Original paper

Assessment Of Her-2/Neu Gene Amplification Status by Chromogenic in Situ Hybridization in Breast Cancer Patients with Equivocal 2+ Her-2/Neu Immunostaining and Its Relation to The Clinic Pathological Parameters

Rasha Abdul Raouf ALSafi [1], Sahira A. Ali [2], Ali Hausin Al-Khafaj[3], Manal Adnan Habib[2]

[1]*Department of Pathology/ College of Medicine/ Karbala University/ Kerbala/ Iraq.*
[2]*Department of Pathology/ College of Medicine/ Baghdad University/ Baghdad/ Iraq.*
[3]*PathologyUnit/ Central Public Health Lab. Ministry of Health Baghdad/ Iraq.*

Abstract

Background: human epidermal growth factor receptor-2 (her2/neu) is related to growth factor receptors with alkaline kinase activity and it is regarded as important prognostic and therapeutic factor that can depended on in breast cancer therapy. HER2/neu expression by immunohistochemistry (IHC) is submitted to a great in terob server inconsistency. Subsequently additional confirmatory tests for assessment of gene alterations and amplification status are needed for patients with early or metastatic breast cancer. In situ hybridization techniques and specifically Chromogenic *in situ* hybridization (CISH) was arise as a practical, cost-effective, and alternative to fluorescent *in situ* hybridization in testing for gene alteration

Aims of the study: was to determinate her2/neu gene amplification in (27) breast cancer cases with equivocal her2/neu (2+) detected by IHC by using dual-color chromogenic in situ hybridization (CISH) method and to compare the results with clinic-pathological parameters of those patients like age, stage, grade, ER, PR and ki-67.

Methods*:* twenty seven breast-cancer cases with equivocal HER2-IHC(2+) test results were prospectively collected and reanalyzedusing dual-color CISH (Zyto- Vision) detection kit for further identification of her2/neu gene amplification.

Results: Cases with equivocal staining for her2/neu were about 27 cases; these cases were involved in chromogenic in situ hybridization technique to determine the status of her2/neu gene. After CISH study 12(44.4%)cases show her2/neu gene amplification (her2/neu/CEN17 ratio > 2.2).In15 (55.5%) cases out of 27 cases, no amplification of the HER2 gene was shown (her2/neu/CEN17 ratio <1.8).

Conclusions: A significant proportion of patients with equivocal IHC(2+) test results show amplification of the HER2 gene by ISH.CISH method has the advantage of diagnosis of gene amplification and revealing tissue histopathology under light microscope.

Key words: chromogenic in situ hybridization, topoisomerase II alpha gene, her2/neu, prognostic markers and breast cancer.

Introduction

Cell proliferation is mediated by many signaling pathways; one of these is through her2/neu activation which is a trans-membrane receptor protein with tyrosine kinase activity.HER2is overexpressed in approximately 25to 30% of breast carcinomas[(1)]at the same time is associated with poor prognosis and is a

*For Correspondence: E-Mail rasha.alsafi78@gmail.com

predictor for responding to treatment. [2]Because trastuzumab is important effective therapy for patients with HER2 gene amplified and over expressing breast carcinomas, so it clear thatspecifyingHER2 status is of great importance.[3]HER2 status determination is depended on many methods the most common and applicable is the identification of the amount ofHER2protein expression on the cell surface by immunohistochemical analysis and the results are scored as 0, 1+, 2+ or 3+ depending on scoring system which comprise both the percentage and quality of membrane staining of tumor cells. Tumors with HER2staining scores of 0 and 1+ are regarded asHER2-negative, 2+ as equivocal and 3+ as HER2-positive.The other is more advanced and more precise method by in situ hybridization techniques whether fluorescent or chromogenic ISH (FISH) or (CISH and SISH-silver enhanced in situ hybridisation) for the detection of either intra-nuclear gene copy number[4] or determination of more specific (gene/chromosome 17) ratio that can specify gene amplification and chromosomal 17 polysomy which indicates the presence of more than two copies of chromosome 17 at the same time.[5]

Cases collection and scoring system

The prospective study was conducted during the period from April 2014 through august 2014. All breast cancer tissue samples of 27 patients gatheredas paraffin embedded tissue blocks at the oncology hospital of breast cancer in AL- Madina Medical City/ Baghdad. All patients undergo modified radical mastectomy with axillary clearance and their specimens were fixedin 4% neutral buffered formalin and were paraffin-embedded and processed for histological diagnosis and subsequent immunohistochemical analysis.

Paraffin blokes were sectioned and processed for histopathological diagnosis of breast carcinoma which established by standard light-microscopic evaluation of sections stained with Hematoxylin and Eosin for each case. Sections from those tumors were reviewed and classified according to the World Health Organization (WHO) classification. [6]Staging of all collected cases of breast cancer done depending on histopathological picture according to American Joint Committee on Cancer (AJCC).[7]Grading of ductal carcinoma was carried out following the recommendations of Scarf, Bloom and Richardson. [8] For all cases immunohistochemical assay were done including four markers (estrogen receptors, progesterone receptor, her2/neu and ki67). According to the results of immunohistochemical analysis, her2/neu equivocal (2+) cases were collected.

Scoring system

Evaluation of tumor marker receptors was done depending on scoring systems. That for estrogen and progesterone receptors (ER, PR) was done depending on Allered scoring system. [9]In concern to her2/neu receptor protein, scoring system involve the evaluation of the intensity and pattern of membranous staining determination of immunoreactivity and this was done by using new recommendations of the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) [10] as bellow:

1. Score 0:no reactivity or membranous reactivity in <10% of the tumor cell.
2. Score 1+: faint or barely perceptible incomplete membranous reactivity in >10% of tumor cells.
3. Score 2+: weak to moderate reactivity of the entire membrane in >10% of the tumor cells as in fig (1).
4. Score of 3+: strong reactivity of the entire membrane in>30% of the tumor cells.

Depending on (ASCO/CAP) protocol published in 2013,[10] the evaluation of HER2/neu gene amplification by ISH (CISH) was used as such:

1. Negative: HER2/neu gene copy number < 4 /nucleus, HER2/neu/CEN 17 <1.8 as in fig (2).
2. Equivocal (suspect positive): HER2/neu gene copy number = 4-6, HER2/neu/CEN 17 between 1.8 - 2.2
3. Positive: HER2/neu gene copy number > or = to 6 or HER2/ neu/CEN17 >2.2 in 20 tumor cells in two different fields as in fig (3).

At least 30 tumor cells from each specimen were counted.

For **Ki-67** evaluation as proliferative index and related to the aggressiveness of the disease, its values are acquired as the percentage of positively stained malignant cells among the total number of malignant cells using the antihuman Ki-67 monoclonal antibody MIB1. [11]

Chromogenic in situ hybridization method:

CISH was performed in all cases (n = 27) applying the protocol of the new dual-color CISH technology of Zy to Vision. This technique allows advanced specificity and less background due to the unique Zy to Vision Repeat Subtraction Technique and is characterized by high sensitivity due to enzyme-coupled polymers for the detection of Her-2⁄neu gene amplification. Since this technique is new and could be of great benefit for routine diagnostics, the so-called ZytoDot_2C protocol is conducted according to the manufacturer's instructions.[12]

1. Preparatory steps:

– Incubate slides at 70_C for 10 min,
– dewaxed twice for 10 min in xylene.
– Hydration followed in graduated concentrations of alcohol [100% (2x), 96% (2x), 70% (2x) Aqua dest (2x)] in a duration for 5 min for each.
– Applying peroxidase in 3% H2O2 (5 min).
– Washing(3x) in Aqua dest for 2.Min.
– Incubation in pre-warmed pretreatment solution at 98_C in a water-bath for 15 min.
– Washed(3x) in Aqua dest for 2 min each.
– Treatment by using pepsin solution was performed for 3.5 min.
– washed (3x) in Aqua dest for 2 min and then air dried for 20 min.

Denaturation and hybridization:

– The ZytoDot 2C SPEC HER2/CEN 17 Probe (P) was vortexed and 10 microliterswas applied to each slide before the slides were cover slipped and sealed with Fixogum.
– Add Horseradish peroxidase (HRP) polymer to detect HER2 and AP polymer detects CEN 17.
– Denaturation was performed on a hot plate at 74_C for 5 min.
– Slides were immediately transferred to a humidity chamber and hybridized overnight in a hybridization oven at 37_C.

Post-hybridization and detection:

– The glue was carefully removed in saline sodium citrate (SSC) buffer for 5 min. The slides were incubated for 5 min in SSC wash buffer at 78_C (water-bath) and then washed (3x) in Aqua dest for 2 min. Blocking solution applied and incubate at room temperature for 10 min. Blocking solution was removed and anti-DIG / anti- DNP mix was applied to the slides. The slides were incubated in a humidity chamber at 37_C for 30 min, followed by washing three times in (1x)wash buffer (TBS) for 2 min. apply HRP Polymer mix and incubate in a humidity chamber at 37_C for 30 min. They were washed three times in (1x) wash buffer TBS for 2 min. AP polymer mix was applied to the slides and they

were incubated in a humidity chamber at 37_C for 30 min, before washing three times in (1x) wash buffer TBS for 2 min.

During the washing steps, an AP-Red Solution was prepared by mixing one drop of AP-Red Solution A and one drop of AP-Red Solution B in a graduated cup and incubating in a dark chamber for 3 min. Six drops of AP-Red Solution C were added, filled up to 1 ml with distilled water, and thoroughly mixed. This AP-Red Solution was applied and incubated in a dark chamberat room temperature for 15 min. The slides were then transferred into a staining jar and washed in running tap water for 2 min. During the washing step, a HRP-Green working Solution was prepared by adding two drops of HRP Green Solution A in a graduated cup and filled up to1 ml with HRP-Green Solution B. Two drops of HRP-Green Solution C were added and thoroughly mixed. This HRP-Green Solution was applied and incubated in a dark chamber at room temperature for 2 min. Afterwards, the slides were washed in (1x) wash buffer TBS for 1 min, counterstained with Mayer's Haematoxylin Solution for 5 seconds, transferred into a staining jarand washed in running tap water for 2 min. In a final step, the slides were dehydrated in 100%ethanol three times for 30 seconds and incubated twice inxylene for 30 seconds. Slides were immediately cover-slipped with mounting medium. CISH was evaluated by a light microscope using (x10), (x20) and (x40)objectives. For signal counting the (x40) objective was used.

Statistical analysis:

SPSS version 16 and Microsoft Office Excel 2007 were used in analysis of these data, Chi-square test was used to study association between any two nominal variables. P-value of less than or equal to 0.05 was considered significant.

Results

Her2/neu receptors protein is a trans-membranous protein and its expression reveals brownish membranous discoloration of the malignant cells. The mean age of the cases was (mean+/- SD), (48.1+/- 10.8).

Her2/neu gene amplification as documented by CISH study of equivocal cases:

About 27cases with equivocal her2/neu immunostaining pattern (A weak to moderate complete membrane staining that forming more than 10% of the tumor cells) were collected and retested by ISH (CISH) for further determination of her2/neu gene status. After CISH study about 12 (44.4%) out of 27 equivocal cases show her2/neu gene amplification, in that 15(55.5%) show no gene amplification as seen in table (1) and figure (4).

Correlation between her2/neu gene status and clinic-pathological parameters:

Correlation between her2/neu gene status and clinicopathological parameters of patients involved in this study were distributed as seen in table (2).In this table it is clear that there was a significant relationship between her2/neu gene amplification and the higher grade of breast cancer. On the other hands there was a significant negative relationship between her2/neu gene amplification and ER status. The relation of her2/neu gene amplification with age, stage, PR and ki-67 was statistically not significant.

Table 1. Samples of 27 patients with breast cancer with equivocal human epidermal growth factor receptor 2 protein test results by immunohistochemistry, retested by chromogenic in situ hybridization.

Equivocal her2/neu by immunostain (2+)	Number	Her2/neu gene CISH	
		Not amplified	amplified
	27	15 (55.5%)	12(44.4%)

Table 2. Correlation between HER2/neu gene status and the available clinico- pathological parameters (Chi-square test).

Clinico-pathological parameters	Her2/neu + N	Her2/neu - N	P value
age categories			Not significant
15-39	1	3	P>0.05
40-44	5	4	
45-49	1	1	
50-54	2	3	
55-59	0	1	
60-64	1	3	
65-69	1	0	
more than 75	1	0	
Stage			Not significant
Stage 1 (3)	2	1	P>0.05
Stage 2 (11)	5	6	
Stage 3 (13)	5	8	
Grade			Significant
G1 (9)	0	9	P<0.05
G2 (13)	7	6	
G3 (5)	5	0	
ER			Significant
ER+ (16)	4	12	Negative
ER - (11)	8	3	P<0.05
PR			Not significant
PR + (18)	6	12	P>0.05
PR- (9)	6	3	
Ki-67			Notsignificant
<14% (7)	1	6	P>0.05
>14% (20)	11	9	

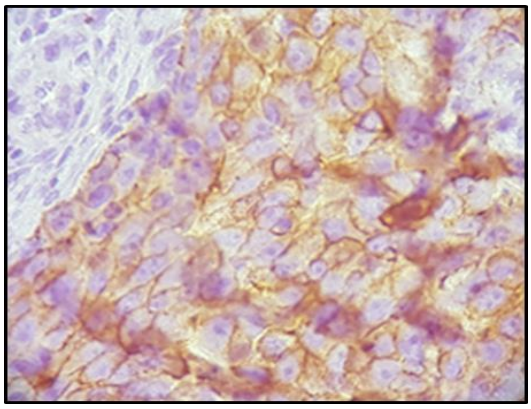

Figure 1. Invasive ductal mammary carcinoma showing positive complete moderately stained cellular membrane for HER2/neu (Score+2), HER2/neu immunostain 10X.

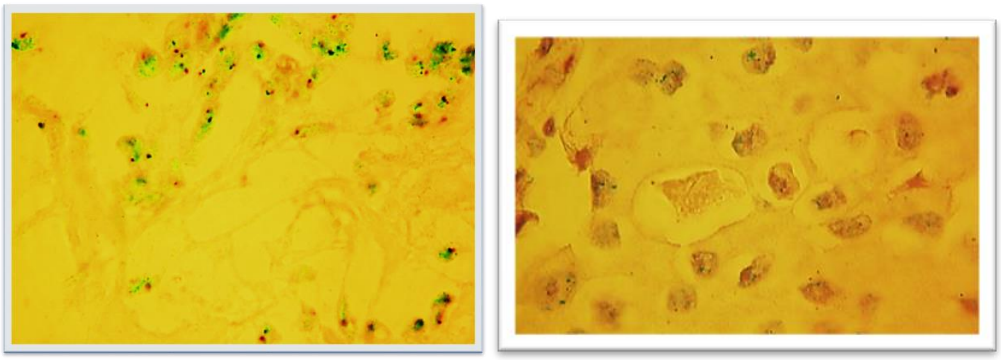

Figure 2. Invasive ductal carcinoma. **Left:** with normal (not amplified) diploid HER-2 gene copy and normal chr.17/CEN. HER-2 gene appears as a dark green colored dot-like signal while the bright red-colored dot signal is that of chr.17 centromere region. **Right:** no amplification, infrequent few small clusters of green intra-nuclear signals, 40X.

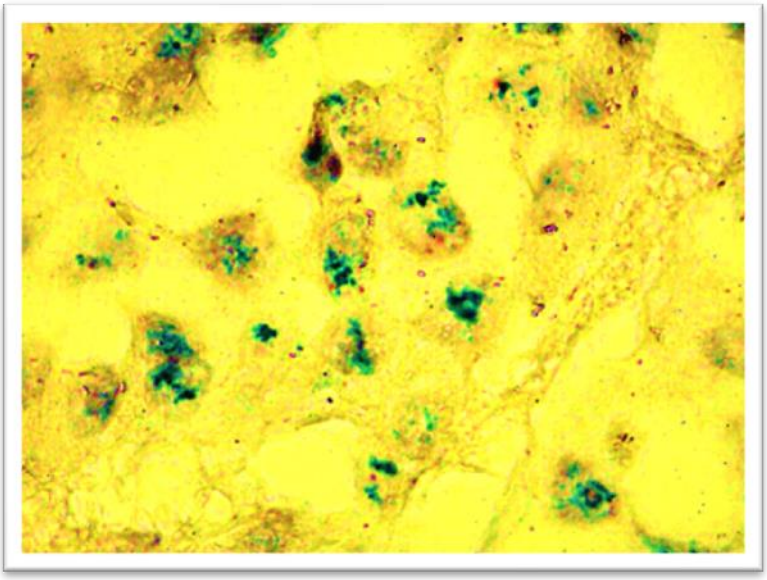

Figure 3. CISH preparation of a tissue section of breast cancer. Picture showing greenish distinct signals of multiple dense clusters of intra nuclear her2/neu gene, this is a case of highly amplified her2/neu gene.Her2/neu/ CEN 17 ratio was more than 2.3(40X).

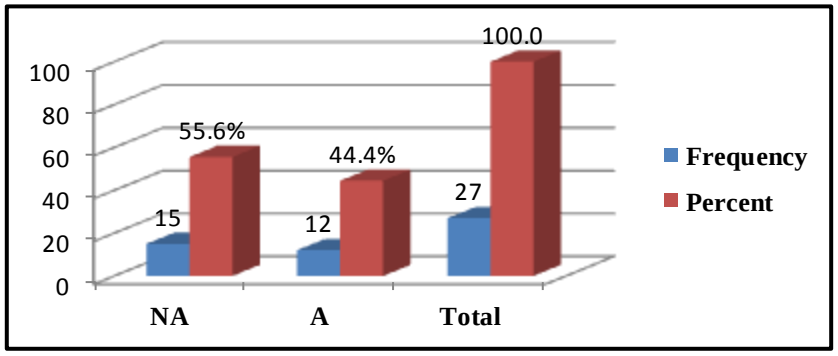

Figure 4. distribution of the cases according to the results of CISH study regarding her2/neu gene amplification. NA (not amplified) and A (amplified).

Discussion

The most popular method for evaluation of the HER2 status is immunohistochemistry. This method is relatively inexpensive and easy to perform in comparison to *in situ* hybridization methods. Because immunostaining results can directly

determine therapeutic decisions, assuring the reliability and reproducibility of the method is necessary. Many factorscan predispose and cause a high degree of inter-laboratory discrepancy in the obtained results. [13] In the IHC method, the most discrepant results are noted in the 2+ group (weak positive), in these cases the evaluation and confirmation by hybridization *insitu* is essential.[13]

According to CISH study about 12(44.4%) out of 27 equivocal cases of her2/neu show her2/neu gene amplification, this result agrees with other study by (Anna Siñczak-Kuta et al, 2007).[14] Fifteen(55.5%) cases with equivocal her2/neu (2+)show negative gene amplification this may be related to technical factors like variation in time fixation, processing conditions that affect the intensity of staining and give such equivocal staining pattern with no gene amplification. In this study, There was a statistical significant relationship between her2/neu gene amplification and the higher grade of breast cancer; these results come in concordance with other studies by (Panjwani P et.al, 2010) [15] and (Kalal Iravathy Goud et al, 2012). [16]Similarly there is an inverse significant relationship between estrogen receptor status and HER-2 gene amplification which is similar to that detected in (Kalal Iravathy Goud et al, 2012)[16] and (Naglaa A.E. Mostafa et al, 2011)[17] this can be explained by the presence of a complex signaling between estrogen receptors and signaling pathways of other growth factors in breast cancer cells.[17]Our study didnot find associations of Her-2 /neu status with the patient age, lymph nodestatus, stage, PR and ki-67 and this may be due to a small sample size, so a larger study may be needed for further confirmation of such relations.

References

1. Owens MA, Horten BC, Da Silva MM. HER2 amplification ratios by fluorescence in situ hybridization and correlation with immunohistochemistry in a cohort of 6556 breast cancer tissues. Clin Breast Cancer 2004; 5:63-9.
2. Hanna WM, Kwok K. Chromogenic in-situ hybridization: a viable alternative to fluorescence in-situ hybridization in the HER2 testing algorithm. Mod Pathol 2006;19:481-7
3. Sybren L Meijer,1 Jelle Wesseling,2,3 Vincent T Smit *et al*. HER2 gene amplification in patients with breast cancer with equivocal IHC results. J Clin Pathol 2011; 64:1069-1072.
4. Kounelis S, Kapranos N, Malamos N, et al. Evaluation of HER2 gene status in breast cancer by chromogenic in situ hybridization: comparison with immunohistochemistry. Anticancer Res 2005;25:939-46.
5. Hyun CL, Lee HE, Kim KS, et al. The effect of chromosome 17 polysomy on HER-2/ neu status in breast cancer. J Clin Pathol 2008;61:317-21.
6. Hans-Peter Sinn, Hans Kreipe. A Brief Overview of the WHO Classification of Breast Tumors, 4th Edition, Focusing on Issues and Updates from the 3rd Edition. Breast Care 2013;8:149–154.
7. AJCC_Cancer_Staging_Manual,_7th_Edition 2010. P347.
8. DC, Harvey JM, Berardo M, Clark GM: Prognostic and predictive factors in breast cancer by immunohistochemical analysis. Mod Pathol 1998, 11:155-168.
9. Allred DC Harvey JM, Berardo M et al. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. Mod Pathol. 1998; 11:155–168.
10. Antonio C. Wolff, M. Elizabeth H. Hammond, David G. Hicks, Mitch Dowsett, Lisa M. McShane, Kimberly H. Allison, Donald C. Allred, John M.S. Bartlett, Michael Bilous, Patrick Fitzgibbons, Wedad Hanna, Robert B. Jenkins, Pamela B. Mangu, Soonmyung Paik, Edith A. Perez, Michael F. Press, Patricia A. Spears, Gail H. Vance, Giuseppe Viale, and Daniel F. Hayes. Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Update. JOURNAL OF CLINICAL ONCOLOGY, VOLUME 31_NUMBER 31_ NOVEMBER 1 2013.
11. Rosai and Ackerman's Surgical Pathology > Volume 2 > 20 - Breast > Carcinoma.
12. Zytovision, Zyto Dot 2C,SPEC HER2/CEN17 Probe Kit , A Practical Approach 2014 .
13. Hayat MA: Comparison of immunohistochemistry, in situ hybridization, fluorescence in situ hybridization and chromogenic in situ hybridization. In: Handbook of immunohistochemistry and in

situ hybridization of human carcinomas. Molecular genetics; Lung and breast carcinomas. Hayat MA, ed, Elsevier Inc 2004, 3–11.

14. Anna Siñczak-Kuta, Romana Tomaszewska, Lucyna Rudnicka-Sosin, Krzysztof Okoñ, Jerzy Stachura. Evaluation of HER2/neu Gene Amplification in Patients with Invasive Breast Carcinoma. Comparison of in situ Hybridization Methods. Pol J Pathol 2007, 58, 1, 41–50.
15. Pierre-Jean Lamy, Frédéric Fina, Caroline Bascoul-Mollevi, Anne-Claire Laberenne, Pierre-Marie Martin, L'Houcine Ouafik, William Jacot. Quantification and clinical relevance of gene amplification at chromosome 17q12-q21 in human epidermal growth factor receptor 2-amplified breast cancers. Breast Cancer Research 2011, 13:R15
16. Wang L, Wang X, Nie X, Ma L. Comparison of fluorescence in situ hybridization and immunohistochemistry for assessment of Her-2 status in breast cancer patients. J Huazhong Univ Sci Technolog Med Sci 2009;29:354–8.
17. Massarweh S, Schiff R. Resistance to endocrine therapy in breast cancer: exploiting estrogen receptor/growth factor signal cross talk. Endocr Relat Cancer 2006;13:S15–24.
18. Panjwani P, Epari S, Karpate A, Shirsat H, Rajsekharan P, Basak R, et al. Assessment of HER-2/*neu*status in breast cancer using fluorescence *in situ* hybridization & immunohistochemistry: Experience of a tertiary cancer referral centre in India. Indian J Med Res. 2010;132:287–94.
19. Kalal Iravathy Goud, Seetha Dayakar, Kolanupaka Vijayalaxmi, Saidam Jangu Babu, and Anand Reddy P. Vijay. Evaluation of HER-2/neu status in breast cancer specimens using immunohisto-chemistry (IHC) & fluorescence in-situ hybridization (FISH) assay. Indian J Med Res. 2012 Mar; 135: 312–317.
20. Naglaa A.E. Mostafa a, Saad S. Eissa a, Dalia M. Belal b, Soheir H. Shoman. Assessment of Her-2/neu gene amplification status in breast carcinoma with equivocal 2+ Her-2/neu immunostaining. Journal of the Egyptian National Cancer Institute 2011; 23, 41–46.