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The Detection of HER2 Gene Amplification in Breast Cancer by upQMPSF

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ABSTRACT

The amplification of human epidermal growth factor receptor 2 (HER2) gene in breast cancer identifies patients eligible for Trastuzumab therapy. immunohistochemistry (IHC) and/or fluorescent in situ hybridization (FISH) are the only FDA approved techniques for evaluating HER2 status. However, Inter-observer variability is still a dilemma. The aim of this study is to develop a new PCR-based technology that is accurate, reliable, less subjective, cost-effective and easier to interpret result. universally-primed Quantitative Multiplex PCR Short Fluorescent Fragments (upQMPSF) identified 3 (18.8%) positive cases and 13 (71.2%) negative. The agreement between FISH and upQMPSF was in 15 of 16 (93.7%) samples with confidence interval (0.7-1.0). upQMPSF may offer an alternative method for the detection of HER2 gene amplification in breast cancer patients, given the small amount of DNA required and faster turnaround result, A larger study is underway to demonstrate the accuracy of cases with equivocal results (FISH 1.8-2.2).

Keywords: *upQMPSF; HER2; FISH; immunohistochemistry; Trastuzumab*

INTRODUCTION

HER2 gene is an oncogene located on chromosome 17q.21.1. amplified in a variety of human cancers and most importantly in 25–30% breast cancer cases. *HER2* gene is considered a valuable breast cancer biomarker that is associated with rapid tumor growth, increased risk of recurrence after surgery, poor response to conventional chemotherapy and lower overall survival rate in patient with invasive carcinoma of the breast (Slamon et al. 1987; Borg et al. 1990; Parkes et al. 1990). Herceptin® (humanized monoclonal antibody) is the recommended therapy for patients with *HER2* positive. Currently, fluorescent in situ hybridization (FISH) and immune histochemistry (IHC) are the only FDA approved methodologies for the detection of *HER2* gene amplification. However, several studies demonstrated a 20% of the samples show discordant *HER2* status between both methods. Additional studies also demonstrated FISH to be more sensitive than IHC in determining *HER2* status (Onody et al. 2001). This controversy is attributed to several factors, including reagent variability, scoring, tissue processing and antigen retrieval. Furthermore, FISH and IHC analysis are microscopic techniques and require intensive and time consuming interpretation. Moreover, these techniques are difficult to standardize across laboratories and are subject to inter-observer variability. Human errors are still a problem among different laboratories or result interpreters even with regard to FISH analysis which make these tests subjective. Moreover, ASCO-CAP 2007 guidelines recommended borderline category for FISH results when the ratio falls in between 1.8 and 2.2 (Bartlett et al. 2001; Tubbs et al. 2001; Moeder et al. 2007; Wolff et al. 2007). There have been many attempts to use different PCR-based methods (Lyon et al. 2001; Sestini et al. 1995; Bièche et al. 1999). However, they are all rated as unsatisfactory in terms of reliability, accuracy of the design. Adding, *HER2* gene amplification has been linked with DNA aneuploidy (Bose et al. 2001; Watters et al.2003). Therefore, low gene amplification may be related to polysomoy of chromosome 17

rather than local *HER2* gene amplification. Thus, including only one internal control from another chromosome will fail to address this possibility. Furthermore, most studies have been using Monoplex PCR (cover only one *HER2* region) (Layfield et al. 2008), which is less informative and insufficient in dealing with inter-assay and inter-sample variability. Nevertheless, all studies demonstrated the feasibility of PCR in replacing FISH and IHC. Considering the inadequacy of current methodologies and the significance of *HER2* amplification in breast cancer treatment, we developed a method addressing the previous limitations of other PCR-based methodologies to achieve improvement of *HER2* amplification detection, named universally-primed Quantitative Multiplex PCR Short Fluorescence Fragments (upQMPSF).

MATERIALS AND METHODS

Patient Samples

Sixteen anonymous human genomic DNA samples were extracted from fresh frozen tissue of breast tumors from Roswell Park Cancer Institute, Buffalo, NY. Since genetic heterogeneity is considered a major challenge using this type of analysis for HER2 amplification test, therefore to limit this effect, HER2 status was analyzed in tumors with matching normal tissues obtained from the same patient. Only sixteen samples with 80% tumor cells were selected for this study, however, other samples with more than moderate inflammatory infiltrate or Ductal Carcinoma In situ (DCIS) were excluded, genomic DNA extraction was performed using (QIAGEN, USA) DNA extraction kit.

Fish

Fluorescent in situ hybridization analysis was performed using PathVysion method at thepathology department at Roswell Park Cancer Institute. HER2 was positive by FISH when HER2: CEP17 ratio was more than 2.2 and negative when this ratio was less than1.8.

upQMPSF

Assay Design

A molecular technique upQMPSF was developed for possible evaluation of *HER2* gene amplification in breast cancer fresh tissue samples. Utilizing one multiplex set of upQMPSF. As shown in (Table.1), primers were designed to amplify four *HER2* entire exons 5, 17,18 and 23, such that it covers the beginning, middle and the end parts of *HER2* gene region, including the antibody Herceptin® binding sites (exons 17-18).

Table1. *upQMPSF multiplex primer set for the detection of HER2 gene amplification*.

Primer Name	Primer Sequence	Product Size (Bps)
c1_17q_f	UF*_tgttcacaggagcactgtgg	141
c1_17q_r	UR*_ccaccagattgtcctgaagc	141
c3_Rad_e5f	UF_tggagaattccgaactggga	177
c3_Rad_e5r	UR_ccatcctaacagatgcttgc	177
her2_e5f	UF_gtgtcctctgacccatctgc	215
her2_e5r	UR_gacaggctggcattggtg	215
C2_17P_F	UF_acaaggctccagggacctgc	244
C2_17P_R	UR_ggaccttcgcgtctgtgca	244
her2_e17f	UF_atggggctcctctcagacc	268
her2_e17r	UR_gcacaccctactgcatctcg	268
her2_e18f	UF_gacccaactaagggcctgat	294
her2_e18r	UR_gaccgttggactcacgagtg	294
her2_e23f	UF_ttgtgatggttgggaggctg	343
her2_e23r	UR_gtttgctccggagagacctg	343

**UF: Universal Forward primer*

**UR: Universal Reverse prime*

We carefully designed the assay to address the limitations of previously reported PCR-based assays, three internal controls were included in the set, 17p and 17q internal control were

located at the centromere flanking region of chr17 in addition to exon 5 of RAD51 gene region as internal control outside the region of chr17, Chr15q.1 (Fig.1).

HER2 gene is located on the long arm of chromosome 17 "red bar", upQMPSF for HER2 primer set contains four amplified products, represent HER2 exons (5, 17, 18 and 23) and three internal controls (17p, 17q "red bars" and Rad51 on chr15), HER2 exon numbers and controls are indicated on the top of the peaks.

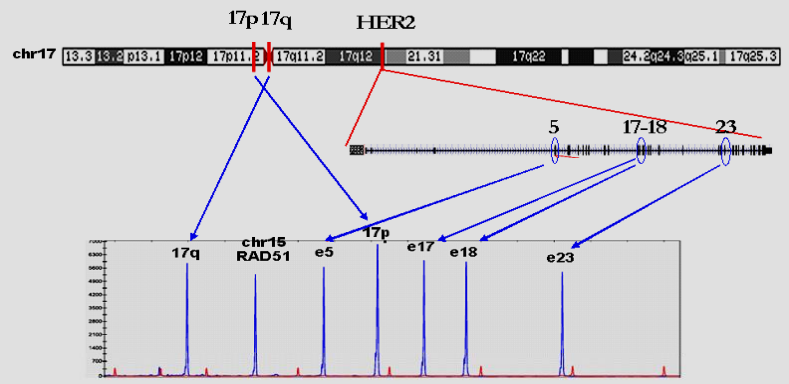

Fig1. *Schematic representation for upQMPSF kit for HER2 amplification detection:*

These internal controls were added to distinguish among copy number changes in regions local to *HER2* gene, involving 17q, and the entire chromosome 17, which were suggested to have different implications in clinical diagnosis of *HER2* positive breast cancer patients. Seven target regions in *HER2* multiplex set were designed to have a product size from 100-300bp to allow use of FFPE samples. To determine the copy number of *HER2* using upQMPSF, we calculated the ratios (R) of the *HER2* peaks vs. the internal control peaks for all three different internal controls in both the tumor and its matching normal samples (Rt and Rn, respectively). A normalized ratio (NR) of Rt/Rn is then calculated as the final upQMPSF score (Table.2).

Table2. *HER2 amplification detection by FISH and upQMPSF:*

Case#	FISH score	Tumor score (Rt)	Normal score (Rn)	Tumor/Normal (NR)
1	0.77	0.73	1.17	0.62
2	0.87	0.66	0.77	0.85
3	0.9	1.25	1.47	0.85
4	0.9	1.01	0.95	1.06
5	0.94	1.4	1.15	1.21
6	1	1.24	1.19	1.04
7	1.29	1.18	1.11	1.06
8	1.3	0.96	0.92	1.04
9	1.4	1.22	0.9	1.35
10	1.5	1.01	0.85	1.18
11	1.5	1.06	0.8	1.32
12	1.5	1.14	0.93	1.22
13	**3.6**	**1.27**	**0.83**	**1.53**
14	4	0.94	0.96	0.97
15	**8**	**2.38**	**0.63**	**3.77**
16	**10**	**5.32**	**1.29**	**4.13**

Bolded samples represent HER2 amplified cases in both (FISH and upQMPSF) techniques, however, case#14 shows disagreement with FISH (highlighted in gray), which is shown as amplified by FISH with a score of 4 and unamplified by upQMPSF with a normalized ratio (NR=Rt/Rn of 0.97).

We expect that the NR values will be around 1 for samples with normal copy number of *HER2*. For example, Tumor samples with extra *HER2* copies will have a score of 1.35 or higher, and

the NR values obtained using different internal controls will inform us about the scope of the amplification.

PCR amplification was performed in two consecutive PCR runs (R1 and R2). In R1, a total volume of 25 ul primer mix (1.5 ul of low concentrated chimerical primers mix, 2.5 ul 10x PCR buffer II, and 0.5 ul *Taq* DNA polymerase) were added to 100-150ng of genomic DNA. Two cycles of PCR amplification were performed under the following conditions: 95°C for 4 m, 94°C for 1 m, 58°C for 1 m, and elongation at 68°C for 1 m. then, 8 cycles of amplification at 62°C for 1 m and final extension at 68°C for 5 m were performed. In R2, 1.75 ul aliquot of R1 products were added to 3.75 ul of high concentrated universal primers (fluorescent forward primer) for a total mixture of 25 ul. The mixer were subjected to 18 amplification cycles, which is empirically chosen to achieve amplification within the linear amplification range. R2 conditions are set as following: 95°C for 4 m, 94°C for 1 m, 48°C for 1 m, 68°C for 1 m and final extension at 68°C for 45 m to reduce adenylation effect, as described by (Azrak 2015). upQMPSF primers sequences were synthesized and purchased from Integrated DNA Technology (IDT), USA.

DNA Fragment Analysis

Fluorescently labeled PCR products from upQMPSF were analyzed on ABI PRISM 3130xl (Applied Biosystem, USA) as per a fragment analysis protocol. 1 ul of PCR product loaded with 10 ul of formamide and 1 ul size standard (Rox500, Applied Biosystem, USA) in capillary plate. Fragements size, fluorescent intensity (peak height), and area size were collected and visualized using GeneMapper software. Downstream quantitative data analysis was performed for calculating the upQMPSF score that represents the normalized ratio (NR).

RESULTS

upQMPSF and HER2 Evaluation

Sixteen genomic DNA samples obtained from breast cancer patients with matching normal breast tissue were analyzed for the detection of *HER2* gene amplification using upQMPSF multiplex set. The presence of *HER2* gene amplification in tumor was clearly indicated by significantly higher upQMPSF score (Fig. 2).

As shown in Table 2, using a upQMPSF score of > 1.35 as a standardized upQMPSF chosen cutoff value for exon copy number gain, as reported by (Azrak 2015). upQMPSF profile in breast cancer patient sample with HER2 amplification. HER2 exons indicated by red arrows, (C1, C2, and C3) are the internal controls (17q, Rad51 and 17p) respectively. Normal sample HER2- (panel A), HER2+ tumor sample (panel B). upQMPSF clearly detected HER2 amplification in tumor in comparison to normal sample.

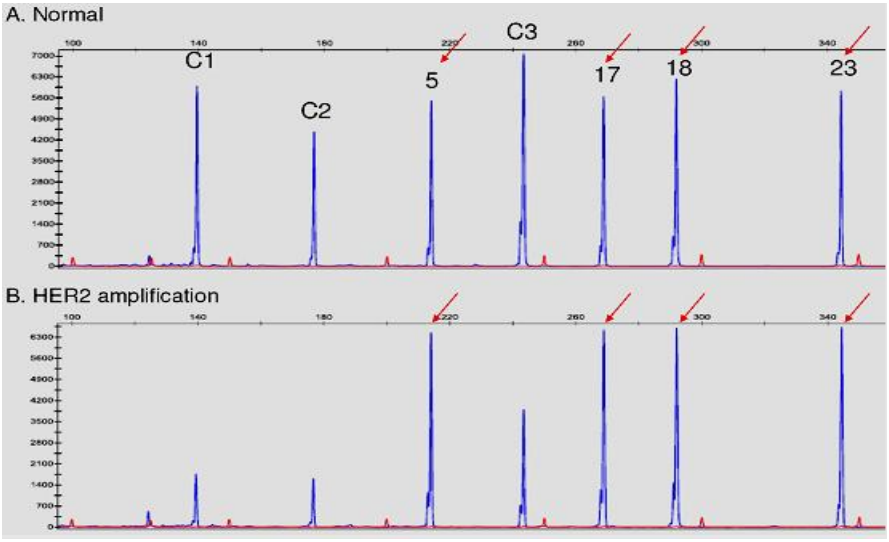

Fig2. *Detection of HER2 amplification using upQMPSF:*

upQMPSF vs Fish

upQMPSF results demonstrated a good overall agreement with the FISH score (15/16 or 93.8%). As documented by FISH, *HER2* amplified cases # 13, 15 and 16 were also found to be amplified by upQMPSF. The only discrepancy between the two methods is in case #14 (Table.2). This case was classified as *HER2* amplification by FISH (no data for IHC) but not

by upQMPSF. In other representation of *HER2* copy number analysis using FISH and upQMPSF scores. FISH with a score of > 2+ for *HER2* amplification has detected four breast cancer patient samples, in contrast, upQMPSF score of 1.35 as a cutoff value has detected only three samples with *HER2* positive using same sample set (Fig.3). This disagreement may be attributed to either a false positive result by FISH or by a high level (>50%) of normal tissue contamination that produced a false negative outcome by upQMPSF. All other breast cancer cases # (1 to 12) that show negative *HER2* amplification based on FISH have also showed normal upQMPSF scores.

(Panel: A) FISH with a score of > 2+ for *HER2* amplification has detected four breast cancer patient samples, in contrast (Panel: B) upQMPSF score =1.35 as a cutoff value has detected only three samples with *HER2* positive.

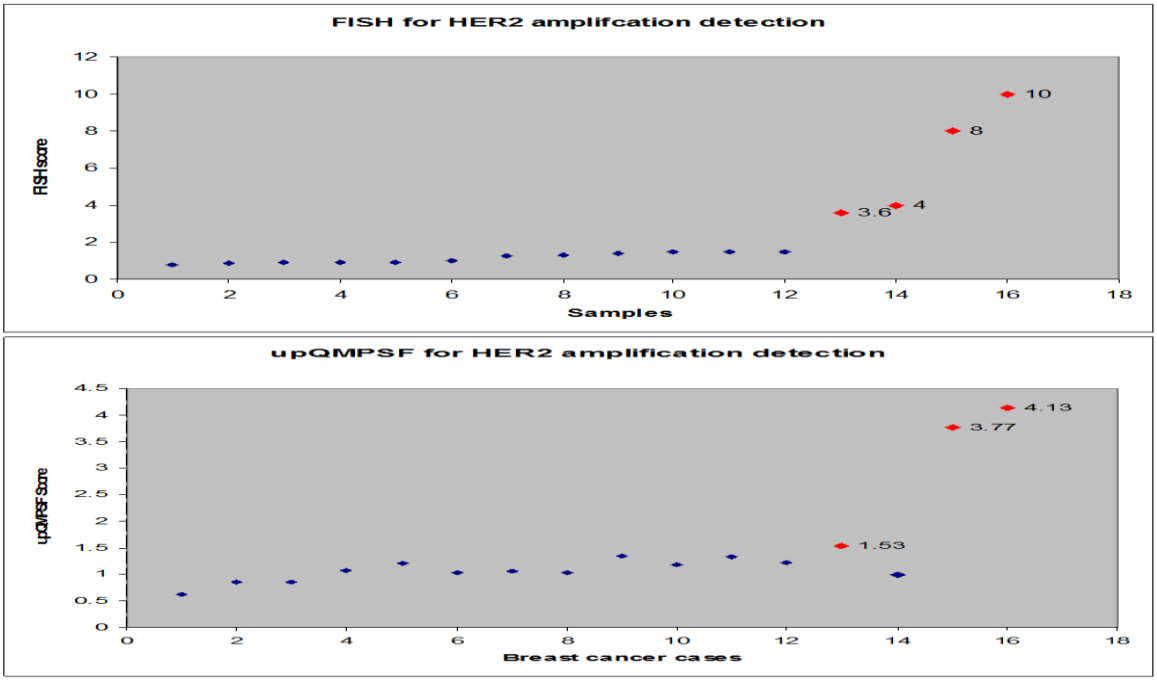

Fig3. *HER2 copy number analysis by FISH and upQMPSF scores*

upQMPSF replicates showed consistent pattern for all tested cases. The result also showed No cases of chromosome17 polysomy were detected in tested cases. The assay was also demonstrated sensitivity and reproducibility in detecting HER2 gene amplification using FFPE samples.

DISCUSSION

IHC and FISH considered the state of the art methods for the detection of *HER2* gene amplification as a prognostic and therapeutic biomarker for breast cancer, however, previous studies demonstrated disagreement between both methods, thus no gold standard method is currently available for this evaluation. On the other hand, PCR-based technologies were previously reported with potential efficacy in detecting such amplification. Therefore, in this study, PCR based technique named upQMPSF was carefully designed to address the limitations of other PCR-based methodologies through increasing the multiplexing capacity by including four *HER2* gene regions and three internal controls in the set, in order to enhance the efficacy of determining *HER2* gene status and chr17 polysomy, previous studies reported Chromosome 17 polysomy in cases with borderline or low *HER2* protein concentrations. Therefore, chromosome 17 polysomy alone may not significantly contribute to *HER2* gene copy number and *HER2* protein overexpression (Liu et al. 2014). In addition, there is not enough clinical data regarding the benefit of trastuzumab therapy in patients with chromosome 17 polysomy (Barberis et al. 2008). Screening sixteen breast cancer patient samples, upQMPSF results demonstrated efficiency in detecting *HER2* gene amplifications in three samples. Moreover, upQMPSF showed disagreement with FISH in one sample. Therefore, upQMPSF offers several advantages over (FISH and IHC) minimal intra-sample and intra-observer variations; 2) quick and easy to use by standardizing the PCR protocol; 3) cost-effective; 4) automatable; and 5) high

sensitivity (requiring as little as 25ng of DNA). The method is also usable with fine-needle biopsy and formalin-fixed paraffin embedded specimens archived samples.

CONCLUSIONS

upQMPSF method may present a significant value in breast cancer treatment as an independent method for validating the FISH and IHC results or even a method for replacing these methods to achieve improved accuracy and quicker turn-around time for the detection of *HER2* gene amplification. Certainly, more extensive comparative analysis with more clinical samples is required in order to fully validate the utility of upQMPSF in clinical test of *HER2* gene amplification, particularly for samples scored in borderline range.

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