26 (23.64%)	
	Unknown	3 (4.76%)	3 (6.38%)	6 (5.45%)	
Treatment plan	Contains CHOP	48 (76.19%)	13 (27.66%)	61 (55.45%)	X^2 = 60.986 , P = 0.000
	Includes GELOXD (excluding CHOP)	0 (0.00%)	32 (68.09%)	32 (29.09%)	
	other	15 (23.81%)	2 (4.26%)	17 (15.45%)	
Therapeutic effect assessment	CR	18 (28.57%)	30 (63.83%)	48 (43.64%)	X^2 = 14.996 , *P* = 0.005
	PR	22 (34.92%)	7 (14.89%)	29 (26.36%)	
	SD	2 (3.17%)	0 (0.00%)	2 (1.82%)	
	PD	13 (20.63%)	5 (10.64%)	18 (16.36%)	
	Unknown	8 (12.70%)	5 (10.64%)	13 (11.82%)	
Survival time	Lost to visit	3 (4.76%)	1 (2.13%)	4 (3.64%)	X^2 = 1.497 , *P* = 0.473
	<60 months	23 (36.51%)	22 (46.81%)	45 (40.91%)	
	>=60 months	37 (58.73%)	24 (51.06%)	61 (55.45%)	
Survival status	Lost to visit	3 (4.76%)	1 (2.13%)	4 (3.64%)	X^2 = 2.754 , *P* = 0.252
	Survival	39 (61.90%)	36 (76.60%)	75 (68.18%)	
	die	21 (33.33%)	10 (21.28%)	31 (28.18%)	

Note: Data in red font indicate statistically significant differences (P < 0.05); the statistical software used was IBM SPSS 26.0.

Appendix 1 | Normality test of quantitative indicators (according to diagnostic classification)

Factor	Diagnostic classification	Kolmogorov - Sminov (V)a			Shapiro Wilke		
		statistics	Degrees of freedom	Significance	statistics	Degrees of freedom	Significance
Age	B cells	0.103	63	0.093	0.968	63	0.099
	NK/T cells	0.103	47	0.200*	0.978	47	0.497
T	B cells	0.086	63	0.200*	0.985	63	0.620
	NK/T cells	0.090	47	0.200*	0.969	47	0.250
CD4	B cells	0.061	63	0.200*	0.975	63	0.229
	NK/T cells	0.065	47	0.200*	0.991	47	0.975
CD8	B cells	0.101	63	0.178	0.962	63	0.050
	NK/T cells	0.067	47	0.200*	0.979	47	0.546
CD4/CD8	B cells	0.102	63	0.099	0.943	63	0.006
	NK/T cells	0.210	47	0.000	0.791	47	0.000
B	B cells	0.087	63	0.200*	0.948	63	0.010
	NK/T cells	0.122	47	0.078	0.935	47	0.012
NK	B cells	0.143	63	0.003	0.942	63	0.005
	NK/T cells	0.103	47	0.200*	0.947	47	0.034
CIK	B cells	0.265	63	0.000	0.518	63	0.000
	NK/T cells	0.208	47	0.000	0.753	47	0.000
TSGF	B cells	0.144	63	0.002	0.910	63	0.000
	NK/T cells	0.102	47	0.200*	0.966	47	0.193
LDH	B cells	0.279	63	0.000	0.538	63	0.000
	NK/T cells	0.247	47	0.000	0.751	47	0.000
Ki67	B cells	0.118	63	0.029	0.945	63	0.007
	NK/T cells	0.147	47	0.013	0.963	47	0.143

** This is the lower bound for true significance.*
a. Reilly significance correction

Appendix 2 | Normality test of quantitative indicators (population)

Category	Kolmogorov - Sminov (V)a			Shapiro Wilke		
	statistics	Degrees of freedom	Significance	statistics	Degrees of freedom	Significance
Age	0.090	110	0.028	0.979	110	0.080
T	0.058	110	0.200*	0.984	110	0.198
CD4	0.034	110	0.200*	0.990	110	0.586
CD8	0.054	110	0.200*	0.985	110	0.253
CD4/CD8	0.146	110	0.000	0.860	110	0.000
B	0.067	110	0.200*	0.956	110	0.001
NK	0.125	110	0.000	0.947	110	0.000
CIK	0.233	110	0.000	0.642	110	0.000
TSGF	0.102	110	0.006	0.939	110	0.000
LDH	0.281	110	0.000	0.463	110	0.000
Ki67	0.131	110	0.000	0.957	110	0.001

** This is the lower bound for true significance.*
a. Reilly significance correction

Table 2 | Analysis of pathological markers in two types of non-Hodgkin's lymphoma (t-test for all variables) (mean (95 CI))

Factors/Indicators	Diffuse large B-cell lymphoma n=63	Nasal NK/T-cell lymphoma n = 4 7	Total population n=110	*t* -test, *P*
Age	53.27 (50.42, 56.12)	42.79 (39.19, 46.38)	48.79 (46.38, 51.21)	t=4.640, P=0.000
T	71.34 (68.50, 74.19)	75.18 (72.46, 77.89)	72.98 (70.98, 74.99)	t=1.899, P=0.060
CD4	36.09 (33.64, 38.54)	36.12 (33.14, 39.09)	36.10 (34.24, 37.96)	t=0.016, P=0.988
CD8	30.76 (28.35, 33.18)	31.90 (28.92, 34.88)	31.25 (29.40, 33.10)	t=0.600, P=0.550
B	7.28 (5.96, 8.61)	7.72 (6.41, 9.04)	7.47 (6.54, 8.40)	t=0.464, P=0.643

Note: Data in red font indicate statistically significant differences (P < 0.05); the statistical software used was IBM SPSS 26.0.

Table 3 | Analysis of pathological markers in two types of non-Hodgkin's lymphoma (non-parametric test) (median (Q25, Q75))

Factors/Indicators	Diffuse large B-cell lymphoma n=63	Nasal NK/T-cell lymphoma n = 4 7	Total population n=110	Mann - Whitney U test, *P*
CD4/CD8	1.21(0.86,1.65)	1.08 (0.82, 1.52)	1.18(0.82,1.64)	U=1376.50, P=0.530
NK	17.70 (11.30, 26.80)	15.10 (9.20, 21.80)	16.10 (10.13, 23.75)	U=1156.00, P=0.050
CIK	1.90 (0.70, 3.70)	3.80 (0.80, 6.30)	2.25(0.70,5.55)	U=1788.50, P=0.063
TSGF	67.80 (53.70, 74.55)	55.70 (41.95, 66.90)	61.91 (47.46, 72.41)	U=1062.50, P=0.012
LDH	262.90 (183.30, 463.10)	209.10 (183.90, 250.90)	227.75 (183.75, 330.38)	U=1159.50, P=0.052
Ki67 (%)	70.00 (50.00, 80.00)	70.00 (50.00, 80.00)	70.00 (50.00, 80.00)	U=1411.00, P=0.671

Note: Data in red font indicate significant differences (P < 0.05); data in bold black font indicate marginally significant differences (P approximately 0.05). Statistical software used was IBM SPSS 26.0 .

Table 4 | Analysis of pathological markers in two types of non-Hodgkin's lymphoma (ANOVA) (mean (95 CI))

Factors/Indicators	Diffuse large B-cell lymphoma n=63	Nasal NK/T-cell lymphoma n = 4 7	Total population n=110	F-test, *P*
age	53.27 (50.42, 56.12)	42.79 (39.19, 46.38)	48.79 (46.38, 51.21)	F=21.529, P=0.000
T	71.34 (68.50, 74.19)	75.18 (72.46, 77.89)	72.98 (70.98, 74.99)	F=3.607, P=0.060
CD4	36.09 (33.64, 38.54)	36.12 (33.14, 39.09)	36.10 (34.24, 37.96)	F=0.000, P=0.988
CD8	30.76 (28.35, 33.18)	31.90 (28.92, 34.88)	31.25 (29.40, 33.10)	F=0.360, P=0.550
CD4/CD8	1.33 (1.17, 1.49)	1.37 (1.10, 1.64)	1.35 (1.20, 1.49)	F=0.071, P=0.791
B	7.28 (5.96, 8.61)	7.72 (6.41, 9.04)	7.47 (6.54, 8.40)	F=0.216, P=0.643
NK	19.48 (16.82, 22.14)	15.69 (13.19, 18.18)	17.86 (15.99, 19.72)	F=4.077, P=0.046
CIK	3.29 (2.01, 4.57)	4.98 (3.33, 6.64)	4.01 (3.00, 5.03)	F=2.712, P=0.103
TSGF	61.84 (57.54, 66.15)	53.67 (47.90, 59.43)	58.35 (54.84, 61.86)	F=5.420, P=0.022
LDH [a]	440.53 (305.30, 575.77)	237.12 (205.99, 268.24)	353.62 (273.67, 433.56)	F=6.540, P=0.012
Ki67	64.86 (60.22, 69.49)	42.79 (39.19, 46.38)	64.23 (60.71, 67.74)	F=0.168, P=0.683

Note: Data in red font indicate statistically significant differences (P < 0.05). The statistical software used was IBM SPSS 26.0 . α indicates unequal variances; in this case, the F-test results are for reference only.

(P = 0.003); DLBCL had more advanced (III-IV) cases, while ENKTL had more early (I) cases (P = 0.000); DLBCL was mainly treated with CHOP-containing regimens, while ENKTL was mainly treated with GELOXD regimens (P = 0.000); DLBCL was mainly characterized by PR, while ENKTL was mainly characterized by CR. ENKTL had a better prognosis (P = 0.005). Therefore, the statistical analysis was determined to focus on "efficacy assessment" as the primary outcome indicator.

Analysis and Comparison of Pathological Indicators in Two Types of Non-Hodgkin Lymphoma

Normality Test of Quantitative Indicators

As shown in Appendices 1 and 2, the normality test results indicate that age, T, CD4, CD8, and B follow an approximately normal distribution (**Appendix 1**). The t-test will be used to compare the means (**Table 2**). CD4/CD8, NK, CIK, TSGF, LDH, and Ki67 do not follow a normal distribution (**Appendix 2**). Nonparametric methods will be used to com-pare the central tendency (median, **Table 3**). Furthermore, this study also used ANOVA (Analysis of Variance) to further validate the above results (**Table 4**).

Comparison of Pathological Markers Between Two Types of non-Hodgkin's Lymphoma

As shown in **Tables 2, 3 and 4**, the differences in age, NK and TSGF between DLBCL and ENKTL were statistically significant ($P<0.05$), LDH showed a marginally significant difference ($P\approx0.05$), while the differences in the other 7 indicators were not statistically significant ($P>0.05$).

KM Survival Analysis of Two Types of non-Hodgkin Lymphoma

As shown in **Figure 2**, the survival curves of DLBCL and ENKTL highly overlap, with no significant difference in survival time and survival rate. Based on this, the direction of statistical analysis was determined again: "efficacy assessment" was used as the main outcome indicator.

Evaluation of the Efficacy of DLBCL and Nasal ENKTL

Evaluation of the Efficacy of DLBCL

Logistic regression analysis was performed first, using univariate regression analysis. Cases with a p-value < 0.10 in univariate regression were then included in multivariate regression analysis. Because CD4/CD8 is a known interaction variable with other factors (CD4, CD8), if CD4 or CD8 was already included in multivariate regression, CD4/ CD8 was not included. A p-value < 0.05 was considered statistically significant in multivariate regression. The analysis results showed that stage, CD8, and LDH were independent risk factors for the efficacy of first-line treatment in diffuse large B-cell lymphoma (higher values indicated poorer outcomes) (**Table 5**). ANOVA analysis showed that CD8, CD4/CD8, and LDH may be associated with the efficacy of first-line treatment for diffuse large B-cell lymphoma; this was consistent with the results of logistic regression analysis (**Table 6**).

Evaluation of the Efficacy of Nasal ENKTL

Logistic regression analysis was performed first, with univariate regression analysis conducted. Cases with a p-value < 0.10 in univariate regression were then included in multivariate regression analysis. Because CD4/CD8 is a known interaction variable with other factors (CD4, CD8), if CD4 or CD8 was already included in multivariate regression, CD4/ CD8 was not included. A p-value < 0.05 was considered statistically significant in multivariate regression. The analysis results showed that stage and total T cell proportion were risk factors for the efficacy of first-line treatment for nasal NK/T-cell lymphoma (higher values indicated poorer outcomes) (**Table 7**). ANOVA analysis showed no factors associated with the efficacy of first-line treatment for nasal NK/T-cell lymphoma. Total T cell proportion showed marginal significance. This may be due to differences between univariate and multivariate analyses; in this case, the results of logical regression were considered definitive (**Table 8**).

Discussion

DLBCL is the most common type of NHL. In China, it accounts for about 40 % of adult NHL cases. The median age of onset is 50 to 70 years, with males slightly more affected than females [10]. In this study, the average age of onset for DLBCL patients was 53 years, which is close to the results of a retrospective analysis of DLBCL patients from the Cancer Hospital of the Chinese Academy of Medical Sciences from 2005 to 2018 (median age of onset was 54 years). The majority of patients were under 60 years old (71.43 %), which is earlier than the median age of onset for patients in the United States during the same period (63 years). The basic treatment for DLBCL is targeted therapy combined with chemotherapy. Rituximab combined with CHOP is the standard first-line treatment regimen (R-CHOP) [11]. In this study, there were more advanced (III-IV) DLBCL cases. 76.19% of DLBCL patients used the R- CHOP regimen, and the CR+PR rate reached 63.49%. Studies have reported that the standard first-line R-CHOP regimen can cure about 60% of DLBCL patients [12].

In China, ENKTL is the most common peripheral T-cell lymphoma, with an incidence rate of approximately 28.16% [10]. ENKTL is more common in men and has a lower age of onset. In this study, the median age of onset for ENKTL was approximately 42.8 years, with a slightly higher rate in men. The tumor is often confined to the nasal cavity or directly invades adjacent structures or tissues. Stage I-II patients ac-

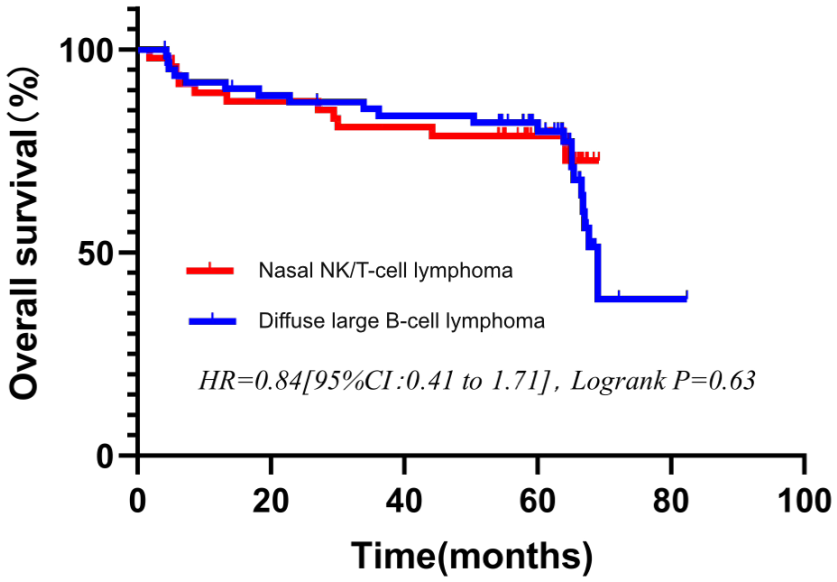


Figure 2 | Kaplan-Meier curves for overall survival in two types of lymphoma

Kaplan - Meier survival curves for overall survival in patients with nasal NK/T - cell lymphoma and diffuse large B - cell lymphoma. The red line represents nasal NK/T - cell lymphoma, and the blue line represents diffuse large B - cell lymphoma. The hazard ratio is 0.84 with a 95% confidence interval of 0.41 to 1.71. The Logrank test gives a value of 0.63, which indicates that there is no statistically significant difference in overall survival between the two groups of lymphoma. The survival curves were plotted using GraphPad Prism 8.0.

Table 5 | Evaluation of treatment efficacy in diffuse large B-cell lymphoma - logistic regression analysis (N=55)

factor	Univariate OR (95% CI)	Single-factor P	Multifactorial OR (95% CI)	Multifactor-P
Gender	0.85 (0.26-2.78)	0.782		
Disease staging (Reference: Stage I)				
Phase II	0.11 (0.01-0.99)	0.049	0.13 (0.01-1.22)	0.074
Phase III	0.05 (0.00-0.59)	0.017	0.07 (0.01-0.92)	0.043
Phase IV	0.07 (0.01-0.58)	0.014	0.20 (0.02-2.11)	0.178
Unknown	0.02 (0.00-0.29)	0.004	0.02 (0.00-0.35)	0.006
age	1.02 (0.96-1.08)	0.491	-	-
T	0.99 (0.94-1.05)	0.709	-	-
CD4	1.05 (0.98-1.12)	0.142	-	-
CD8	0.92 (0.86-0.99)	0.016	0.94 (0.88-1.00)	0.047
CD4/CD8	4.13 (1.12-15.21)	0.033	-	-
B	1.08 (0.95-1.23)	0.221	-	-
NK	1.00 (0.95-1.06)	0.942	-	-
CIK	1.00 (0.90-1.12)	0.957	-	-
TSGF	0.98 (0.94 - 1.02)	0.302	-	-
LDH	1.00 (0.99-1.00)	0.032	1.00 (0.99-1.00)	0.021
Ki67	0.05 (0.00-2.10)	0.115	-	-

Table 6 | Diffuse large B-cell lymphoma - pathological marker analysis

Factors/Indicators	S D or P D n=15	P R or CR n=40	Total population n=55	*F* -test, *P*
age	50.73 (45.53, 55.93)	52.90 (49.43, 56.37)	52.31(49.49,55.13)	F=0.466, P=0.498
T	73.31 (66.04, 80.59)	72.08 (68.82, 75.35)	72.42(69.46,75.38)	F=0.135, P=0.714
CD4	32.97 (26.78, 39.16)	37.54 (34.44, 40.64)	36.29 (33.53, 39.05)	F=2.233, P=0.141
CD8	36.62 (30.86, 42.38)	29.28 (26.51, 32.04)	31.28 (28.67, 33.88)	F=7.048, P=0.010
CD4/CD8	1.00 (0.73, 1.28)	1.43 (1.22, 1.64)	1.32 (1.14, 1.49)	F=5.237, P=0.026
B	5.49 (2.90, 8.08)	7.40 (5.72, 9.08)	6.88 (5.49, 8.26)	F=1.526, P=0.222
NK	18.58 (11.61, 25.55)	18.81 (15.71, 21.90)	18.75 (15.93, 21.56)	F=0.005, P=0.943
CIK	3.31(1.34,5.27)	3.39 (1.50, 5.29)	3.37 (1.92, 4.82)	F=0.003, P=0.958
TSGF	66.63 (56.75, 76.51)	61.66 (56.85, 66.46)	63.01 (58.73, 67.29)	F=1.077, P=0.304
LDH	756.75 (275.98, 1237.53)	278.26 (226.29, 330.24)	408.76 (270.72, 546.80)	F=11.429, P=0.001
Ki67	0.70 (0.59, 0.81)	0.61(0.55,0.67)	0.64 (0.59, 0.69)	F=2.599, P=0.113

Note: Data in red font indicate statistically significant differences (P < 0.05); the statistical software used was IBM SPSS 26.0.

Table 7 | Evaluation of treatment efficacy for nasal NK/T-cell lymphoma Logistics regression analysis (N=42)

factor	Univariate OR (95% CI)	Single-factor P	Multifactorial OR (95% CI)	Multifactor-P
Gender	NR	0.998		
Disease staging (Reference: Stage I)				
Phase II	0.13 (0.01-1.43)	0.095	0.03 (0.00-0.88)	0.042
Phase III	NR	NR	NR	NR
Phase IV	0.09 (0.00-1.98)	0.126	0.00 (0.00-0.59)	0.033
Unknown	NR	NR	NR	NR
age	0.92 (0.82-1.02)	0.121	-	-
T	0.89 (0.78-1.02)	0.088	0.74 (0.56-1.00)	0.049
CD4	0.94 (0.85-1.03)	0.184	-	-
CD8	0.99 (0.91-1.08)	0.792	-	-
CD4/CD8	0.60 (0.28-1.33)	0.209	-	-
B	1.05 (0.84-1.31)	0.658	-	-
NK	1.16 (0.97-1.38)	0.109	-	-
CIK	0.95 (0.83-1.09)	0.478	-	-
TSGF	0.99 (0.94-1.04)	0.642	-	-
LDH	1.00 (0.99-1.01)	0.885	-	-
Ki67	0.68 (0.01-85.97)	0.875	-	-

Note: The logistic regression statistical analysis software used is IBM SPSS 26.0 ; NR indicates that the numerical value cannot be specifically calculated (the denominator in the proportion is 0).

Table 8 | Nasal Cavity NK/T Cell Lymphopathological Indicators Analysis

Factors/Indicators	S D or P D n=5	P R or CR n=37	Total population n=42	*F* -test, *P*
Age	50.40 (44.22, 56.58)	41.46 (37.50, 45.42)	42.52 (38.90, 46.15)	F=2.715, P=0.107
T	82.10 (72.18, 92.03)	74.18(71.17,77.19)	75.12(72.25,77.99)	F=3.459, P=0.070
CD4	41.90 (20.36, 63.44)	35.21(32.14,38.29)	36.01 (32.76, 39.26)	F=1.848, P=0.182
CD8	33.18 (10.58, 55.78)	31.86 (28.67, 35.05)	32.02 (28.71, 35.33)	F=0.067, P=0.798
CD4/CD8	1.92 (-0.19, 4.03)	1.31 (1.03, 1.59)	1.39 (1.08, 1.69)	F=1.750, P=0.193
B	7.12 (3.86, 10.38)	8.08 (6.48, 9.68)	7.96 (6.54, 9.39)	F=0.188, P=0.667
NK	9.52 (-2.09, 21.13)	16.27 (13.62, 18.92)	15.47 (12.88, 18.06)	F=3.059, P=0.088
CIK	6.74 (-5.34, 18.82)	4.76 (3.01, 6.50)	4.99 (3.18, 6.80)	F=0.507, P=0.481
TSGF	56.87 (38.47, 75.26)	52.51(45.69,59.34)	53.03 (46.86, 59.20)	F=0.209, P=0.650
LDH	227.84 (191.79, 263.89)	235.12 (197.41, 272.84)	234.26 (201.09, 267.42)	F=0.020, P=0.888
Ki67	0.64 (0.43, 0.85)	0.63 (0.56, 0.69)	0.63 (0.57, 0.69)	F=0.023, P=0.879

Note: Data in red font indicate statistically significant differences (P < 0.05); the statistical software used was IBM SPSS 26.0 .

count for 70%-90%, and Stage III-IV patients account for 10%-30% [13, 14]. The results of this study are consistent with this, with Stage I-II accounting for 82.98%. Studies have reported that although the disease is mainly localized (Stage I and II) at onset, its efficacy is poor and the overall prognosis is bad. In this study, the survival rate of cases with a survival time of more than 5 years reached more than 50%. The recommended treatment for ENKTL is chemotherapy regimens based on L-asparaginase or pegaspargase, including the P-GemOx regimen (gemcitabine + pegaspargase + oxaliplatin), the DDGP regimen (cisplatin + dexamethasone + gemc-itabine + pegaspargase), the dose-adjusted SMILE regimen (methotrexate + leucovorin + ifosfamide + mesna + dexam-ethasone + etoposide + L- asparaginase)and the AspaMetDex regimen (pegaspargase + high-dose methotrexate + dexam-ethasone), etc. [10]. In this study, most ENKTL patients used the GELOXD regimen (68.09%), which was similar to the P-GemOx regimen. The study showed that this regimen was more effective, less toxic, and more tolerable than chemo-therapy regimens such as SMILE, Hyper-CVAD, and Hy-per- CCVP [15]. In this study, ENKTL patients were mainly CR.

DLBCL and ENKTL include factors such as age, distant lymph node invasion, stage, primary extranasal tumor, and LDH. This study newly included lymphocyte subsets and TSGF, indicators that are reported in the literature to be closely related to tumor prognosis, in order to discover more sensitive and reliable prognostic biomarkers or supplement existing prognostic factors. Studies have found that the occurrence and development of tumors are related to changes in peripheral blood lymphocyte subsets [16-18]. Lymphocyte subsets can reflect the body's immune status, have certain

value in the diagnosis of benign and malignant tumors, and are related to clinicopathological features such as lymph node metastasis, clinical stage, and molecular subtyping [19-21]. Studies have reported that lymphocyte subsets can predict the response to neoadjuvant chemoimmunotherapy in NSCLC [22]. Therefore, monitoring lymphocyte subsets in vivo is helpful for the auxiliary diagnosis and disease analysis of cancer in clinical practice.

In this study, the proportion of NK cells, TSGF, and LDH expression were all higher in DLBCL than in ENKTL. Ki67 expression showed no significant difference. Since there was no significant difference in survival time and survival rate between DLBCL and ENKTL in this study, we further analyzed the correlation between various factors and disease treatment efficacy. The results showed that stage, CD8+ T cell proportion, and LDH were independent risk factors for first-line treatment efficacy in DLBCL; stage and total T cell proportion were risk factors for first-line treatment efficacy in ENKTL. Stage and LDH, as classic prognostic factors, have been widely reported and included in guidelines [10,23]. Lymphocyte subsets can serve as new prognostic markers to supplement existing models.

Conclusion

The distribution of peripheral blood lymphocyte subsets is closely related to the efficacy of first-line treatment for DLBCL and nasal ENKTL. By detecting the distribution of peripheral blood lymphocyte subsets before treatment, the efficacy of first-line treatment for DLBCL and nasal ENKTL can be predicted, thereby guiding doctors to adjust the treatment plan.

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An Analysis on Prevalence and Risk Factors of Chronic Kidney Disease in Yongzhou Region

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KEYWORDS

Chronic Kidney Disease;
Prevalence;
Risk Factors;
Cross-Sectional Study

ABSTRACT

Objective This study is to investigate the prevalence of chronic kidney disease (CKD) and its major risk factors among the adult population in Yongzhou, Hunan Province, it provides a scientific basis for developing targeted CKD prevention and management strategies in this region.

Methods A cross-sectional study was conducted from January 2024 to July 2025, involving participants from the nephrology outpatient clinic and health check-up center of Yongzhou Central Hospital. All participants underwent questionnaires, physical examinations, and laboratory tests. The collected data were statistically analyzed.

Results A total of 2,571 eligible participants were enrolled in this study. The participants comprised 1,167 patients from the nephrology outpatient clinic and 1,404 individuals from the health examination center, with a mean age of 52.16 ± 14.23 years (50.36 ± 15.82 years for males and 54.09 ± 12.18 years for females). Chi-square tests revealed that the differences in the prevalence of CKD across groups stratified by age, BMI, physical labor level, alcohol consumption, hypertension, diabetes, dyslipidemia, and hyperuricemia were statistically significant in the total population. Logistic regression analysis identified increasing age, lack of physical labor, hypertension, diabetes, dyslipidemia, and hyperuricemia as independent risk factors for CKD.

Conclusion Although the prevalence of CKD among adults in Yongzhou is slightly lower than the national average, the absolute disease burden remains substantial and is closely associated with multiple modifiable metabolic risk factors. Early screening and comprehensive management of risk factors such as hypertension, diabetes, obesity, and hyperuricemia should be strengthened to delay the onset and progression of CKD.

Introduction

Chronic Kidney Disease (CKD) is a major non communicable disease characterized by progressive damage to the structure or function of the kidneys, and has become an increasingly severe public health challenge worldwide. Its course is insidious, and early symptoms are often not obvious, resulting in a large number of patients not receiving timely diagnosis and intervention. Once end-stage renal disease (ESRD) progresses, patients will face a heavy burden of dialysis or kidney transplantation, accompanied by a soaring risk of cardiovascular events and a significant increase in all-cause mortality. This not only seriously damages individual health and quality of life, but also puts enormous pressure on social medical resources. According to data from the Global Burden of Disease (GBD) study, the total number of CKD patients worldwide has exceeded 800 million, with an overall

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prevalence rate close to 10% and showing a continuous upward trend. In China, with the acceleration of the aging process of the population, the sharp increase in the prevalence of metabolic diseases (such as diabetes and hypertension) and profound changes in environmental and lifestyle factors, the prevalence of CKD is particularly severe [3]. Thoroughly analyzing the risk factors of CKD is the cornerstone of developing effective prevention and control strategies. Therefore, this study aims to understand the characteristics and epidemiological factors of CKD in Yongzhou area, and provide certain data support for the prevention and treatment of CKD.

Materials and Methods

Population

The data source for this study is patients and physical examination personnel who visited in Departmentthe Nephrology of Yongzhou Central Hospital from January 2024 to July 2025. The selected individuals had complete personal information and ultimately agreed to participate in this study, age at 18 - 65 years, the effective sample number is 2571. All enrolled individuals had signed informed consent forms, understood all the contents of this study, and voluntarily participated in this study.

Method

This study consists of questionnaire survey and laboratory examination. The content of the questionnaire includes basic personal information such as gender, height, weight, marital status and education level, life habit risk factors such as smoking, drinking, diet type, and sleep duration, and chronic disease risk factors such as hypertension, diabetes, hyperuricemia, and dyslipidemia. The laboratory examination includes the determination of serum biochemical indicators and urine protein, including blood glucose, blood uric acid, blood lipids, urine α1 microglobulin, urine α2 macroglobulin, urine immunoglobulin G and other indicators.

Diagnostic Criteria and Related Indicators

Personal basic information, lifestyle habits, and chronic disease status were obtained through face-to-face paper questionnaire surveys. According to the National Occupational Classification Dictionary of the People's Republic of China, this study classifies agricultural, forestry, animal husbandry, and fishery workers and laborers as manual laborers in industry and agriculture, while other occupations are defined as non industry and agriculture.

The investigator recorded the height (M), weight (KG), blood pressure (mmHg) and other indicators of the respondents on site according to the operating procedures. According to the current industry standard "Adult Weight Determination" (WS/T 428-2013), the Body Mass Index (BMI) of adults in China should be maintained at $18.5 \leq BMI < 24.0$. $BMI < 18.5$ indicates underweight, $24.0 \leq BMI < 28.0$ indicates overweight, and $BMI \geq 28.0$ indicates obesity. Blood pressure was measured using the Omron HBP-9021J fully automatic electronic blood pressure monitor. According to the "Chinese Guidelines for the Prevention and Treatment of Hypertension (Revised 2024 Edition)" [4], the upper arm blood pressure of the subjects was recorded after resting quietly for at least 5 minutes in a sitting position. The measurements were repeated twice every minute. If there was a difference of more than 10 mmHg between the two readings of systolic or diastolic blood pressure, the measurements were taken again and the average of the three readings was recorded. Hypertension is defined as having a systolic blood pressure of ≥ 140mmHg and/or a diastolic blood pressure of ≥ 90mmHg, or having been diagnosed with hypertension by a township (community), or higher level hospital and taking medication in the past 2 weeks without the use of antihypertensive drugs. The blood sugar, blood uric acid, blood lipids and other related serum biochemical indicators were measured by Siemens Advia 1800 biochemical analyzer. Among them, diabetes patients refer to those with fasting blood glucose ≥ 7.0 mmol/L and/or blood glucose ≥ 11.1 mmol/L 2 hours after the oral glucose tolerance test (OGTT) or those who have been diagnosed as diabetes by township (community) level hospitals or above. Patients with hyperuricemia, whether male or female, have blood uric acid levels greater than 420 μmol/L; Abnormal blood lipids refer to the "Chinese Blood Lipid Management Guidelines (Primary Edition 2024)" [5], with total cholesterol ≥ 6.2mmol/L or triglycerides ≥ 2.3mmol/L or low-density lipoprotein cholesterol ≥ 4.1mmol/L or high-density lipoprotein cholesterol<1.0mmol/L. The urine protein was detected using the Huasheng H-1000 fully automatic dry chemistry urine analyzer, and the results were expressed as negative (-), weakly positive (±), and positive (1+, 2+, and 3+), respectively. The urine protein was measured using the Doctoral TD240 fully automatic specific protein analyzer. According to the Clinical Practice Guidelines for Evaluation and Management of Chronic Kidney Disease (2024) published by Kidney Disease: Improving Global Outcomes (KDIGO), CKD is defined as the presence of proteinuria or a urinary albumin/creatinine ratio (UACR) greater than 30 mg/g. The relevant information on family history of chronic kidney disease, history of kidney stones, etc. comes from the self-report and past medical history of the respondents.

The inclusion criteria for this study are: (1) Permanent residents who are over 18 years old and have resided in Yongzhou City for no less than 6 months; (2) Complete basic information and able to provide clinical samples required for this study; (3) Clear consciousness, basic communication skills, able to cooperate in completing questionnaire surveys

and physical examinations. The exclusion criteria are: (1) Patients with end-stage renal disease; (2) Patients with acute kidney injury; (3) Severe organ dysfunction, including severe heart failure, severe liver failure, advanced malignant tumors, etc; (4) Pregnant women.

Statistical Analysis

SPSS 25.0 statistical software was used to perform statistical analysis on the data of this study. The measurement data was represented by $\bar{x}\pm s$, and t-test was used to compare the differences between groups. Count data was represented by frequency or rate, and comparison of differences between groups was performed using the chi square test. Among them, comparison of ordered multi class data was performed using the linear trend chi square test. The risk factors for CKD were analyzed using logistic regression. The test level is all $\alpha=0.05$.

Results

Basic Information Analysis

This study included a total of 2571 individuals, of whom 56.98% individuals were female, 43.02% were male. The average age was 52.16 ± 14.23 years, with 50.36 ± 15.82 years for males and 54.09 ± 12.18 years for females. The cultural level of the respondents was mainly junior high school or below (50.14%), high school (33.06%), and college or above (16.80%). The prevalence rates of hypertension, diabetes, hyperuricemia and dyslipidemia were 27.69%, 20.65%, 18.51%, 16.45% and 12.80% respectively. Men have higher levels of blood uric acid than women; Total cholesterol, triglycerides, and low-density lipoprotein cholesterol were lower in females, and the differences were statistically significant ($P<0.05$) (**Table 1–4**).

Prevalence of Chronic Kidney Disease in Various Populations

In this study, CKD patients accounted for 9.26% (238/2571), with female CKD patients accounting for 9.28% (136/1465) and male patients accounting for 9.22% (102/1106), there is no statistical difference between the male and female groups. Among all the participants included in the study, there was a significant correlation ($P < 0.001$) between the prevalence of CKD and age and BMI, while the relationship between BMI levels in females and CKD was not significant. Meanwhile, there is no significant correlation between educational level and sleep duration and the incidence of CKD. Smoking and alcohol consumption, as unhealthy habits, are not significantly associated with CKD, while the latter shows a higher risk of CKD in the male population. Hypertension, diabetes, dyslipidemia and hyperuricemia, as the influencing factors of CKD related diseases, are all significantly related to the prevalence of CKD (**Table 5**).

Analysis of Various Influencing Factors and the Risk of Chronic Kidney Disease

Based on various lifestyles and chronic disease conditions, including age, educational level BMI, physical labor, smoking, drinking, sleep duration, hypertension, diabetes, dyslipidemia and hyperuricemia were taken as independent variables. Perform binary logistic regression analysis with CKD as the dependent variable and the health level of each influencing factor as a reference. The results showed that the increase of age, non manual workers, hypertension, diabetes, dyslipidemia and hyperuricemia were all associated with the high risk of CKD (**Table 6**).

Discussion

CKD has become an important public health issue in worldwide. Some studies have shown that the incidence of CKD has been gradually increasing in the past 30 years. From 1990 to 2019, the number of CKD cases doubled in worldwide, and the mortality and disability adjusted life years (DALYs) have also been increasing year by year. This indicates that CKD has become a serious public health problem worldwide. The growth of incidence rate and mortality is closely related to the high incidence of primary diseases, such as population aging, diabetes and hypertension, and constitutes a vicious circle of mutual correlation and intensification [8-10]. A survey conducted in developed countries shows that CKD and ESRD impose a heavy medical burden on the healthcare system [11]. In China, with the development of social economy and the transformation of residents' lifestyles, the prevalence of CKD is also severe. Diabetes nephropathy and hypertensive glomerular arteriosclerosis have become the primary cause of disease replacing primary glomerulonephritis, and the transformation of this disease spectrum is similar to that of western countries [12]. In this context, it is of great practical significance to conduct an analysis of the current prevalence and risk factors of CKD in Yongzhou district, a specific region. The aim of this study is to clarify the disease burden and unique risk characteristics of CKD in the region, providing scientific basis for the development of regional early screening and precise prevention and control strategies, in order to address this increasingly severe public health challenge.

The data from this study shows that the prevalence of CKD among adults in Yongzhou City is 9.26%, which is lower than the global estimated prevalence of 13.4% (11.7-15.1%) [13], and compared with data from other regions around the world, it is lower than 22.48% in Bangladesh [14] and 13.24% in India [15]. Another survey on CKD prevalence based on 16 Asian countries showed significant differences in the overall CKD prevalence rate among countries, ranging from 7.0% to 34.3% [16]. Compared with the domestic research, it is lower than 12.72% in Liaoning Province [17] and 9.97% in Henan Province [18]. This indicates

Table 1 | Age, BMI, blood pressure, glucose and cholesterol of participants

Groups	N	Age	BMI	Systolic blood pressure	Diastolic blood pressure	Fasting blood glucose	Total cholesterol
	(year)	(kg/m^2)	(mmHg)	(mmHg)	(mmol/L)	(mmol/L)	(year)
Male	1106	45.36±15.82	25.35±6.28	135.21±20.12	81.45±12.56	5.67±1.41	4.53±0.81
Female	1465	48.09±12.18	23.54±7.68	135.08±18.54	78.14±11.49	5.65±1.32	4.77±0.93
Total	2571	46.16±14.23	24.41±6.98	135.15±19.33	79.80±12.03	5.66±1.36	4.65±0.87

Table 2 | Triglycerides, high- and low-density lipoprotein, blood uric acid, α1 and α2, microglobulin, immunoglobulin G, retinol binding protein and transferrin of participants

Groups	Triglycerides	High-density lipoprotein	Low-density lipoprotein	Blood uric acid	Urine α1 microglobulin	Urine α2 macroglobulin	Immunoglobulin G	Retinol binding protein	Transferrin
	(mmol/L)	(mmol/L)	(mmol/L)	(μmol/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)
Male	1.61±1.12	1.36±0.42	2.58±0.72	325.32±85.15	31.26±53.54	2.24±7.56	5.37±35.41	1.55±2.83	490.21±1049.53
Female	1.69±1.15	1.39±0.48	2.76±0.82	260.21±77.41	24.27±58.54	1.35±2.49	4.25±29.32	1.72±2.92	674.21±1328.75
Total	1.64±1.14	1.37±0.45	2.68±0.77	292.765±81.28	27.43±55.12	1.79±5.03	4.66±33.36	1.65±2.87	544.21±1128.14

Table 3 | Educational level, smoke, drinking, and manual labor of participant

Group	Educational level			Smoking	Drinking	Manual labor
	Junior high school and below	High school	College or above			
Male	701 (27.27%)	312 (12.14%)	93 (3.62%)	426 (16.57%)	153 (5.95%)	356 (13.85%)
Female	879 (34.19%)	395 (15.36%)	191 (7.43%)	217 (8.44%)	78 (3.03%)	339 (13.19%)
Total	1289 (50.14%)	850 (33.06%)	432 (16.80%)	643 (25.01%)	231 (8.9%)	695 (27.03%)

Table 4 | Sleep, CDK family, kidney stone history, hypertension, diabetes, hyperuricemia and dyslipidemia of participant

Group	Sleep duration			CDK Family history	History of kidney stones	Hypertension	Diabetes	Hyperuricemia	Dyslipidemia
	< 6h	6~8	>8						
Male	271 (10.54%)	714 (27.77%)	121 (4.71%)	75 (2.92%)	186 (7.23%)	328 (12.76%)	241 (9.37%)	228 (8.87%)	201 (7.82%)
Female	428 (16.65%)	802 (31.19%)	235 (9.14%)	79 (3.07%)	234 (9.10%)	384 (14.94%)	290 (11.28%)	248 (9.65%)	222 (8.63%)
Total	699 (27.19%)	1516 (58.97%)	356 (13.85%)	154 (5.99%)	420 (16.34%)	712 (27.69%)	531 (20.65%)	476 (18.51%)	423 (16.45%)

Note: The data in the table are mean ± standard deviation; The data in parentheses is the rate (%), and the data outside parentheses is the number of examples

that there are significant regional differences in the prevalence of CKD, which may be closely related to factors such as public awareness of CKD, aging population, socio-economic factors, dietary and cultural habits, occupational and environmental exposure [19-21]. In addition, the prevalence of CKD in women was slightly higher than that in men in this study, which is consistent with the results of other similar studies. The reasons for this are related to physiological differences between genders and the effects of sex hormones on kidney function [22, 23].

Simultaneously, the results of this study also showed that the growth of age, lack of physical labor, hypertension, diabetes, dyslipidemia, and hyperuricemia accelerated the occurrence and development of CKD to a certain extent through logistic regression. Hypertension, diabetes, dyslipidemia and hyperuricemia are closely related to CKD in different regions, and they are risk factors for each other, which is consistent with the results of relevant studies in the same period [24]. However, the negative correlation between physical labor and CKD disease is not commonly seen in related studies, which contradicts the conclusion obtained from a similar study [25]. China has a large population, and the problem of population aging is becoming increasingly serious, so the above disease groups are growing day by day. It is necessary

Table 5 | CKD prevalence among populations with different baseline characteristics

Baseline characteristics	total（n=2571）	Male（n=1106）	Female（n=1465）
Age（Year）			
＜30	46（1.79）	19（0.74）	27（1.05）
-30	63（2.45）	27（1.05）	36（1.40）
-50	129（5.02）	56（2.18）	73（2.84）
χ^2	93.23	49.72	44.62
P	＜0.001	＜0.001	＜0.001
Educational level			
Junior high school and below	109（4.24）	56（2.18）	87（3.38）
High school	85（3.31）	34（1.32）	27（1.05）
College degree or above	44（1.71）	12（0.47）	22（0.86）
χ^2	1.70	3.78	0.02
P	0.192	0.052	0.887
BMI			
Underweight	21（0.82）	9（0.35）	12（0.47）
Normal weight	97（3.77）	39（1.52）	58（2.26）
Overweight	89（3.46）	35（1.36）	54（2.1）
Obesity	31（1.21）	19（0.74）	12（0.47）
χ^2	30.32	41.46	3.63
P	＜0.001	＜0.001	0.057
Manual labor			
No	96（3.73）	46（1.79）	50（1.94）
Yes	142（5.52）	56（2.18）	86（3.35）
χ^2	23.54	8.58	15.65
P	＜0.001	0.003	＜0.001
Smoking			
No	62（2.41）	49（1.91）	13（0.51）
Yes	176（6.85）	53（2.06）	123（4.78）
χ^2	0.15	4.30	3.28
P	0.697	0.038	0.070
Drinking Wine			
No	193（7.51）	66（2.57）	127（4.94）
Yes	45（1.75）	36（1.4）	9（0.35）
χ^2	31.58	43.41	0.50
P	＜0.001	＜0.001	0.48
Sleep duration			
＜6	65（2.53）	26（1.01）	39（1.52）
-6	129（5.02）	63（2.45）	66（2.57）
-8	44（1.71）	13（0.51）	31（1.21）
χ^2	1.363	0.022	1.830
P	0.243	0.881	0.176
Hypertension			
No	145（5.64）	57（2.22）	88（3.42）
Yes	93（3.62）	45（1.75）	48（1.87）
χ^2	144.64	37.05	114.86
P	＜0.001	＜0.001	＜0.001
Diabetes			
No	162（6.30）	71（2.76）	91（3.54）
Yes	76（2.96）	31（1.21）	45（1.75）
χ^2	20.36	7.25	31.37
P	＜0.001	＜0.001	＜0.001
Dyslipidemia			
No	145（5.64）	65（2.53）	80（3.11）
Yes	93（3.62）	37（1.44）	56（2.18）
χ^2	97.65	24.76	78.96
P	＜0.001	＜0.001	＜0.001
Hyperuricemia			
No	174（6.77）	67（2.61）	107（4.16）
Yes	64（2.49）	35（1.36）	29（1.13）
χ^2	12.20	12.89	2.06
P	＜0.001	＜0.001	0.151

Note: The data outside parentheses represents the number of CKD cases, while the data inside parentheses represents the composition ratio (%)

Table 6 | Logistic regression analysis of various influencing factors and CKD risk

Lifestyle	β value	SE	χ^2 value	OR（95%）	P
Age	0.021	0.006	12.250	1.021（1.009~1.033）	＜0.001
Educational level					
Junior high school and below				1.000	
High school	0.154	0.092	2.802	1.25（0.818~1.909）	0.094
College degree or above	0.217	0.156	1.935	1.25（0.818~1.909）	0.164
BMI					
Underweight				1.000	
Normal weight	-0.217	0.285	0.580	0.805（0.46~1.407）	0.446
Overweight	-0.316	0.257	1.512	0.729（0.441~1.206）	0.219
Obesity	-0.364	0.247	2.172	0.695（0.428~1.128）	0.141
Manual labor					
No				1.000	
Yes	-0.528	0.248	4.533	0.590（0.363~0.959）	0.033
Smoking					
No				1.000	
Yes	0.033	0.214	0.024	1.034（0.679~1.572）	0.877
Drinking Wine					
No				1.000	
Yes	0.047	0.138	0.116	1.048（0.8~1.374）	0.733
Sleep duration					
＜6				1.000	
-6	0.167	0.318	0.276	1.182（0.634~2.204）	0.599
-8	0.223	0.216	1.066	1.25（0.818~1.909）	0.302
Hypertension					
No				1.000	
Yes	0.685	0.096	50.914	1.984（1.644~2.394）	＜0.001
Diabetes					
No				1.000	
Yes	0.746	0.174	18.381	2.109（1.499~2.965）	＜0.001
Dyslipidemia					
No				1.000	
Yes	0.513	0.096	28.556	1.67（1.384~2.016）	＜0.001
Hyperuricemia					
No				1.000	
Yes	0.261	0.085	9.429	1.298（1.099~1.534）	0.002

to prevent hypertension, diabetes and other risk factors at the source while preventing chronic kidney disease. Correct cognition, healthy lifestyle, and early intervention measures are of great significance in reducing the risk of CKD.

In summary, although the prevalence of CKD among adults in Yongzhou City is relatively lower than in other regions, it is still necessary to pay attention to the prevention and control of this disease. It is recommended to develop and promote multi-level and forward-looking comprehensive prevention. The control strategies should come from the following aspects: **1)** Strengthen early screening and diagnosis. Focusing on high-risk groups of CKD such as hypertension, diabetes and hyperuricemia, regular UACR and serum creatinine detection will be carried out to improve the early detection rate. **2)** Carry out systematic health education. Enhance public awareness of CKD risk factors and prevention measures through various channels, advocate for low salt, low-fat, low purine diets and healthy lifestyles, and improve

group health literacy. **3)** Enhance the capacity of primary healthcare services. Strengthen the capacity building of grassroots medical institutions in CKD screening, diagnosis, treatment, and long-term management, and promote the realization of "early detection, early diagnosis, and early treatment". Implement standardized follow-up and management for confirmed patients, delay the progression of ESRD, and reduce the medical burden on families and society. **4)** Promote interdisciplinary collaboration management. Establish a diagnosis and treatment model led by nephrology, with collaboration from nutrition, cardiovascular, endocrinology, and other departments, to provide comprehensive interventions for patient comorbidities and optimize treatment plans. **5)** Improve the construction of regional chronic disease information platforms, achieve dynamic monitoring and sharing of CKD patient data, and support precise prevention and control as well as policy formulation. 6. Encourage high-risk populations to undergo regular physical examinations, improve disease screening coverage, effectively move the checkpoint forward, combine prevention and treatment, and effectively curb the growth trend of CKD.

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The Role and Significance of *BRCA1* c.4712delT Mutation in Ovarian Cancer

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KEYWORDS

Ovarian Cancer;
BRCA1 Gene;
Gene Mutation;
Clinical Diagnosis

ABSTRACT

Objective: To investigate the mutation status of BRCA1 c.4712delT in patients with sporadic ovarian cancer and to analyze its clinical significance in relation to clinical indicators such as HE4 and CA125.

Methods: 1) The BRCA1 c.4712delT mutation status in patients with sporadic ovarian cancer and healthy individuals was detected using Restriction Fragment Length Polymorphism (RFLP) and Sanger sequencing. **2)**Statistical analyses were conducted to assess differences in age, tumor markers, and coagulation function between ovarian cancer patients and healthy individuals, as well as between BRCA1 c.4712delT-mutated and non-mutated ovarian cancer patients.

Results: This study enrolled 209 ovarian cancer patients and 213 healthy individuals undergoing routine health checkups. In sporadic ovarian cancer patients, the BRCA1 c.4712delT mutation frequency was 1.67%, slightly higher than the 0.47% frequency in the healthy population; however, the difference was not statistically significant (P = 0.103). Levels of age, HE4, and CA125 showed statistically significant differences between ovarian cancer patients and healthy individuals. No statistically significant differences were observed in age, HE4, CA125, PT, APTT, TT, FIB, D-D, personal cancer history, family history, lymph node metastasis, pathological grade, or clinical stage between the BRCA1 c.4712delT-mutated and non-mutated groups. Bioinformatics analysis predicted that the BRCA1 c.4712delT mutation is deleterious. An ovarian cancer risk model established based on age, HE4, CA125 levels, and BRCA1 c.4712delT mutation status demonstrated that the combined model incorporating all factors yielded the best diagnostic efficacy for predicting ovarian cancer risk.

Conclusions: 1) The mutation frequency of BRCA1 c.4712delT in ovarian cancer patients is 1.67%. **2)**Changes in serum levels of the tumor markers HE4 and CA125 serve as effective indicators for the early diagnosis of ovarian cancer. **3)**BRCA1 c.4712delT mutation detection is a potential indicator for the early diagnosis of ovarian cancer.

Introduction

Ovarian cancer is one of the most lethal malignancies of the female reproductive system and is characterized by high heterogeneity. Its biological behavior, molecular characteristics, and clinical outcomes differ markedly across histological types. According to the 2020 fifth edition of the World Health

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Organization (WHO) classification, ovarian cancer is clearly divided into five major subtypes: high-grade serous carcinoma (HGSC, 70%), endometrioid carcinoma (EC, 10%), clear cell carcinoma (CCC, 10%), mucinous carcinoma (MC, 3%), and low-grade serous carcinoma (LGSC, <5%). Each subtype possesses distinct epidemiological profiles, molecular alterations, and prognostic features. Globally, both the incidence and mortality of ovarian cancer rank among the highest in female cancers. In 2020, there were approximately 314,000 new cases and 207,000 deaths worldwide, with China accounting for 17.6% of global cases (approximately 57,000 new cases). Current diagnostic approaches primarily include imaging methods (transvaginal ultrasound, CT, and MRI), tumor markers (CA125 and HE4), and pathological diagnosis as the gold standard. Due to the insidious nature of early symptoms and the lack of effective screening methods, 75% of patients are diagnosed at advanced stages (stage III/IV), resulting in a 5-year survival rate of only 30%–40%. Studies have established that germline *BRCA1/2* mutations are closely associated with the occurrence and metastasis of ovarian cancer, suggesting that *BRCA1/2* mutation testing may serve as an effective approach for early clinical diagnosis.

Breast cancer susceptibility genes (*BRCA*) are critical tumor suppressor and cancer predisposition genes, primarily including *BRCA1* and *BRCA2* . *BRCA1* is located on chromosome 17 (17q12–21) and encodes 1,863 amino acids, while *BRCA2* is located on chromosome 13 (13q12–13) and encodes 3,418 amino acids. Both proteins maintain genomic stability by interacting with proteins such as RAD51 and PALB2 to repair DNA double-strand breaks via homologous recombination (HR), and they also regulate the cell cycle and transcription. When *BRCA1/2* are inactivated by mutation, HR repair is compromised, leading to increased genomic instability and promoting carcinogenesis. Furthermore, cells with *BRCA1/2* mutations exhibit significantly increased sensitivity to platinum-based drugs and PARP inhibitors, which informs clinical treatment strategies and prognosis. Women carrying pathogenic *BRCA1* mutations have a lifetime breast cancer risk exceeding 60% and an ovarian cancer risk ranging from 39.0% to 58.0%. Among the various *BRCA* mutations, *BRCA1* c.4712delT is a high-frequency deleterious germline mutation site.

The c.4712delT mutation is a frameshift mutation caused by the deletion of a thymine (T), resulting in a shift of the reading frame. This mutation typically leads to a premature termination codon, generating a truncated protein. Consequently, the BRCT domain of the BRCA1 protein may be lost, impairing its ability to bind DNA repair proteins and resulting in defective DNA repair. As a loss-of-function mutation, c.4712delT is closely associated with early-onset breast cancer (<45 years) and familial ovarian cancer.

Genome-wide association studies (GWAS) are statistical approaches used to identify genetic variants associated with specific phenotypes or diseases by scanning genome-wide single-nucleotide polymorphisms (SNPs) in large cohorts. To date, GWAS has identified approximately 27 genomic regions associated with epithelial ovarian cancer (EOC) risk, including key loci repeatedly validated across studies, such as 8q21 (rs11782652), 17q12 (rs757210), and 9p22 (rs3814113). Insertions and deletions refer to the addition or removal of one or more nucleotides in a DNA sequence; *BRCA1* c.4712delT belongs to this category. Studies indicate that the frequency of the *BRCA1* T-deletion in the Chinese population is 6.8%, significantly higher than the 3.5% reported in the BIC database for other populations. Therefore, in-depth research on this specific mutation is of substantial clinical significance.

In addition to genetic testing, early clinical diagnosis of ovarian cancer relies heavily on tumor markers. CA125 (carbohydrate antigen 125), encoded by the *MUC16* gene, is a high-molecular-weight mucin-type glycoprotein. Serum CA125 levels exceed 35 U/mL in 83% of ovarian cancer patients, and its fluctuations correlate with disease progression or remission. HE4 (human epididymis protein 4), encoded by the *WFDC2* gene, is another crucial biomarker. HE4 is significantly overexpressed in ovarian cancer (especially serous and endometrioid carcinomas). A meta-analysis showed that HE4 has a sensitivity of 73% and specificity of 89% for diagnosing ovarian cancer, which is superior to CA125 (sensitivity 74%, specificity 82%). The combined detection of CA125 and HE4 further improves diagnostic sensitivity and specificity.

Beyond detection methods, exploring the relationship between clinical indicators such as coagulation function and ovarian cancer provides insight into disease progression. Commonly used coagulation indicators include prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), fibrinogen (FIB), and D-dimer (D-D). This study uses these indicators to explore the association between coagulation function and the *BRCA1* c.4712delT mutation in ovarian cancer patients.

The present study utilizes experimental methods to analyze differences in the *BRCA1* c.4712delT mutation frequency between ovarian cancer patients and healthy individuals. By statistically analyzing clinical data—including age, tumor markers, coagulation function, and pathology—this study aims to elucidate the role of this mutation in ovarian cancer development and identify new avenues for early diagnosis.

Materials and Methods

Clinical Specimens

This study collected 2 mL of EDTA-anticoagulated whole blood and associated clinical data from 209 ovarian cancer patients (excluding 17 cases) treated at Hunan Cancer Hospital between December 23, 2024, and April 22, 2025. Addi-

Table 1 | Primers for detection of BRCA1 c.4712delT site mutation

Primer Name	Direction	Sequence
4712F	Forward	5'-ACGTGTTAAGGTGTTTGCTACA-3'
4712R	Reverse	5'-GACTCTGGGGCTCTGTCTTC-3'

Table 2 | PCR amplification reaction system

Component	Volume
DNA Template	2 μL
Forward Primer (10 μM)	0.5 μL
Reverse Primer (10 μM)	0.5 μL
PCR SuperMix	10 μL
Nuclease-free Water	7 μL
Total Volume	20 μL

Table 3 | PCR amplification program

Temperature	Time	
94℃	5 min	
94℃	30 sec	35 cycles
58℃	30 sec	
72℃	30 sec	
72℃	5 min	

Table 4 | DNA enzyme digestion reaction system

Component	Volume
ddH_2O	16.5 μL
10×FuniCut® Color Buffer	3 μL
Substrate DNA	10 μL
FuniCut® Earl	0.5 μL
Total	30 μL

tionally, samples and data were collected from 213 healthy individuals (excluding 29 cases) undergoing physical examinations between February 5, 2025, and April 11, 2025, totaling 422 participants.

- **Inclusion criteria for the experimental group: 1)** Clinically and pathologically diagnosed with malignant ovarian tumors; **2)** Complete medical records.
- **Inclusion criteria for the control group: 1)** No history of malignant ovarian tumors; **2)** Age between 20 and 60 years; **3)** Valid clinical data. All pathological diagnoses were confirmed by experienced pathologists. This study was approved by the Medical Ethics Committee of Hunan Cancer Hospital, and informed consent was obtained from all participants.

Reagents

Blood DNA extraction kits (spin column type) were purchased from Tiangen Biotech. PCR amplification reagents (2×EasyTaq® PCR SuperMix +dye) were purchased from TransGen Biotech. PCR primers were synthesized by Tsingke Biotechnology. The restriction enzyme FuniCut® Earl was purchased from Yeasen Biotechnology. Agarose (BIOWEST) and Tris-acetate electrophoresis buffer (50×TAE) were purchased from Aibowei Biotechnology.

Experimental Methods

PCR Amplification

Primers were designed based on the upstream and downstream sequences of the *BRCA1* c.4712delT mutation site (**Table 1**). PCR amplification was performed using the system and program outlined in **Tables 2 and 3**.

Enzyme Digestion

Prepare the system reaction mixture according to the sample addition order in Table 4 (perform on ice). The reaction mixture was prepared on ice according to **Table 4**. The mixture was incubated at 37℃ for 30 minutes, followed by incubation at 80℃ for 20 minutes to inactivate the enzyme.

Electrophoresis

Electrophoresis was conducted at a constant voltage of 120 V for 30 minutes. Wild-type samples displayed two clear bands (approximately 50 bp and 250 bp). Samples showing a single band (~300 bp) or three bands of different lengths were flagged as potential mutations.

Sanger Sequencing & Analysis

Samples suspected of mutation based on electrophoresis were subjected to Sanger sequencing. The resulting sequences were aligned with the NCBI reference sequence for *BRCA1* (NC_000017.11) to determine the genotype (wild-type, heterozygous, or homozygous).

Statistical Analysis

Data analysis was performed using SPSS 27.0. Normality was assessed using the Shapiro-Wilk test. Quantitative data (e.g., age, HE4, CA125, coagulation markers) did not follow a normal distribution and are presented as mean ± standard deviation (X ± SD); comparisons were made using the Mann-Whitney U test. Ordinal data (pathological grade, clinical stage) were analyzed using the Mann-Whitney U test. Categorical data (cancer history, family history, metastasis) are expressed as frequencies (n, %) and were compared using the Chi-square test or Fisher's exact test. Logistic regression was used to construct risk assessment models. A P -value < 0.05

Table 5 | Summary of clinical data for ovarian cancer patients (experimental group) (n = 209)

Indicator	Category	N(%)
Age	≤ 40	17 (8.13)
	41–50	55 (26.32)
	51–60	88 (42.11)
	≥ 61	49 (23.44)
	Mean	54.06 ± 10.70
Histological subtype	Serous carcinoma	169 (80.86)
	Mucinous carcinoma	5 (2.39)
	Endometrioid carcinoma	5 (2.39)
	Clear cell carcinoma	18 (8.61)
	Other	11 (5.26)
	Unknown	1 (0.48)
Clinical stage	I	21 (10.05)
	II	27 (12.92)
	III	93 (44.50)
	IV	55 (26.32)
	Unknown	13 (6.22)
Histologic grade	Low grade	6 (2.87)
	Intermediate grade	2 (0.96)
	High grade	163 (77.99)
	Unknown	38 (18.18)
Lymph node metastasis	Yes	99 (47.37)
	No	79 (37.80)
	Unknown	31 (14.83)

Table 6 | Comparative analysis between the experimental group and the control group.

Indicator	experimental group (n=209)	control group (n=213)	Z	P
Age	54.06 ± 10.70	34.41 ± 12.05	-13.142	<0.001
HE4	532.52 ± 505.01	47.42 ± 8.96	-8.543	<0.001
CA125	1492.42 ± 4156.48	13.86 ± 6.44	-12.748	<0.001

Table 7 | Comparative analysis of genotypes between the experimental group and the control group

Genotype	Experimental group	Control group	P
AA	202	211	
A-	7	2	
Total	209	213	0.103

Table 8 | Bioinformatics prediction results for the BRCA1 c.4712delT variant

Items	Results
Gene	*BRCA1*
Nucleotide change	*BRCA1* c.4712delT
Exon	exon16
Effect on protein	p.E1571fs
variant_function	frameshift substitution
dbSNP ID	rs886037790
Mutation type	Frameshift del
Clinically Importance	Yes
Previously reported	BIC/ClinVar/UMD/LOVD
Ethnicity	Eastern-European, Chinese, Han, None Specified, None-Specified, Not Specified, Western
MetaSVM/MetaLR/SIFT/Poly-Phen	deleterious

Table 10 | Summary of baseline clinical data in the mutant group (n = 7)

Indicator	Category	N (%)
Age	≤ 40	1 (14.29)
	41–50	2 (28.57)
	51–60	4 (57.14)
	≥ 61	0 (0.00)
	Mean (years)	50.29 ± 8.08
Histological subtype	Serous carcinoma	5 (71.43)
	Mucinous carcinoma	0 (0.00)
	Endometrioid carcinoma	0 (0.00)
	Clear cell carcinoma	2 (28.57)
Histological subtype	Other	0 (0.00)
	Unknown	0 (0.00)
Clinical stage	I	1 (14.29)
	II	1 (14.29)
	III	5 (71.43)
	IV	0 (0.00)
	Unknown	0 (0.00)
Histologic grade	Low grade	0 (0.00)
	Intermediate grade	0 (0.00)
	High grade	5 (71.43)
	Unknown	2 (28.57)
Lymph node metastasis	Yes	3 (42.86)
	No	4 (57.14)
Personal history of cancer	Yes	0 (0.00)
	No	7 (100.00)
HBOC-associated tumors	Yes	1 (14.29)
	No	6 (85.71)
Family history	Yes	3 (42.86)

Table 9 | Clinical data of seven patients with the mutant genotype

ID	H46	H74	H87	H165	H168	H172	H173
Age (years)	60	54	38	45	51	59	45
Histological type	Serous adenocarcinoma	Clear cell carcinoma	Serous adenocarcinoma	Serous adenocarcinoma	Clear cell carcinoma	Serous adenocarcinoma	Serous adenocarcinoma
Histologic grade	High grade	Not reported	High grade	High grade	Not reported	High grade	High grade
Clinical stage	III	I	III	II	III	III	III
Family history of cancer	No	Yes	No	Yes	No	No	Yes
HBOC syndrome	No	No	No	No	No	No	Yes
Lymph node metastasis	No	No	Yes	No	No	Yes	Yes

Table 11 | Comparative analysis of clinical data between the mutant group and the wild-type group

Indicator	Mutant group (n = 7)	Wild-type group (n = 202)	Z	P
Age (years)	50.29 ± 8.08	54.19 ± 10.77	-1.059	0.290
HE4	316.93 ± 425.50	533.72 ± 487.53	-1.538	0.124
CA125	1882.82 ± 1937.76	1212.96 ± 1868.83	-0.297	0.766
PT	11.31 ± 0.81	11.62 ± 0.84	-1.212	0.225
APTT	26.10 ± 2.89	27.14 ± 3.20	-0.601	0.548
TT	20.10 ± 7.90	16.96 ± 1.71	-1.224	0.221
FIB	4.65 ± 1.96	4.84 ± 6.72	-0.057	0.954
D-dimer (D-D)	4.30 ± 4.21	3.69 ± 3.83	-0.235	0.814
Personal history of cancer	0 (0)	11 (5.4%)	—	1.000
Family history	3 (42.9%)	56 (27.7%)	—	0.406
Lymph node metastasis	3 (42.9%)	95 (55.6%)	—	0.636
Histologic differentiation	—	—	-0.536	0.592
Well differentiated	0 (0)	6 (3%)	—	—
Moderately differentiated	0 (0)	2 (1%)	—	—
Poorly differentiated	7 (100%)	194 (96%)	—	—
Clinical stage	—	—	-1.255	0.210
Stage I	1 (14.3%)	19 (10.1%)	—	—
Stage II	1 (14.3%)	25 (13.2%)	—	—
Stage III	5 (71.4%)	91 (48.1%)	—	—
Stage IV	0 (0)	54 (28.6%)	—	—

was considered statistically significant.

Table 12 | Statistical analysis and correlation between serological biomarkers and clinical characteristics in the experimental group

Indicator	Category	N (%)	HE4 (mean ± SD)	CA125 (mean ± SD)
Age	≤ 40	17 (8.13)	390.66 ± 539.39	751.44 ± 1145.35
	41–50	55 (26.32)	421.96 ± 503.52	1102.33 ± 1632.96
	51–60	88 (42.11)	556.80 ± 501.85	1514.13 ± 2335.46
	≥ 61	49 (23.44)	642.52 ± 487.35	2148.34 ± 7793.06
	P		0.009	0.367
Histological subtype	Serous carcinoma	169 (80.86)	599.02 ± 514.28	1716.39 ± 4555.03
	Mucinous carcinoma	5 (2.39)	133.27 ± 171.89	80.10 ± 61.89
	Endometrioid carcinoma	5 (2.39)	51.82 ± 13.96	224.48 ± 413.37
	Clear cell carcinoma	18 (8.61)	164.70 ± 197.37	531.66 ± 1387.91
	Other	11 (5.26)	309.05 ± 367.22	603.79 ± 1251.82
	Unknown	1 (0.48)	1312.7 ± 0	4111 ± 0
	P		< 0.001	< 0.001
Clinical stage	I	21 (10.05)	129.26 ± 155.25	124.80 ± 172.13
	II	27 (12.92)	202.83 ± 289.97	622.48 ± 1434.86
	III	93 (44.50)	611.08 ± 487.23	2150.08 ± 5958.27
	IV	55 (26.32)	660.86 ± 527.37	1480.73 ± 1688.11
	Unknown	13 (6.22)	679.58 ± 647.87	853.05 ± 1084.73
	P		< 0.001	< 0.001
Histologic grade	Low grade	6 (2.87)	227.62 ± 228.31	36.01 ± 17.65
	Intermediate grade	2 (0.96)	194.90 ± 0	2061.09 ± 2860.83
	High grade	163 (77.99)	615.32 ± 509.38	1748.15 ± 4630.47
	Unknown	38 (18.18)	236.58 ± 381.98	595.46 ± 1243.34
	P		< 0.001	< 0.001
Lymph node metastasis	Yes	99 (47.37)	590.17 ± 487.35	2148.41 ± 5718.41
	No	79 (37.80)	353.23 ± 418.76	558.94 ± 1230.92
	P		< 0.001	< 0.001
Personal history of cancer	Yes	11 (5.26)	645.91 ± 618.65	722.52 ± 856.14
	No	196 (93.78)	525.31 ± 498.21	1550.33 ± 4281.63
	P		0.672	0.630
HBOC syndrome	Yes	15 (7.18)	636.75 ± 580.42	1845.85 ± 2792.01
	No	191 (91.39)	523.93 ± 499.27	1463.71 ± 4272.79
	P		0.462	0.305
Family history	Yes	59 (28.23)	482.94 ± 481.41	1199.81 ± 1882.68
	No	147 (70.33)	554.77 ± 515.55	1608.63 ± 4802.48
	P		0.345	0.990

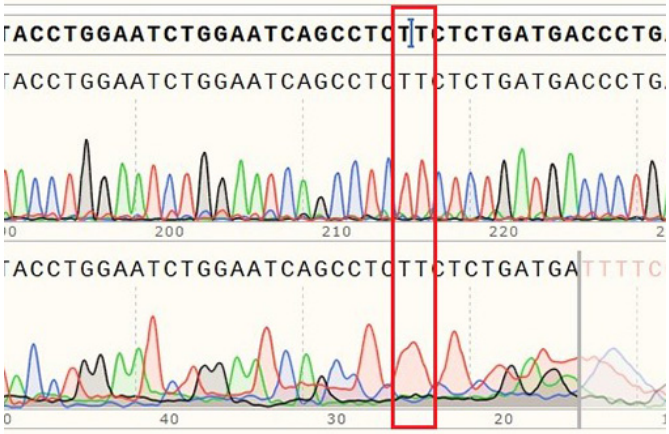


Figure 1 | Wild-type Sanger sequencing chromatogram and sequence alignment

Note: (1) The bold text at the top indicates the reference sequence BRCA1 (NC_000017.11). (2) The two chromatograms, from top to bottom, correspond to the forward and reverse sequences, respectively. (3) The four colored peaks represent the four nucleotides. (4) The target site is indicated within the red box.

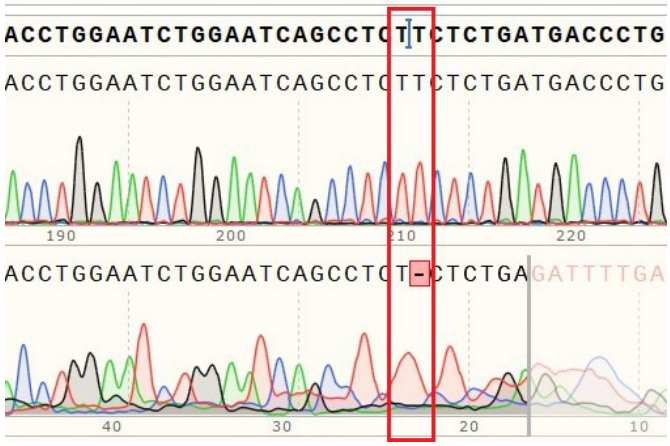


Figure 2 | Heterozygous mutant Sanger sequencing chromatogram and sequence alignment

Note: (1) The bold text at the top indicates the reference sequence BRCA1 (NC_000017.11). (2) The two chromatograms, from top to bottom, correspond to the forward and reverse sequences, respectively. (3) The four colored peaks represent the four nucleotides. (4) The target site is indicated within the red box.

Results

Comparative Analysis Between Experimental and Control Groups

The clinical characteristics of the 209 sporadic ovarian cancer patients are summarized in **Table 5**. The mean age was 54.06 ± 10.70 years. The predominant histological type was serous carcinoma (80.86%), and the majority of patients had high-grade tumors (77.99%). Most patients were diagnosed at advanced clinical stages (Stage III: 44.50%; Stage IV: 26.32%), and lymph node metastasis was common (47.37% confirmed yes).

Comparative analysis (**Table 6**) revealed that Age ($P < 0.001$), HE4 ($P < 0.001$), and CA125 ($P < 0.001$) were significantly higher in the experimental group compared to the healthy control group.

BRCA1 c.4712delT Mutation Status

The electrophoresis results indicated 18 samples in the experimental group and 10 samples in the control group with suspected mutations and/or failed restriction enzyme digestion, for a total of 28 samples. Sanger sequencing was performed on these 28 samples, and the resulting nucleotide sequences were compared with the BRCA1 reference sequence in the database (NC_000017.11). In the experimental group, 9 cases were wild type, 7 cases were heterozygous mutations, 0 cases were homozygous mutations, and 2 cases were indeterminate (excluded). In the control group, 7 cases were wild type, 2 cases were heterozygous mutations, 0 cases were homozygous mutations, and 1 case was indeterminate (excluded). The mutation frequency of BRCA1 c.4712delT was 1.67% (7/418) in the experimental group and 0.47% (2/426) in the control group. **Figure 1** shows the sequencing result for the wild-type genotype, and **Figure 2** shows the sequencing result for the heterozygous mutant genotype.

The experimental results were summarized and subjected to statistical analysis (**Table 7**), showing that the difference in genotype distribution between the experimental group and the control group was not statistically significant ($P > 0.05$).

In the preliminary phase of this project, the protein functional impact of the BRCA1 c.4712delT variant was evaluated using the bioinformatics prediction tools MetaSVM and MetaLR, as well as the online tools SIFT (http://sift.jcvi.org/) and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2) (**Table 8**). The predictions from MetaSVM, MetaLR, SIFT, and PolyPhen-2 all indicated that this germline variant is deleterious, suggesting that it impairs the function of the encoded protein. Specifically, alterations were identified in amino acids downstream of the 1571st amino acid in exon 16 of BRCA1, resulting in truncation of the BRCA1 protein beyond this position and loss of normal BRCA1 function. This truncation is expected to hinder the interaction of BRCA1 with homologous recombination repair–related proteins such as RAD51 and PALB2, thereby disrupting key processes including homologous recombination repair of DNA double-strand breaks, cell-cycle regulation, and transcriptional regulation. It may further impair centrosome duplication control, leading to centrosome dysregulation, genomic instability, and malignant cellular transformation, ultimately contributing to carcinogenesis.

Comparative Analysis Between Genotype Groups

The experimental group was stratified by genotype into a mutant subgroup and a wild-type subgroup. The basic clinical information of the mutant subgroup was summarized and

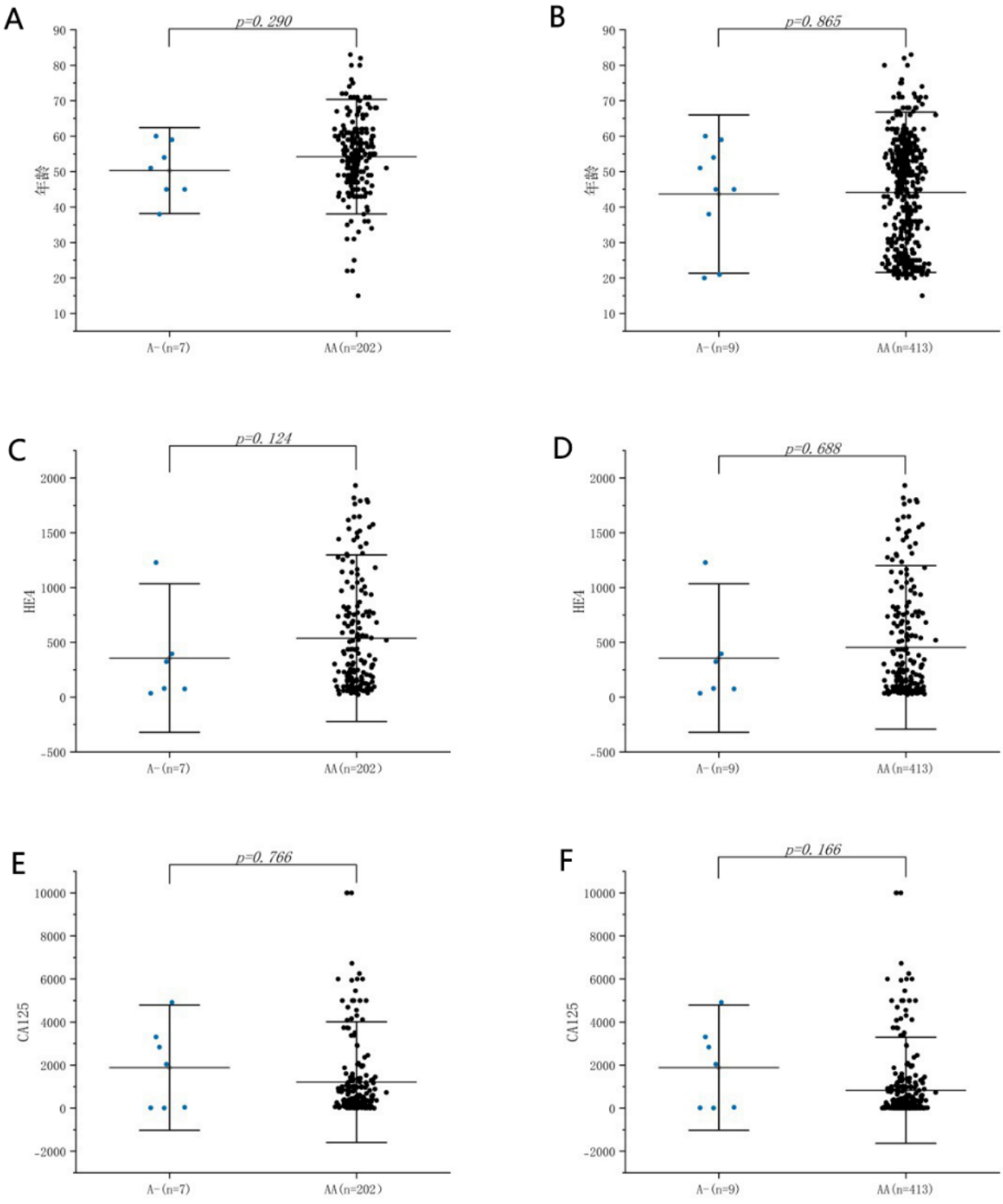


Figure 3 | Comparative analysis of clinical parameters across genotype groups

Note: (1) Panels A, C, and E present comparisons between the mutant and wild-type subgroups within the experimental group, whereas Panels B, D, and F present comparisons between the mutant and wild-type subgroups in the pooled dataset combining the experimental and control groups. (2) The x-axis indicates genotype, where A− denotes the heterozygous mutant genotype and AA denotes the wild-type genotype. (3) The lines in each panel represent the mean and standard deviation for the corresponding group.

Table 13 | Predictive performance of logistic regression models for ovarian cancer

Model variables	Sensitivity	Specificity	AUC
HE4	0.891	0.973	0.945
CA125	0.871	1.000	0.958
Age	0.919	0.723	0.870
HE4, CA125	0.935	1.000	0.988
HE4, Age	0.870	1.000	0.950
CA125, Age	0.919	0.968	0.978
HE4, CA125, Age	0.935	1.000	0.989
HE4, CA125, Age, Genotype	0.946	1.000	0.991

analyzed (**Tables 9 and 10**). Differences between the two subgroups were then compared across 13 indicators, including age, HE4, CA125, PT, APTT, TT, FIB, D-dimer, personal history of cancer, family history, lymph node metastasis, histologic grade, and clinical stage. Among these, age, HE4, CA125, PT, APTT, TT, FIB, and D-dimer were continuous variables and are presented as mean ± SD; personal history of cancer, family history, and lymph node metastasis were categorical variables and are presented as n (%); histologic grade and clinical stage were ordinal variables and are presented by category as n (%). Statistical analyses showed that none of these indicators differed significantly between the mutant and wild-type subgroups ($P > 0.05$). The comparative results for all indicators are shown in **Table 11**.

According to the clinical characteristics of patients with the mutation (Table 9), the predominant pathological subtype was high-grade serous adenocarcinoma, and most cases were at stage III.

In addition, this study further evaluated differences in age, HE4, and CA125 across genotypes within the experimental group, and in the pooled dataset combining the experimental and control groups (**Figure 3**).

Statistical Analysis of Associations Between Serological Biomarkers and Clinical Characteristics in Ovarian Cancer

To evaluate the clinical utility of HE4 and CA125 as serological tumor biomarkers, this study examined the associations between these two markers and the clinical and pathological characteristics of patients with ovarian cancer. As shown in **Table 12**, HE4 differed significantly across age groups, histological subtypes, clinical stages, histologic grades, and lymph node metastasis status ($P < 0.05$), whereas no significant differences were observed according to personal history of cancer, HBOC syndrome, or family history ($P > 0.05$). CA125 differed significantly across histological subtypes, clinical stages, histologic grades, and lymph node metastasis status ($P < 0.05$), but showed no significant differences across age groups or according to personal history of cancer, HBOC syndrome, or family history ($P > 0.05$). These findings indicate that HE4 and CA125 effectively discriminate between pathological characteristics and may aid in the diagnosis of ovarian cancer.

Disease Risk Assessment Model

To further investigate the effects of age, HE4 level, CA125 level, and the presence of the BRCA1 c.4712delT variant on ovarian cancer status, a logistic regression–based risk assessment model was developed (**Table 13**; **Figure 4**). The combined model using HE4 and CA125 demonstrated markedly better predictive performance (AUC = 0.988, sensitivity = 0.935, specificity = 1.000) than either biomarker alone, indicating that their combined use provides a more reliable clinical diagnostic indicator than HE4 or CA125 individually. In addition, compared with the joint model including HE4, CA125, and age, incorporating genotype as an additional predictor further improved performance (AUC = 0.991, sensitivity = 0.946, specificity = 1.000), yielding the best diagnostic performance among the evaluated ovarian cancer risk prediction models.

Discussion

Among the various BRCA1 variants, BRCA1 c.4712delT is regarded as a high-frequency deleterious germline variant. Investigating differences in its mutation rate between ovarian cancer patients and healthy individuals is important for evaluating its potential utility as an early detection marker for ovarian cancer. A previous study reported a BRCA1 c.4712delT mutation rate of 5% (2/40) among ovarian cancer patients with hereditary breast and ovarian cancer (HBOC) syndrome in Hunan Province. In the present study, the mutation rate in the experimental group was 3.35% (7/209), with a mutation frequency of 1.67% (7/418); in the control group, the mutation rate was 0.94% (2/213), with a mutation frequency of 0.47% (2/426). The mutation rate observed in the experimental group was lower than that reported in the above study. This may be explained by two factors. First, the EDTA-anticoagulated whole-blood samples collected in this study were obtained from ovarian cancer patients with different histological subtypes and were not strictly limited to HBOC patients, resulting in fewer inclusion restrictions. Sec-

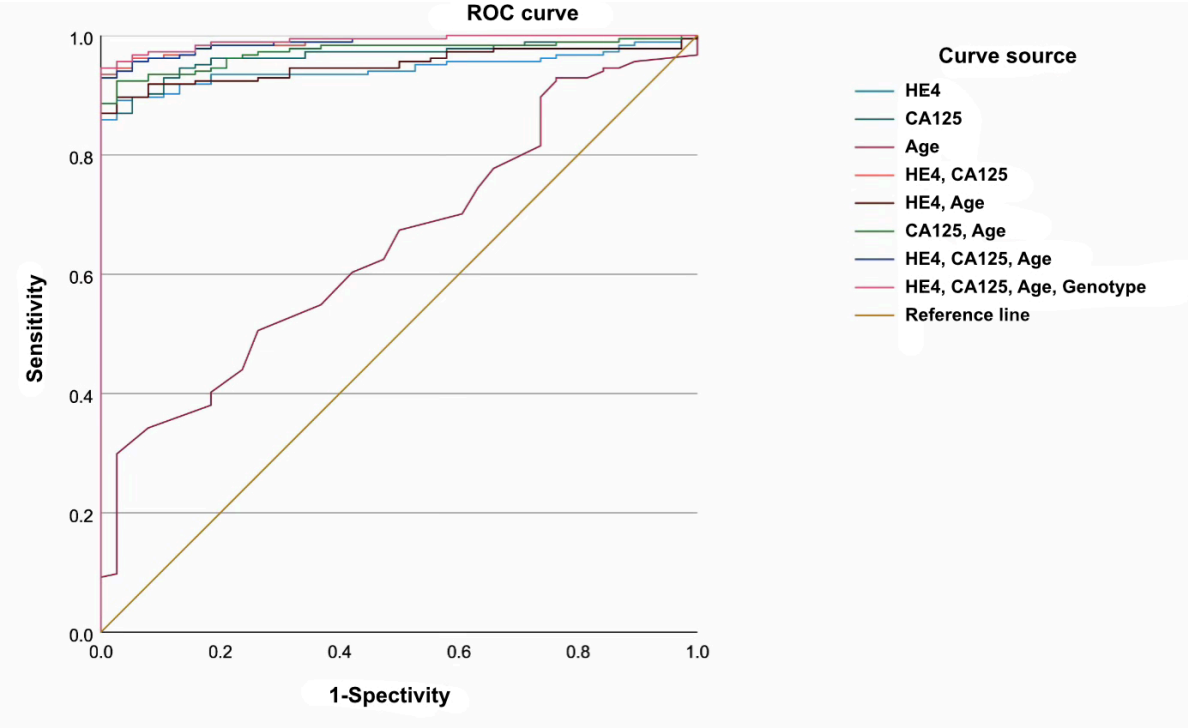


Figure 4 | ROC curves of logistic regression models for ovarian cancer

ond, the sample size in this study was limited, and the influence of random variation on the results cannot be excluded. Moreover, statistical analysis showed no significant difference in mutation rate between the experimental and control groups ($P > 0.05$), suggesting that BRCA1 c.4712delT is not significantly associated with ovarian cancer status; this finding is clearly inconsistent with the expert consensus on BRCA germline variant screening in Chinese populations [8]. Nevertheless, although the difference was not statistically significant, an apparent upward trend in the BRCA1 c.4712delT mutation rate was observed in the experimental group compared with the control group. The lack of statistical significance may still be attributable to the relatively small sample size.

In this cohort of 209 ovarian cancer patients, analysis of BRCA variants in whole-blood samples in relation to clinical characteristics indicated that, because ovarian cancer lacks routine screening and effective early detection strategies, most patients are already at an advanced stage at initial diagnosis. Given the high proportions of serous adenocarcinoma (80.86%), advanced FIGO stage (III and IV, 70.82%), and lymph node metastasis among middle-aged and older patients in this study, these features may partly explain the relatively high BRCA mutation frequency observed. Bioinformatic predictions suggest that the BRCA1 c.4712delT variant alters amino acids downstream of the 1571st amino acid in exon 16 of BRCA1, leading to truncation of the BRCA1 protein beyond this position and loss of normal protein function. This truncation is expected to impede binding to homologous recombination repair–related proteins such as RAD51 and PALB2, thereby disrupting key processes including homologous recombination repair of DNA double-strand breaks, cell-cycle regulation, and transcriptional regulation. It may further impair centrosome duplication control, resulting in centrosome dysregulation, genomic instability, and malignant cellular transformation, ultimately contributing to tumorigenesis. Clinically, mutation carriers in this study were predominantly diagnosed with high-grade serous adenocarcinoma and were mainly classified as stage III, which to some extent suggests an association between this variant and pathological characteristics. In addition, logistic regression modeling showed that including genotype improved the predictive performance for ovarian cancer risk, supporting the possibility that BRCA1 c.4712delT may contribute to ovarian cancer development and may have potential as an early diagnostic biomarker. Increasing the sample size in future studies may help to further verify this association.

In the experiment to determine the genotype of each sample, this study first employed the restriction fragment length polymorphism method, and conducted a preliminary screening for whether a mutation occurred by visually observing the bands obtained from electrophoresis. The screened samples were then subjected to Sanger sequencing, and the final determination was made as to whether the BRCA1 c.4712delT

mutation occurred and whether the mutation type was a homozygous mutation or a heterozygous mutation. The advantage of performing electrophoresis first and sequencing afterward is that it can reduce experimental costs and is more suitable for the detection of large batches of samples; the disadvantage is that the electrophoresis results need to be judged by visual observation, which has strong subjective dependence, and the possibility of missing mutant samples is higher than performing Sanger sequencing for all samples, thereby possibly leading to a lower mutation rate obtained in the experiment, which may also be one of the reasons why the mutation rate in the experimental group in this study was relatively low.

Based on the clinical data collected in this study and the statistical analysis results, the differences in HE4 levels and CA125 levels between the experimental group and the control group were statistically significant ($P < 0.05$), and the differences between the two markers across pathological subtype, clinical stage, histologic grade, and lymph node metastasis status were all significant ($P < 0.05$), indicating that HE4 and CA125 can significantly reflect the differences in the occurrence and progression of ovarian cancer among different patients, which is consistent with various previous studies [18]. In addition, the predictive model performance of the combined use of HE4 and CA125 was superior (AUC = 0.988, sensitivity = 0.935, specificity = 1), indicating that their combined use can more effectively predict the occurrence and progression of ovarian cancer. Both HE4 and CA125 are relatively mature clinical biomarkers for ovarian malignant tumors, and elevated levels of these markers are of important significance for the diagnosis of ovarian cancer; this study further corroborates this point.

When this study compared the indicators between the mutant group and the wild-type group, the results showed that there were no statistically significant differences between the two groups in 13 indicators, including age, HE4, CA125, PT, APTT, TT, FIB, D-D, personal history of cancer, family history, lymph node metastasis status, histologic grade, and clinical stage ($P > 0.05$), indicating that the BRCA1 c.4712delT mutation has no significant effect on tumor marker levels, coagulation function, lymph node metastasis status, histologic grade, or clinical stage in ovarian cancer patients, and has no clear association with age, which is inconsistent with the expected results. This may be due to the too small number of mutant samples and the lack of effective clinical data, such that intergroup differences could not be well reflected. Even so, compared with the wild-type group, the CA125 level in the mutant group still showed a relatively obvious upward trend. In subsequent experiments, further increasing the sample size and the amount of clinical data is expected to convert this trend into a statistically significant difference.

In summary, there are significant differences in HE4 and CA125 levels between the ovarian cancer patient population and the healthy population, further corroborating the effectiveness of HE4 and CA125 as biomarkers for ovarian malignant tumors. In addition, detection of the BRCA1 c.4712delT mutation, as a potential early diagnostic method for ovarian cancer, has demonstrated certain potential, and further investigation of the effects of this type of mutation on ovarian cancer risk and various clinical indicators is of important significance. This study has limitations in the detection method and sample size; subsequent experiments should expand the sample size and improve the experimental methods to enhance the reliability of the experimental results.

Conclusions

1) The mutation frequency of BRCA1 c.4712delT in ovarian cancer patients was 1.67% (7/418);
2) Changes in the serum levels of the tumor markers HE4 and CA125 can serve as effective indicators for the early diagnosis of ovarian cancer;
3) Detection of the BRCA1 c.4712delT variant is a potentially useful indicator for the early diagnosis of ovarian cancer.

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Research article

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Evaluation of the Inhibitory Effect of 2,5-Dimethyl-Celecoxib on Tamoxifen-Sensitive and -Resistant Human Breast Cancer Cells (MCF-7)

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KEYWORDS

Tamoxifen Resistance;
DMC;
DNA Damage;
MCM7

ABSTRACT

Acquired endocrine-resistance has become a big clinical challenge in breast cancer endocrine therapy.We previously reported that simvastatin inhibited TamR cell growth by reducing the expression of minichromosome maintenance protein 7 (MCM7) and retinoblastoma protein (Rb), which caused a significant up-regulation of γH2AX expression and subsequently induces DNA damage.Our previous studies have demonstrated that 2,5-dimethyl-celecoxib (DMC) can significantly reduce MCM7 and Rb protein expression in both MCF-7 and MCF-7/TamR cell lines. Aimed to investigate the effect of DMC on the proliferation of TAM-sensitive and -resistant breast cancer cell lines as well as to evaluate the possible underlying inhibitory mechanism, MTT and apoptosis analysis were performed to detect cell proliferation and apoptosis.Western blotting assays were performed to analyse the protein expression levels of cell cycle and apoptosis regulators. Furthermore, immunofluorescence and comet assays were carried out to explore the mechanism of DNA damage.Finally, *in vivo* experiments are performed to verify the results of *in vitro* experiments. The results demonstrated that DMC inhibited the proliferation and increased the apoptosis of both TAM-sensitive and -resistant breast cancer cells *in vitro*. In addition, DMC was observed to down-regulate Rb/MCM7 and induce DNA damage, particularly when used in combination with TAM. Notably,DMC also proved to have the same effect *in vivo* model. In summary , the growth inhibition generated by DMC may be achieved by inhibiting the protein expression of Rb/MCM7 and subsequently inducing DNA damage. This study provides a novel strategy for the treatment of TamR breast cancer patients in the clinical setting.

Introduction

Breast cancer is the most commonly diagnosed malignancy and a main cause of mortality among women worldwide(1). Endocrine therapy is the main choice for postoperative adjuvant treatment and has emerged as an advanced palliative treatment for ER-positive breast cancer(2, 3).Tamoxifen (TAM) is one of the most widely prescribed selective ER modulators and a gold standard endocrine treatment for all stages of breast cancer(4, 5). However, after 5 years of endocrine therapy with TAM, one-third of patients have been reported to develop drug resistance during or after chemo-

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therapy(6, 7). Acquired endocrine-resistance has become a big clinical challenge in breast cancer endocrine therapy and no effective solution has been reported. Therefore, understanding the underlying mechanism responsible for resistance during the course of endocrine therapy is critical and has important clinical value.

2,5-Dimethyl-celecoxib (DMC) is a methylated derivative of celecoxib(8). Despite the lack of selective cyclooxygenase-2 inhibition, DMC exhibits a tumor inhibition activity that is 20–50% higher than that of celecoxib, and it is devoid of cardiovascular toxicity(9). Many studies have showed that DMC has significant antitumor effects in several cancers, such as colorectal cancer, gastric cancer, lung cancer, human leukemia, glioblastoma, multiple myeloma, Burkitt's lymphoma, and breast cancer(10-19). However, whether DMC inhibits the proliferation of TAM-resistant (TamR) breast cancer cells has not been reported yet. We previously reported that simvastatin inhibits TamR cell growth by reducing the expression of MCM7/Rb, which causes a significant upregulation of γH2AX expression and subsequently induces DNA damage(20). In addition, our previous studies have demonstrated that DMC can significantly reduce MCM7 and Rb protein expression in MCF-7 and TamR cell lines.

In our research, we aimed to determine the effects of DMC on the proliferation and apoptosis of TAM-sensitive and -resistant breast cancer cell lines, as well as to evaluate the possible mechanism to provide a theoretical basis for the clinical application of DMC in the treatment of TamR breast cancer. In this research, as a model of human breast cancer, the cell line MCF-7 and the TamR cell line (MCF-7/TamR) were cultured *in vitro* and treated with various concentrations of DMC for different incubation periods. The effects of DMC on cell proliferation and apoptosis were investigated. The western blotting assays were performed to analyse the protein expression levels of cell cycle and apoptosis regulators.Furthermore, immunofluorescence and comet assays were carried out to explore the mechanism of DNA damage.Finally, *in vivo* animal experiments further validate *in vitro* results.

Materials and Methods

Cells and Reagents

MCF-7 cells were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences.MCF-7/TamR was derived from MCF-7 as previously described(20). Dimethyl sulfoxide (DMSO), TAM, and DMC were obtained from Sigma-Aldrich (USA). Fetal bovine serum (FBS) and charcoal-dextran-stripped FBS were obtained from Gibco (USA). High-glucose Dulbecco's modified Eagle's medium (DMEM) and phenol-free DMEM were purchased from Hyclone (USA).

MTT Assay

The cells(5 x 103 cells/well) were cultured in 96-well plates. After 24h, the cells were incubated with a series of concentrations of DMC and TAM. The treatment was performed either alone or in combination for different time periods (24, 48, 72 h). A 10-μl aliquot of MTT solution (5 mg/ml) was added and incubated at 37 °C for 4 h. After removing the medium, 100 μl of DMSO was added and shaken. Absorbance values were measured by using the microplate reader at 490 nm.

Flow Cytometric Analysis

Apoptosis was determined by using PE Annexin V apoptosis detection kit according to the manufacturer's guidelines. In brief, the cultured cells at a concentration of 5 × 104 cells/well in 6-well plates were washed twice with PBS and then resuspended in 100 μl of Annexin V binding buffer (1 × 106 cells/ml). Next, 5 μl each of PE Annexin V and 7-aminoactinomycin were added to the cells, the cells were incubated (30 min, in the dark), and staining and flow cytometric analysis were performed.

Western Blotting

Western blotting assays were carried out according to reported protocols, with slight modifications(21). Antibodies against p-Rb, γ-H2AX, p-ATM, p-CHK-2, Bcl-2, survivin, cleaved caspase 3, and PARP were diluted at 1:1000 (Cell Signaling Technology, USA) . Antibodies against Rb, MCM7, and PCNA were diluted at 1:500 (Santa Cruz, USA).

Immunofluorescence Assays

After treatment with drugs for 24h, the cells were each treated with 4% paraformaldehyde and 0.2% Triton X-100 for 10 min, and 5% BSA was blocked for 1 hour, then incubated with the primary antibody γH2AX at 4 °C overnight. Next day, incubated with an Alexa Fluor 488 anti-mouse secondary antibody for 1 h. Finally, the cells were stained with DAPI (5 μg/mL) and images were taken by a confocal fluorescence microscope (Leica SP5II).

Neutral Comet Assays

Neutral comet assays were carried out by using Trevigen's Comet Assay Reagent Kit according to the manufacturer's guidelines.Briefly, cells (106) were cultured in 6-well culture plates. An aliquot of 10 μl of cells at a concentration of 1 × 105 cells/ml was suspended in PBS solution, and the resulting suspension was mixed with 100 μl of molten LMAgarose at 37 °C in a 1:10 (v/v) ratio, followed by immediate loading of sample (50 μl) onto a CometSlide, which was kept at 4 °C for 30 min before subsequent experimentation. DNA staining was performed with SYBR® Gold, and visualization was carried out using epifluorescence microscopy (Leica DMi8, Germany). Finally, 50 cells per group were analyzed and quantified using CASP1.2.3 beta1 software.

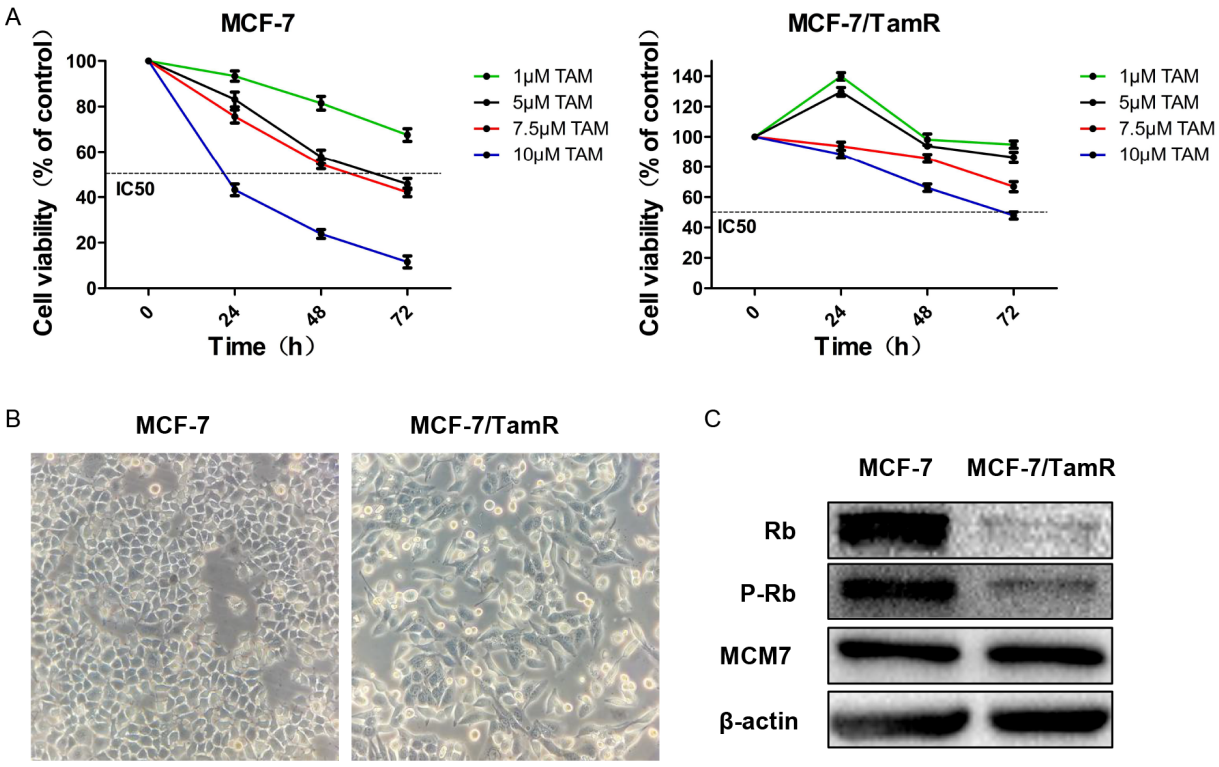


Figure 1 | Characteristics of MCF-7 and MCF-7/TamR cell lines

In Vivo Tumor Experiment

All animal experiments were done by protocols approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Xi'an Jiaotong University and four-week-old SCID-Beige female mice were from the Experimental Animal Center of the Medical College of Xi'an Jiaotong University. In this experiment, 5×106 MCF-7 and MCF-7/TamR cells were suspended in 100ml of PBS and injected into SCID female mice fat pad. At one weeks after injection,Tamoxifen (5 mg/kg in corn oil) and DMC (10 mg/kg in corn oil) was treated for 10 days by gavage .After 7 days , tumors were removed and photographed. The tumor were surgically fixed by using 4% paraformaldehyde for immunohistochemistry.

Immunohistochemistry

4μm sections of tissue wax block were attached to a glass slide, dewaxed by xylene, dehydrated by gradient alcohol, and blocked endogenous peroxidase activity, and then subjected to high temperature antigen retrieval in sodium citrate buffer.After cooling to room temperature, added 1:50 dilutions of primary antibody (MCM7, Rb) were incubated overnight at 4 °C in a refrigerator.The second day, horseradish peroxidase (HRP)-labeled secondary antibody was added, DAB staining,, hematoxylin counterstained and mounted.

Statistical Analysis

The data were expressed as the means ± standard deviation using GraphPad Prism software, version 5.00 (GraphPad Software, La Jolla, CA, USA, www.graphpad). The unpaired Student's t-test was carried out to compare two groups, and two-way analysis of variance and Dunnett's multiple comparison tests were used for multiple comparisons. $P < 0.05$ was considered statistically significant.

Results

Characteristics of MCF-7 and MCF-7/TamR Cell Lines

Initially, we evaluated the characteristics of the TamR cells using the MTT assay and western blotting analysis. The results of the MTT assay indicated that the half maximal inhibitory concentration (IC50) values of TAM in the MCF7 and MCF-7/TamR cell lines were 4.392 μM and 9.800 μM, respectively. Furthermore, the TAM resistance factor (RF) of MCF-7/TamR cell line was 2.231 (**Figure 1A**). Meanwhile, the morphology of the MCF-7 and MCF-7/TamR cell lines indicated that epithelial-mesenchymal transition-like changes were observed in the TamR cells (**Figure 1B**).Furthermore, western blotting assays revealed that the protein expression levels of p-Rb and Rb in the MCF-7/TamR cells were lower than those in the parental cells (**Figure 1C**). From above re-

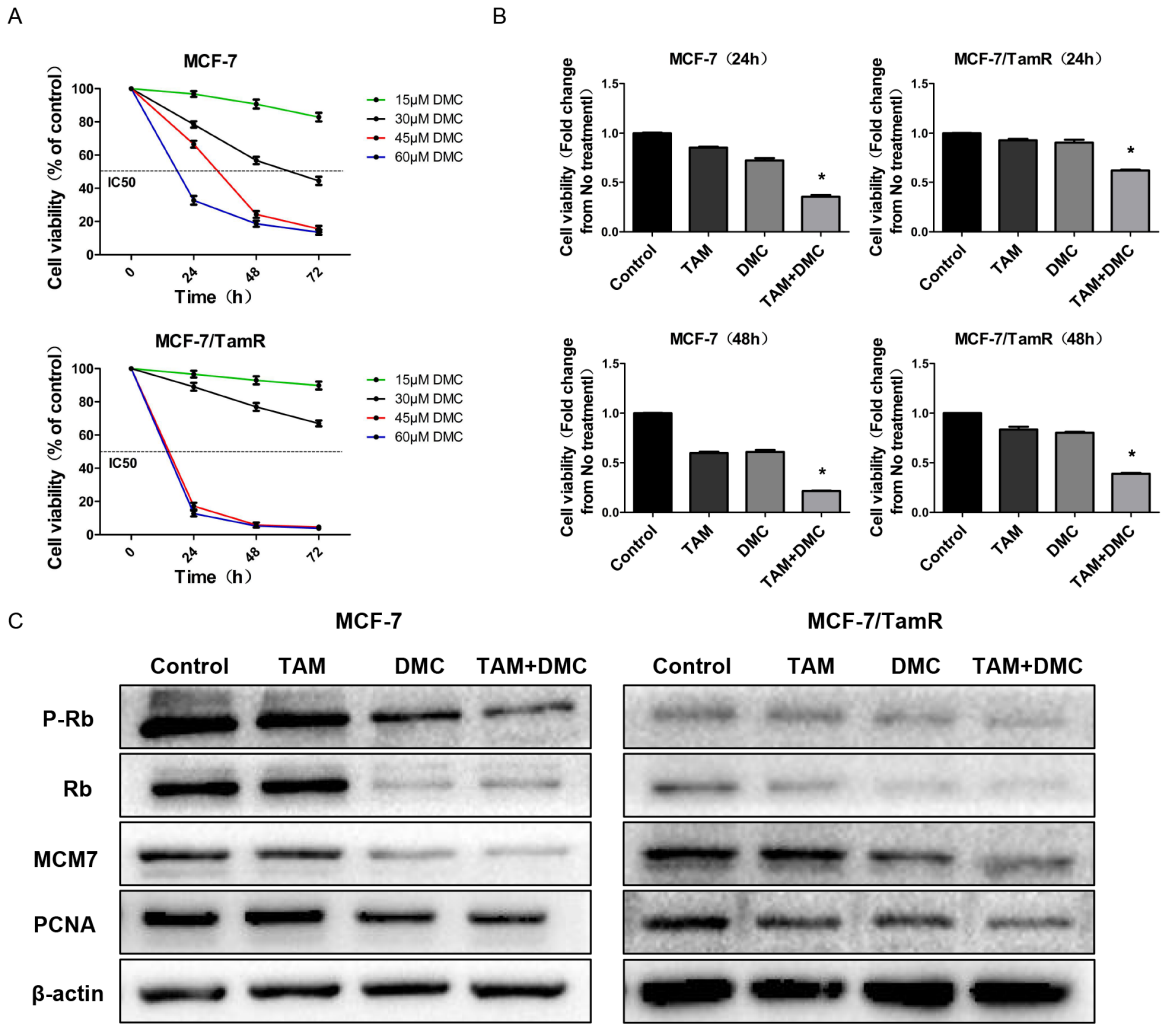


Figure 2 | DMC combined with TAM inhibits the growth of MCF-7 and MCF-7/TamR cell lines

*(A) MCF-7 and MCF-7/TamR cell lines were incubated with a series concentrations of DMC, and the MTT analysis were used to detect the proliferation inhibition effect of DMC on the cells. The concentration of DMC was 30 μM for the subsequent experiments. (B) DMC (30 μM) alone or combination with TAM (5 μM) was added to MCF-7 cells, and DMC (30 μM) alone or combination with TAM (7.5 μM) was applied to MCF-7/TamR cells; the cell proliferation was detected by the MTT analysis after 24 and 48 h. n = 3, *P < 0.05.(C) The expression of the cell cycle-related proteins Rb, p-Rb, MCM7, and PCNA were detected by Western blotting analysis.*

sults, our MCF-7/TamR cells has the characteristics of TAM-resistant cells.

DMC Combined With TAM Inhibits the Growth of MCF-7 and MCF-7/TamR Cell Lines

MCF-7 and MCF-7/TamR cell lines were treated with various concentrations of DMC (15, 30, 45, and 60 μM) for different incubation periods (24, 48, 72 h).As seen, the results showed that with an increase of DMC dose and incubation time, the proliferation rate was significantly inhibited in a time- and dose-dependent manner. Interestingly, when the DMC concentrations increased to a certain level(45 μM), the viability of MCF-7/TamR cells was significantly reduced, possibly due to the significant down-regulation of MCMs complex under the condition that the cell cycle progression was out of control due to Rb deficiency, resulting in lethal DNA damage further induced apoptosis(**Figure 2A**). Next, MCF-7 and MCF-7/TamR cell lines were incubated with a series of TAM and DMC concentrations either alone or in combination. The results of the MTT assay indicated that the cell viability of MCF-7 cells was inhibited significantly when they were treated with DMC (30 μM) and TAM (5 μM) for 48 h or 72 h; similarly, the cell viability of MCF-7/TamR cells was inhibited significantly when they were treated with DMC (30 μM) and TAM (7.5 μM) (**Figure 2B**). These two drug combinations were used in subsequent experiments. Furthermore, the protein expression levels of p-Rb, Rb, MCM7, and PCNA in MCF-7 and MCF-7/TamR cell lines were down regulated after treatment with DMC combined

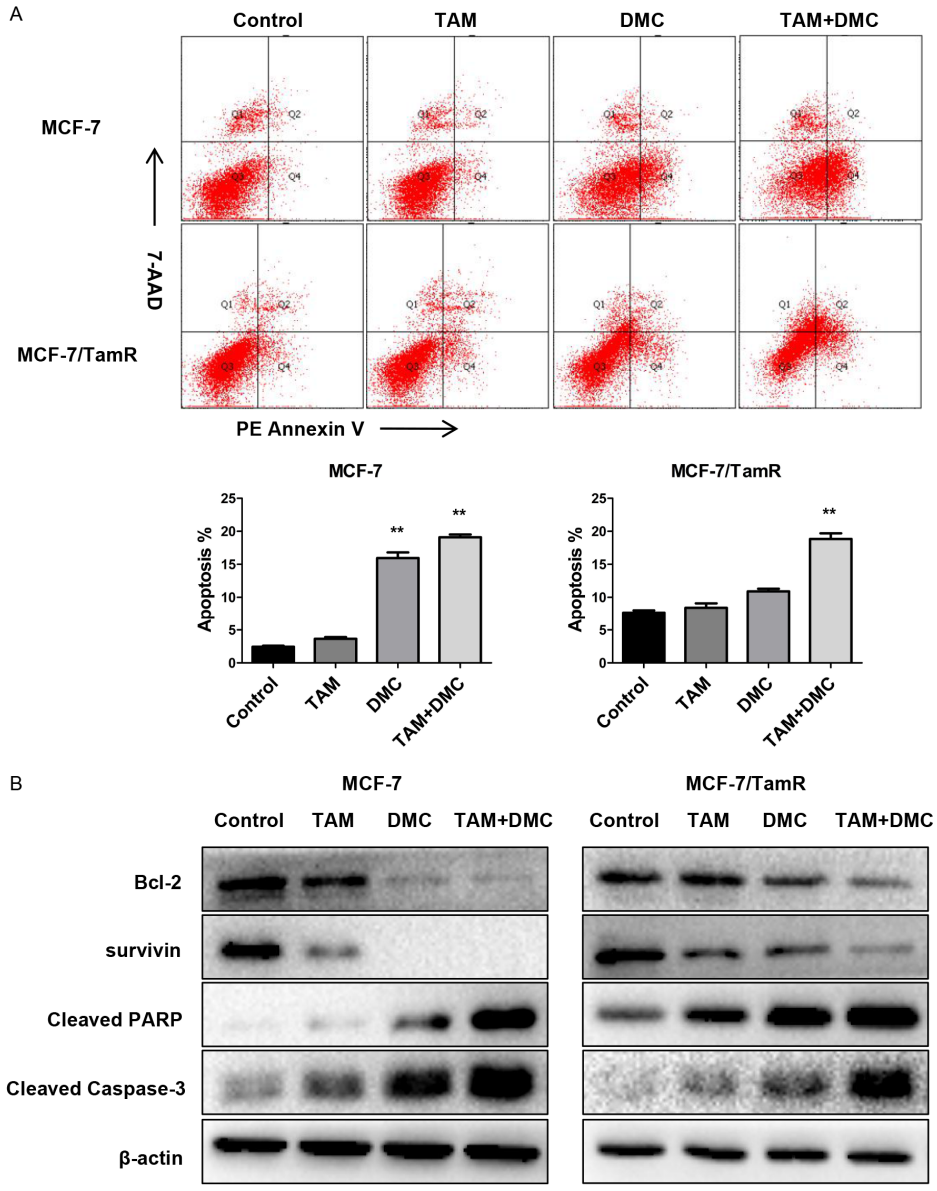


Figure 3 | DMC combined with TAM induces apoptosis in MCF-7 and MCF-7/TamR cell lines

*(A) Flow cytometry was performed to analyze the apoptosis of both cell lines after DMC treatment alone or in combination with TAM. n = 3, **P < 0.01. (B) The protein expression levels of cleaved caspase 3, Bcl2, cleaved PARP, and survivin were measured by western blotting analysis.*

with TAM for 48 h (**Figure 2C**). It is well known that the lost of Rb is related with increase cellular proliferation, but the simultaneous decreased in Rb and MCM7 inhibited cell proliferation(22). This is consistent with our experimental results. These results suggest that DMC combined with TAM inhibits the growth of MCF-7 and MCF-7/TamR cell lines.

DMC Combined With TAM Induces Apoptosis in MCF-7 and MCF-7/TamR Cell Lines

The flow cytometry assay showed that incubated with DMC combined with TAM for 48h caused the number of apoptotic cells for both MCF7 and MCF7/TamR cell lines to be increased (**Figure 3A**).Furthermore, the protein expression levels of cleaved caspase 3 and cleaved PARP were up-regulated, whereas Bcl2 and survivin were down-regulated obviously in the two cell lines after treatment (**Figure 3B**). In summary, DMC combined with TAM induces apoptosis in both MCF7 and TamR cell lines.

DMC Combined With TAM Upregulates γH2AX and Induces DNA Damage in MCF-7 and MCF-7/TamR Cell Lines

Our previous research indicated that DNA damage induced by inhibiting the protein expression of Rb and MCM7

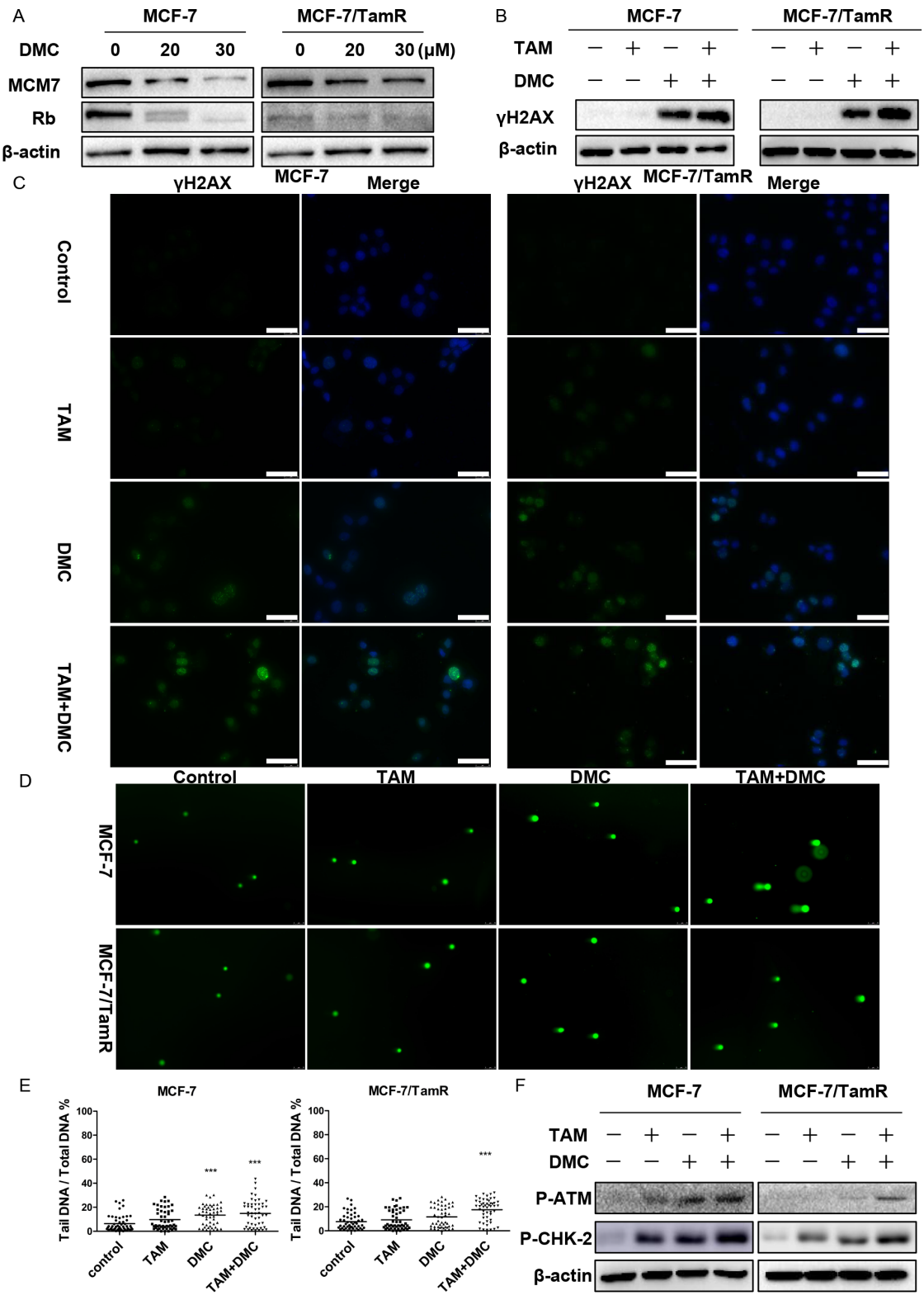


Figure 4 | DMC combined with TAM upregulates γH2AX and induces DNA damage in MCF-7 and MCF-7/TamR cell lines

*(A) MCF-7 and MCF-7/TamR cell lines were incubated with different doses (0, 20, and 30 μM) of DMC for 24 h, and the total cellular proteins of the cell extracts were analyzed by western blotting. (B) MCF-7 and MCF-7/TamR cell lines were incubated with control, TAM (5 μM)/(7.5 μM), DMC (30 μM), or TAM (5 μM) plus DMC (30 μM)/TAM (7.5 μM) plus DMC (30 μM) for 24 h, and the total cellular proteins were extracted for western blotting analysis. (C) MCF-7 and MCF-7/TamR cell lines were treated as described above and immunofluorescence assays were performed. The bar represents 100 μm. (D) The two cells were treated as described above and neutral comet assay was carried out. The bar, 100 μm. (E) Quantitative analysis of comet tail DNA content/total DNA content (%) in 50 cells per group.n = 3, ***P < 0.001. (F)Two cells were treated as described above, and the extracted total cellular proteins were analyzed by western blotting.*

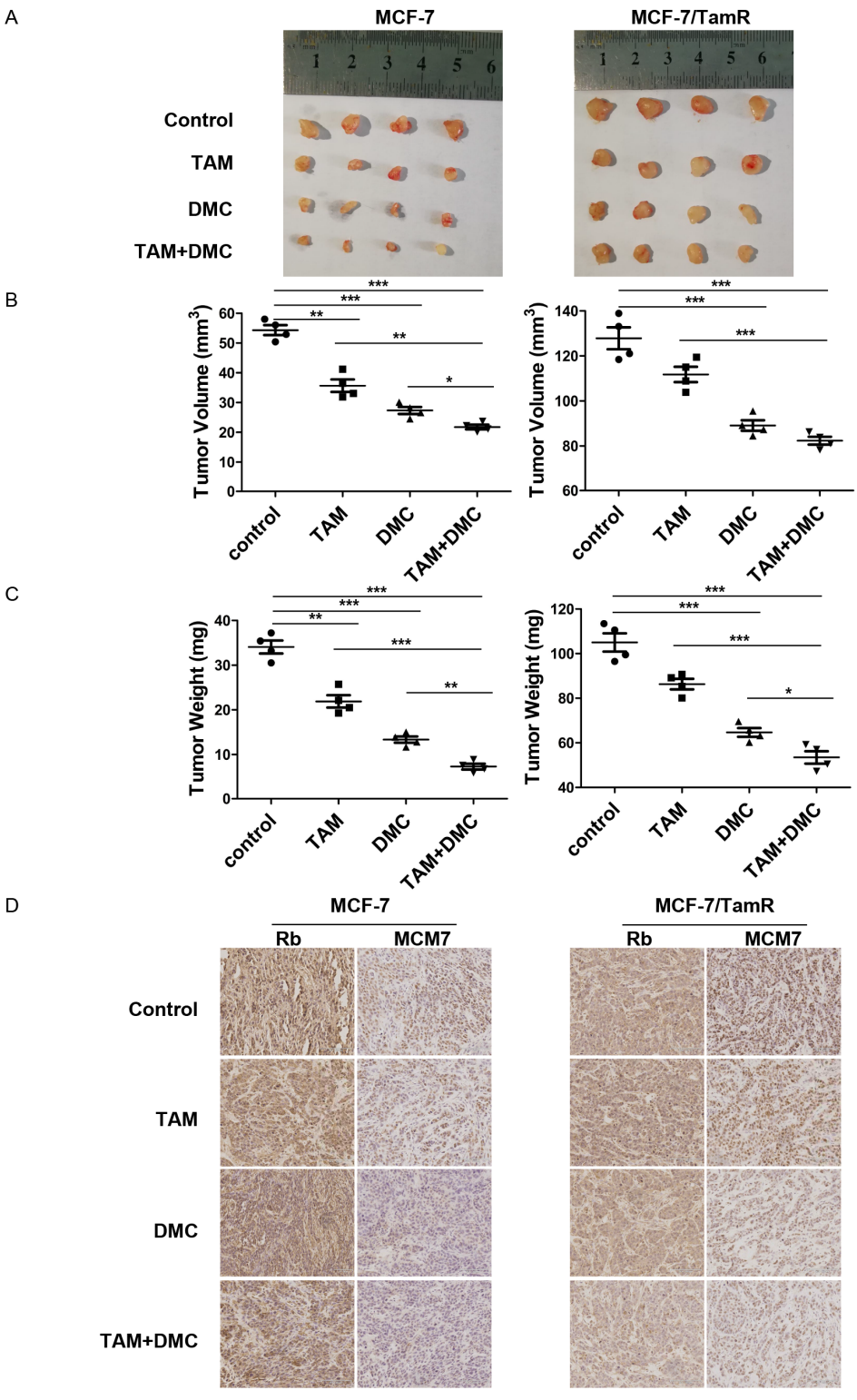


Figure 5 | DMC combined with TAM inhibits the growth of MCF-7 and MCF-7/TamR cells *in vivo*

*The xenograft tumors model were established by injecting 5 x 106 MCF-7 and MCF-7 / TamR cells into the SCID / Beige mice fat pad. After seven days, mice were treated with TAM (5 mg/kg) / DMC (10 mg/kg) alone or combination for 10 days by gavage. Tumor size (A,B), tumor weight (C) and immunochemical staining with RB and MCM7 antibodies(D); The bar,100 μm. *p < 0.05, **p < 0.01 and ***p < 0.001.*

could strongly inhibit the proliferation of TamR cells(22). Consistently, the western blotting assay results indicated that the protein expression levels of Rb and MCM7 were down-regulated significantly after incubated with DMC for 24 h (**Figure 4A**). The γH2AX expression was increased significantly after incubated with DMC for 24 h and further increased upon treatment with a combination of DMC and TAM (**Figure 4B**). In addition, the immunofluorescence assay showed consistent experimental results with the western blot assay results (**Figure 4C**). Neutral comet assays were used to detect double-stranded breaks, and the results showed that severe DNA damage was caused in MCF-7 and MCF-7/TamR cell lines after treatment with DMC alone or in combination with TAM for 24 h (**Figure 4D,E**). Finally, western blotting assays demonstrated that the protein expression of the DNA damage checkpoint proteins p-ATM and p-Chk2 was up-regulated after a 24h treatment with DMC combined with TAM (**Figure 4F**). All of these results demonstrate that DMC combined with TAM up-regulates γH2AX and induces DNA damage in MCF-7 and MCF-7/TamR cell lines.

DMC Combined With TAM Inhibits the Growth of MCF-7 and MCF-7/TamR Cells *in Vivo*

To explore the *in vivo* effects of DMC, we established a xenograft tumor model in SCID / Beige mice using MCF7 and MCF7 / TamR cell lines.When tumors were touchable,mice were treated with TAM (5 mg/kg)/DMC (10 mg/kg) alone or combination for 10 days by gavage. The xenograft tumors were significantly inhibited after DMC alone or TAM combined with DMC treatment(**Figure 5A to C**).In addition ,the result of immunochemistry showed that Rb and MCM7 expression were reduced after DMC alone or combined with TAM treatment *in vivo*(**Figure 5D**).Therefore, our results revealed that DMC can be consistent with *in vitro* experiments by inhibiting Rb/MCM7 expression and inhibiting tumor growth *in vivo*.

Discussion

In recent years, the traditional nonsteroidal anti-inflammatory drug celecoxib has become a hot spot for anti-tumor research as it causes cell cycle arrest, inhibits human breast cancer cell proliferation, promotes apoptosis, and also inhibits migration and invasion. Additionally, it has been shown to be a beneficial therapeutic in patients with an increased sensitivity to radiotherapy and chemotherapy(23-26). However, its mechanism of action involves COX-2 inhibition, which may cause severe cardiovascular toxicity if used for a long period of time(27). DMC, a methylated derivative of celecoxib, has shown a tumor suppression activity that is 20–50% higher than that of celecoxib; in addition, it lacks COX-2 inhibitory activity and also is devoid of cardiovascular toxicity. It has been revealed that DMC plays a role in inhibiting cell cycle progression and inducing apoptosis in human leukemia cells(14). Moreover, it has demonstrated cytotoxic and antitumor effects both *in vitro* and *in vivo* by downregulating the expression of the antiapoptotic protein survivin and subsequently inducing apoptosis(28).

In our study, we also observed that DMC significantly inhibited proliferation and induced apoptosis in MCF-7 and MCF-7/TamR breast cancer cell lines. It has been revealed that DMC has drug-sensitizing effects when used in combination with ABT-737 to increase the sensitivity of gastric cancer cells as well as with imatinib to treat colorectal cancer(10, 12). Our study indicated that the combination of DMC with TAM had a further enhancement on proliferation inhibition and apoptosis induction. Additionally, DMC has shown antiangiogenic activity in the tumor vasculature both *in vivo* and *in vitro*; thus, it plays an antitumor effect(29). In breast cancer, DMC has demonstrated a killing effect on triple-negative and chemotherapy-resistant breast cancer cells by aggravating endoplasmic reticulum stress(19, 30), but its role in TAM resistance of breast cancer has not been reported. In our research, we examined the mechanism of action of DMC on MCF-7 and MCF-7/TamR cell lines , the results indicated that DMC down-regulated the protein expression of Rb and MCM7 but up-regulated γH2AX and also induced DNA damage in MCF-7 and MCF-7/TamR cell lines. Interestingly, in combination with TAM, these effects were further enhanced. Notably,DMC also proved to have the same effect *in vivo* model, our results revealed that DMC can be consistent with *in vitro* experiments by inhibiting Rb/MCM7 expression and inhibiting tumor growth *in vivo*. Unlike celecoxib, DMC has not been studied in humans, therefore, further assessment of the *in vitro* mechanism would be of great significance provided that *in vivo* experiments are also performed. In addition, appropriate preclinical testing should be performed to further verify the effects.

In conclusion, we observed that DMC inhibited proliferation and induced apoptosis of TAM-sensitive and -resistant human breast cancer cells (MCF-7) both *in vitro* and *in vivo*. Moreover, DMC significantly downregulated Rb and MCM7 expression and induced DNA damage in both cell lines. Our data suggest that growth inhibition generated by DMC may be achieved by inhibiting the protein expression of Rb and MCM7 and subsequently inducing DNA damage.This study provides a novel strategy for the treatment of TamR breast cancer patients in the clinical setting.

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Conflict of interest statement The authors declare no competing interests.

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