RESEARCH ARTICLE

Quantification of Her-2/Neu Gene in Breast Cancer Patients using Real Time-Polymerase Chain Reaction (Q-PCR) and Correlation with Immunohistochemistry Findings

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Abstract

Background: HER-2/neu is a proto-oncogene that encodes a transmembrane tyrosine kinase growth factor which is crucial for stimulating growth and cellular motility. Overexpression of HER-2/neu is observed in 10-35% of human breast cancers and is associated with pathogenesis, prognosis as well as response to therapy. Given the imperative role of HER-2/neu overexpression in breast cancer, it is important to determine the magnitude of amplification which may facilitate a better prognosis as well as personalized therapy in affected patients. In this study, we determined HER-2/neu protein expression by immunohistochemistry (IHC) concurrently with HER-2/neu DNA amplification by quantitative real time-polymerase chain reaction (Q-PCR). Materials and Methods: A total of 53 paired tissue samples from breast cancer patients were frozen-sectioned to characterize the tumour and normal tissues. Only tissues with 80% tumour cells were used in this study. For confirmation, Q-PCR was used to determine the HER-2/neu DNA amplification. Results: We found 20/53 (37.7%) of the tumour tissues to be positive for HER-2/neu protein overexpression using IHC. Out of these twenty, only 9/53 (17%) cases were in agreement with the Q-PCR results. The concordance rate between IHC and Q-PCR was 79.3%. Approximately 20.7% of positive IHC cases showed no HER-2/neu gene amplification using Q-PCR. Conclusion: In conclusion, IHC can be used as an initial screening method for detection of the HER-2/neu protein overexpression. Techniques such as Q-PCR should be employed to verify the IHC results for uncertain cases as well as determination of HER-2/neu gene amplification

Keywords: Breast cancer - HER-2/neu gene quantification - real time - polymerase chain reaction (Q-PCR)

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Introduction

Breast cancer is the most frequent cancer in women in most parts of the world. Approximately 1.1 million of women in the world were diagnosed with breast cancer every year and 410,100 died from the disease (Jemal et al., 2010). According to the Malaysia Cancer Statistics 2007, there were 3,242 female breast cancer cases diagnosed and reported to the National Cancer Registry (Omar et al., 2011). This figure accounted for 18.1% of all cancer cases reported (Omar et al., 2011). The Chinese showed the highest incidence rate with age-standardised rate (ASR) of 38.1 per 100,000 population followed by Indian and Malay with 33.7 and 25.4 per population respectively (Omar et al., 2011).

The aetiology of breast cancer is multifactorial, with genetic, environmental and reproductive factors interacting in a complex way. Genetic factors involved mutations in several genes such as BRCA1, BRCA2 and P53 (Metcalfe et al., 2010; Denisov et al., 2011). In addition to mutations in these genes, alterations in proto-oncogenes and tumour suppressor genes have been reported in human breast cancer, eg HER-2/neu oncogene (Carney et al., 2007; Dowsett et al., 2007). HER-2/neu gene encodes for a 185-kDa transmembrane tyrosine kinase growth factor receptor that belongs to the epidermal growth factor receptor family (Nistor et al., 2006). The gene is located at the long arm of chromosome 17 (q21) and it is expressed in the epithelial cells (Bofin et al., 2004). Amplification of HER-2/neu gene has been studied intensively and reported in many cancers (Bofin et al., 2004; Carney et al., 2007; Dowsett et al., 2007; Gown, 2008). In breast cancer, the HER-2/neu amplification can be detected in 25% to 35% and it is strongly associated with a poor prognosis (Nistor

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et al., 2006). Patients where tumours exhibit HER-2/neu amplification also showed an increased recurrence risk and shorter survival rates (Nistor et al., 2006).Therefore, the detection of HER-2/neu is invaluable where it can be used as a predictive marker of patient responsiveness to therapy including chemotherapy, hormonal therapy and therapeutic anti-HER-2 antibodies.

HER-2/neu gene amplification can be determined using several techniques such as immunohistochemistry (IHC), fluorescence in situ hybridization (FISH) and chromogenic in situ hybridization (CISH) (Carney et al., 2007; Rosa et al., 2009). To date, only two methods have been approved by the FDA, where IHC is used as a screening method for the identification of the positive and negative HER-2 protein overexpression cases (Rosa et al., 2009). Consequently, FISH is utilized in cases with undefined IHC status (2+ IHC positive cases).

Advancement of the PCR technology results in the development of LightCycler HER-2/neu DNA Quantification Kit using real-time PCR Light Cycler (Roche Molecular Biochemical, Germany). This method offers an inexpensive, rapid and accurate quantification of HER-2/neu amplification compared to IHC, FISH and CISH. Given the clinical importance of HER-2/neu amplification in the breast cancer patients, we determined the HER2/neu protein status using IHC and subsequently confirmed by real-time PCR based assay.

Materials and Methods

Samples collection

The study protocol was approved by The Research and Ethics Committee of the Universiti Kebangsaan Malaysia Medical Centre (UKMMC). A total of 53 breast cancer patients were enrolled for this study. Prior consent was obtained from the patients. Paired tissue samples comprising of normal and tumour were dissected from the patient who underwent mastectomy. Table 1 shows the clinical and demographic data of the breast cancer patients involved in this study. The tissue samples were snap frozen into the liquid nitrogen and stored in -80°C until further use.

Frozen section and histopathologic analysis

Frozen section was performed on the tissues using a cryostat, Microm HM550 (ThermoScientific, USA). Subsequently, haematoxylin & eosin (H&E) staining was carried out followed by a histological examination to determine the status of the tissue samples. Tumour tissues with more than 80% of the malignant cells and normal tissues which were free from malignant cells were characterized as representative samples.

DNA Extraction

The total genomic DNA was prepared using a commercial available kit as described by the manufacturer (Qiagen DNA Mini Kit, Germany). The DNA samples were separated on a 1.2% agarose gel and their purities were determined by the spectrophotometer (A260/A280).

Real time - polymerase chain reaction (Q-PCR) of the HER-2 DNA

Quantitative measurement of HER-2 DNA was performed using the LightCycler Her2/neu DNA quantification kit on a Light Cycler Instrument (Roche Diagnostics, Germany). A 112 bp fragment of HER-2/neu gene and a 133 bp fragment of reference gene from the human genomic DNA were amplified by the polymerase chain reaction (PCR) with specific primers. The amplicons were simultaneously detected using the specific pairs of hybridization probes. Each pair of the hybridization probes consists of two different oligonucleotides that hybridize to an internal sequence of the amplified fragment during the annealing phase of the amplification cycle. One probe was labeled at the 5'- end with a LightCycler -Red fluorophore (LightCycler-Red 640 or LightCycler-Red 705). To avoid extension, this probe was modified at the 3'-end by phosphorylation. The other probe was labeled at the 3'-end with the LightCycler - Fluorescein. These two probes came in close proximity resulting in fluorescence resonance energy transfer (FRET) between the two fluorophores after hybridization to the template DNA. During FRET, the LightCyler-Fluorescein, the donor fluorophore which is the excited energy was transferred to the LightCycler Instrument. In addition, a part of the excitation energy was transferred to the LightCycler-Red, the acceptor fluorophore. The emitted fluorescence of the LightCycler-Red fluorophore was then measured by the Light Cycler Instrument. Briefly, all components for Q-PCR were prepared as follows, 9µl of H20 PCR-grade, 2µl of LC-HER2/neu Detection Mix 10x concentration, 2µl of LC-HER2/neu Reference Gene Detection Mix 10x concentration and 2µl of LC-HER2/neu Enzyme Master Mix in a total volume of 15µl. The master mix was pipetted into a precooled LightCycler capillary and 5 µl of the DNA template was added. The capillaries were centrifuged at 3000rpm and placed in the rotor of the LightCycler Instrument. The Q-PCR was performed as follows, denaturation step at 95˚C for 10 mins, followed by 45 cycles of initial temperature at 95˚C for 10 secs, annealing at 58˚C for 10 secs and final extension of 72˚C for 10 secs. The cooling step involved incubation for 30 secs at 40˚C.

Q-PCR data analysis

The calculation of the relative amount of HER-2 was performed by the LightCycler Relative Quantification Software (Roche Molecular Biochemicals, Germany). The final result was expressed as a ratio of T:R in the sample relative to the ratio of the T:R in the Calibrator DNA. The ratio of T:R in the LightCycler – Calibrator DNA provided with the kit has a value of 1.00. Ratio of < 2.00 means the sample is negative for HER2/neu DNA amplification whereas ratio of > 2.00 means it is positive for HER2/neu DNA amplification (T=target gene; R=reference gene).

Immunohistochemistry (IHC) technique

The commercially available immunohistochemical staining kit, HercepTest and the Dako Autostainer was used for staining of the histological sections (DakoCytomation, Denmark). Briefly, 3μm section from each case was deparaffinized and rehydrated through a descending alcohol series. The slides were subjected

to heat-induced apitope retrieval by immersing them in preheated 95˚C DAKO Target Retrieval Solution followed by heating in water bath for 40 minutes at 95˚C. The slides were then cooled down at room temperature for 20 minutes and incubated with primary antibody for 30 minutes at room temperature. Subsequently, the slides were incubated with LSAB+ Detection System (labeled streptavidin biotin immunoperoxidase, DAKO) according to the manufacturer's instructions. Diaminobendizine was used as a chromogen and the slides were counterstained with hematoxylin. The results were expressed as 0 with no expression and the degree of overexpression of the HER-2/neu were indicated as positive (1+, 2+ and 3+).

Results

Patients demographic

In total, 53 breast cancer patients were included in this study. The age ranged between 30 to 75 years old (mean aged = 50.4 years). The prevalence of breast cancer in this study was highest in Malays, followed by Chinese and Indians with 35/53 (66%), 10/53 (18.9%) and 7/53 (13.2%) respectively.

Estrogen, progestrone and HER-2/neu status in breast cancer tissues using immunohistochemistry (IHC)

Frozen section was performed in all breast cancer tissues to determine the representative samples with 80% tumour (Figure 1). Table 1 shows the estrogen, progesterone and HER-2/neu status in the breast cancer tissues studied. For estrogen receptor, about 37 (69.8%) samples showed positive results. Approximately, 33 (62.3%) of the tumour tissues appeared to be positive progesterone receptor. The tumour tissues with positive HER-2/neu were identified in 20 (37.7%) of all cases.

Table 2 shows comparison between the HER-2/neu identification using IHC compared to Q-PCR. 20/53 (37%) of the breast cancer patients were positive for HER-2 protein expression using the immunohistochemistry (IHC). However, Q-PCR HER-2/neu DNA amplification showed only 9 (17%) of the tumour tissues were positive which was correlated with the IHC results. The concordance rate between IHC and Q-PCR was 79.3% as shown in Table 2. 11 (20%) of the tumour tissues showed positive HER-2/neu protein overexpression by IHC but absence for HER-2/neu DNA amplification where the Q-PCR results showed less than 2. Comparison between IHC and Q-PCR of HER-2 /neu DNA was shown as in table 2. Table 3 showed tumour tissues with positive HER-2/neu protein overexpression and DNA amplification by IHC and Q-PCR respectively. The Q-PCR results ranges

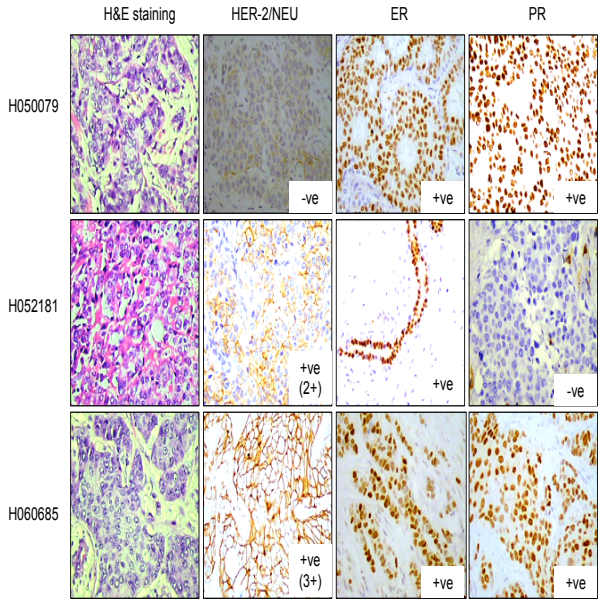

Figure 1. Haematoxylin and Eosin (H&E) Staining of Breast Cancer Tissues H050079, H052181 and H060685 Showed 80% Tumour. Immunohistochemistry (IHC) of tumour tissues with HER-2/neu, estrogen (ER) and progestron receptor (PR)

Table 1. Estrogen, Progesterone and CERB-B2/HER2 Status in the Breast Cancer Patients Studied

Description		n (%)
Estrogen receptor status	Negative	16 (30.2)
	Positive	37 (69.8)
Progesteron receptor status	Negative	20 (37.7)
	Positive	33 (62.3)
CERB-B2/ HER-2 status	Negative	32 (60.4)
	Positive	20 (37.7)
	NA	1 (1.8)

Table 2. Comparison of IHC and Real-time HER-2 Protein Expression and DNA Amplification Status in 53 Tumour Tissues of Breast Cancer Patients

	HER2 gene amplification (n=53)	
IHC	No amplification (%)	High amplification (%)
Negative	33 (62.3%)	0
Positive	11 (18.9%)	9 (17.0%)

Table 3. Comparison between IHC and Real-time HER-2 Protein Expression and DNA Amplification Status in 53 Tumour Tissues of Breast Cancer Patients According to the IHC Classification

		Q-PCR (Tumour tissues)		
IHC	n (%)	HER-2/neu ratio (Mean, range)	Ratio <2.00 n (%)	Ratio <2.00 n (%)
0 or 1+	34 (64.2)	1.56 (1.37-1.81)	34 (64.1)	-
2+	4 (7.5)	1.52 (1.34-1.61)	4 (7.5)	-
3+	14 (26.4)	2.90 (1.39-8.91)	5 (35.7)	9 (64.3%)
NA	1 (1.9)	1.56	1 (1.9)	-
Total	53			

Table 4. Real-time HER-2/neu DNA Amplification Results in Breast Cancer Patients with Positive IHC

Patient	Diagnosis	ER	PR	HER-2	Q-PCR_NT	Q-PCR_TT
1	IDC	N	N	P (3+)	1.77	8.91
2	IDC	P	P	P (3+)	1.62	3.27
3	IDC	N	N	P (3+)	1.85	3.92
4	IDC	P	N	P (3+)	1.59	2.84
5	IDC	N	N	P	1.48	2.51
6	IDC	N	N	P	1.44	2.48
7	IDC	P	P	P (3+)	1.8	3.66
8	IDC	P	P	P (3+)	1.65	3.6
9	IDC	P	P	P (3+)	1.44	2.25

*IDC: Infiltrating ductal carcinoma, ER: Estrogen receptor, PR: Progesteron receptor, NT: Normal tissue, TT: Tumour tissue

between 2.25 to 8.91 for tumour tissues and as expected the normal tissues showed results < 2.0.

Discussion

HER-2/neu overexpression has been observed in 10% to 35% of the human breast cancer cases (Goud et al., 2013). In addition to being a prognostic indicator, HER-2/neu overexpression is also important in determining treatment strategies for breast cancer patients where overexpression may be associated with decreased or adverse effect to anti hormonal therapy (Fehm et al., 2007). Moreover, the degree of HER-2/neu amplication is linked with survival hence the accurate assessment of HER-2/neu is crucial to determine therapeutic strategy in the breast cancer patients (Mendoza et al., 2012).

Several methods can be used to determine the HER-2/neu DNA amplification and protein overexpression including fluorescence in situ hybridization (FISH), immunohistochemistry (IHC) (Moelans et al., 2011), chromogenic in situ hybridization (CISH) which are semiquantitative (Tse et al., 2005; Rosa et al., 2009). In addition, quantitative real time-polymerase chain reaction (Q-PCR) can be used for quantitative measurement of the HER-2/neu DNA/RNA (Nistor et al., 2006). Current practice uses two different methods to confirm the HER-2/neu protein overexpression and DNA amplification in the breast cancer patients. However, FISH and IHC are the most common methods used and have been approved by the US Food and Drug Administration (FDA) (Bofin et al., 2004). The standard method to identify the HER-2/neu status is immunohistochemical technique (IHC) whereby the HER-2/neu protein expression is detected (Carney et al., 2007). IHC uses different antibodies with different binding affinities and different epitope specificities, thereby creating differences in HER-2/neu overexpression rates (Nistor et al., 2006). In addition, the HER-2/neu overexpression scoring systems differ and often rely on the subjective measures of staining intensity and pattern (Nistor et al., 2006). Therefore, IHC can only be the initial screening strategy to distinguish between the positive and negative cases of HER-2/neu amplification in the breast cancer patients (Carney et al., 2007). Although FISH is a gold standard for detection of the HER-2/neu amplification, this method does not assess the gene expression and cannot identify cases in which the gene product is overexpressed in the absent of gene amplification. Chromagenic in situ hybridization (CISH) is an alternative method to FISH where it produces a permanent staining and samples can be archived indefinitely (Rosa et al., 2009). CISH is a combination of both in situ hybridization technology and chromogenic signal detection of IHC. In CISH, a DNA probe is detected using a simple IHC-like peroxidise reaction which can be visualized by ordinary microscope (Nistor et al., 2006). CISH also relies on subjective interpretation which could lead to misinterpretation of the results (Nistor et al., 2006). However, cell morphology can be easily analysed and identified. The only quantitative technique is quantitative-real time polymerase chain reaction (Q-PCR) which offers more accurate, reliable and simple method in detecting HER-2/neu amplification (Tse et al., 2005).

In this study, we determined the HER-2/neu overexpression using immunohistochemistry (IHC) as an initial screening process. Consequently, Q-PCR using LightCycler (Roche Diagnostic, Germany) was performed to confirm the IHC results as well as to determine the degree of HER-2/neu DNA amplification. LightCycler combines real-time PCR and fluorescent detection using sequence-specific hybridization probes which are labeled with fluorophores. These fluorescent labeled probes are used to monitor the accumulation of the PCR products during the log phase (Wittwer et al., 2001) and allow end-point analysis via the melting curve (Lyon, 2001). The only drawback using this technique is that the tumour and normal tissues must be validated prior to Q-PCR analysis. We validated the tumour and normal tissues using haematoxylin and eosin staining. This is crucial to determine the representative tumour and normal (>80% tumour) tissues without contamination of other tissues such as fat and connective tissues. However, this limitation based on tumour heterogeneity can be abolished using laser capture microdissection (LCM) method where the tumour and normal tissues can be dissected simultaneously. Since we do not have the LCM, only tissues with 80% normal or tumour were used. The results of the present study showed that 20/53 (37.7%) of the tumour tissues were positive for HER-2/neu protein overexpression which was determined by the IHC. However, only 9/53 (17%) of the tumour tissues demonstrated positive HER-2/neu DNA amplification. The present study revealed that 79.3% cases were concordance between IHC and Q-PCR. This is in agreement with another study performed by Rosa and colleagues where they demonstrated concordance rate between IHC and Q-PCR at 78.9% (Rosa et al., 2009). Although several techniques can be used in order to determine the HER-2/neu gene expression, Q-PCR is more convenient, easy and rapid compared to IHC, FISH and CISH. Nistor and colleagues used FISH and Q-PCR to determine the HER-2/neu amplification in breast tumours that received IHC score of 2+ (Nistor et al., 2006). They showed that Q-PCR is more sensitive compared to FISH where 3% of negative cases by IHC were detected as positive HER-2/neu amplification by Q-PCR. However, the concordance between IHC and Q-PCR were high with 10% FISH+/Q-PCR+ and 82% FISH-/Q-PCR-. They concluded that HER-2/neu detection using Q-PCR was more accurate and reproducible compared to IHC.

Despite intensive efforts to establish the standard methods for detecting HER-2/neu overexpression either by IHC, FISH and CISH, false-positive or false-negative results are still arising. These methods use fixed tumour tissues where formalin fixation may cause damage to the HER-2/neu epitope. In IHC, conditions such as tissue processing, reagent variability, antigen retrieval methods, scoring interpretation, tumour heterogeneity may contribute to false-positive or false-negative results. In this study, IHC showed 11/53 (20.8%) of false positive results where Q-PCR revealed absence of HER-2/neu DNA amplification. Out of these 11 cases, five samples were scored as 3+, four samples were classified as 2+ and two patients with positive (unknown score). In IHC, an

expression of 0 or +1 was characterized as HER-2/neu negative whereas HER-2/neu positive scored 2+ or 3+. However, score of 2+ is uncertain where other methods such as FISH and Q-PCR are needed to confirm the amplification of HER-2/neu gene.

In conclusion, IHC can be used in the laboratory set up to determine the HER-2/neu overexpression as a screening method. In addition, methods including FISH, CISH or Q-PCR should be employed to confirm the IHC results. It is also recommended that laboratories offering the HER-2/neu test should demonstrate 95% concordance using another validated test for positive and negative assay value

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