

Original Article

The Expression of Biomarkers in Invasive Ductal Carcinoma of Breast Through Tissue Array Method And Its Comparison with Clinicopathologic Features

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ABSTRACT

Background and Objective: In this study, we explored expression rate, some biomarkers affecting the prognosis of the breast carcinoma, and the relationship between these markers and clinicopathologic features of the disease as well as the relationship between each of these markers through a tissue array technique.

Materials and Methods: This study was an observational and cross-sectional study. From 100 breast samples which had been diagnosed as invasive ductal carcinoma, blocks were prepared through a tissue array method and were stained by monoclonal antibodies of the markers. All data were analyzed using SPSS program.

Results: The appearance rate of EGF-R marker had a direct relationship with the degree of malignancy ($p=0.026$), metallothionein marker with the mean number of mitosis ($p=0.044$), sialyl-Tn marker with the macroscopic size of tumor ($p=0.036$), the appearance of cyclin B1 marker with the appearance of metallothionein marker ($p=0.012$), and the appearance rate of EGF-R marker had a reverse relationship with Nm23 ($p=0.020$).

Conclusion: Through investigating the relationship between some biomarkers such as EGF-R and metallothionein and the clinicopathogenic features of tumor or the relationship between each marker and the other parameters, we can assess the state of invasion and metastasis process or the degree of its malignancy or determine its prognosis.

Key words: Breast, Carcinoma, Tissue array, Biologic markers

Introduction

Application of various prognostic factors of breast carcinoma has been taken into consideration for determining the prognosis and the response to the medicinal therapies as well as choosing the appropriate therapeutic method. So far several prognostic factors such as the size of the tumor, tumor grade, metastasis to the lymph node, and distant metastasis and some

biologic markers have been recognized (1). At present, TNM staging method, of which the status of involvement of lymph nodes is a part, is the most effective prognostic method which prognosticates the longevity of the patients affected with breast cancer (2,3). Determining the molecules which are related to oncogenesis, distinction, invasion, and metastasis of breast cancer, and using a combination of expression

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of these biomarkers and the clinicopathologic features can be a more reliable method for estimating the invasion capability of tumor, the risk level of metastasis, determining the prognosis, and choosing a better therapeutic strategy and can suggest better and newer therapeutic methods. So far, several factors such as oncogen Her-2/neu, ki-67 cell proliferation factor, and Bcl-2 apoptosis suppressor factor have been recognized as prognostic factors (2,4). Although the expression of Nm23 in tumoral cells has also been recognized as a tumor suppressor factor and metastasis suppressor, but it is not recognized as a definite factor (1). The investigations carried out on factors such as EGF-R, sialyl-tn, metallothionein, and cyclin B1 have resulted in contradictory results. On the other hand, through investigating the relationship between these markers and with other clinicopathogenic and prognostic factors on which there has been a consensus, their role and significance in estimating the capability of tumoral invasion, metastasis risk, and their application in choosing an appropriate therapeutic method and determining the prognosis can be discovered. A method that can be used for investigation of this biomarker is tissue array which is regarded as one of the IHC methods in which a high number of small pieces of the tissue are concurrently put on a slide and analyzed through IHC. One of its advantages is that it is economical (4). Therefore, in this study, the expression of EGF-R, metallothionein, Nm23, sialyl-Tn, and cyclin B1 markers in invasive ductal carcinoma of breast were investigated through a tissue array method and were compared with clinicopathologic features.

Materials and Methods

This study was an observational cross-sectional investigation. The population of this study includes 100 samples of invasive ductal carcinoma (1385) of breast from the women who referred to Cancer Institute of Imam Khomeini hospital complex. After the fixation of the samples, a paraffin block was prepared from each sample of the carcinoma of breast of women which was used later to prepare the tissue array blocks. Twelve cases of invasive ductal carcinoma of breast were eliminated from the investigation because of containing residual tumoral tissue, 5 cases for the relapse of carcinoma, one case because of autolytic changes and one case because of the incidence of carcinoma in a lactating breast. Then, the proportion of the involved lymph nodes to the

total number of lymph nodes taken from the patient, tumor grade, nuclear grade, histologic grade, mitotic grade, vascular invasion, and the microscopic size of the tumor were investigated, verified and specified. Other data such as age and sex were also extracted from the files of the patients. Then, the appropriate areas including invasive carcinoma with the desired fixation and having the highest grade were marked on the paraffin blocks to be analyzed through IHC, and using punch instruments, skin biopsy was extracted in the aforementioned areas of cylindrical tumoral tissue pieces with a thickness of 0.3 cm. Then, these tissue pieces were placed in new paraffin blocks through tissue array method, and 11 cylindrical tissue pieces were placed in each paraffin block. Four-micron tissue cuts were prepared from tissue array and after being H&E-stained, they were examined to confirm the existence of invasive tumoral tissue. Three-micron tissue section were also prepared from tissue array paraffin blocks and were stained by monoclonal antibody for EGF-R, metallothionein, Nm23, sialyl-Tn, cyclin B1 markers and through immunohistochemistry (IHC) method. In the IHC staining phase, the positive and negative controls of all biologic markers were used. After collecting the data, the frequency of various variants such as the size of tumor, tumor grade, nuclear grade, histologic grade, mitotic count, metastasis to the lymph node, vascular invasion, and the rate and intensity of the incidence of biologic markers were investigated and the age-based distribution of the patients was specified. Then, data were analyzed using SPSS software (Chi-square, student's t-test, analysis of variance with Yates correction, and ANOVA). In this study, a p-value less than 0.05 considered as significant.

Results

From a total number of 100 invasive ductal carcinomas (IDC) of breast that were analyzed, 67 cases were from mastectomy, 8 cases from quadranectiony, 5 cases from lumpectomy and 20 cases from excision of the tumoral mass. Mean age of patients was 48.3 years old. Within the 100 tumors, the largest and smallest size of tumor was 1 and 13 cm respectively, and the average size of tumor was 4 cm. From 100 subject cases of carcinoma, in 14 cases the sampling has not been done with the axillary lymph nodes, and in other 86 cases which could be analyzed, 24 (27.9%) cases did not have lymph node involvement, and in 62 (72.1%) cases the lymph

node involvement was observed. Among 86 cases of carcinoma in which sampling has been done with the axillary lymph nodes, 24 cases (27.9%) cases were without lymph node involvement, 22 cases (25.6%) had 1-3 lymph nodes involved and 40 cases (46.5%) had more than 3 lymph nodes involved. Based on tumor grade analyses, 36 cases (36%) were grade III, 42 cases (42%) as grade II, and 22 cases (22%) as grade I. From a total of 100 cases, 35 cases (35%) had 11 mitoses or less in 10 HPF, 39 cases (39%) between 12-22 mitoses in 10 HPF and 26 (26%) cases had 23 mitoses or more in 10 HPF. Based on nuclear grade analyses, out of 100 cases, 20 cases (20%) showed grade I or mild, 48 cases (48%) grade II or moderate, and 32 cases (32%) grade III or severe. According to the histological grade analyses, in 2 cases (2%) the rate of tubule formation was in more than 75% of the tumoral mass (grade I), in 21 cases (21%) was between 10-75% of the tumoral mass (grade II), and in 77 cases, it was observed in less than 10% of the tumoral mass (grade III). From 100 cases, 36 cases (36%) did not have vascular invasion and 64 cases (64%) had vascular invasion.

The expression of biomarkers is shown in Table 1. The investigation of the relationship of the tumor grade with the expression of EGF-R marker through the application of Chi-square test demonstrated a significant statistical relationship between these two variants ($p=0.026$), so that the expression of EGF-R in tumors with higher tumor grades, i.e. grades II and III was higher. In the investigation of the relationship

of the expression of EGF-R marker with the other various variants, no significant relationship was observed.

In the investigation of the relationship of tumor grade, macroscopic and microscopic size of tumor, metastasis to LN and the number of LNs with tumoral involvement, number of mitoses, age grouping, nuclear grade or histologic grade, there was no significant relationship with the expression of Nm23 marker within these variants. There was also a significant relationship between the expression of metallothionein marker and the average number of mitoses ($p=0.034$). The average number of mitoses in metallothionein-positive cases was 21.9/10 HPF which was significantly higher than the metallothionein-negative cases that was 16.3/10 HPF. The positivity of metallothionein was also significantly higher in those cases that mitosis was more than 11/10 HPF. In the investigation of the relationship of expression of metallothionein biomarker and the other various variants, no relationship was observed. In addition, no relationship was observed between the expression of sialyl-Tn marker and the average macroscopic and microscopic size of tumor using student's t-test, but after grouping the macroscopic size of tumor into two groups; i.e. 2 cm or less, larger than 2 cm and using Fisher exact test, there was a significant relationship between these two variants ($p=0.036$), so that in 100% of cases that were sialyl-Tn-positive, the size of tumor was larger than 2 cm. Such a relationship was not found out about the microscopic size of tumor.

Table 1. The expression of biomarkers in studied specimens (as prevalence and percentage)

Marker	Negative	Positive	Number of excluded specimens
EGF-R	Prevalence 54	43	3
	Percentage 55.7	44.3	
Nm23	Prevalence 27	67	6
	Percentage 28.7	71.3	
Metallothionein	Prevalence 67	27	6
	Percentage 71.3	28.7	
Sialyl-Tn	Prevalence 83	14	3
	Percentage 85.6	14.4	
Cyclin B1	Prevalence 49	47	4
	Percentage 51	49	

* Cases that could not be analyzed due to the loss of tumoral tissue during the staining procedure

In the investigation of the relationship of the expression of sialyl-Tn marker with the other various variants, no significant relationship was observed. In the investigation of the relationship of the incidence of cyclin-B1 biomarker with tumor grade, macroscopic and microscopic size of tumor, metastasis to LN and the number of LNs with tumoral involvement, average number of mitoses, vascular invasion, age grouping, nuclear grade or histological grade, no significant relationship was observed. There was also a significant relationship between the expression of cyclin B1 and metallothionein using Chi-square test ($p=0.012$), so that in 61.7% of cases, the incidence of these two biomarkers conformed to each other. There was also a significant converse relationship between the expression of EGF-R marker and Nm23 using Chi-square test ($p=0.020$), so that in 55.3% of cases, the expression of one marker was accompanied with the non-incidence of the other.

Discussion

In this study, the appearance of EGF-R was observed in 44.3% of invasive ductal carcinoma of breast and in the previous investigations, this rate was reported to be between 14% and 66% of IDC of breast (5,6). The investigation between the relationship of tumor grade with the rate and intensity of the incidence of EGF-R marker using Chi-square test revealed a significant statistical relationship between these two variants ($p=0.026$). This finding was in conformity to the results obtained in most of the previous studies (6-12). Just in one of the previous studies which were carried out, Gohring et al investigated 244 cases of invasive carcinoma of breast and did not observe a relationship between EGF-R incidence and tumor grade (13).

In a large number of previous studies, there has been a significant relationship between the increase of appearance of EGF-R and the existence of metastasis to LN (6,9,11,12,14); however, in our investigation and some other previous studies such as Wiseman et al (10) which was carried out on 242 cases through a tissue array method and the study of Gohring et al (13) which was carried out on 244 cases, and the study of Suzuki et al (14) which was carried out on 64 cases, no relationship was found out with metastasis to LN. The cause of such a discrepancy in the results might be due to the difference in the time interval between the beginning of carcinoma to the date of sampling which may intervene with the metastasis to LN, or

due to the variety in the method of investigation or the number of samples. In our study, like most of other recent studies, i.e. Wiseman et al (10) and Rampaul et al on 254 cases of invasive ductal carcinoma of breast, no significant relationship was observed between the increase in the appearance of EGF-R and the large size of tumor but in some older studies (5,6,8), a significant relationship was found out between these two parameters.

Nivy et al (7) carried out an investigation on 192 cases of IDC of breast and observed a significant relationship between the increase of EGF-R appearance and the existence of vascular invasion, but such a relationship was not found out in our investigation. Okamura et al (9) investigated 65 cases of invasive carcinoma of breast and observed a significant relationship between the increase of EGF-R appearance and an increase in the number of mitoses, but such a relationship was not observed between these two variants in our study. In the investigation of Soares et al (6) which was carried out on 86 cases of invasive ductal carcinoma of breast, the rate of EGF-R appearance in lower ages was significantly higher, but in our study no such relationship was observed for the age of the patients. The relationship that was found between an increase in EGF-R appearance and the higher tumor grade in our study and most of previous investigations and the relationship found between the rate of appearance of this marker and the existence of metastasis to LN in the previous studies suggests a significant role for EGF-R in the invasion process and the spread of tumor. In this study, a decrease in the appearance of Nm23 was also observed in 28.7% of cases of invasive ductal carcinoma of breast and in the previous studies (15,16) and a decrease of Nm23 was reported in 28-61% of cases of invasive carcinoma of breast.

In this study like some previous studies such as the investigation of Midullac et al (17) on 71 cases of IDC of breast and Gohring et al on 325 cases, no significant relationship was observed between a decrease in the appearance of Nm23 and the existence of metastasis to LN and the number of involved LNs. Whereas in other investigations including Kaya et al (18) on 27 cases and Terasaki-Fukuzawa et al (19) on 44 cases and Ding et al (20) on 102 cases, a significant relationship was found out with the existence of metastasis to LN. The results of this study, unlike the previous studies, cast doubt on the role of Nm23 as a metastasis suppressor factor.

Generally, in most of these studies, a small sample

size has been used. It seems necessary to carry out more investigations with higher amounts of samples to reach a definite result on the relationship of Nm23 with metastasis to LN. In this study, no significant statistical relationship was observed between the rate of appearance of Nm23 and the age of the patients, however in the investigations of Belev et al (21), the frequency of Nm23-negative tumors was significantly higher in those with ages higher than 50 years. Previous investigations revealed that an increase in the appearance of cyclin B1 marker had a significant relationship with the increase of mitotic index (22), however in this study no relationship was found out with the mitosis number. No significant statistical relationship was found out for other investigated clinicopathologic features. Considering the above-mentioned results, it seems that cyclin B1 has a limited value as a biologic marker and prognostic factor in invasive carcinoma of breast. In this study, the appearance of metallothionein was observed in 28.7% of cases of invasive ductal carcinoma of breast; in the previous investigations, its appearance was reported in 26-100% of cases (23). In the investigations carried out so far, a significant role has been suggested for metallothionein in the basic cellular procedures such as proliferation and apoptosis (23). In this study, a significant relationship was observed between the increase in the appearance rate of this marker and the average number of mitosis ($p=0.034$) as well as with the number of mitosis as grouping ($p=0.044$). In the investigation of Haerslev et al (24) on 478 cases, the same relationship was observed. In the previous studies, the appearance of sialyl-Tn was reported in 16-85% of cases of invasive carcinoma of breast, and in this study, the appearance of this marker was reported in 14.4% of cases. According to the results of the previous studies regarding carcinogenesis of the breast tumors, the appearance of sialyl-Tn occurs in primary phases and it seems that a biomarker is the transformation of malignancy (25). In this study, just like the results of the investigations of Leivonen et al (26), a significant relationship was observed between the increase in the appearance of the marker and larger size of tumor ($p=0.036$). Unlike the studies of Cho Sh et al (25) and like Lmai et al (26), in this study a significant relationship was not found out between the increase in the appearance of this marker and high

nuclear pleomorphism; and like other studies (26,27), no significant relationship was found out with the age of the patients. The existing difference between the results of different investigations can be due to the difference in clones of the antibodies which have been used, because there is the possibility of difference in their specificity. For example, in some investigations, antibody clone B72.3 (25) and in some other clone C1282 (26) and clone HB-stn (28) were used. In this study, clone HB-stn was used. On the whole, the diversity of results which have been observed about the relationship of the appearance of sialyl-Tn and the features related to the oncogenes, invasion and metastasis of invasive carcinoma of breast suggest the potential role of this marker in the biology of carcinoma of breast, therefore, a wider range of investigations is suggested to be done to determine the role of sialyl-Tn in this regard. In this study, a significant relationship was found out between the rate of appearance of cyclin B1 and metallothionein markers and the appearance of these two biologic markers conformed to each other. In the investigations of Jin et al (29-32), there was a significant relationship between the increase of appearance of metallothionein and the increase of proliferation of tumoral cells of breast carcinoma which had been determined by Ki-67 marker. Since cyclin B1 is a marker related to the cellular proliferation, the existence of the relationship between these two markers can confirm the previous findings. In this study, a statistical significant converse relationship was observed between the rate of appearance of EGF-R marker and Nm23 marker. Considering the relationship between the increase in the appearance of EGF-R and the higher tumor grade observed in this study and most of previous investigations as well as the relationship observed in most of previous studies between this marker and the existence of metastasis to LN and the significant role which has been suggested for the EGF-R in the invasion process and spread of tumor, its accompaniment with the decrease of appearance of Nm23 which has been identified as a metastasis suppressor factor can be justified. Although in this study no significant relationship was found out between the appearance of Nm23 and metastasis to LN, in the investigation of Soares et al (6) carried out on 86 cases of breast carcinoma, a significant relationship was observed between the increase in

the appearance of EGF-R and sialyl-tn marker, but in this study, such a relationship was not observed. In this study, like Ioachim et al (30,33,35) on 98 cases of invasive breast cancer, no significant statistical relationship was observed between the appearance of metallothionein and EGF-R.

Conclusion

The relationship between some but not all biologic markers including the direct relationship between EGF-R and the tumor grade as well as the existence of metastasis to LN or the relationship of metallothionein with the number of mitoses as a cellular proliferation marker or the relationship of sialyl-tn with tumor size can be used in specifying tumor grade for determining its prognosis and we suggest the application of these biologic markers in assigning the therapeutic strategy.

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