Clinical, Pathological and Molecular Characteristics of Newly Diagnosed Breast Cancers

J. PRAUSOVÁ[1*], T. ECKSCHLAGER[2], M.MRHALOVÁ[3], A. SLOBODNÍK[4], K. KUBÁČKOVÁ[1], S. VINAKURAU[1], R. KODET[3]

[1]Radiotherapy- Oncology Department, University Hospital Motol, Charles University, V Uvalu 84,CZ 150 00, Prague, Czech Republic, e-mail:jana.prausova@fnmotol.cz; [2]Paediatric Clinic of Oncology and Hematology,University Hospital Motol, Charles University, Prague, V Uvalu 84, 150 00, Prague, Czech Republic; [3]Department of Pathology and Molecular Medicine, University Hospital Motol, Charles University, V Uvalu 84, 150 00 Prague, Czech Republic; [4]Institute of Biostatistics and Analysis, Masaryk's University, Kamenice 3, 625 00 Brno, Czech Republic

Received August 17, 2006

Selection of breast carcinoma therapy is based on standard prognostic markers, such as tumor size, infiltration of regional lymph nodes, tumor grade, and expression of hormonal receptors. Insufficient treatment results stimulate a search for new markers which may lead to a more precise characterization of these tumors and to a more effective treatment. In our study we determined essential clinical and histopathological characteristics of non-metastasizing breast cancer - primary tumor size, involvement of the regional lymph nodes, expression of hormonal receptors and a status of ERBB-2 protein (HER-2), DNA ploidy, and their possible inter-correlation. In this study 77 patients were analyzed. The mean age was 59.3 years. Tumor stage T1 was found in 53%, T2 in 39%, T3 in 5% of patients. 57% of patients did not show any metastases in the axillary lymph nodes. A higher tumor grade 3 was seen mainly in larger tumors, in 62% of T2 and 66% of T3 tumors; 77% of carcinomas expressed hormonal receptors. HER-2 expression was shown in 21 T1 tumors, 13 T2 tumors, and 1 T3 tumor. 47 tumors were diploid. 13 T1 tumors, 14 T2 tumors, and 2 T3 tumors were aneuploid. Any significant correlation among staging T, N and ERBB-2 expression, hormonal receptors expression, tumor grade and DNA ploidy was found.

Key words: Breast Cancer, Hormonal receptor, ERB-2 expression, Ploidity

Carcinoma of the mammary gland is the most frequent malignant tumor in women in developed countries. There are marked geographic variations in incidence of this carcinoma. It occurs most frequently in Caucasian women living in developed countries on northern hemisphere. Life style in these areas is often accompanied with early menarche and postmenopausal obesity. These factors are responsible for a prolonged estrogen exposition on to mammary gland. Estrogen exposition is associated with increased risk of breast cancer development. Late first pregnancy, low birth rate and short breast feeding period which are characteristics of a modern life style also contribute to an increased frequency of breast cancer. However, recent statistical data show a slight decrease of mortality. This phenomenon has several causes – implementation of new treatment procedures, early diagnosis attributed to a more widely used self-examination, screening mammography, and also public information about the disease.

Appropriate initial treatment has a crucial importance for prognosis. The treatment modality is based on prognostic factors. Stage of lymph node infiltration, expression of estrogen receptors (ER), size and grade of tumor differentiation represent standard factors for therapy selection. Clinical practice, as well as unsatisfactory treatment results, lead to a search for new parameters which may make treatment planning more precise and may enable individualization of oncology treatment.

In our study, we focused on possible correlation among standard factors, ERBB-2 protein expression, and cellular ploidy. The amount of tumor DNA is considered to be one of possible prognostic indicators. According to the data published so far, aneuploid DNA in breast cancer portends a worse prognosis; such patients have more frequently affected lymph nodes, larger tumors (T2 and T3), higher grade, and absence

*Corresponding author

of estrogen receptors (ER) and progesteron receptors (PR) [1]. Only a minority of studies did not find any relation to prognosis or to other prognostic factors [2, 3].

ERBB-2 (HER2) protein is one of the *erb* receptor family members. It consists of an extracellular binding domain, transmembrane segment, and intracellular domain showing a tyrosin kinase (TK) activity. TK is one of key enzymes in signalling pathways facilitating stimuli necessary for cell growth, differentiation, cell division and adhesiveness. A gene coding for ERBB-2 protein is located on chromosome 17 (17p21) and is called ERBB2 (HUGO Gene Nomenclature Committee) (synonyms: HER/2 or HER/2/neu or c-erb-2).

The ERBB2 gene was first identified in 1981 as transforming *neu* gene in rats with chemically induced neuroblastoma. Its human homolog was isolated in 1985.

After binding a ligand, receptor homodimerization or heterodimerization with other HER family members occurs and the tyrosin kinase intracellular signal pathway is activated.

First method introduced for a detection of ERBB-2 protein expression was immunohistochemistry on formalin fixed / paraffin embedded tissue sections using antibodies against ERBB-2 protein. Although various primary antibodies and detection systems have been designed, Dako HercepTest kit is a currently used standardized system for diagnostics which has been approved for use by FDA (Federal Drug Administration).

Based on expression intensity of ERBB-2 protein, tumors are divided into four groups. Tumors of score 0 show no ERBB-2 protein positivity or positivity in up to 10% tumor cells. 1+ tumors show presence of the protein in more than 10% of tumor cells but the positivity is not complete on the tumor cell membranes. 2+ tumors show a mild to medium complete positivity and 3+ tumors show a strong positivity of ERBB-2 protein. 0 and 1+ tumors are evaluated as negative, 2+ and 3+ tumors are considered positive. In case of an ambiguous or borderline positivity of HER2 protein expression, FISH (fluorescence *in situ* hybridization) is used for evaluation of ERBB2 gene copy numbers. Directly labelled mixture of locus specific probe for ERBB2 gene and centromeric probe for enumeration of chromosome 17 is commonly used. Increased copy number (amplification) of ERBB2 gene mostly correlates with elevated ERBB-2 protein expression. 0 and 1+ tumors are not indicated for the trastuzumab (Herceptin®, Genentech) treatment (targeted ERBB-2 protein antibody). 2+ tumors should also include FISH examination and 3+ tumors are indicated for Herceptin treatment.

Based on our previous clinical experience, patients can be divided into several groups according to ERBB-2 protein expression.

The first group includes patients with 3+ ERBB-2 protein expression which correlates with ERBB2 gene strong amplification. The second group includes patients with tumors expressing ERBB-2 protein 2+ but with no or weak ERBB2 gene amplification, i.e. patients which should not be indicated for Herceptin treatment. However, in some patients showing a strong membrane 3+ ERBB-2 protein positivity, no gene amplification occurs and vice versa, in some cases, strong amplification of the gene is accompanied with a weak protein expression [4]. These are grey zone patient groups which reflect complexity of intracellular processes, and represent a most problematic category from a therapeutic standpoint. In accordance with these findings, only about 20-40% of patients eligible for Herceptin treatment gain certain profit from the treatment. These data are based on treatment results of patients with advanced metastatic carcinoma.

Material and methods

We evaluated patients who were examined from 1999 to 2004 at the Radiotherapy-Oncology Department of Motol Hospital and whose molecular-pathology diagnosis was performed at the Department of Pathology and Molecular Medicine. All patients were subject to radical modified mastectomy with exenteration of the axillary lymph nodes. Clinical stage was determined according to the TMN WHO classification: T0 is any ductal carcinoma in situ, T1 tumor size up to 2 cm, T2 tumor size from 2 to 5 cm, T3 tumor larger than 5 cm, T4 tumor fixed to the chest wall or to skin, inflammatory carcinoma. A total of 77 patients were included into this study. The mean age of patients was 59.3 years, the youngest patient was 33 and the oldest was 88 years old.

Cytometric DNA analysis. The tumor cells were retrieved from paraffin embedded tissues. 2-4 sections (depending on the size of embedded tissue) of 50 μm were prepared from each paraffin block. With each case a 3 μm thin section was placed on histological slide and stained with hematoxylin-eosin for verification of the presence of tumor cells. Thick sections in a vial were used for the DNA analysis. Deparaffinization and preparation of naked nuclei suspension was performed by a modified method according to Robinson [5]. Sections were deparaffinized in xylene and re-hydrated by descending line of ethanol. The sections were then stored overnight at 4°C in distilled water, cut into small pieces and incubated while stirring with 0.25% solution of pepsin in diluted hydrochloric acid until disintegration of tissue particles (0.5-2 h). 25 μl of liquid suspension was placed into cytometric tube, 25 μl of permeabilization agent (solution of gentle detergent permeabilizing cell and nuclear membrane) and 500 μl of a dye agent (solution of propidium iodide and RNAse) were added from DNA Prep Reagent Kit (Becman Coulter) [5]. Samples were incubated for at least 30 min and then measured on flow cytometer FACSCalibur (BD). Evaluation of the DNA index and the percentage of individual phases of the cell cycle was performed using ModFit program.

Immunohistochemical determination of protein expression. Histological sections made from formalin-fixed, paraffin-embedded tissue were stained with hematoxylin-eosin. Once diagnosis and carcinoma grading were determined, a suitable tissue for immunohistochemical examination was selected.

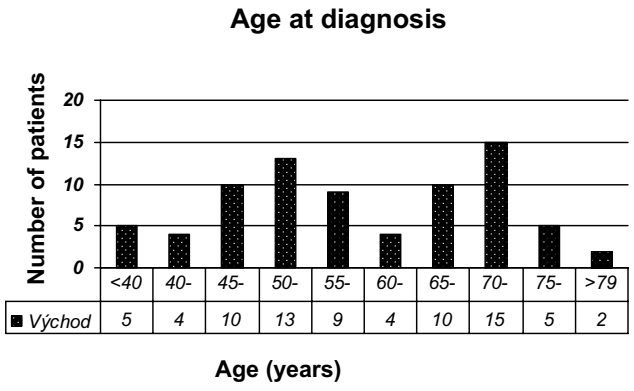

Figure 1

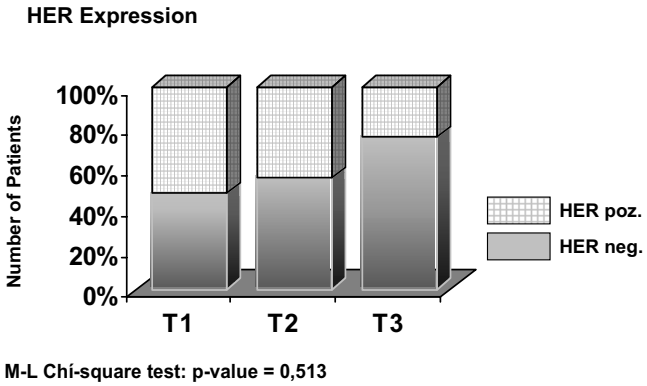

Figure 2

ERBB-2 protein expression was determined using a standardized HercepTest system (Dako) according to the user manual. Expression of estrogen receptors alpha and progesterone receptors was determined using mouse monoclonal antibodies (clones 1D5 and PgR636, demasking of antigenic epitopes by boiling in 10 mM citrate buffer pH 6.0; Dako).

We estimated a relation by correlation among individual tumor characteristics, such as primary tumor size, infiltration of the regional lymph nodes, expression of hormonal receptors, DNA ploidy, and ERBB-2 protein expression.

Results

This study included mainly patients with initial carcinoma stages, T1 tumor stage was diagnosed in 41 patients, T2 in 30 patients, T3 in 4 patients. In 2 patients, primary tumor extent was not registered. 27 patients were diagnosed with involvement of the regional axillary lymph nodes, 44 patients showed no signs of lymph node metastases. Regional lymph nodes were tumor free in 6 patients. Poorly differentiated tumors (grade 3) slightly prevailed: 37.7% patients, grade 2 tumors were found in 32.4% patients, and grade 1 tumors in 10.4% patients. With respect to nodes involvement there was no correlation among histopathology grade and primary tumor size and nodal affection was carried out. The results are summarized in Table 1 and Fig. 1. Estrogen receptor alpha positivity as determined by immunohistochemistry was found in 75 patients, while progesterone receptor positivity was detected in 58 patients. Immunohistochemical examination of ERBB-2 protein expression was carried out in 73 patients. Strong ERBB-2 protein expression (3+) was observed in 13 patients, moderate (2+) in 22 patients, and weak (evaluated as negative) (1+) or negative (0+) in 38 patients.

Relation of ERBB-2 protein expression, tumor grade, hormonal receptors, and DNA ploidy to the T staging is depicted in Tables 2, 3 and Fig. 2, 3. The only statistically significant correlation was found between the tumor grade and tumor size. Tumors having up to 2 cm in diameter (T1) showed significantly more frequently grade 1 or 2 ($p < 0,05$, test χ^2 as contingency 2x2). The correlation between expression of ERBB-2 protein and T staging was not statistically signifi-

Table 1

Number of Patients		**77**
Age		
averager (SD)		**59,3**
median		57,0
min-max		33-88
T		
T1	**41**	(53,2%)
T2	**30**	(39,0%)
T3	**4**	(5,2%)
	2	(2,6%)
N		
N0	**44**	(57,1%)
N1	**26**	(33,8%)
N2	**1**	(1,3%)
unknown	**6**	(7,8%)
G		
G1	**8**	(10,4%)
G2	**25**	(32,4%)
G3	**29**	(37,7%)
unknown	**15**	(19,5%)

Table 2

	T1	**T2**	**T3**
ER-/PR-	4	8	1
ER-/PR+	4	2	0
ER+/PR-	10	6	2
ER+/PR+	23	12	1
	41	28	4

M-L Chí-square test: p-value = 0,384

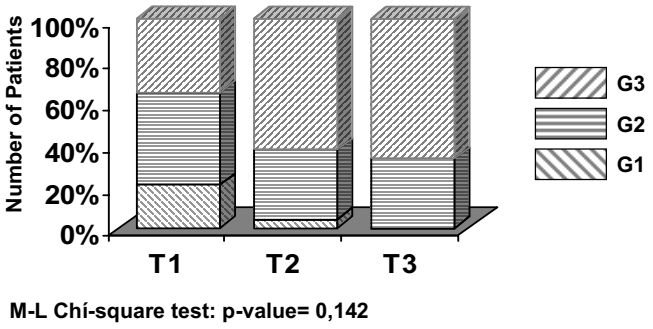

Figure 3

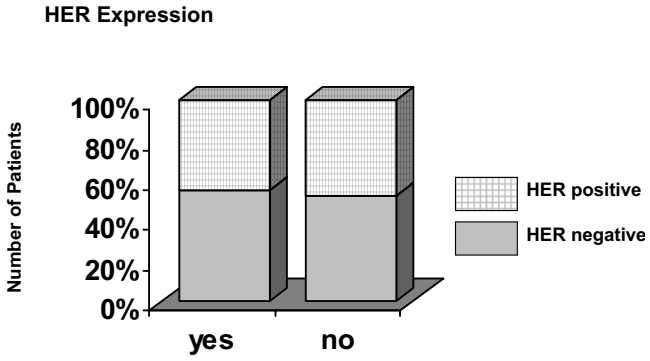

Figure 4

Table 3

	T1	T2	T3
Yes	13	14	2
No	28	16	2
	41	30	4

M-L Chí-square test: p-value = 0,394

Table 4

Aneuploidiya	Yes	No
G1	3	5
G2	7	18
G3	14	15
	24	38

M-L Chí-square test: p-value = 0,307

Table 5

	G1	G2	G3
HER negativity	2 (25,0%)	14 (60,9%)	13 (44,8%)
HER positivity	6 (75,0%)	9 (39,1%)	16 (55,2%)
	8 (100%)	23 (100%)	29 (100%)

M-L Chí-square test : p-value = 0,180

cant. No statistically significant correlation has been proved between expression of hormonal receptors and tumor size. But tumor T1 and T2 mainly expressed one or both hormonal receptors. Despite no statistical correlation between tumor size and DNA ploidy, tumors T1 and T2 were obviously more often DNA diploid. The correlation between ERBB-2 protein expression and the number of involved nodes was not statistically significant, neither was the correlation between hormonal receptor, DNA ploidy and the number of involved nodes. 66.7% patients showing progesterone receptors expression were ERBB-2 protein negative, 50% patients showing estrogen receptor expression were ERBB-2 protein negative and 57,2% showing both estrogen and progesterone expression were ERBB-2 protein negative. However, the results were not statistically significant. DNA aneuploid tumors were ERBB-2 protein negative in 55.6% patients, DNA diploid tumors were EBBB-2 protein positive in 48% (Fig. 4) and DNA aneuploidy prevailed in poorly differentiated tumors grade 2 and 3. (Tab. 4).

The correlation between tumor grade and ERBB-2 is depicted in Tab. 5. Half of the tumors expressing estrogen receptors alpha and progesterone receptors were DNA diploid. The correlation between DNA ploidy and hormonal receptors was not statistically significant. To evaluate the relation between DNA aneuploidy and the age, the patients were categorized into three groups (30-49, 50-74, and >74). In the group of patients aged 50-74 years, there was a significantly higher number of patients with DNA aneuploidy when compared to the other two groups. Among patients both younger and older than the mentioned group diploid patients prevailed. There was however no correlation between age category and DNA aneuploidy.

Discussion

In this study a correlation among primary tumor size, involvement of axillary nodes, histological grade, expression of hormonal receptors, expression of ERBB-2 protein and DNA aneuploidy was analyzed. The only significant correlation was found between tumor size and histological grade. Tumors smaller than 2 cm were well- or medium-differentiated, i.e. those showing a better prognosis. According to Lundin et al. only 2% of tumors stage T1 develop distant metastases [6]. ERBB-2 protein positivity was shown in lower stages of the disease T1 in 52.5% and T2 in 44.8%. According to the published data, ERBB2 gene amplification or ERBB-2 protein over-expression is identified in 10-40% of breast cancers [7] and it is associated with a worse prognosis [8] and with resistance to tamoxifen hormonal treatment [9]. In accordance with these data, the largest group of patients with poorly differentiated tumors in our study

was ERBB-2 protein positive (55.2%). Poorly differentiated tumors grade 3 prevailed in tumors larger than 2 cm (T2, T3). 82% of all tumors expressed estrogen or progesterone receptors. Tumors larger than 2 cm, T2 28.6%, were in most cases hormonal receptor-negative. Tumors stage T1 expressed either estrogen or progesterone receptors. The majority of patients have no nodal involvement. Further investigation will be performed for correlation between lymphangioinvasion, expression of ERBB-2, expression of hormonal receptors and DNA ploidy.

Some studies have shown a prognostic significance of estrogen and progesterone receptors. In 5-year interval from breast cancer diagnosis, women with a tumor expressing hormonal receptors have 5 to 10% lower risk of recurrence [10]. This advantage, however, decreases with the observation time. A review including 1500 patients showed an additional importance of PR determination in ER-positive patients. Expression of ER and PR is the best prognostic factor to evaluate response to tamoxifen treatment.

Most ERBB-2 protein-negative tumors were found in patients ER-/PR+ and ER+/PR+. Flow cytometry studies in the recent decades have revealed the importance of DNA analysis of ploidy, cell cycle and brought valuable information for diagnostics, treatment, and prognosis of cancer patients [11, 12]. Sometimes it is difficult to distinguish between nearly diploid tumor cell population and a non-neoplastic stroma, such as fibroblasts and leukocytes using flow cytometry [13, 14]. In our study, we have not shown any statistically significant correlation between more advanced stage of the disease and DNA aneuploidy. Tumors smaller than 2 cm were mostly diploid (68%). Higher stage tumors showed no differences in DNA aneuploidy (T2 46%, T3 50%). Tumors expressing hormonal receptors were mainly DNA diploid. This finding suggests that aneuploidy may be a late event in the tumor progression which is in accordance with the data published by Ferrero et al. [13]. There is evidence showing correlation between DNA aneuploidy and histological type of tumor or differentiation grade of the tumor [15, 16]. This evidence corresponds with our finding that DNA aneuploidy occurs mostly in poorly differentiated tumors. ERBB2 gene amplification and ERBB-2 protein over-expression followed by loss of hormonal receptors expression has been described in DNA aneuploid tumors which correlates with a more aggressive biological tumor behavior [6, 16, 17]. DNA aneuploidy has been associated with a loss of functional wild type p53 [18, 19], which is considered to be one of the early genetic events in tumor cells leading to accumulation of further mutations.

A higher proportion of DNA aneuploid tumors in the medium age category found in this study may be explained by less frequent chromosomal aberrations in younger patients. Most correlations in our study were not statistically significant. This may be due to a relatively small number of patients. Ratio of DNA diploid and aneuploid tumors in particular groups (T, grade, hormonal receptors) was very similar to that found in larger patient groups showing statistically significant differences [1]. Another cause may lay in statistical methods used in this study. In 2004 Banerjee et al. published an article defining prognostic groups in breast cancer using non-parametric statistical method, called “recursive partitioning (RP)” [20]. Contrary to conventional regression Cox-analysis where the parameters must be defined in advance, RP automatically finds important correlations.

Conclusion

Although no correlation between histopathological findings and clinical characteristics was found, a tendency of correlation between DNA ploidy and tumor stage, expression of hormonal receptors and expression of ERBB-2 protein showed similarity to other published studies with statistical significance among more factors. An improvement of treatment procedures can be achieved by further studies of intracellular mechanisms responsible for malignant transformation and growth. Search for their correlation with clinical findings may lead not only to more precise diagnostics but also to more accurate tumor type determination and, therefore, tailoring and targeting anticancer therapy.

References

[1] MOUREAU-ZABOTTO L, BOUCHET C, CESARI D, et al. Combined flow cytometric determination of S-phase fraction and DNA ploidy is an independent prognostic factor in node-negative invasive breast carcinoma: analysisof series of 271 patients with stage I and II breast cancer. Breast Cancer Res Treat, 2005; 91: 61–71

[2] O´REILLY SM, CAMPLEJOHN RS, BARNES DM et al: Node-negative breast cancer. Prognostic subgroups defined by tumor size and flow cytometry. J Clin Oncol, 1990; 8:2040–2046

[3] KHAN E, MAPARA Z, KHAN S, et al. DNA ploidy analyses in 218 consecutive Pakistani breast cancer patients: Does it add anything. Pathol Oncol Research, 2001; 7: 125–128

[4] MRHALOVÁ, M., KODET, R., KALINOVÁ, et al. Relative quantification of ERRB2 mRNA in invasive duct carcinoma of the breast: Correlation with ERRB-2 protein expression and ERRB2 gene copy number. Pathol. Res. Pract. 199: 453–461, 2003

[5] ECKSCHLAGER T, PILÁT D, KODET R. et al.: DNA ploidy in neuroblastoma. Neoplasma, 1996; 41:23–26

[6] LUNDIN J, LUNDIN M, HOLLI K, et al. Omission of Histologic Grading From Clinical Decision Making May Result in Overuse of Adjuvant Therapies in Breast Cancer: Results From a Nationwide Study. JCO; 2001:29–36

[7] SLAMON DJ, CLARK GM, SONG SG, et al. Human breast cancor: correlation of relapse and surfoval with amplification of the HER-2/neu onocogene. Science 1987, 235:177–182

[8] ROSS JS, FLETCHER JA, LINETE GP et al. The HER-2/neu gene and protein in breast cancer 2003: biomarker and target of therapy. Oncologist 2003, 8:307–325

[9] SHOU J, MASSAREH S, OSBORNE CK, et al. Mechanism of tamoxifen resistance: increased estrogen receptor-HER2/neu Gross-talk in ER/HER-2positive Brest cancor. J Natl Cancer Inst 2004, 96:926–935

[10] ELLEDGE RM, FUQUA SA. Estrogen and progesteron receptors. JR Harris, ed. Philadelphia. Lippincott Williams & Wilkins, 20004:471

[11] McGUIRE WL, Clark GM. Prognostic factors and treatment decisions in axillary- node-negative breast cancer. N Engl J Med 1992, 326: 1756–1761

[12] SHANKEY TV, RABINOVITCH PS,BAGWELL B et al. Guidelines for implementation of clinical DNA cytometry. Cytometry 1993, 14: 472–477

[13] FERRERO M, SPYRATOS F, LE DOUSSAL V, et al.Flow cytometric analysis of DNA content and keratins by using CK7, CK8, CK18, CK19, and KL1 monoclonal antibodies in benign and malignant human breast tumors. Cytometry 1990, 11: 716–724.

[14] VISCHER DW, ZARBO RJ, JABCOBSEN G et al. Multiparametric deoxyribonucleic acid and cell cycle analysis of breast carcinomas by flow cytometry. Clinocopathologic correlations. Lab Invest 1990, 62: 370–378

[15] RENNSTAM K, BALDETORP BO, KYTÖLÄ S, et al. Chromosomal rearrangements and oncogene amplification precede aneuploidization in the genetic evolution of breast cancer. Cancer Research 2001,61: 1214–1219

[16] SMITH CHA, POLLICE AA, GU LP, BROWN A, et al. Correlation amon p53, HER-2/neu, and ras overexpression and aneuploidity by multiparameter flow cytometry in human breast cancer: Evidence for a common phenotypic evolutionary pattern in infiltrating ductal carcinomas. Clinical Cancer Research 2000, 6: 112–126.

[17] REVILLION F, BONNETERRE J, AND PEYRAT, JP. ERBB2 oncogene in human breast cancer and its clinical significance. Europ. J. Cancer, 1998, 34: 791–808

[18] SHACKNEY, S, AND SHANKEY, T: Common patterns of genetic evolution in human solid tumors. Cytometry, 1997, 29:1–27

[19] DONEHOWER L. Genetic instability in animal tumorigenic models. Cancer Surv., 1997, 29: 29–352.

[20] BANERJEE M, GEORGIE J, SONG EY, et al. Based Model for Breast Cancer Prognostication. JCO 2004; 13, 2567–2575.