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Relationship between molecular classification and clinicopathological features of young and medium-elderly breast cancer patients

Lili Li[1], Jing Zhao[2], Yufen An[3], Yunxiang Zhang[1], Zhimin Wei[2]

[1]Department of Pathology, Weifang People's Hospital, Weifang 261041, China

[2]Department of Pathology, Affiliated Hospital of Qiingdao University, Qingdao 266061, China

[3]Clinical Pathology of Qingdao University Medical College, Qingdao 266003, China

Abstract: **Young breast cancer patients are a special group. And the research about this group is very seldom. To investigate the relationship between molecular classification and clinicopathological features of young breast cancer patients, and the differences of those features between young and middle aged patients. A total of 2488 incident cases of invasive breast cancer were identified from the Affiliated Hospital of Qingdao University between April 2012 and August 2015. To analyze the relationship between molecular subtypes and histologic grade, tumor size, lymph node involvement, the expressions of EGFR, Topo Ⅱα, Ki-67 of 177 cases of young breast cancer patients (<or＝35 years old) were selected in the observation group.. 2311 cases of middle aged breast cancer patients (>or＝45 years old) during the same period were served as the control group. A meaningful correlation was detected between molecular subtypes and histologic grade, tumor size, the expressions of EGFR, Topo Ⅱα, Ki-67 in young breast cancer patients (Z=11. 321−38. 371, χ2=31. 886, F=27. 241, P<0. 05). In addition, the histologic grade, the expressions of Topo Ⅱα and Ki-67 were statistically different between young and elderly breast cancer patients (P<0. 05), especially in Luminal B subtype. Significant differences in histologic grade of Luminal B subtype, the expression of Topo Ⅱα of Luminal B (HER2-) subtype and the expression of Ki-67 of Luminal B (HER2+) subtype were observed between young and middle aged breast cancer patients (Z=-2. 151～-2. 027, t =2. 129, P<0. 05). The molecular classification of young breast cancer patients is related to their pathological features. Some pathological characteristics of Luminal B subtype of young breast cancer patients are unique compared to the middle aged ones. It is very important to estimate prognosis and formulate individualized treatment plan based on the precise medical treatment.**

Keywords: Young breast cancer; Molecular classification; Clinicopathological features; Immunohistochemistry; FISH

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*Corresponding Author: Zhimin Wei, weizmqyfy@126.com

1. Introduction

Breast cancer is the most common malignant tumor in women. In recent years, the incidence of female breast cancer continues to rise and become common among young. Breast cancer has become one of the most common causes of cancer death in young women. At present, the age limit of young breast cancer is not clear. Most studies defined the young breast cancer patients as less than or equal to 35 years old when diagnosed with breast carcinoma at the first time[1]. Breast cancer is highly heterogeneous. Sometimes, the response and prognosis of patients with same morphology is different. With the development of molecular biotechnology, molecular classification provides a new perspective for individual treatment and prognosis of breast cancer.

2. Study population

Patients were eligible for the study if they were Han females in the Affiliated Hospital of Qingdao University between April 2012 to 2015 August and made a definite diagnosis of invasive breast cancer by pathology for the first time, and were accepted modified radical mastectomy for breast cancer. There were 2488 cases, primary unilateral solitary invasive breast cancer patients. All patients were women without any treatment before surgery. Among them, there were 177 cases young breast cancer patients, 2311 cases middle-aged breast cancer patients in the same period. The youngest patient was 20 years old, and the oldest patient was 95 years old.

Inclusion Criteria: Patients treated at the Affiliated Hospital of Qingdao University that had information about the tumor size, lymph node status of their primary tumor.

Exclusion Criteria: Patients with prior malignancy and if there was evidence of metastatic disease or post-chemotherapy were excluded.

3. Methods

We used Immunohistochemistry (IHC) PV9000 method to test the expressions of estrogen receptor (ER, 1:200), progesterone receptor (PR, 1:200), epidermal growth factor receptor (EGFR, 1:100), topoisomerase Ⅱα (Topo Ⅱα, 1:150) and Ki-67 (1:150) in breast tumor. The antibodies were all

rabbit anti human monoclonal antibodies. We used hot fix that was repairing 20min in sodium citrate (PH9. 0) with 100 centigrade high temperature. Positive breast cancer tissue sections were used as positive control. PBS buffer instead of first antibody was used as negative control. Immunohistochemical antibody and auxiliary reagents were purchased from Beijing Zhongshan Jinqiao Biological Technology Limited company. We used Fluorescence in situ hybridization (FISH) method and human epidermal growth factor 2 (HER2) gene detection reagent box to test HER2. After the slide pretreatment, reagent was added at a temperature of 85°C and degenerated for 5 minutes, then incubated overnight at 37°C. After hybridization it was washed and counter stained. FISH detection kit was purchased from Guangzhou LBP Medicine Science & Technology Limited company.

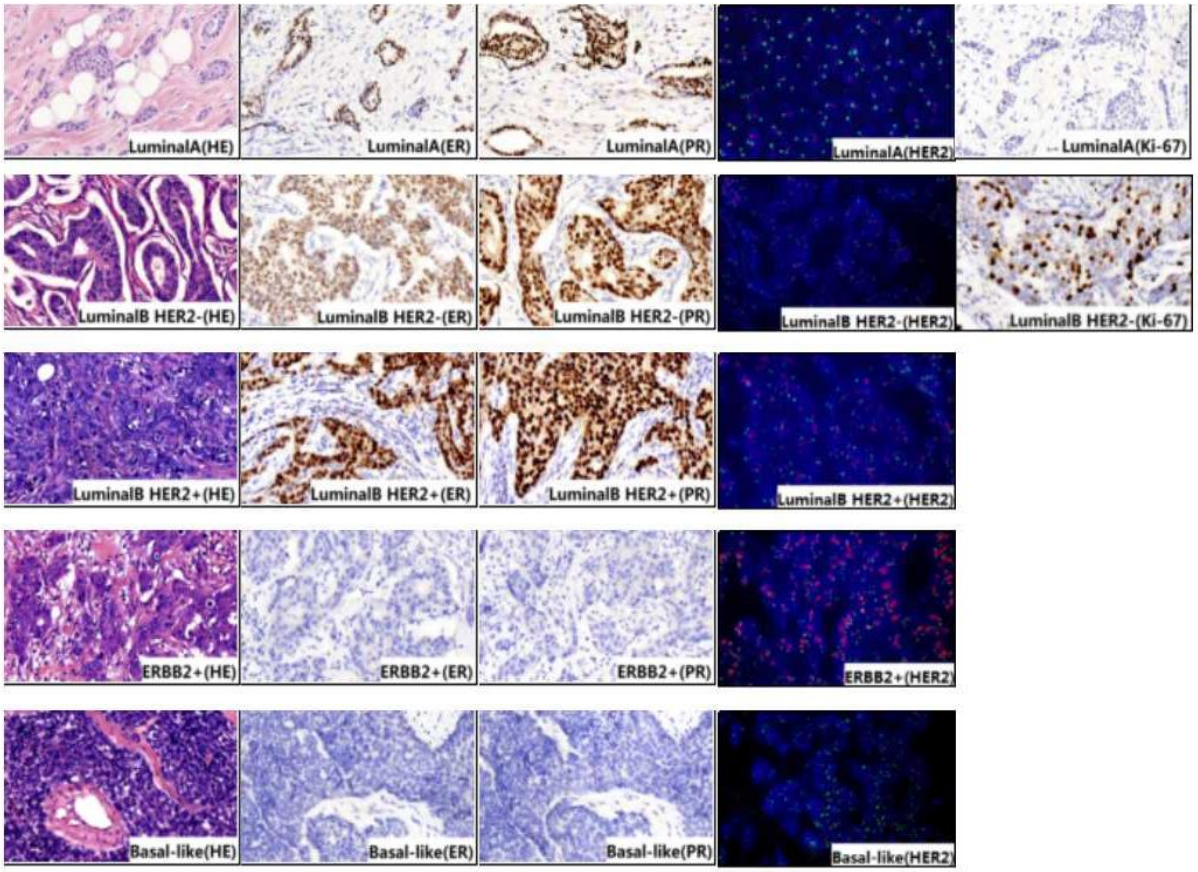

Figure 1. Representative cases for each molecular subtype of young breast cancer (Immunohistochemical staining, original magnification ×400; FISH staining, original magnification ×1000).

3.1. Result criterion

Tumors were considered ER and PR positive if the staining of the nuclei of rumor cells were more than 1%[2]. EGFR was positive if more than or equal to 1% tumor cell membrane were stained. Topo Ⅱα stained in the nucleus. Each section were observed 10 high mirror field randomly, if the positive cell number was less than 1% then it is negative, 1% ~ 25% for grade Ⅰ, 26% ~ 50% for grade Ⅱ, 51% ~ 75% for grade Ⅲ, 76% ~ 100% for grade Ⅳ. The positive staining of Ki-67 was localized in the nucleus at a high magnification, to count equal to or more than 500 tumor cells, without considering the the tinting strength according to the proportion of positive cells to calculate the positive rate. The test score standard of HER2 by FISH referred to the 2016 edition of breast cancer HER2 testing guidelines 3.

In this work, subtypes were classified by IHC and FISH staining according to St. Gallen subtypes as follows: Luminal A, ER and / or PR(+), HER2 (-) , and Ki-67% < 14%; Luminal B HER2-neu negative, ER and /or PR(+), HER2 (-), and Ki-67% ≥14%; Luminal B HER2-neu positive, ER and / or PR(+), HER2 (+); non-luminal HER2-neu positive, ER (-), PR (-) , HER2 (+); Basal-like, ER (-), PR (-), HER2 (-).

The histological classification was based on the classification criteria of WHO in 2012, and it was evaluated from three aspects of the formation of the gland, the nuclear polymorphism and nuclear division.

3.2. Statistical analysis

Rank test and the Chi-square test was used for count data. Single factor analysis of variance and t test was used for measurement data. P<0.05 means the difference has statistical significance. All statistical analysis were performed by SPSS 21.0.

4. Results

4.1. The difference of molecular classification between young and middle-aged breast cancer patients

Among the 177 enrolled young patients with breast cancer, the distribution of subtypes was Luminal A, 18. 08%; Luminal B(HER2-), 44. 07%, luminal B (HER2+), 15. 82%; ERBB2+, 8. 47% and Basal-like, 13. 56%. The presenting characteristics of the population were presented in Table 1. The images of each molecular classification of young breast cancer patients were presented in Figure 1.

Table 1. The difference of molecular classification between young and middle-aged breast cancer patients (n(x/%))

Group	Luminal A	Luminal B (HER2-)	Luminal B (HER2+)	ERBB2+	Basal-like	value
young	32(18.08)	78(44.07)	28(15.82)	15(8.47)	24(13.56)	χ^2=6.425
medium-elderly	529(22.89)	943(40.80)	254(10.99)	249(10.77)	336(14.54)	*P*=0.170

4.2. Relationship between molecular subtypes and pathological characteristics of young breast cancer

Among the 5 subtypes there were significant differences in the distribution of histologic grade, tumor size, EGFR, Topo Ⅱα and Ki-67 proliferation index between different molecular subtypes of young breast cancer patients (Z=11.321-38.371, χ2=31.886, F=27.241, P<0.05), but there was no statistical significance in the nodal status (Z=6.549, P=0.162). See Figure 2, Table 2.

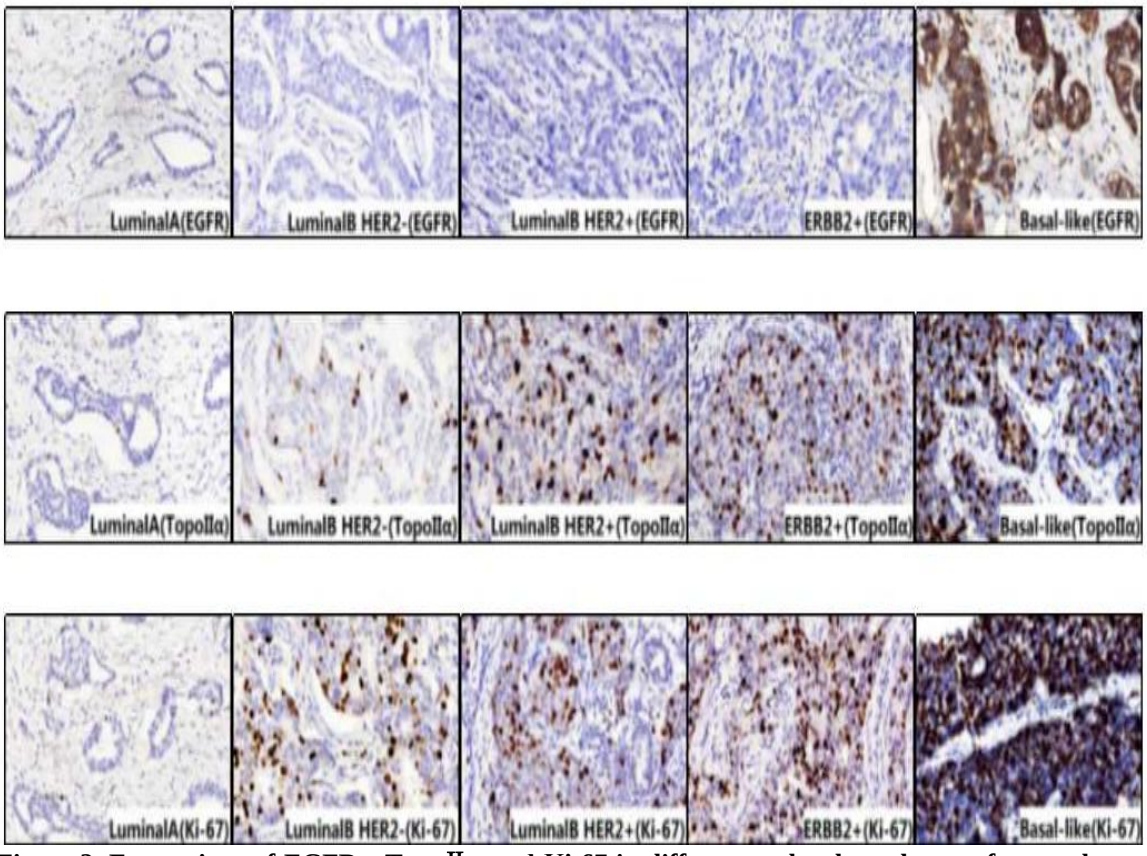

Figure 2. Expressions of EGFR、Topo Ⅱα and Ki-67 in different molecular subtype of young breast cancer (Immunohistochemical staining, original magnification ×400).

Table 2. The Relationship between Molecular Subtypes and Pathological Characteristics of Young Breast Cancer (n(x/%))

Characteristic	Luminal A	Luminal B (HER2-)	Luminal B (HER2+)	ERBB2+	Basal-like	value
histologic grade						
Ⅰ	6(18.8)	1(1.3)	0(0.0)	0(0.0)	1(4.2)	Z=38.371
Ⅱ	22(68.8)	50(64.1)	10(35.7)	3(20.0)	6(25.0)	P<0.001
Ⅲ	4(12.5)	27(34.6)	18(64.3)	12(80.0)	17(70.8)	
Tumor size						
T1	26(81.3)	45(57.7)	14(50.0)	6(40.0)	11(45.8)	Z=11.321
T2	6(18.8)	31(39.7)	12(42.9)	9(60.0)	12(50.0)	P=0.023
T3+T4	0(0.0)	2(2.6)	2(7.1)	0(0.0)	1(4.2)	
Nodal status						
N0	19(59.4)	41(52.6)	14(50.0)	4(26.7)	15(62.5)	Z=6.549
N1	7(21.9)	18(23.1)	5(17.9)	5(33.3)	5(20.8)	P=0.162
N2	5(15.6)	11(14.1)	6(21.4)	3(20.0)	3(12.5)	
N3	1(3.1)	8(10.3)	3(10.7)	3(20.0)	1(4.2)	
EGFR status						
-	31(96.9)	70(89.7)	25(89.3)	9(60.0)	12(50.0)	χ^2=31.886
+	1(3.1)	8(10.3)	3(10.7)	6(40.0)	12(50.0)	P<0.001
Topo Ⅱα status						
-	2(6.3)	0(0.0)	0(0.0)	1(6.7)	0(0.0)	Z=32.168
Ⅰ	30(93.8)	49(62.8)	15(53.6)	6(40.0)	8(33.3)	P<0.001
Ⅱ	0(0.0)	24(30.8)	11(39.3)	8(53.3)	11(45.8)	
Ⅲ+Ⅳ	0(0.0)	5(6.4)	2(7.1)	0(0.0)	5(20.8)	
Ki-67(%)index	8.66±2.21	35.64±16.12	43.39±23.14	46.00±19.57	54.63±26.07	F=27.241 P<0.001

4.3. The difference between young and middle-aged breast cancer patients with different molecular subtypes

There were statistically significant differences in tissue histologic grade, Topo Ⅱα and the expression of Ki-67 between young and medium-elderly breast cancer patients (Z=-3.006, Z=-2.801, t=2.822, P＜0.05), but there was no statistically difference in tumor size, lymph node metastasis and the expression of EGFR(Z=-1.162, Z=-1.898, χ^2= 0.704, P ＞ 0.05). There was statistically significant difference in histological grading of Luminal B subtype between young and middle-aged breast cancer patients (Z=-2.027, P=0.043; Z=-2.151, P=0.032). Significant differences in the expression of Topo Ⅱα of Luminal B (HER2-) subtype (Z=-2.111, P=0.035) and the expression of Ki-67 of Luminal B (HER2+) subtype (t =2.129, P=0.034) were observed between young and middle-aged breast cancer patients. There was no statistically significant difference in other molecular classification or indexes between young and middle-aged breast cancer patients, (Z=-1.622～-0.220, χ^2=0.096-1.687, t=0.823～1.417, P＞0.05). See Figure 3, Table 3.

Table 3. Pathological differences in molecular subtypes between young and middle-aged breast cancer(n(x/%))

Characteristic	Luminal A		value	Luminal B(HER2-)		value	Luminal B(HER2+)		value
	young	Middle-aged		young	Middle-aged		young	Middle-aged	
histologic grade									
I	6(18.8)	98(18.5)	Z=-0.942	1(1.3)	33(3.5)	Z=-2.027	0(0.0)	8(3.1)	Z=-2.151
Ⅱ	22(68.8)	413(78.1)	P=0.346	50(64.1)	674(71.5)	P=0.043	10(35.7)	135(53.1)	P=0.032
Ⅲ	4(12.5)	18(3.4)		27(34.6)	236(25.0)		18(64.3)	111(43.7)	
Tumor size									
T1	26(81.3)	386(73.0)	Z=-1.046	45(57.7)	601(63.7)	Z=-1.144	14(50.0)	126(49.6)	Z=-0.220
T2	6(18.8)	139(26.3)	P=0.296	31(39.7)	332(35.2)	P=0.253	12(42.9)	123(48.4)	P=0.826
T3+T4	0(0.0)	4(0.8)		2(2.6)	10(1.1)		2(7.1)	5(2.0)	
Nodal status									
N0	19(59.4)	343(64.8)	Z=-0.692	41(52.6)	534(56.6)	Z=-1.004	14(50.0)	133(52.4)	Z=-0.450
N1	7(21.9)	119(22.5)	P=0.489	18(23.1)	236(25.0)	P=0.315	5(17.9)	62(24.4)	P=0.653
N2	5(15.6)	35(6.6)		11(14.1)	108(11.5)		6(21.4)	29(11.4)	
N3	1(3.1)	32(6.0)		8(10.3)	65(6.9)		3(10.7)	30(11.8)	
EGFR status									
-	31(96.9)	518(97.9)	χ^2=0.158	70(89.7)	879(93.2)	χ^2=1.323	25(89.3)	232(91.3)	χ^2=0.132
+	1(3.1)	11(2.1)	P=0.509	8(10.3)	64(6.8)	P=0.250	3(10.7)	22(8.7)	P=0.990
Topo Ⅱ status									
-	2(6.3)	27(5.1)	Z=-0.397	0(0.0)	13(1.4)	Z=-2.111	0(0.0)	2(0.8)	Z=-0.568
I	30(93.8)	499(94.3)	P=0.691	49(62.8)	675(71.6)	P=0.035	15(53.6)	147(57.9)	P=0.570
Ⅱ	0(0.0)	3(0.6)		24(30.8)	221(23.4)		11(39.3)	90(35.4)	
Ⅲ+Ⅳ	0(0.0)	0(0.0)		5(6.4)	34(3.6)		2(7.1)	15(5.9)	
Ki-67(%)index	8.66±2.21	8.32±2.64	t=0.823	35.64±16.12	33.23±15.91	t=1.271	43.39±23.14	35.98±16.78	t=2.129
			P=0.416			P=0.207			P=0.034

Characteristic	ERBB2+		value	Basal-like		value	Ki-67		value
	young	Middle-aged		young	Middle-aged		young	Middle-aged	
histologic grade									
Ⅰ	0(0.0)	3(1.2)	Z=-1.537	1(4.2)	8(2.4)	Z=-0.329	8(4.5)	150(6.5)	Z=-3.006
Ⅱ	3(20.0)	96(38.6)	P=0.124	6(25.0)	81(24.1)	P=0.742	91(51.4)	1399(60.5)	P=0.003
Ⅲ	12(80.0)	150(60.2)		17(70.8)	247(73.5)		78(44.1)	762(33.0)	
Tumor size									
T1	6(40.0)	123(49.4)	Z=-0.648	11(45.8)	185(55.1)	Z=-0.954	102(57.6)	1421(61.5)	Z=-1.162
T2	9(60.0)	123(49.4)	P=0.517	12(50.0)	145(43.2)	P=0.340	70(39.5)	862(37.3)	P=0.245
T3+T4	0(0.0)	3(1.2)		1(4.2)	6(1.8)		5(2.8)	28(1.2)	
Nodal status									
N0	4(26.7)	125(50.2)	Z=-1.622	15(62.5)	222(66.1)	Z=-0.343	93(52.5)	1357(58.7)	Z=-1.898
N1	5(33.3)	52(20.9)	P=0.105	5(20.8)	64(19.0)	P=0.732	40(22.6)	533(23.1)	P=0.058
N2	3(20.0)	42(16.9)		3(12.5)	35(10.4)		28(15.8)	249(10.8)	
N3	3(20.0)	30(12.0)		1(4.2)	15(4.5)		16(9.0)	172(7.4)	
EGFR status									
-	9(60.0)	187(75.1)	χ^2=1.687	12(50.0)	157(46.7)	χ^2=0.096	147(83.1)	1973(85.4)	χ^2=0.704
+	6(40.0)	62(24.9)	P=0.320	12(50.0)	179(53.3)	P=0.756	30(16.9)	338(14.6)	P=0.401
Topo Ⅱ status									
-	1(6.7)	14(5.6)	Z=-0.628	0(0.0)	14(4.2)	Z=-1.396	3(1.7)	70(3.0)	Z=-2.801
Ⅰ	6(40.0)	134(53.8)	P=0.530	8(33.3)	146(43.5)	P=0.163	108(61.0)	1601(69.3)	P=0.005
Ⅱ	8(53.3)	88(35.3)		11(45.8)	122(36.3)		54(30.5)	524(22.7)	
Ⅲ+Ⅳ	0(0.0)	13(5.2)		5(20.8)	54(16.1)		12(6.8)	116(5.0)	
Ki-67(%)index	46.00±19.57	38.62±20.05	t=1.417	54.63±26.07	49.50±24.45	t=0.934	35.44±22.72	30.78±21.07	t=2.822
			P=0.176			P=0.359			P=0.005

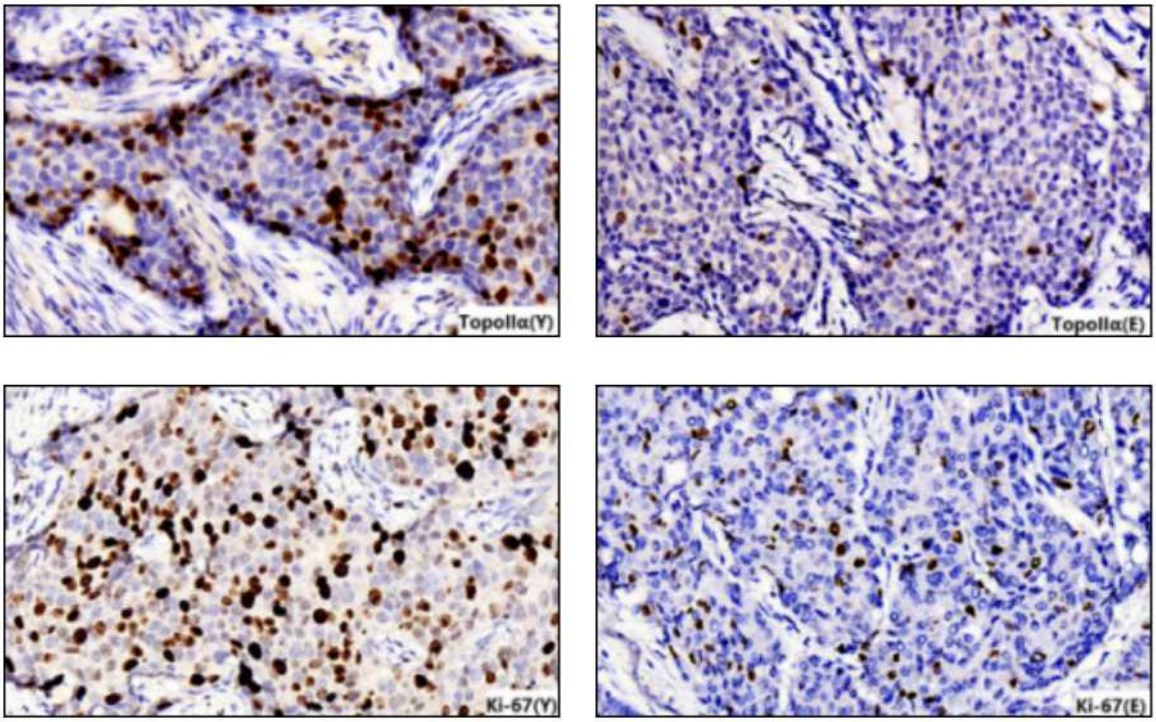

Figure 3. Expressions of TopoⅡα and Ki-67 in Luminal B subtype of young and middle-aged breast cancer (Immunohistochemical staining, original magnification×400).

5. Discussion

5.1 The difference of molecular subtypes between young and middle-aged breast cancer patients

The molecular classification of breast cancer was presented firstly by Perou[4] through the study of cDNA microarray in 2000. And it made the level of classification of breast cancer became molecular biology from tissue morphology. Gene expression profiling is the gold standard for the molecular classification of breast cancer. Carey proved that immunohistochemistry can replace gene chip technology to categorize breast cancer analogous in molecular profiling. It is of great clinical importance because IHC is cost affordable, fast, practical, and does not require an ultra-specialized laboratory facility. After that, many domestic and foreign studies combined the molecular subtypes with treatment and prognosis to add and perfect the molecular subtypes of breast cancer[5-8].

The research results showed that Luminal B was the mainly molecular subtype both in young and middle-aged breast cancer patients, especially Luminal B (HER2-) subtype occupied 44.07% in the young group, followed by Luminal A subtype, ERBB2+ subtype and Basal-like subtype were less. The proportion of Luminal B subtype in the young group was higher than that in middle-aged group. But there was no statistically significant difference in molecular subtype between young and middle-aged breast cancer patients (χ^2=6.425, P=0.170). These findings were in accordance with the study of Lian Zhenqiang[9]. But it was in contrast with the study of Ihemelandu[10]. Luminal A was the most common subtype breast cancer. In young breast cancer patients, Basal-like was the most common subtype and Luminal B accounted for only a small part. This difference relate to the adjustment of classification standard. Race and environment are also affecting the molecular subtype of breast cancer[11,12].

5.2. Relationship between molecular subtypes and pathological characteristics of young breast cancer

The tissue histological grading of breast cancer is basis on morphological and molecular classification according to the characteristics of gene. Many studies confirmed that there were some connections between them. It was shown that the organization grade was significantly higher of Luminal B (HER2+) subtype, ERBB2+ subtype and Basal-like subtype than that of Luminal A and Luminal B (HER2-) subtypes in young breast cancer. The former is mainly basis on histology Ⅲ, and the latter is mainly basis on histology Ⅱ.

The size of primary tumor size determines the clinical pathological stage, and the stage relates to prognosis closely. In the research of Ihemelandu[10], different molecular subtypes of breast cancer had significant difference in tumor size. At present, the generally point of view was that the tumor size of

luminal subtypes were smaller, and the tumor size of ERBB^{2+} and Basal-like subtypes were larger. These point of views consist with our study. We also found that, in young breast cancer patients, the maximum diameter of luminal subtypes are mainly equal to or less than 2cm, and that of ERBB^{2+} and Basal-like tumors are mainly more than 2cm but equal to or less than 5cm.

EGFR is a growth promoting factor of the tumor's vascular, it is over expression can inhibit cell's apoptosis, promote tumor genesis and metastasis, and it is a biological indicator of poor prognosis of breast cancer[13]. This study showed in young patients with breast cancer the positive rate of EGFR of ERBB2+ and Basal-like subtypes were significantly higher than that of Luminal A and Luminal B (HER2-) subtypes, and the positive rate of EGFR in Basal-like subtype was almost high. The study has shown that the over expression of EGFR was associated with poor prognosis in Basal-like subtype[14].

Topo Ⅱα can regulate cell proliferation and growth, and it has important role in the stability of gene and genetic[15]. The research found that the overexpression of Topo Ⅱα predicted shorter disease-free survival period[16]. In young breast cancer patients of our study, the expression of Topo Ⅱα in Luminal A subtype was significantly lower than that of other subtypes. The positive rate of Topo Ⅱα in Luminal subtypes were mainly less than or equal to 25%, while that of ERBB2+ subtype and Basal-like subtype were more than 25%.

Ki-67 is a nuclear protein and expresses in G1, G2, S and M stages of the cell cycle, it does not express in G0 stage. Ki-67 involved in the regulation of the cell cycle, the processing of ribosomal RNA and the synthesis of DNA etc. The higher expression of Ki-67, the poorer prognosis in breast cancer[17]. Nishimura etc[18] analyzed 3652 cases of breast cancer patients and showed that the Ki-67 index in Luminal A subtype was 17%, in Luminal B subtype was 29%, in ERBB2+ subtype was 40% and in Basal-like subtype was 50%. Our experimental results and the above research were almost consistent. In this study, the Ki-67 proliferation index increased in turn in Luminal A, Luminal B (HER2-), Luminal B (HER2+), ERBB2+ and Basal-like subtypes of young breast cancer. Moreover, the Ki-67 index of Luminal A was significantly lower than that of other subtypes, and Basal-like subtype was significantly higher than that of luminal subtypes.

Lymphatic metastasis is a adverse prognostic factor in breast cancer. The earlier and the more of transfer, the worse of prognosis[19,20]. This study showed that, lymph node metastasis was prone to happen in ERBB2+ subtype of young breast cancer, but there was no statistical significance deference in the lymph node metastasis in every molecular subtype (Z=6.549, P=0.162). This result was consistent with the result of Lian Zhenqiang[9], but it was different from the conclusion of Carey etc11. In different studies, the clinical characteristics of breast cancer relate to race, environment and other factors[12], and it leads to inconsistent results.

Analysing the above indicators comprehensively, we can find that there were significant differences in the degree of malignancy and prognosis with different molecular subtype of young breast cancer. Patients with Basal-like and ERBB^{2+} subtypes had the worst prognosis. And patients with Luminal A subtype had the best prognosis. The prognosis of patients with Luminal B (HER2-) was better than that of Luminal B (HER2+). This was consistent with the results of most scholars[4,10-12].

5.3. The difference between young and middle-aged breast cancer patients with different molecular subtypes

Young breast cancer patients have dense glands, their tumors are not easy to find early. The tumor's blood supply is abundant, and it is affected by hormone secretion and metabolism. Compared to middle-aged patients, the breast tumors in young patients grow faster, more aggressive and the prognosis is poorer. The results of this study showed that, the breast cancer in young patients had higher histological grading, Topo Ⅱα expression rate and Ki-67 proliferation index than that of middle-aged patients. These differences were statistically significant. And the difference mainly reflected in Luminal B subtype, see Table 3 and Figure 3.

The histology grade Ⅲ tumors in Luminal B subtype of young breast cancer was more common than that of middle-aged patients. The expression rate of Topo Ⅱα in young breast cancer was higher than that in middle-aged patients with Luminal B (HER2-) subtype. The Ki-67 index of young breast cancer was higher than that of middle-aged patients with Luminal B (HER2+) subtype (43.39% VS 35.98%). The histological grading is an important indicator to influence the prognosis of patients with breast cancer. The higher the grade, the worse the prognosis[21]. Topo Ⅱα is the key enzyme of DNA replication, the higher expression rate, the more obvious malignant biological behavior of the tumor[22]. Ki-67 is closely relate to cell proliferation, which can be used as an indicator to judge the prognosis of breast cancer patients, and it's high expression suggests poor prognosis[23]. The above showed that the young breast cancer patients had adverse biological behavior and clinical pathological characteristics than middle-aged breast cancer patients. So age can be used as a risk factor for the prognosis of breast cancer. This was consistent with the results of Anders[24].

6. Conclusion

To sum up, young breast cancer patients are a special group. They have more adverse elements than middle-aged patients. To refine an in-depth research for molecular classification of breast cancer, combined with the pathogenic and clinical pathological characteristics of young breast cancer has important significance for correct judgment of young breast cancer patients' prognosis and realization in precise medicine based on individual comprehensive therapy.

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