Serum Level of HER-2 Extracellular Domain in Iranian Patients with Breast Cancer: A Follow-up Study

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ABSTRACT

Background: A soluble form of HER-2/neu extracellular domain (sHER-2) is reported to be released in the sera of metastatic breast cancer patients. **Objective:** To measure the level of sHER-2 in sera of 115 breast cancer patients. **Methods:** Serial samples of 27 patients with metastasis, 18 non-metastatic patients, 15 patients in stage 0/I and 14 patients with accompanying benign breast disease were also included in this study. **Results:** No significant difference was observed between sHER-2 level in the pre-operative sera of breast cancer patients and that of healthy individuals. Only 8 out of 27 patients whom later developed metastasis showed elevated levels of sHER-2 in their first serum sample. However, a trend of increase in the level of sHER-2 was observed in 14 (51.8%) of 27 metastatic sera before clinical diagnosis of the metastasis. A significant association between sHER-2 positive status and vascular invasion of the tumor was observed (P = 0.02). In addition, significant correlation of sHER-2 level with CEA (highest r = 0.74) and CA 15.3 (highest r = 0.74) tumor marker levels in the serial sera were observed. The mean time from sHER-2 positivity to tumor metastasis was calculated to be 98 days (range = 29-174). **Conclusion:** Our results indicate that a relatively high percentage of Iranian patients with breast cancer show an elevated level of sHER-2 in their sera before clinical diagnosis of the tumor metastasis. Therefore, measuring the level of this oncoprotein, not only helps physicians in monitoring the patients during HERCEPTIN^{TM} therapy, but also can be helpful in choosing more aggressive treatments at the early satges of tumor metastasis.

Keywords: Breast Cancer, HER-2, Iranian, Serum

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INTRODUCTION

Molecular biomarkers have long been used for different purposes in clinical settings. Among the most useful biomarkers are those that can be used as prognostic or predictive markers. HER-2/neu molecule is a member of Epidermal Growth Factor Receptor (EGFR) family which has been used both as a prognostic and predictive biomarker in breast cancer (1, 2, 3, 4, 5). Increased expression of HER-2/neu in breast cancer cells results in transactivation of EGFR family receptors with an expanded repertoire of substrates and signaling responses and also stabilizing and recycling of EGFR-HER-2 heterodimers (6,7). Therefore the overexpression of HER-2/neu in breast tumors is considered a poor prognostic factor (8, 9, 10). In addition, evaluation of HER-2/neu expression in breast cancer patients can aid physicians in choosing the appropriate type of adjuvant therapy (11). A more promising use of HER-2/neu biomarker came into existence after approval of HERCEPTIN^{TM}, a humanized monoclonal antibody, for treatment of breast tumors with HER-2/neu overexpression.

In spite of being very useful in the selection of patients who benefit from HERCEPTIN^{TM} treatment, the traditional immunohistochemical detection of membranous HER-2/neu overexpression in breast cancer patients cannot help physicians in monitoring the patient's status after surgical removal tumor. However, several studies showed that the extracellular domain of this molecule is released into sera of 9-52% of breast cancer patients with regional and distant metastasis (12,13,14) and its level in the circulation is reported to correlate with the immunohistochemical detection of this protein in breast tumors (15). Serum level of circulatory soluble HER-2 (sHER-2) molecule is therefore suggested to be a prognostic marker in metastatic breast carcinoma and is a candidate marker for early detection and response to therapy in breast cancer (15, 16).

Overexpression of the HER-2 protein has been reported in 20-30% of malignant tissues of breast cancer patients (17). We have shown previously that HER-2 overexpression is common and associated with a shorter-disease free survival in Iranian patients with breast cancer (17). In the present study, we determined the level of extracellular domain of HER-2 in the sera of Iranian breast cancer patients and investigated its potential usefulness in predicting the clinical course of the disease after removal of tumor mass.

SUBJECTS AND METHODS

In total, 115 patients whom referred to the Referral Breast Cancer Clinic, Shiraz University of Medical Sciences, for surgery and 43 healthy women were included in this study. Sera were collected before and every 3 months after surgery for 24 consecutive months (between January 2001 and January 2003) and were kept at –20°C until use. Of the 115 patients whom the study started with, 41 patients were excluded from further follow up, due to lack of consecutive sera. The level of soluble HER-2 molecule in the first sera of all patients and in the serial sera (where available) of 27 metastatic , 18 non-metastatic breast cancer patients, 14 patients with accompanying fibrocystic breast disease and 15 patients in the stages 0/I was measured using a commercial sandwich ELISA assay (IBL, Germany). The ELISA assay was performed according to the manufacturer's instructions. The same method was used to

determine the level of sHER-2 in the sera of control subjects who were age/sex/ethnicity matched females referred to the Zeinabiye Gynecologic Clinic for routine check up and were clinically diagnosed to be healthy. The cut off level of sHER-2 positivity (6.8 ng/ml) was calculated as mean + 2 SD of sHER-2 level in 43 normal individuals. Due to the high variation in the level of sHER-2, Geometric mean level (GMT) of sHER-2 was calculated and used for further analysis. Demographic and clinical data of the patients were recorded in a questionnaire at the time of sampling and during follow-up.

Indirect immunoperoxidase method was used for detection of estrogen receptor (ER) and progesterone receptor (PR) on formalin fixed paraffin-embedded 5 μm sections. Estrogen and progesterone receptor assays were carried out by linked avidin-streptavidin method as described by the manufacturer (DAKO, Denmark). The carcinoembryonic antigen (CEA) and CA15.3 levels had previously been determined by commercial ELISA assays (IBL, Germany) according to the manufacturer's instructions (Talei et al. unpublished data). CEA levels greater than 5 ng/ml and CA15.3 levels greater than 30 IU/ml were considered as positive.

Statistical Analysis. Using SPSS software (version 10, SPSS Inc., Chicago, IL., USA) and EPI Info 2000 software (public domain application), independent samples t-test was used to compare the mean level of sHER-2 between groups. Chi-square, Fisher exact and Spearman's rho tests were used to analyze the correlations. Mann-Whitney test was used to compare the mean level of sHER-2 between metastatic and non-metastatic patients.

RESULTS

All patients were females aged between 26 to 74 years (mean ± S.D. = 47.9 ± 9.8 years). The mean age of control subjects was 42.5 ± 6.7 years. Only 11(9.6%) patients had a positive family history of cancer and 6(5.2%) patients had a positive history of previous cancer(s). Of 115 patients, 61(53%) were post-menopausal and 51 (44%) were pre-menopausal. For 3 (3%) patients the menopausal status was not clear. The histologic type of tumor was found to be 103 (90%) ductal, 5 (4%) lobular, 4 (3%) medullary and 3 (3%) unknown. Of the 115 patients, 1 (0.9%) was in stage 0, 14 (12.2%) were in stage I, 75 (65.2%) were in stage II and 25 (21.7%) were in stage III.

None of the patients with accompanying fibrocystic breast disease had a family history of cancer or history of other cancer(s). Of these 14 patients, 6 (43%) were post-menopausal and 7(50%) were pre-menopausal. One (7%) of the patients menopausal status was not known. All but one of them had ductal carcinoma type of the tumor. Among the patients whom were in stage 0/I disease, only one patient had lobular carcinoma and all others had ductal carcinoma of breast. In this group 7 patients were post-menopausal, 7 were pre-menopausal and one was of unknown status. Only one of them had a positive family history of cancer and none had a positive history of previous cancer(s).

Only 4 out of 43 normal females showed a level of sHER-2 in their sera. The mean level of sHER-2 in sera of 43 normal individuals and in the first sera of 115 breast cancer patients were found to be 4.82 ± 1.62 ng/ml and 14.23 ± 2.88 ng/ml, respec-

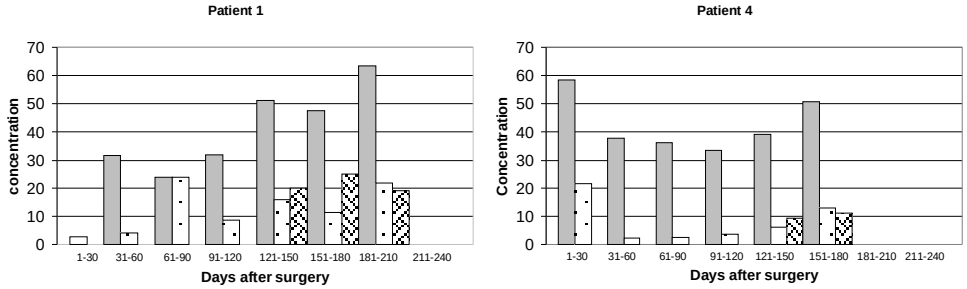
tively. There was no significant difference between the mean level of sHER-2 in sera of patients and controls (P = 0.46). Of the 14 patients with accompanying fibrocystic breast disease, only one patient had a low level of sHER-2 in her first serum (i.e. 13.79 ng/ml). This was the only patient in this group who showed an increasing trend of sHER-2 in her sera during the follow-up. Unfortunately the patient passed away after 4 samplings (in 9 months period). It is worth mentioning that the level of CEA and CA15-3 tumor markers in her serum showed an increasing pattern, too (table 1). None of the patients with stages 0/I disease had a detectable level of sHER-2 in their sera.

Table 1. Comparative level of tumor markers in 4 consecutive sera of a breast cancer patient with accompanying benign breast disease

	sHER-2 (ng/ml)	CA 15-3 (IU/ml)	CEA (ng/ml)
Sample 1	13.79	20	26.1
Sample 2	62.12	375	86.6
Sample 3	85.23	421.3	47.8
Sample 4	126	392.2	52.2

The clinical characteristics of the studied metastatic and non-metastatic patients are shown in table 2. No significant difference was observed between metastatic and non-metastatic patients in relation to menopausal status, tumor size, ER and PR status, perineural invasion and vascular invasion of the tumor, however, a trend of difference was observed according to the stage and nodal status (P = 0.06). Due to the small number of patients in groups, patients with stages I and II disease were compared to those with stages III and IV in metastatic and non-metastatic patients. Fisher exact test revealed a significant difference between the two groups (P = 0.001).

Of 18 non-metastatic patients, only one had a low level of soluble HER-2 in her first (4.35 ng/ml) and last (2.09 ng/ml) collected sera. In contrast, 8 (29.6%) of 27 metastatic patients had elevated levels of soluble HER-2 in the first serum samples (mean ± S.D. = 16.57 ± 3.02 ng/ml, P=0.04). A trend of continuous rise in soluble HER-2 level during the tumor progression and metastasis was observed. In addition, 14 (51.8%) of 27 metastatic patients showed sHER-2 in their sera (figure 1) before metastasis of the tumor. In this regard a significant association was observed between vascular invasion and sHER-2 positivity in the patients' first post-operative sera (P = 0.024). Among the 14 patients whom were sHER-2 positive, 5 were pre-menopausal and 8 were post-menopausal, while the number of pre-menopausal and post-menopausal sHER-2 negative metastatic patients was 7 and 5, respectively. Menopausal status for one of the sHER-2 positive patients and one of the sHER-2 negative patients was not known. Accordingly, there was no correlation between menopausal status and sHER-2 positive status in sera of metastatic patients (P = 0.60).



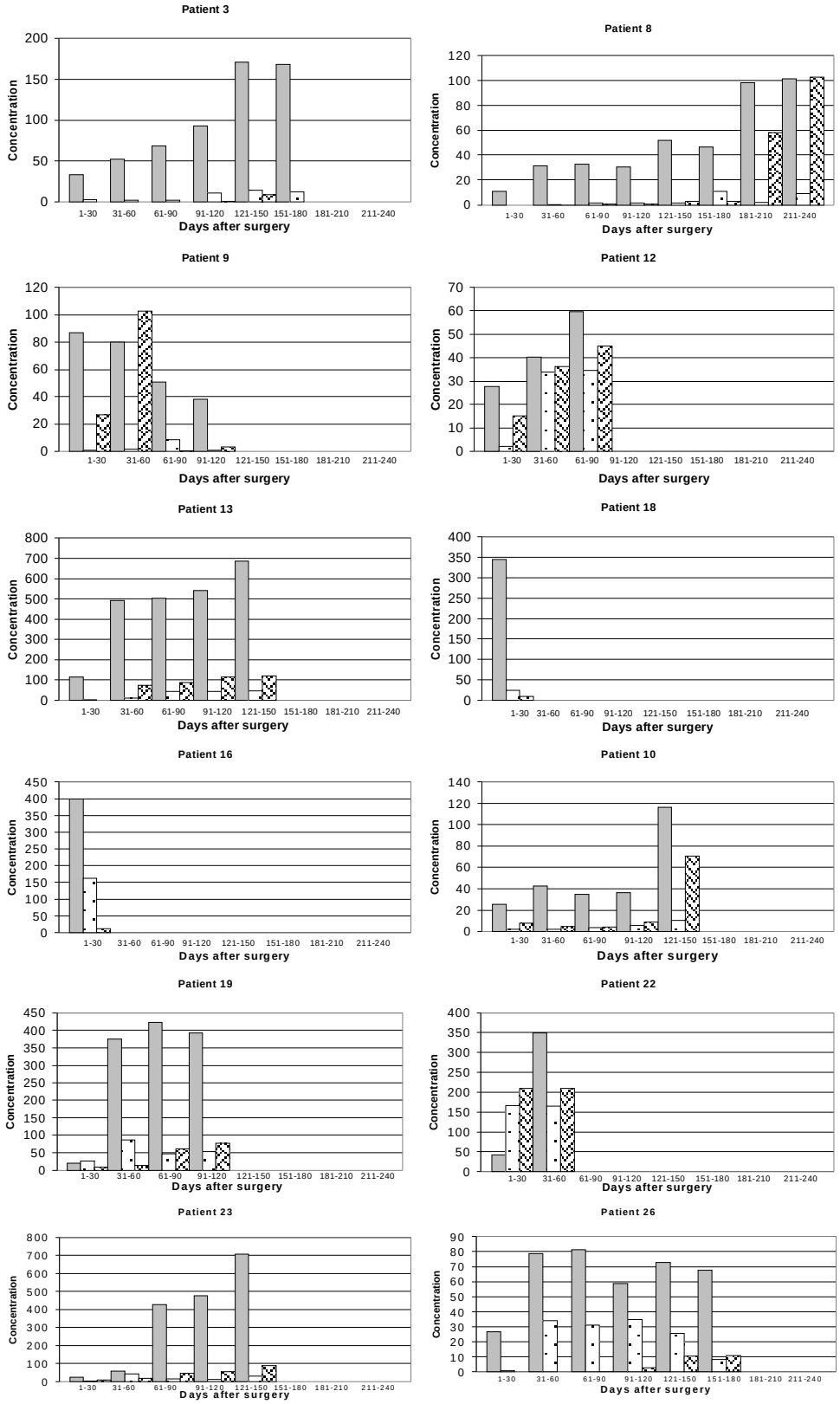


Figure 1. Levels of CA15.3 (IU/ml), CEA (ng/ml), and SHER-2 (ng/ml) in 14 SHER-2 positive metastatic patients in a 24 months period of follow-up. The samples were collected every three months. Legends: CEA ▩ CA15.3 ⁘ SHER-2 ▨

Table 2. The clinical characteristics of 27 metastatic and 18 non-metastatic breast cancer patients

	Metastatic	Non-metastatic
Menopause		
Pre	12	7
Post	13	11
Unknown	2	0
Family history of cancer		
Pos	2	0
Neg	25	18
History of previous cancers		
Pos	2	0
Neg	25	18
Histologic type		
Ductal	27	17
Lobular	0	0
Medulary	0	1
Unknown	0	0
Nodal involvement		
Pos	14	8
Neg	8	1
Unknown	5	9
Stage		
I	4	2
II	11	14
III	12	0
IV	0	0
Unknown	0	2
Tumor size		
T0	1	0
T1	4	3
T2	13	13
T3	6	1
T4	3	0
Unknown	0	1
Perineural invasion		
Pos	7	3
Neg	15	10
Unknown	5	5
Vascular invasion		
Pos	11	6
Neg	12	8
Unknown	4	4
Histological grade		
I	3	5
II	14	6
III	3	2
Unknown	7	5
Estrogen receptor		
Pos	13	8
Neg	11	9
Unknown	3	1
Progesterone receptor		
Pos	15	10
Neg	9	7
Unknown	3	1
ER/PR double status		
Pos/Pos	11	7
Neg/Neg	7	6
Pos/Neg	2	1
Neg/Pos	4	3
Unknown	3	1
Total	27	18

The mean time from soluble HER-2 positivity to diagnosis of metastasis was found to be 98 days (range: 29-174 days, figure 2). Among metastatic breast cancer patients, 7 had bone metastasis, 8 had lung metastasis, 10 had liver metastasis and 2

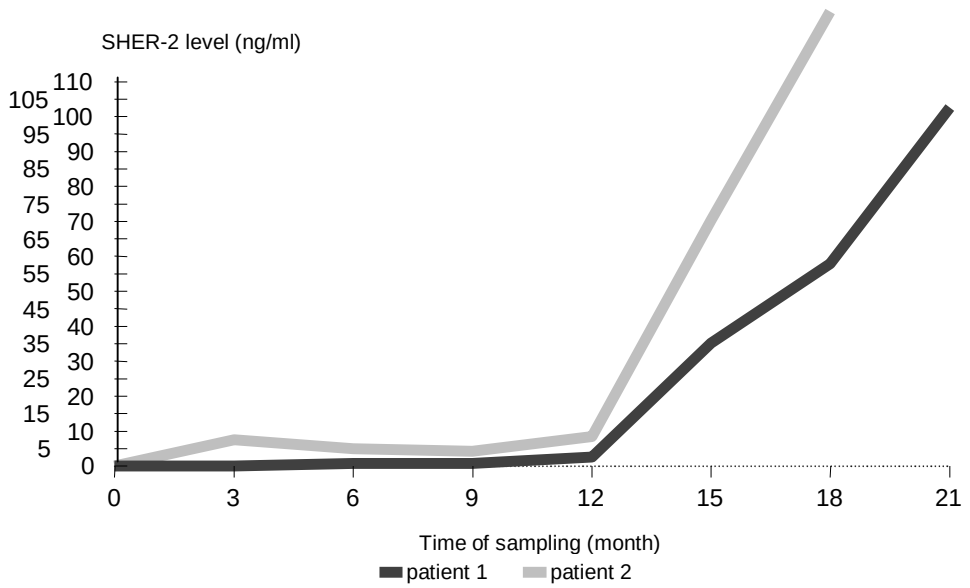


Figure 2. The increasing trend of SHER-2 in sera of 2 metastatic patients before detection of lung metastasis. The arrow shows the time that metastasis was clinically detected.

had brain metastasis. The rate of soluble HER-2 positivity was higher in patients with liver metastasis, however, the difference was not statistically significant (P=0.53, table 3). Three metastatic patients were sHER-2/ER/PR positive and three were sHER-2/PR positive, however, only one non-metastatic patient was sHER-2/ER/PR positive. None of the patients who developed distant metastasis in the course of follow up were in stage IV at the time of diagnosis; however, 7 sHER-2 positive and 5 sHER-2 negative patients were in stage III. The numbers of sHER-2 positive and sHER-2 negative metastatic patients in stage II were 7 and 4, respectively. The only 4 metastatic patients in stage I were all sHER-2 negative. No difference was observed between metastatic patients in early breast cancer (stages I/II) and locally advanced breast cancer (stage III) according to the sHER-2 status (P = 0.83).

Table3. The Serum HER-2 positivity in different metastatic patterns

	Soluble HER-2 positive*	Soluble HER-2 negative	Total
Bone metastasis	3	4	7
Lung metastasis	3	5	8
Liver metastasis	7	3	10
Brain metastasis	1	1	2
Total	14	13	27

There was a positive correlation between levels of sHER-2 and CA15.3 in the second, third, fourth and fifth serum samples (n = 25, 23, 21, and 18, respectively), however, the correlation between sHER-2 and CEA was only observed in the second, third, and fifth sera. The highest level of correlation between sHER-2 and CA15.3 was obtained in the fourth serum samples (r = 0.74, P = 0.0001, n = 21), while the highest level of correlation between sHER-2 and CEA was observed in the fifth serum samples (r = 0.72, P = 0.001, n = 18). The highest level of correlation between CEA and CA15.3 level was observed in the fourth sera (r = 0.69, P = 0.0001). No

correlation was observed between any of the tested tumor markers in the sixth (n = 15), seventh (n = 10) and eighth (n = 5) serum samples.
There was also no correlation between sHER-2 level in any of the serial samples and age, age at diagnosis, marital status, children number, number of gestations, menopausal status, stage, tumor size, nodal status, ER and PR expression, histological grade and nuclear grade.

DISCUSSION

In this study, elevated pre-operative levels of sHER-2 (>6.8 ng/ml) were observed in 1 out of 43 Iranian healthy women, 0 out of 14 breast cancer patients with accompanying fibrocystic disease of breast, 0 out of 15 breast cancer patients with stages 0/I disease, and 8 out of 27 breast cancer patients who further developed metastasis. These results are in accordance with the reported data of other investigations (18, 19, 20, 21). We observed no significant difference in the mean sHER-2 level in the pre-operative sera of 115 Iranian breast cancer patients and the mean sHER-2 level in sera of Iranian healthy women. However, a trend of increase in the level of sHER-2 in sera of 51.8% of the patients who later developed distant metastases was observed. This increase correlated well with the level of CA15.3, however, the correlation with the CEA level was only seen in some of the series. Similarly, in a previous study by Krainer et al. a highly significant correlation between sHER-2 level and CA15.3 tumor marker was observed in 62 patients with advanced disease (20). However, the reported correlations between HER-2 level and CEA are mostly related to early stages of the disease (22, 23). Accordingly, in our data the level of correlation between sHER-2 and CA15.3 was higher than that of sHER-2 and CEA, as a whole. This observation might, at least in part, be a result of higher specificity of CA15.3 for metastatic breast tumors compared to CEA (24).
Comparing the level of sHER-2 in the pre-operative sera of metastatic (n = 27) and non-metastatic (n = 18) patients revealed a significant difference which was in accordance with the results obtained by Classen et al. (25). In addition a significant association between vascular invasion and sHER-2 positivity in the first post-operative sera shows a higher aggressiveness of tumors in patients with elevated sHER-2 levels.
The average lead time to detection of distant metastasis of the tumor was found to be 98 days which is less than the reported values from other studies (26). In addition, our patients had higher pre-operative sHER-2 levels (7.52-209.80 ng/ml) compared to other studies (27). One of the possible reasons of this lower prediction power might be the high aggressive nature of tumors in our patients or the late referral of the patients to breast clinics. However, the latter is unlikely because only 21.7% of 115 breast cancer patients in our study were in stage III compared to the 34.3 % of stage III patients in the study of Imoto et al. (27).
Results of this study indicate that a high percentage (51.8%) of metastatic breast cancer patients develop elevated levels of sHER-2 in their sera and this elevation correlates well with other markers of tumor aggressiveness. More than being a marker of tumor progression and metastasis, sHER-2 may also have impacts on the tumor escape from the naturally developed anti-HER-2 antibodies and may interfere with the HERCEPTIN^{TM} therapy of the tumors (19). In this regard, retaining the signaling

ability by the truncated intracellular tyrosine kinase of HER-2/neu molecule is noteworthy (28, 29). It is also suggested that the shedding process is a result of activation state of the tumor as well as tumor burden and the expression status of the primary tumor (30). There are also evidences that HER-2/neu overexpression can even occur *de novo* in metastatic tissues of breast carcinomas which are further subjected to the selection process (31, 32). It is therefore promising that measurement of sHER-2 in sera of breast cancer patients not only assists physicians in monitoring and follow-up but also provides information on a subset of patients whose tumor cells might change their pattern of HER-2 expression and need reconsideration of the treatment.

In conclusion, the increased serum levels of sHER-2 in a high percentage of metastatic patients supports the possibility of using the serum ED/HER-2 measurement to monitor breast cancer patients undergoing surgical treatment, hormonal therapy and chemotherapy as well as those requiring HERCEPTIN™ therapy (33). Since HERCEPTIN™ is a new medicine in the Iranian market, knowing the status of HER-2/neu overexpression and ED/HER-2 level in patients' circulation may be useful in future use of this therapy in our settings.

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