Biochemical, pathological and genetical studies for *HER*-2/neu oncogene in cancer breast patients

(Received : 22.11. 2006 ; Accepted 4. 12. 2007)

A.N. Sharaf*; N. Mokhtar; S.M. Mostafa* and M. Moawad****
* Department of Genetics, Faculty of Agriculture, Cairo University, Giza, Egypt.
** Department of Surgical Pathology, National Cancer Institute, Cairo University, Egypt.

ABSTRACT

Breast carcinoma ranks as first malignancy affecting females, contributing 33% of all female cancers. The C-erbB-2 is a class of oncogenes prevalent in breast cancer that play a role in cancer development. This research was performed to assess HER-2/neu oncogene amplification by semi-quantitative PCR technology, and HER-2/neu expression using immunohistochemical (IHC) staining. Electron microscopy made a contribution to the final diagnosis in the only case of invasive duct carcinoma. HER-2/neu gene amplification positive by PCR was detected in 40% of invasive duct breast cancer and showed a significant correlation between HER-2/neu gene amplification by PCR and HER-2/neu gene expression by IHC. Breast tumor specimens from 20 patients invasive duct carcinoma were studied; 10 without radiotherapy treatment and 10 after radiotherapy treatment. The study revealed a significant increase in chromosomal breaks and chromosomal rearrangements in breast cancer patients. There are also increases in chromosomal aberrations in patients received radiotherapy compared with patients not received radiotherapy. Cytogenetic study could be used as a prognostic factor in some breast cancer cases.

Keywords: *Breast cancer, HER-2/neu gene, chromosomal aberrations, radiotherapy.*

INTRODUCTION

Breast cancer is ranking number one after urinary bladder tumors and malignant lymphomas in Egypt. Breast carcinoma constitutes 33% of all female's cancers (El-Bolkainy *et al.*, 2005). Over the past two decades, electron microscopy (EM) has been extensively used in the diagnostic biologic behavior of human neoplasms. Both transmission and scanning studies have led to a more basic understanding of the structure–function relationships in tumors. Furthermore, EM studies have provided a much better appreciation of the light microscopic images that form the basis of diagnostic pathology. Moreover, ultrastructural studies have provided special information of practical diagnostic value in selected instances (Mackay, 1999). Immunohistochemistry (IHC) techniques are widely used in diagnostic histopathology to help re-differentiate the light microscopically undifferentiated tumors. *HER-2/neu* amplification or over expression is an important independent prognostic indicator in breast carcinoma, identifying a subset of patients with poor prognosis. Also, patients with C-erbB-2 positive metastasis lesions appear to have more aggressive. clinical course, which can be detected by polymerase chain reaction (PCR) technique (Kim *et al.*, 2002). Radiations that penetrate tissues are of two types: (a) ionizing radiations such as neutrons, alpha particles, electrons, X-rays,

gamma rays; (b) non ionizing radiations of low frequency waves such as UV light (Perez *et al.*, 2004).

The aim of this investigation was to study histopathologic and ultrastructural parameters using light and electron microscopy in normal, benign, and malignant breast cancer cases. Immunohistochemical analysis of *HER-2/ neu* gene status was assessed and confirmed by amplification using semi-quantitative PCR technique. Correlation with all previously assessed factors was considered. Chromosomal aberrations caused by radiation therapy were also studied using Giemsa staining.

MATERIALS AND METHODS

Patients

The present study was performed on 45 diagnosed female breast cancer patients visited the Surgical Department, National Cancer Institute(NCI), Cairo University, during the period from 2002 to 2004. Their ages ranged from 27 to 65 years. The tumor tissues were fixed in 10% neutral bufferedformalin (18 to 24 hr), and processed for histological, immunohistological analyses and DNA extraction. The fresh tumor tissues were divided into fragments for analysis of chromosomal aberrations. Patients were randomly allocated to one of the following groups: (a) ten patients with breast cancer were γ- irradiated using conventional fractionation (200Gy/6MV), and (b)-ten patients received no irradiation.

Histological diagnosis

For histological diagnosis, tissues were fixed in 4% phosphate-buffered formalin and routinely processed to wax. Paraffin sections (5 μm) were stained with heamatoxilene and eosin and examined with the microscope. For electron microscopy examination, paraffin embedded tissues (small pieces of tissue about 1 mm) were fixed in 2.5% phosphate-buffered glutaraldehyde for 4 hr at 4°C. The tissues were subsequently osmicated, dehydrated and embedded in resin. Ultra thin sections having a thickness of 70-80 nm were counterstained with lead citrate and uranyl acetate before being examined by transmission electron microscope (Bancroft and Gamble, 2002).

Immunostaining

The over expression of *HER-2/neu* protein was examined immunohistochemically using autostainer machines. *HER-2/neu* was categorized into negative showing membrane staining (<10% of the tumor cells), and positive showing staining of the entire membrane (> 10 % of the tumor cells) (Boenisch *et al.*, 2001).

DNA extraction and PCR analysis

DNA extraction from paraffin sections was carried out according to Coombs *et al.* (1999). Gene copy determination using polymerase chain reaction (PCR) was followed (Lőnn *et al.*, 1995). A 100 μl PCR was prepared containing 10 μl of 10x buffer, 200 mM of dNTPs , 1 mM of each primer (erbB2/thymidine kinase), 6 μl 25 mM MgCℓ2, 5 μl *Taq* DNA polymerase (2.5μ). Then, the volume of 100 μl mix is added to each sample (1μg) DNA. Finally, the samples were loaded in the thermal cycler blocks. Primers (25-mers) were obtained from Gibco BRL, USA. Two primers were used to amplify part of the erbB2 and thymidine kinase as shown (Table 1). PCR was performed in the thermal cycler, Roobycycler gradient 96 Stratagene. Initially, samples were heated for 5 min at 94 °C for denaturation, and then cycled 20 times at 94°C for 1 min, 56 °C for 2 min, and 72 °C for 3 min, followed by a final extension cycle at 72 °C for 5 min.

Table (1): Primers used to amplify HER-2/neu gene.

Primer name	Nucleotide sequence	Expected size (bp)
erbB2-P1	5`-CACCTGTGAGGCTTCGAAGCTGCAG-3`	217
erbB2-p2	5`-GGATATCCAGGAGGTGCAGGGCTAC-3`	
TK-P1	5`-CTCTGGGAACAACTCTGGGATGAGG-3`	136
TK-P2	5`-ACTCAGGTGGTCCCAGGAAGTGTGG-3`	

(Lönn *et al.*, 1995).

Assessment of *HER-2/neu* gene amplification

DNA was extracted from formalin fixed paraffin embedded tissues; from normal patients (negative control); from two cases of advanced breast cancer (positive). Semiquantitation of *HER-2/neu* gene by co-amplification of *HER-2/neu* gene and *thymidine kinase* as a control gene was assessed. Gel was analyzed by a documentation system, using phoenix 1D software V5.1 to measure the density of each amplified fragment.

Cytogenetic study

The tumor tissue was cultured in complete culture medium (RPMI, Gibco), under complete aseptic conditions. To each culture tube, 0.02 ml of colcemid 13 RC added ,tube incubated overnight ; Their, the hypotonic solution (KCl) and freshly prepared fixative (3 parts methanol and one part glacial acetic acid) were added. Then, for each tube, a few drops of the suspension were allowed to fall on a clean cold slide. For routine screening of metaphases, slides were stained with conventional Giemsa for 3 min, then transferred to tap water for washing. Using the oil immersion objective, chromosomal aberrations were scored and metaphase spreads were photographed (Barch *et al.*, 1997).

RESULTS AND DISCUSSION

Histology/electron microscopy

Light microscope was used to study the cases of malignant breast lesions, from invasive ductal carcinoma of the breast by hematoxelin and eosin stain (Fig. 1a). Malignant cells and high level of mitotic division were observed. Ultrasructural examination of the neoplastic cells showed mucous secretory granules and exhibited a prominent golgi rough endoplasmic reticulum and intercellular lumina with junctions (Fig.1b).

Immunohistochemistry and molecular analysis

A total of 45 patients were examined for the presence of the *HER-2/neu* gene in breast tissues cases by PCR analysis. An amplified fragment of 217 bp was detected by PCR in 18 cases that represent 40% of all the studied cases (Fig. 2a and Table. 2). Immunohistochemistry assay was performed to confirm the expression of *HER-2/neu* gene in the tissues of the 18 PCR positive cases. The expression of the *HER-2/neu* gene was detected in 16 cases that represent 97% of the PCR positive cases. (Fig. 2b and Table. 2).

Table (2): Correlation between HER-2/neu amplified by PCR and diagnosed by immunohistochemistry.

HER-2/neu expression by IHC	HER-2/neu Amplification (PCR)				P- value
	+ve		-ve		
	No. cases (18)	%	No. cases (27)	%	
+ve	16	93	0	0	<0.001
-ve	2	3	27	100	

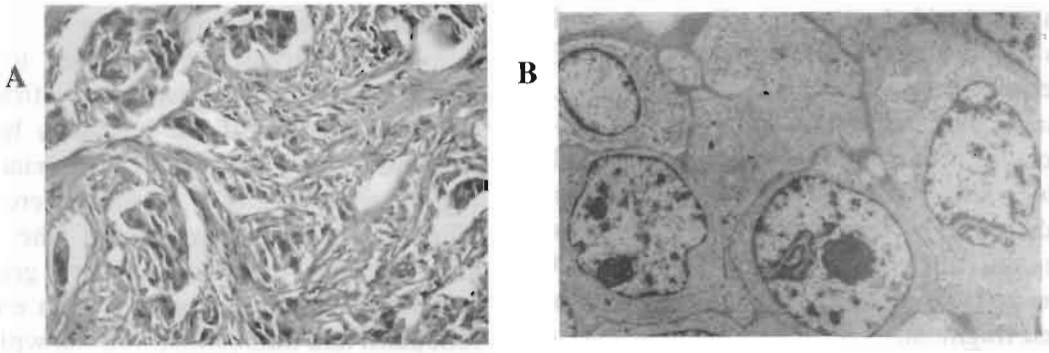

Fig. (1):Histology study. (a) A case of IDC by hematoxelin and eosin stain (X 400) showing mitosis, and (b) Ultra structure of (IDC) (x17000) showing microvilli.

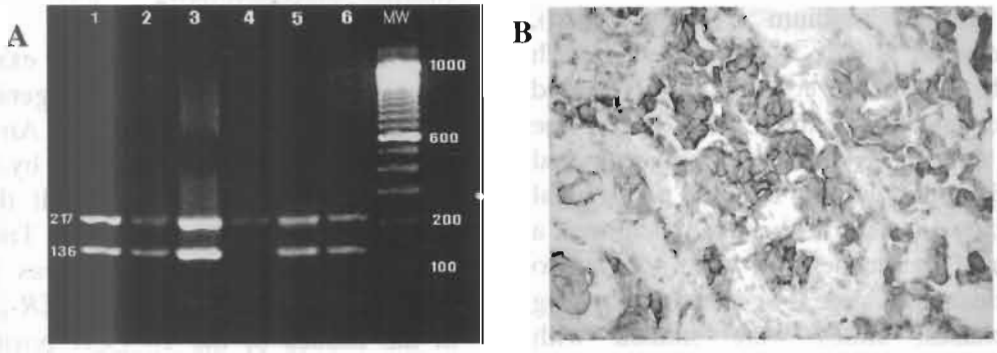

Fig. (2): Detection of HER-2/neu gene and its expression in IDC. (a) Electrophoresis separation of HER-2/neu gene (217 bp) and control gene thymidine kinase (136 bp) PCR amplified fragments on 2% agarose gel. Lanes 1, 3, 5&6: IDC positive and lanes 2 and 4 negative for HER-2/neu gene amplification. Lane 7: 100 bp molecular weight DNA standard and (b) IDC showing strong expressing membranous reaction of HER-2/neu (X400).

Cytogenetic study

The results of chromosomal aberrations are shown in Table (3). In the control group, the chromosomal breaks are very rare, whereas rearrangements and numerical aberration were completely absent (Fig. 3b). In the group of benign tumors, the frequency of chromosomal breaks is twice those of the control group (Fig.

3a). In the same direction, the chromosomal rearrangements (including dicentric and ring forms) were non significant, while numerical aberrations are completely absent. In the present study, a significant increase in numerical aberrations was detected, chromosomal breaks and chromosomal rearrangements were observed in cancer patients with and without gamma irradiation treatment (Fig. 3c & 3d). Moreover, this significant increase was very obvious in cancer patients with γ-irradiation compared with cancer patients without γ–irradiation.

Table (3): Numerical and structural aberration frequencies in tissues of control, benign, and cancer patients with and without gamma ir radiation.

Group	No, patients	Numerical aberrations	Types of aberration	
			Chromosomal breaks	Chromosomal rearrangements
Control	10	0.0	0.4±0.16	0.0
Benign patients	10	0.0	0.8±0.24	0.2±0.17
Malignant without γ irradiation	10	1.0 ±0.36*	3.0 ±0.62*	2.2*±0.50
Malignant with γ irradiation	10	1.2±0.42*	6±1.40*	4.0* ± 0.9

* Significantly different from control P<0.05

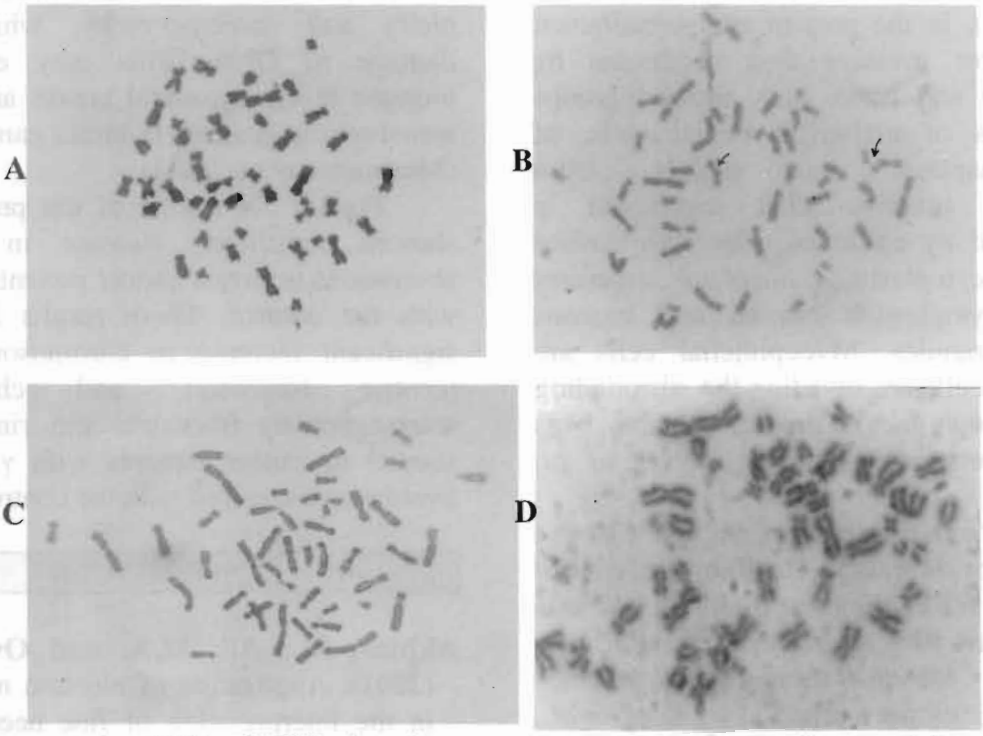

Fig.(3): Cytogenetic study of IDC. (a) Control female metaphase; (b) metaphase of benign patients; (c) metaphase of malignant patients without γ-irradiation, and (d) metaphase in cells of γ-irradiated patients.

Breast cancer is a clinically and pathologically heterogonous disease. There have been growing interest in the use of biological markers in the diagnosis of breast cancer. Many researches have focused on the identification of functional markers that play a causative role in the development and progression of breast cancer. *C-erbB-2 (HER 2/ neu)*, is a class of oncogenes playing an important role in breast cancer development and differentiation. The average age of female patients reported in this study was 49.6 years.

Invasive duct carcinoma cases with these cell types can usually be detected by both light and electron microscopy. Ultra structural techniques have been widely applied to different human neoplasmas, but only a few reports have been published on tumors (Akhtar *et al.*, 2001). In the present study, malignant breast tissues invasive duct carcinoma by hematoxelin and eosin stain showed groups and clusters of malignant ductal cells, of highly anaplasia and mitosis. Ultra structurally, invasive duct carcinoma is characterized by epithelial cells with surface microvilli, cytoplasm filamentous structures with intracytoplasmic lumina and mucous secretory granules. Myoepithelial cells are absent, and cells are invading the surrounding stroma, through gaps in the basal lamina. This was in agreement with Hadjisavvas *et al.* (2002).

HER-2neu gene amplified by PCR and IHC was not expressed at all in the control cases, while *HER-2/neu* amplified by PCR was expressed in 40% and its expression was positive in 36% in breast cancer patients. Similar findings were reported by Borg *et al.*, (2002). In the present study using semiquntitative PCR, the *HER-2/neu* gene amplification was detected in 40% of paraffin embedded IDC breast cancer patients. In concordance with these results, Slamon *et al.* (2001) reported a prevalence rate for *HER-2/neu* of 30%. Both authors used differential semiquantitative PCR methodology. In the present study, *C-erbB-2* gene was detected by PCR amplification and immunohistochemical techniques. Both techniques showed almost the same sensitivity. All the immunohistochemical positive tumors for *C-erbB-2* gene gave a heavy band by PCR, except two negative cases by IHC, which showed positive bands by PCR. This could be due to the low level of DNA in such cases leading to low levels of translated proteins, which was not enough for immunohis-tochemical detection; or alternatively, the protein was not expressed at all. The super oxide radicals and the metabolic product H_2O_2 lead to fat oxidation in cell membrane may have caused mutagenicity and carcinogenicity, which induce damage to DNA. This may explain the increase in chromosomal breaks and chromosomal rearrangements in breast cancer patients (Morimotor *et al.*, 2003).

Finally the results of the present study showed significant increase in numerical aberrations in breast cancer patients compared with the control. These results indicated a significant increase in chromosomal breaks (centric fragments) and chromosomal rearrangements (dicentric and ring chromosomes) in cancer patients with γ irradiation treatments compared with the control.

REFERENCES

Akhtar, M.; Ali, M.A. and Owen, E.W. (2001). Application of electron microscopes in the interpretation of fine needle biopsy. Cancer Res., 48: 2458-2463.

Bancroft, J.D. and Gamble, M. (2002). Theory and Practice of Histological techniques, Fifth Edition;Elserviser science limited, 689-691.

Barch, M.; Knutsen, T. and Spurbeck, J.(1997). The AG Cytogenetics Laboratory Manual, 3rd Edition, Lippncott-Raven.Philadelphia. New York, 210-215.

Boenisch, T.; Farmilo, A.; Stead, R.; Key, M.; Welcher, R.; Harvey, R. and Atwood, K. (2001). Hand Book Immunohistochemecal Staining Methods.3rd Edition, Dako.

Borg, A.; Zhang, Q.X.; Alm, P.; Olsson, H. and Sellberg, G. (2002). The retinoblastoma gene in breast cancer: allele loss is not correlated with loss of gene protein expression. Cancer Res. 52:2991-2994.

Coombs, N. J.; Gouph, A. C. and Primrose, J. N. (1999). Optimization of DNA and RNA extraction from archival formalin fixed tissue. Nucleic acid Res., 27 (16):12-18.

El-Bolkainy, M.; Noh, A. and El-Bolkainy, T. (2005). Breast cancer in topographic pathology of cancer, 3rd Edition. Al-Asdekaa Graphics Center Cairo, 81-88.

Hadjisavvas, A.; Mikelliou, C.; Zenios, A.; Zovani, I. and Kyriacou, K. (2002). Correlation between morphology, and molecular pathology in hereditary and sporadic breast cancer cases. Ultrastructural Pathology. 26: 237-244.

Kim , R.; Tanabe, K.; Uchida, Y. and Toge, T. (2002). The role of HER-2/neu oncoprotein in drug-sensitivity in breast cancer, Oncol Rep., 9(1): 3-9.

Lőnn, U.; Lőnn, S.; Nilsson, B. and Stenkvist, B. (1995). Prognostic value of C-erbB-2 and Myc amplification in breast cancer imprints. Cancer, 75(11): 2681-2687.

Mackay, B. (1999). Diagnostic electronmicroscopy of tumours. Guest editor,Bruce Mackay,Texas,Washington: 49-65.

Morimotor, K.; Wolf, S. and Kovzumi, A. (2003). Induction of sister-chromatid exchanges in human lymphocytes by microsomal activation of benzene metabolites. Mutation Research., 119: 355-360.

Perez, C.A.; Brady, L.W.; Halperin, E. C. and Schmidt-Ullrich, R. K. (2004). Principles and practice of radiation oncology, Lippincott Williams and Wilkins, 96-137.

Slamon, D. J.; Layland-Jones, B.; Shak, S. and Fuchs, H. (2001). Use of chemotherapy plus a monoclonal antibody against HER-2/neu for metastatic breast cancer that overexpress HER-2.N. Engl. J. Med., 344(11): 783-792.

الملخص العربي

التحاليل البيو كيميا ئية والباثولوجية والدراسات الوراثية للجين المسرطن (هير-٢/نيو) في مرضى سرطان الثدى

أ حمد نجيب شرف * نادية مختار** سمير محمد مصطفى * محمود معوض**
*قسم الوراثة – كلية الزراعة – جا معة القا هرة
**قسم الباثولوجى – معهد الأورام - جا معة القا هرة

يعتبر سرطان الثدى فى المرتبة الأولى بالنسبة لجميع أنواع السرطانات الاخرى فى جمهورية مصر العربية و ذ لك من واقع سجلات المعهد القومى للأورام- جامعة القاهرة. يهدف هذا البحث الى دراسة الجين المسرطن هير -٢/ نيو. تم دراسة الجين هير -٢/ نيو بطريقتين هما فصل و تكبير الدناDNA amplification بواسطة تفاعل البلمرة المتسلسل (PCR) وطريقة الصبغات المناعية Immunohistochemically فى ٤٥ سيدة مصابة بسرطان الثدى و تتراوح اعمارهن بين ٢٧-٦٥ عاما بمتوسط قدره حوالى٥٠ عاما. و بمقارنة الطريقتين اللتين استخدمتا فى تحديد الجين هير -٢/ نيو وهى الـ PCR والصبغات المناعية وجد انهما تقريبا بنفس الحساسية .أ ثبتت النتا نج أن الطرق الكيميائية الحيوية يمكن استخدامها باستعمال الصبغات المناعية كطريقة لعمل مسح لوجود جين هير -٢/ نيو بين مرضى سرطان الثدى مع إستعمال طريقة الـ PCR كطريقة تأكيدية للحالات السلبية. وقد أثبتت النتائج أن طريقة تفاعل البلمرة المتسلسل من الناحية ا لوراثية هى من الطرق التأكيدية فى مرضى سرطان الثدى.وقد تم دراسة الكروموسومات فى ٢٠ سيدة مصابة بسرطان الثدى، منهن ١٠مرضى تم معالجتهن بالعلاج الاشعاعى واظهرت النتائج ان المريضات اللاتى تم معالجتهن بالاشعاع بهن تغيرات وطفرات كروموسومية بنسبة أكبر من المريضات اللاتى لم يعالجن بالاشعاع ويتضح من ذلك ان استخدام العلاج الاشعاعى فى حالات أورام الثدى يكون له بعض التأثيرات السلبية على كروموسومات خلايا انسجة الثدى. واظهرت النتائج ان دراسة الكروموسومات هى وسيلة مهمة لاكتشاف التغيرات التى حدثت مثل الكسور فى الكروموسومية واعادة الالتحام وظهور الشكل الحلقى لبعض الكروموسومات ووجود طفرات عديدة على مستوى الكروموسومات قد تؤثر على طريقة العلاج فى بعض حالات سرطان الثدى .