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Direct resequencing of the complete *ERBB2* coding sequence reveals an absence of activating mutations in *ERBB2* amplified breast cancer.

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Abstract

Gene amplification is among the most common cytogenetic abnormalities that cause cancer. One of the most clinically important gene amplifications in human cancer causes extensive reduplication of *ERBB2* (*ERBB2*). A variety of cancers also occasionally harbor somatic mutations in *ERBB2*. Gene amplification and activating mutations both have predictive value for clinical response to targeted inhibitors. Since the number of gene copies in an amplicon may exceed 100, and since amplicons may encompass multiple genes, high resolution analysis of gene amplifications poses considerable technical challenges. We have overcome this obstacle by using emulsion-based resequencing to determine the sequence of many independently-amplified individual DNA molecules in parallel. We used this high throughput sequencing technology to analyze *ERBB2* mutational status in five *ERBB2* amplified cell lines and two tumors. Genomic DNA was isolated and the 28 exons of *ERBB2* were independently amplified. Amplicons were then pooled at equimolar ratios, subjected to emulsion PCR (emPCR) and finally to picotiter plate pyrosequencing. High quality sequence data was obtained for all amplicons analyzed and no activating mutations within *ERBB2* were identified. Although we did not find activating mutations within the multiple copies of *ERBB2* in these samples, the results establish the utility of this technology as a feasible and cost-effective approach for high resolution analysis of amplified genes.

Keywords

ERBB2 amplified breast cancer; mutation; emulsion PCR; picotiter plate pyrosequencing

INTRODUCTION

Receptor tyrosine kinases (RTK's) function at the apex of signaling cascades that regulate cell survival and cell growth. Dysregulation of RTK signaling commonly causes initiation and/or progression of tumors, and RTKs are important targets for cancer therapy (Stern 2000; Adjei and Hidalgo 2005). RTKs can be activated in cancer by autocrine or paracrine production of their growth factor agonists, and by dysregulation in splice site selection or posttranslational processing. Protein structure changes are also caused by missense and nonsense mutations, and small insertions and deletions (indels). Hotspots for mutations in RTKs include the juxtamembrane, transmembrane, and catalytic domains. Often, RTKs are activated by chromosomal aberrations including gene amplification, resulting in excessive gene copy number and high level protein expression, or translocations that induce gene fusions leading to abnormal regulation or formation of abnormal proteins.

Aside from their importance in cancer causation, RTK mutations are increasingly important in prediction of response to RTK-targeted drugs. Triage of tumors for the presence of *BCR-Abi* fusion, *KIT* mutations, or *ERBB2* amplification has been important in selection of patients who respond to Imatinib (Gleevec) or Trastuzumab (Herceptin) (Giaccone and Rodriguez 2005; Meric-Bernstam and Hung 2006). However, prediction of response to tyrosine kinase inhibitors (TKI's) is complex. For example, although mutation status of the epidermal growth factor receptor (EGFR) has predictive value for clinical response to the EGFR inhibitors gefinitib (Iressa) and erlotinib (Tarceva) (Lynch et al. 2004; Paez et al. 2004; Pao et al. 2004; Bell et al. 2005; Kobayashi et al. 2005; Thomas et al. 2005), EGFR mutations do not absolutely predict response, and not all responders have mutations (Giaccone and Rodriguez 2005).

ERBB2 (neu; ErbB2) is a member of the Epidermal Growth Factor receptor (EGFR) (ErbB1) family of RTKs, which also includes genes encoding the EGFR, ERBB3, and ERBB4. Ligand binding to these receptors induces receptor homodimerization and heterodimerization with other members of the family, phosphorylation, and activation of numerous and diverse downstream signaling cascades. Although no ligand for ERBB2 has been identified, it is the preferred heterodimerization partner for the other ErbBs once they are activated by ligand binding (Yarden and Sliwkowski 2001). ERBB2 is overexpressed and occasionally mutated in a variety of human tumors, including breast, ovary, lung, gastric, and oral cancers. *ERBB2* is amplified in approximately twenty-five percent of breast cancers, and this amplification is associated with poor prognosis (Slamon et al. 1987; Slamon et al. 1989; Stern 2000). The humanized monoclonal antibody, Trastuzumab (Herceptin), binds the extracellular domain of ERBB2 and is used for the treatment of women with breast cancer judged "ERBB2-positive" by fluorescence in situ hybridization (to detect high gene copy number) or immunohistochemistry (to detect high ERBB2 protein expression) (Meric-Bernstam and Hung 2006; Nahta and Esteva 2006). Recently it has been reported that a variety of cancers also occasionally harbor somatic mutations in *ERBB2* that might contribute to the development of cancer (Stephens et al. 2004; Shigematsu et al. 2005; Bekaii-Saab et al. 2006; Buttitta et al. 2006; Lee et al. 2006).

Although HER-2 positivity is important in identification of women who may benefit from trastuzumab, only a subset of these women respond to treatment (Meric-Bernstam and Hung 2006; Nahta and Esteva 2006). And, we have determined that there is considerably heterogeneity in signaling activity of *ERBB2* in HER-2-positive tumors measured with a monoclonal antibody that recognizes *ERBB2* phosphorylation at Tyr1248 (Thor et al. 2000). One possible explanation is that the copies of *ERBB2* in amplified tumors are themselves heterogeneous, since the high gene copy number provides fertile ground for development of further genetic alterations. Thus, it has not been determined whether any of the numerous amplified copies of the *ERBB2* gene in a single tumor also harbor dominant mutations that would affect *ERBB2* activity and predict clinical response to TKI's. Previously, the technical challenge in answering this question was the extensive reduplication of *ERBB2*, with as many as 20 or more gene copies per tumor (Slamon et al. 1987; Kallioniemi et al. 1992). If only a subpopulation, possibly even a single copy of *ERBB2* in the amplified set is mutated, such mutations would be difficult to detect without prior knowledge of the nature of the mutations. With *ERBB2* copy numbers in some tumors rising above twenty to fifty, the frequency of minor alleles could be five percent or lower, well below the threshold for detection by conventional chain termination resequencing methods (Sanger et al. 1977). Heteroduplex and conformational-shift methods, which are useful for identification of minor DNA sequence variants, are cumbersome and do not detect all mutations (Cotton RGH 1998; van Ommen et al. 1999). One approach to circumventing these problems is the use of high-throughput DNA sequencing methodology, which facilitates detection of minor alleles in a population by parallel resequencing of numerous individually-sampled DNA molecules (Thomas et al. 2005; Thomas et al. 2006). In this study we have searched for *ERBB2* mutations in genomic DNA isolated from four breast cancer cell lines (BT474, SKBR3, MDA-MB345 and MDA-MB461 (Kraus et al. 1987; Kallioniemi et al. 1992)), and one ovarian cancer cell line (SKOV3 (King BL 1992)), all known to have amplified *ERBB2*, by emulsion polymerase chain reaction (PCR) based sequencing.

METHODS

Cell lines and tissue samples.

BT474, SKBR3, SKOV3, MDAMB361 and MDAMB453 cell lines were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, sodium pyruvate, L-glutamine and penicillin-streptomycin in a 5% CO₂ 37° incubator. *ERBB2*-positive formaldehyde-fixed paraffin-embedded sections of (FFPE) breast tumors were obtained from Critical Technologies for Tissues Services of the Yale Cancer Center.

Isolation of DNA.

DNA was isolated from cells grown in culture or from FFPE tumor specimens using the QIAmp tissue extraction kit (Qiagen) according to the manufacturers' instructions.

Polymerase Chain Reaction.

Genomic DNA from the five cell lines and two tumors were amplified with 29 primer pairs covering exons 1–27, including the 3'UTR. All PCR reactions were set up in a sterile laminar flow hood using dedicated instruments. ~50 ng template DNA was mixed with 10

uM dNTP's, 20 nM each primer, 5% PCR grade DMSO (Pierce), Pfu Ultra II Fusion HS DNA polymerase (Stratagene) and 1X Pfu buffer and brought up to 50 uL with ultra pure molecular biology grade ddH₂O (Eppendorf). Reactions were denatured at 94° for 3 minutes, followed by 40 cycles of 94° 1 minute, 59–72° for 45 seconds, and 72° for 1 minute, with a final extension at 72° for 5 minutes. 5 uL of the sample was subjected to agarose gel electrophoresis to check for purity and yield, and the remaining products were purified through a Qiagen min-elute column. If necessary, PCR products were gel extracted before purification to minimize contaminating PCR artifacts. Amplicons were analyzed on a BioAgilent analyzer to check for quality and yield.

Sequencing and data interpretation.

Sequencing was performed as previously described (Margulies et al. 2005). The reference sequence for primer design and data analysis was Ensembl gene ENSG00000141736, transcript variant one. Analysis was performed as described (Margulies et al. 2005; Thomas et al. 2006). Any variations identified were compared to NCBI (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=snp>), and Ensembl (http://www.ensembl.org/Homo_sapiens/genesnpview?db=core;gene=ENSG00000141736) SNP databases. Cutoffs for sequencing quality were 76% at 98% accuracy over 200 bases.

Site directed mutagenesis:

HEX20 (ERBB2 exon 20) G>T: The ERBB2 exon 20 amplicon obtained from BT474 was ligated into the pJet1.2/blunt cloning plasmid (Fermentas). A single base pair substitution (G>T) was introduced by site directed mutagenesis using the primers: 5'-CTGGGCATCTGCCTTACATCCACGGTGCAGCT-3' and 5'-CCAGCTGCACCGTGATGTAAGGCAGATGCCC-3'. HEX20 GT and HEX20 WT were digested out of pJet1.2 with BglII. Digests were purified through a Qiagen column. Concentrations were determined using a DNA 1000 chip (Agilent) and confirmed by OD. HEX20 G>T was serially diluted into HEX20 WT and sequencing was performed as described (above and (Thomas et al. 2006))

pCMV ERBB2 A890S:

A 1.4 kb NdeI-XhoI fragment of pCMV-ERBB2 was PCR amplified and ligated into the pJet1.2/blunt. A single base pair substitution was introduced by site directed mutagenesis using the primers: 5'-CATCAAGTGGATGTCGCTGG and 5'-GAGAATGGACTCCAGCGACATC. Pjet1.2/blunt-ERBB2 G>T was then digested with NdeI and AfeI and the ERBB2 fragment ligated back into a pCMV-ERBB2 expression plasmid and verified by sequencing.

Transient transfections, immunoprecipitations, and in vitro kinase assays:

COS-7 cells were transfected with FuGene (Roche) according to manufacturers instructions. After 48 hours cells were lysed with EBC (50 mM Tris pH 7.4, 120 mM NaCl, 0.5% NP-40) supplemented with Complete protease inhibitor tablet minus EDTA (Roche). Protein levels were normalized using a Bio-rad Dc protein assay reagent. Lysates were immunoprecipitated with Oncogene Science ERBB2 mAb-5 and ImmunoGold protein A/G

(Pierce). IP's were washed with TG buffer (1% Triton X-100, 10% glycerol, plus aprotinin (Sigma) in CMF PBS) and resuspended in ERBB2 Kinase Buffer (40 mM Hepes pH 7.4, 3 mM MnCl₂, 10 mM MgCl₂, 0.05% Triton X-100). rATP (Promega) was added (100 μ M) for the indicated times at room temperature, and reactions were stopped by the addition of sample buffer (62.5 mM Tris pH 6.8, 4% glycerol, 2% SDS, 0.1% bromophenol blue, 4% betamercaptoethanol) and heating to 95°C for 5 min. Immune complexes were separated on a 4–12% bis tris gel (Invitrogen), transferred onto PVDF (Millipore) and blotted with anti-phosphotyrosine antibody 4G10 (Upstate) and then reprobbed with anti-ERBB2 pAb (Santa Cruz).

RESULTS

Genomic DNA was isolated and the 27 exons of *ERBB2* as well as the 3' untranslated region (UTR) were amplified by polymerase chain reaction (PCR). Additionally, to identify whether this technology might be beneficial in the clinical setting where mutational analysis might help in determining treatment options, two formalin-fixed paraffin-embedded (FFPE) *ERBB2*⁺ breast cancer tumor specimens were analyzed. These blocks had a low tissue content but we were able to obtain enough DNA to generate sufficiently pure amplicons to resequence all but three exons which code for portions of the protein that lie within the cytoplasmic domain (exons 1, 13 and 15).

PCR primer pairs are listed in Supplementary Table 1. Primers were designed against intronic sequences to generate amplicons averaging 220 bp in length, including the anchor sequences on both forward and reverse primers which are required for the 454 sequencing platform. Amplicons were pooled at equimolar ratios and subjected to emulsion-based PCR and picotiter plate pyrosequencing (Thomas et al. 2006).

To establish the limits of sensitivity for detecting single base pair variations, we introduced a single base pair substitution into a *ERBB2* exon 20 (HEX20) amplicon. The HEX20 GT was serially diluted into HEX20 WT and these four-fold dilutions were subjected to emulsion-based PCR and picotiter plate pyrosequencing (Thomas et al. 2006) (Figure 1). High coverage sequencing reads were obtained (35,000–46,000 reads) and the observed frequency of the single base pair substitution was plotted against the relative degree of dilution. A linear correlation was observed between measured and expected variation frequencies down to allele frequencies below 0.08% ($R^2 = 0.9984$).

Sequence variants were considered valid when they appeared in sequencing reads in both the forward and reverse direction at a minimum frequency of greater than 0.04% (Thomas et al. 2006). With only a few exceptions, a minimum of 1000 high quality reads was obtained from each cell line for each exon, with an average of 9445 reads per exon (Supplementary Table 2). 76% of reads achieved 98% accuracy over 200 bases. A complete list of variations identified is summarized in Table 1. MDAMB453 cells and Tumor A had an A>G substitution within exon 17 that results in a well-known Ile655Val polymorphism within the *ERBB2* juxtamembrane domain (Papewalis et al. 1991; Fleishman et al. 2002). We identified a variation within exon 7 of SKOV3 cells which would result in a Cys295Tyr substitution. Exon 7 encodes a region within the extracellular domain between the two

ligand binding domains. MDA MB361 cells showed a low frequency deletion within exon 5 that would result in the expression of a truncated protein predicted to terminate at aa198, within the extracellular domain I. With this small sample size and without matched normal tissue it is not possible to distinguish SNPs from de novo mutations, however, to our knowledge, none of these variants has previously been reported. All of these variations occurred outside of the catalytic domain. However, of particular interest was one low frequency substitution within exon 22 of Tumor A. This G25345T substitution results in an Ala890Ser substitution. This residue falls within the conserved WMALE tyrosine kinase signature motif. To determine whether this substitution has any effect on kinase activity, an expression construct harboring this single point mutation was generated and expressed in COS-7 cells. In vitro immune complex kinase assays on the altered protein show that the A890S substitution reduces kinase activity compared to the wild type ERBB2 (Figure 2). Moreover, we were unable to reliably reproduce this sequencing result. Although the variation was found in two separate sequencing runs with the same amplicon, resequencing of an independently PCR amplified amplicon failed to identify this variant, suggesting that the variant was correctly identified in the sequencing reaction but that it arose as a result of PCR polymerase infidelity or emulsion PCR artifact.

In addition, since we used intronic sequencing primers, we identified one previously reported intronic single nucleotide polymorphism (SNP) between exons 17 and 18 (G23509A; rs903506) in MDA-MB362, MDA-MB453 and SKOV3 cells, and two novel intronic variants, or possibly rare SNPs; one C-G substitution between exons 5 and 6 in MDA-MB361 cells (C10068G), and one C-A substitution between exons 24 and 25 in Tumor A (C26688A).

DISCUSSION

Previous studies have searched for *ERBB2* mutations in breast tumors and cell lines either using a candidate mutation approach, resequencing of pooled amplicons, or resequencing of individual *ERBB2* alleles from tumors with gene amplification. For example, Stephens *et al* resequenced the complete *ERBB2* coding sequence in 173 tumors, 18 of which were breast, and the kinase domain in 303 cancers (56 breast) and 235 cell lines (9 breast, none of those sequenced in this report). No mutations were found in any of the breast tumors or cell lines, and amplification status in the breast tumors and cell lines was not indicated (Stephens *et al*. 2004). Recently, Lee *et al* used single stranded conformation polymorphism (SSCP) to look for mutations in exons 18–23, which encode for the ERBB2 kinase domain, in 378 tumors, 94 of which were breast tumors. While they did find mutations in 4 of the breast tumors, they reported that none of these four tumors had amplified *ERBB2* (Lee *et al*. 2006). Given that others have found low frequency mutations in ERBB2 in a variety of tumor specimens (Stephens *et al*. 2004; Shigematsu *et al*. 2005; Bekaii-Saab *et al*. 2006; Buttitta *et al*. 2006; Lee *et al*. 2006), a larger study of *ERBB2* mutational status in ERBB2 positive fresh tumor specimens could be valuable, and is feasible using this approach.

Whether activating mutations would predict response to Herceptin or TKIs in development remains to be determined. Based on our results with this limited number of tumors and cell lines, it would seem that amplification and mutation are mutually exclusive events and a

failure to respond initially to Herceptin cannot be explained by the presence of mutated active protein. Acquired Herceptin resistance may occur by mutation, and can be studied using similar techniques. Although we didn't find activating mutations in the *ERBB2* gene in this report, mutations have been reported that could affect ERBB2 kinase activity and as such further studies into *ERBB2* mutational status and sensitivity to inhibition by targeted inhibitors in *ERBB2* positive tumors would be beneficial.

We believe this approach to be an effective and reliable new method for identifying mutations in target genes. In addition to the fact that this technology is extremely sensitive, there are several other advantages over conventional mutation detection techniques. The foremost is that many of these classic technologies (SSCP, heteroduplex analyses, chemical or enzymatic cleavage of mismatch, and hybridization with sequence specific oligonucleotide probes (SSOP)) do not identify the mutation, but only provide evidence for the existence of a mutation (Cotton RGH 1998). Ultimately, direct sequencing is still required, and the older methodologies can be considered as a pre-screen for mutation identification. Since most of these classical techniques begin with PCR amplification, the direct resequencing approach effectively eliminates the middle step. Additionally, with direct sequencing the number of mutations that can be detected within a gene is unlimited, and there are no restrictions as to the type of mutation that can be detected, whereas many of the earlier screening methods are unable to detect all categories of nucleotide substitutions and indels. One exception is for the detection of variations following homopolymer stretches of >7 nucleotides, but these occur very rarely within the genome, and not at all within the *ERBB2* coding sequence. Another exception would be the detection of large genomic rearrangements, for example deletions of entire exons leading to the EGFR variant III mutation (Pedersen et al. 2001). Furthermore, while classical mutation detection techniques are relatively inexpensive, optimization of the experimental conditions and interpretation of the data can be problematic and require some experience. Finally, in clinical specimens with small tumor loads, it is possible to obtain enough starting material for direct resequencing using this approach.

In summary, although it is well established that *ERBB2* gene amplification contributes significantly to tumor development, it has also become clear that in some cancers low frequency activation mutations might contribute to tumorigenesis as well. Although in our samples *ERBB2* gene amplification and mutation were mutually exclusive, dominant mutations within the amplicon may well be important, either in tumor initiation, progression, or development of Herceptin resistance. The techniques presented here outline a new approach that could be very useful in future studies on RTK mutational analyses in tumor development and in response to targeted therapy, and, more generally, for analysis by resequencing of gene amplicons.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1: Sensitivity of detection of single base pair variations. HEX20 GT containing a single base pair substitution was serially diluted into HEX20 WT and subjected to sequencing. Inset shows a magnification of the lower part of the curve.

Figure 2. ERBB2 A890S results in a decrease in ERBB2 kinase activity. pCMV ERBB2 Vector, Wild type, or A890S was expressed in COS-7 cells. ERBB2 was immunoprecipitated and immune complexes subjected to in vitro kinase assays. ERBB2 kinase activity was measured by anti-phosphotyrosine western blot. Blots were reprobed for ERBB2.

Table 1:

Variations identified within Her2 coding region.

Exon	Type	Mutation	Frequency	# Reads	Alt. Residues	cell/tumor :
5	deletion	TC	3.1%	10070	M198X	MDAMB361
7	non-syn. Sub.	G/A	3.2%	6318	C295Y	SKOV3
17	non-syn. Sub.	A/G	80%	38483	I655V	MDAMB453
17	non-syn. Sub.	A/G	80%	2880	I655V	Tumor A
22	non-syn. Sub.	G/T	1%	2148	A890S	Tumor A