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Quantifying Insulin Receptor Isoform Expression in FFPE Breast Tumors

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Abstract

Background—The development of predictive biomarkers for IGF targeted anti-cancer therapeutics remains a critical unmet need. The insulin receptor A isoform (InsR-A) has been identified as a possible biomarker candidate but quantification of InsR-A in widely available formalin fixed paraffin embedded (FFPE) tissues is complicated by its similarities with the metabolic signaling insulin receptor isoform B (InsR-B). In the present study, qPCR based assays specific for InsR-A, InsR-B and IGF-1R were developed for use in FFPE tissues and tested for feasible use in clinical archived FFPE estrogen receptor (ER) + and ER– breast cancer tumors.

Design—FFPE compatible primer sets were designed with amplicon sizes of less than 60 base pairs and validated for target specificity, assay repeatability and amplification efficiency. FFPE tumors from ER+ (n=83) and ER– (n=64) primary untreated breast cancers, and ER+ hormone refractory (HR ER+) (n=61) breast cancers were identified for feasibility testing. The feasible use of InsR-A and InsR-B qPCRs were tested using all tumor groups and the feasibility of IGF-1R qPCR was determined using HR ER+ tumors.

Results—All qPCR assays were highly reproducible with amplification efficiencies between 96–104% over a 6 log range with limits of detection of 4 or 5 copies per reaction. Greater than 90% of samples were successfully amplified using InsR-A, InsR-B or IGF-1R qPCR primer sets and greater than 88% of samples tested amplified both InsR isoforms or both isoforms and IGF-1R. InsR-A was the predominant isoform in 82% ER+, 68% ER– and 100% HR ER+ breast cancer. Exploratory analyses demonstrated significantly more InsR-A expression in ER+ and HR ER+ groups compared to InsR-B (ER+ p< 0.05, HR ER+ p< 0.0005) and both groups had greater InsR-A expression when compared to ER– tumors (ER+ p< 0.0005, HR ER+ p< 0.05). IGF-1R expression of HR ER+ tumors was lower than InsR-A (p<0.0005) but higher than InsR-B (p<0.0005). The InsR-B expression of HR ER+ tumors was significantly reduced compared other

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tumor subgroups (ER+ and ER-, $p < 0.0005$) and lead to a significant elevation of HR ER+ InsR-A:InsR-B ratios (ER+ and ER-, $p < 0.0005$).

Conclusions—The validated, highly sensitive InsR-A and InsR-B qPCR based assays presented here are the first to demonstrate the feasible amplification of InsR isoforms in FFPE tissues. Quantification data generated from this feasibility study indicating InsR-A is more predominant than InsR-B in breast cancer support the use of these assays for further investigation of InsR-A and InsR-B as predictive biomarkers for IGF targeted therapeutics.

Keywords

Real-time; qPCR; breast cancer; receptor; insulin; receptor; IGF Type 1; monoclonal antibodies; therapeutic use; protein kinase inhibitors

INTRODUCTION

The insulin receptor (InsR) is a transmembrane protein composed of 2 covalently bound dimers each with a ligand binding α and tyrosine kinase active β subunit¹. The InsR dimer is expressed as an A (InsR-A) or B (InsR-B) isoform through alternate splicing of the insulin receptor gene's exon 11 and assembled post translationally as a functional homodimeric (A/A, B/B) or heterodimeric receptor (A/B)². As a homodimer, the full length isoform, InsR-B (+exon 11), binds insulin to maintain glucose metabolism and transport but can induce proliferative and pro-survival signaling in hyperinsulinemic conditions^{3–6}. The shorter InsR-A (–exon 11) isoform is truncated by 12 amino acid residues (717–728) near the c-terminus of the α subunit and receptor homo/heterodimers are activated by IGF-II to initiate IRS mediated proliferative, pro-survival, and metastatic signaling (PI3K and MAPK pathways)^{1,2,7}. However, understanding InsR component of IGF signaling is further complicated by the heterodimerization of InsR with the type-1 insulin-like growth factor receptor (IGF-1R) which form functional IGF-I and IGF-II responsive receptor hybrids (IGF-IR/InsR-A and InsR-A/InsR-B)⁸.

The predominance of IGF signaling in cancer has driven the development of IGF targeted anti-cancer therapeutics^{9,10}. In breast cancer, primary and secondary resistance to hormone and HER2 targeted therapies has led to the co-targeting of these pathways with the IGF signaling pathway^{8,9,11–15}. The most developed IGF targeted therapies employ monoclonal antibodies (mAb) to block IGF-IR, mediated IGF-I and IGF-II signaling, and induce the internalization/degradation of IGF-1R protein^{9,10,16,17}. However, IGF-II signaling is only partially blocked by mAb inhibitors due to its high affinity for both IGF-1R and InsR-A^{9,11,17,18}. Phase I clinical trials with these inhibitors have demonstrated tolerability and preliminary evidence of activity, but the metabolic consequences of these therapies, hyperglycemia, hyperinsulinemia, and elevated levels of growth hormone, can incite upregulation of IGF-II/InsR-A signaling as one mechanism of therapy resistance^{11,19–21}. As such, InsR-A has emerged as one possible biomarker for both the selection and monitoring of IGF-1R targetable patient populations and provides a rationale for co-targeting InsR-A^{4,10,20,22–28}. The more recently developed small-molecule IGF-1/insulin tyrosine kinase inhibitors (TKI) and dual IGF-I/IGF-II ligand targeting antibody therapies have a more comprehensive IGF targeting design and may prove to be more optimal in blocking IGF signaling if an IGF-II/InsR-A tumor proliferation and survival signaling component proves to be significant^{29,30}.

Functional InsR/IGF-1R hybrid receptors have been described in breast and many types of cancer⁵. In breast cancer, immunohistochemical studies measuring total InsR protein expression have suggested a correlation between elevated InsR expression and positive outcomes in primary and node negative cancers, and poor outcomes in advanced cancers,

but it is uncertain whether IGF signaling measured in these studies is predominantly driven by InsR or IGF-1R as immunohistochemical reagents capable of differentiating InsR-A from InsR-B proteins have not been developed^{18,24,25,31}. Alternative methods for the detection of InsR-A and InsR-B based on quantitative and qualitative PCR have been developed to address this need, but these assays rely upon the availability of fresh frozen or RNA preserved tissues and have not been adopted for testing in the clinical setting^{7,32-34}. In the clinic, diagnostic tissues are universally preserved through the FFPE process, but this type of tissue preservation can present significant compatibility issues with qPCR based assays. During formalin fixation process, variations in sample size, permeability, and type can result in variable levels of RNA-protein cross-linkage, RNA base modification, and RNA fragmentation which can be further exacerbated by archival storage³⁵⁻³⁷. Specialized kits for FFPE RNA isolation can reverse some of the fixation induced damage but the residual damage can strongly inhibit reverse transcription and result in variable lengths of transcribed cDNA³⁵. Under these fragmented cDNA conditions the amplicon size of a primer set is critical for successful amplification and primer set amplicons of 60 base pairs have an 80% success rate in FFPE cDNA³⁵. InsR isoform specific qPCR assays compatible with FFPE sample types would aid in defining IGF signaling by significantly broadening the number of samples available and enabling the correlation of results with cancer pathology, but previously published InsR primer sets use larger amplicons and limit their reliable use to non-FFPE sample types^{35,38}.

To address this need, qPCR InsR-A, InsR-B, and IGF-1R assays with primer designs compatible with FFPE, RNA-preserved, and frozen sample types were developed³⁹. Each assay was validated using established minimum information for publication of quantitative real-time PCR experiments (MIEQ) guidelines⁴⁰. The feasibility of measuring target gene expression was tested using clinically archived estrogen receptor positive (ER+) and negative (ER-) primary untreated breast tumors, and ER+ hormone refractory tumors (HR ER+) breast tumors.

MATERIALS AND METHODS

Primer design and standard template generation

GenBank accession numbers NM_001079817 (InsR-A), NM_000208 (InsR-B), and NM_000875 (IGF-1R) were used to identify target specific primer sets. To insure FFPE sample compatibility InsR isoform and IGF-1R specific primers having amplicons of 50 base pairs (InsR-A: F- GTTTTCGTCCCCAGGCCATC, R- CCAACATCGCCAAGGGACCT³⁹), 42 base pairs (InsR-B: F- CACTGGTGCCGAGGACCCTA, R-GACCTGCGTTTCCGAGATGG³⁹), and 48 base pairs (IGF-1R: F- GAGCAGCTAGAAGGGAATTAC, R- AAGTTCTGGTTGTCGAGGA) were designed using Oligo 6 Primer Analysis Software (Molecular Biology Insights, Inc. Cascade, CO.), and checked for specificity using Primer-Blast (www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHomeAd). Primer sets were synthesized at Mayo Foundation's Advanced Genomics Technology Center (AGTC) (Rochester, MN) and the identity of PCR products from each primer set were confirmed by sequencing (AGTC).

IGF-1R standard template was produced by PCR cloning and purified by agarose gel electrophoresis. InsR isoform specific standard templates were identified from homologous regions of InsR-A (404bp) and -B (440bp) using Oligo 6 Primer Analysis Software. Isoform specific sequences were synthesized and individually cloned into pUCminusMCS plasmids by Blue Heron Biotechnology (Bothell, WA.) (Figure 1A-B). Sequence identity and cloning were validated by Blue Heron and resulting plasmids were supplied in purified form. Log₁₀ dilutions of each assay standard were made in 0.01M Tris-HCL pH 8.0 using the molecular weight and OD₂₆₀ readings. Each standard was amplified by PCR across a 5 log₁₀ range of

input and visually examined by agarose gel electrophoresis to confirm reactions produced a single product of the expected molecular weight (Figure 1A–B, IGF-1R not shown).

InsR and IGF-1R qPCR assay validation

All qPCR validation and breast cancer sample reactions were assembled in triplicate using ABI SybrGreen PCR core reagents kit (Applied Biosystems, Carlsbad, CA) and run on ABI 7900HT Fast Real-Time PCR System and copy number calculation performed using Sequence Detection Systems software v2.4 (Applied Biosystems). Ten assay replicates were used to determine amplification efficiency, slope, intercept, R squared, intra-assay variation, and overall CV for each qPCR assay between 10^1 and 10^6 copies per reaction. InsR-A and -B qPCR assay were additionally tested for isoform crossreactivity using between 10^1 and 10^6 copies of opposing isoform plasmid per reaction. Five assay replicates were used to measure assay recovery for all assays. Isoform specific interference was assessed for InsR-A and InsR-B qPCR assays using non-target to target isoform ratios of 1, 10 and 100. Each assays limit of detection (LOD) was tested using 8 assay replicates. Points with detection levels of 10 copies or less were completed using 6 replicates per assay (n=48). The LOD of each assay was determined empirically using the lowest input copy number at which 95% of assay replicates reported target specific melt curves

Breast cancer specimen power analysis and selection

Based on our preliminary data with cell lines and data from the literature, we hypothesize that 75% of breast cancer samples had a predominance ($A/A+B > 0.5$) of InsR-A^{4,41}. Based on this, we determined 200 patients would give a $\pm 6.1\%$ 95% confidence interval around 75%, if this was the true predominance. Thus, if the true predominance of isoform A is 57% or greater, 200 samples would be sufficient to determine if the majority of patients were InsR-A predominant ($p < 0.05$). All samples were collected with approval of Mayo Clinic Institutional Review Board, Rochester MN. 208 breast cancer blocks were identified from patients with 80% or more tumor tissue present in the paraffin archives using the Mayo Clinic Breast Cancer Tissue Registry. A total of 208 (147 primary untreated ER+ [n=83], and ER– [n=64], and 61 HR ER+) breast cancer blocks were identified for this study. All other analyses, including dependence of ER expression, were exploratory.

RNA isolation and generation of cDNA from FFPE samples

ER+ and ER– breast cancer tumor RNA was isolated from 3 tissue core biopsy punches taken from tumor rich areas of FFPE tissue blocks⁴². To preserve the integrity of HR ER+ sample blocks for future studies RNA was isolated from 10–20, 5 μ m sections cut from each block. Total RNA was isolated from FFPE samples using the Qiagen RNeasy FFPE Kit reagents either manually (HR ER+) or with QIAcube instrumentation (ER+ and ER–) (Qiagen, Valencia CA) following vendor's standard protocols. All isolated FFPE RNA was treated with DNase (Qiagen) and re-purified using RNeasy Mini Kit (Qiagen) as described in the manufactures clean-up protocol. RNA concentration and purity were determined spectrophotometrically ($OD_{260/280} > 1.8$) (Nano Drop 2000 spectrophotometer, Thermo Scientific, Waltham MA). Between 300–500ng of FFPE tissue RNA was reverse transcribed using ABI High capacity RNA to cDNA kit (Applied Biosystem, Carlsbad, CA) as described in the manufacturer's instructions and resulting cDNA was diluted 1:5 in molecular grade RNase/DNase free H₂O and stored at –20C prior to use.

InsR qPCR assay of FFPE ER+ and ER– breast cancer samples

FFPE RNA quality cannot be measured directly by fragmentation based methods; therefore, the quality FFPE RNA was assessed within each group post reverse-transcriptionally through ribosomal protein 19 (RPL19) expression. Normalization ratios for each sample

were calculated ($N_{\text{sample}} = \text{RPL19}_{\text{median}}/\text{RPL19}_{\text{sample}}$) and compared to the group mean. Samples with RPL19 normalization ratios greater than +3.5 standard deviations (SD) (approximately 99.9% confidence interval) from the mean were excluded from further analysis. The standard replicates for each qPCR assay were examined for amplification efficiencies between 95–105% and all standards and sample replicate data were analyzed for product specific melt curves. Sample or standard replicates which did not conform to these parameters were removed from the analysis. Gene transcript copy numbers from each conforming replicate were normalized to RNA input and RPL19 gene expression before being reported as copies of target gene per μg RNA.

Sample Analysis and Statistics

Outlier analysis of RPL19 normalization values was completed using Grubbs' sample outlier test (GraphPad online calculator website (<http://graphpad.com/quickcalcs/Grubbs1.cfm>)). Samples reporting measurable values of InsR-A and -B were used for InsR isoform analysis and samples reporting with measurable levels of InsR-A, -B and IGF-1R were used for IGF-1R and InsR comparisons. The following analyses were performed using GraphPad Prism software v5.04 (La Jolla, CA). Differences in assay recovery were tested for significance using the Friedman repeated measures test with Dunns post test. Differences in global isoform expression were tested for significance using the Mann-Whitney U- test and group specific expression differences were tested with the Kruskal-Wallis one-way ANOVA and Dunns Multiple Comparison post test. The number of InsR-A predominant samples were tested using the Wilcoxon Signed Rank Test (H_0 InsR-A predominance = 50%).

RESULTS

FFPE qPCR primer design

The InsR isoforms have nearly identical nucleic acid sequences which can be differentiated by the presence or absence of the 36 bp region encoding exon 11. InsR-A and InsR-B primer sets were designed to amplify isoform specific areas of 50 bp within the exon 11 region (Figure 1A–B). IGF-1R primers were designed to amplify an IGF-1R specific 48 bp region crossing the junction between exons 5 and 6. All primer pairs produced specific single PCR reaction products within a 5- $\log_{10}$ dilution range of standard template input: InsR-A (50 bp, Figure 1A), InsR-B (42 bp, Figure 1B) and IGF-1R (48 bp, data not shown).

qPCR performance

InsR-A, InsR-B and IGF-1R qPCR assays were each reproducible, specific, and highly sensitive over a broad range of input. Results from 10 template standard runs across a minimum 6 log range are summarized in Table 1. The detection limits per reaction were 5 copies for InsR-A and 4 copies for both InsR-B and IGF-1R. Each assay reported amplification efficiencies between 96% and 104% and an intra and overall variation below 5% (Figure 2).

InsR isoform qPCR specificity

To determine the specificity of each qPCR reaction for its target isoform, cross reactivity experiments were performed using InsR-A or -B primer sets using their opposing isoform template. This testing detected some minor cross reactivity of the InsR-A qPCR primers with very high levels InsR-B template ($10^{4.4}$ copies per reaction, Figure 3A). Under these conditions, 0.189% of the total InsR-B template was reported as InsR-A. InsR-B cross reactivity could be eliminated by dilution as input levels of InsR-B less than $10^{4.4}$ copies were not cross reactive. Reciprocal testing of InsR-B qPCR found no cross reactivity with InsR-A template plasmid (Figure 3B).

InsR isoform qPCR interdependency

As it is likely that both InsR-A and InsR-B isoforms will exist together in clinical samples, the recovery of both isoforms was evaluated in the presence of the opposing isoform. Specifically, the recovery of InsR-A was determined in the presence of increasing amounts of InsR-B template (Figure 4A) and InsR-B in the presence of increasing InsR-A template (Figure 4B). Each assay averaged greater than 94% recovery of the target template when tested with opposing isoform to target plasmid ratios of 1, 10, and 100 (Figure 4A–B).

InsR isoform expression in FFPE breast cancer tissues

The FFPE RNA quality of each sample was indirectly evaluated post reverse transcriptionally using RPL19 normalization values. Samples with excessive RNA fragmentation and or residual RNA crosslinking would be expected to report elevated normalization values by under performing in the RPL19 qPCR assay. Statistical analysis of all the samples found 206/208 with RPL19 normalization values within a 99.9% confidence interval of the mean. Two samples, which were minimally amplified by the RPL19 qPCR assay, produced outlying normalization values ($p=2.57 \times 10^{-7}$, $p=1.48 \times 10^{-49}$) consistent with a high level of residual fixation induced RNA damage, and were removed from the analysis.

Automated and manual methods of RNA isolation utilizing the same reagents and workflow generated a similar proportion of samples with detectable InsR-A and -B (automated 127/147, manual 56/61, $p=0.277$). InsR-A and InsR-B were measurable in 93.6% (193/206) and 91.7% (189/206) of samples respectively (Table 1.) and both isoforms were reported in 88.8% (183/206) of samples (Table 2). IGF-1R expression was measurable in 91.8% (56/61) of HR ER+ tumors (Table 1) and all three targets were reported in 88.5% (54/61) of samples tested (Table 2).

Matched InsR-A and -B isoform expression was measured in 74 ER+, 53 ER–, and 56 HR ER+ breast tumors and data are summarized in Table 2. The primary analysis revealed that InsR-A was the predominant isoform in 83% of breast cancer samples (95% CI; 78%, 89%). In ER+, ER– and HR ER+ breast cancer, the InsR-A predominance (95% CI) was 82% (74%, 91%), 68% (55%, 81%) and 100% (n/a, n/a). Additional exploratory analyses were performed, including determination of InsR-A versus -B in all samples ($n=183$), which detected a greater level of InsR-A expression overall compared to -B ($p<0.0001$, Figure 5A). Cumulative isoform expression (InsR-A + InsR-B) was significantly greater in ER+ tumors compared to ER– and HR ER+ groups ($p<0.0005$, Figure 5A). Within tumor groups, InsR-A expression was significantly greater in ER+ and HR ER+ tumors compared to InsR-B (ER+ $p<0.05$, HR ER+ $p<0.0005$, Figure 5B), but no significant differences in isoform expression were detected in ER– tumors. Across tumor groups, ER+ and HR ER+ tumors had similar InsR-A expression and both expressed higher levels InsR-A than ER– tumors (ER+ $p<0.0005$, HR ER+ $p<0.05$, Figure 5B). InsR-B expression was comparable between ER+ and ER– tumor groups but strikingly lower in HR ER+ tumors (ER+ and ER– $p<0.0005$, Figure 5B). This reduction in InsR-B expression resulted in an elevation of InsR-A:InsR-B ratios in HR ER+ tumors by more than a 12-fold compared to other groups ($p<0.0005$, Table 2, Figure 5C). As hormone and IGF-1R targeting therapy combinations have been shown to restore hormone sensitivity in resistant cancer cells^{43,44}, IGF-1R was also explored in HR ER+ tumors. InsR-A, -B, and IGF-1R were measurable in 88.5% (54/61) HR ER+ tumors (Table 2) and IGF-1R expression was significantly greater than InsR-B but lower than InsR-A (InsR-A > IGF-1R > InsR-B, $p<0.0005$, Figure 5D).

DISCUSSION

The development of predictive biomarkers for IGF targeted anti-cancer therapeutics remains a critical unmet need. The InsR-A has been identified as a possible biomarker candidate but its quantification in widely available FFPE tissues is complicated by its similarities with the metabolic signaling insulin receptor isoform, InsR-B. The present study is the first to demonstrate MIEQ validated, InsR-A and InsR-B qPCR primer pairs compatible in FFPE sample types and show feasible use in clinically archived breast tumors⁴⁰. In the research setting, PCR based detection of InsR-A and InsR-B have been adopted in place of protein based methods, but these assays have not been widely used for clinical studies due to their dependence on fresh, frozen or RNA preserved samples types^{7,32–34}. Primer sets compatibility with FFPE tissue cDNA is highly dependent upon a limiting amplicon size to 60 base pairs or less³⁵. The InsR-A, -B, and IGF1-R qPCR primer sets presented here were designed with an amplicons of 50 base pairs or less. Previously reported InsR primer sets exceed this amplicon boundary and data demonstrating their feasible use in FFPE sample types has not been shown.

InsR-A, -B, and IGF-1R primer sets underwent extensive validation and testing to ensure sensitivity and specificity for their respective assay targets (Table 1). Of note is the rigorous isoform cross reactivity testing completed with the InsR-A and -B assays (Figure 3A). Testing of InsR-B primer set showed no crossreactivity when over 10^6 copies of InsR-A plasmid template was added per reaction. Reciprocal testing with the InsR-A primer set identified a very minimal level of crossreactivity (0.188%) when $10^{4.4}$ or more copies of InsR-B plasmid template were input per reaction. In this example, an InsR-A deficient sample with $10^{4.4}$ copies of InsR-B would misreport the detection of 47 copies of InsR-A, and an InsR-B to InsR-A ratio of 532. Breast tumor samples from this study reported a maximum InsR-B to InsR-A ratio of 5 suggesting much lower isoform ratios will exist in clinical samples. Given the consistent recovery of InsR-A and InsR-B (Figure 4A–B) when testing more physiologic isoform ratios (1, 10, and 100), the low level of observed low level of InsR-B plasmid crossreactivity in the InsR-A qPCR assay is not significant and it is unlikely that the observed crossreactivity would obscure assay sensitivity and specificity for InsR-A under physiologic conditions.

Feasibility data generated from FFPE samples support the use of these qPCR assays with FFPE samples types. Overall more than 88% of samples had measurable levels of InsR-A, and -B, or InsR-A, -B and IGF-1R (HR ER+ tumors). Although tumor sample RNA was purified manually and by automation, any processing differences were minimized by using the same core reagents and work flow for both procedures. Resulting RNA from manual and automated purification methods produced similar proportions of samples (91% and 86% respectively) with measurable levels of both InsR-A and -B.

Predominance data from this preliminary breast cancer study show InsR-A is a more significantly expressed component of total InsR expression than InsR-B. The observed expression of InsR-A and -B in ER+ and HR ER+ breast tumors is of particular interest as a considerable proportion of ER+ breast tumors will have *de novo* resistance to hormonal therapies via ligand-independent ER cross-talk with IGF-1R or other activated growth factor receptors^{13,45,46}. Combining hormone therapies with IGF-1R inhibitors has restored hormone sensitivity to cancer cells and recent work showing IGF-II mediated activation and nuclear translocation of ER has further implicated IGF-1R and a possible role for InsR-A in this process^{12,21,47,48}. HR ER+ tumors examined in this study expressed significantly greater levels of InsR-A vs IGF-1R (Figure 5D). Resistance to IGF-1R inhibition has been associated with elevated InsR:IGF-1R ratios in human breast cancer cells²⁷. The median InsR-A:InsR-B ratio of HR ER+ tumors in the present study (20.98) was 12.5 fold greater

than ER+ tumors and 17.5 fold greater than ER– tumors (Table 2, Figure 5C). The observed perturbation of InsR-A:InsR-B ratios in HR ER+ tumors was due to a significant drop in InsR-B expression and similar reductions of InsR-B have been previously reported³². While correlative InsR isoform work remains to be done, these data suggest a greater potential for IGF-II/InsR-A signaling in HR ER+ breast cancers and may help explain the positive and negative correlation of InsR protein expression with disease free survival in early and node negative cancers and invasive cancers respectively^{24,25,31}.

Developing biomarkers which define IGF responsive and resistant patient populations is critical for the future success of IGF targeted therapeutics^{9,11,19,20,49,50}. Clinical testing in the absence of IGF specific biomarkers has confounded study results, contributed to the closure of two large phase III trials and resulted in the distancing of some drug companies from further development of IGF targeting programs^{9,20,47,49,51}. Data presented here and by others support further testing of InsR-A and InsR-B as biomarker candidates. The described InsR isoform assays are the first to demonstrate compatibility with FFPE sample types and the feasibility of measuring expression in FFPE patient tissues. As such these assays offer a significant enhancement in clinical utility for predictive biomarker testing of patients receiving IGF targeted therapies.

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Figure 1A

Figure 1. InsR PCR primer design and standard plasmid template construction

InsR-A and B isoform templates were synthesized and cloned into pUCminusMCS plasmid vectors. (A) InsR-A qPCR primers amplify a 50 bp region within the 404 bp encoded by exons 10 and 12. (B) InsR-B qPCR primers amplify a 42 bp region of the 440 bp encoded by exons 10, 11, and 12. A 20 bp ladder is shown.

Figure 2. InsR-A, -B, and IGF-1R qPCR amplification curves

A best fit line (—) and a 95% confidence intervals (---) are shown. Data points are graphed as mean±SD. ○: InsR-A, □: InsR-B, and ◇: IGF-1R.

Figure 3A

Figure 3B

Figure 3. InsR isoform qPCR assay specificity and cross-reactivity testing

Data are graphed as mean $\pm$ SD. The InsR-A and InsR-B qPCR assays tested across a 6 log range of InsR-A or InsR-B plasmid input. (A) InsR-A qPCR. (B) InsR-B qPCR.

Figure 4A

Figure 4. InsR isoform qPCR assay recovery

Data are graphed as mean±SD. For reference, calculated input bars of theoretical values are displayed. (A) InsR-A assay recovery: B:A = InsR-B to InsR-A input ratio. (B) InsR-B assay recovery: A:B = InsR-A to InsR-B input ratio.

Figure 5A all samples isoform and group cumulative comparison

Figure 5B All groups

Figure 5. InsR isoform expression of ER+, ER-, and HR ER+ breast tumors

Data are graphed as a median within a 50% interquartile range box plot with whiskers extending from 10–90% of the reported data range. (A) Cumulative and isoform specific InsR expression of FFPE breast tumors. (B) InsR isoform expression of FFPE tumor ER subgroups. (C) InsR isoform expression ratios. (D) IGF-1R expression of HR ER+ breast tumors. +++p<0.0001, ***p<0.0005, * p<0.05

Table 1

qPCR Validation Data

qPCR Assay	InsR-A	InsR-B	IGF-1R
Amplification Efficiency Ave $\pm$ SD, (n=10)	100.71% $\pm$ 3.28%	99.98% $\pm$ 2.32%	100.49% $\pm$ 1.74%
Slope Ave $\pm$ SD, (n=10)	-3.305 $\pm$ 0.077	-3.322 $\pm$ 0.054	-3.310 $\pm$ 0.041
Intercept Ave $\pm$ SD, (n=10)	36.97 $\pm$ 0.170	39.88 $\pm$ 0.376	37.34 $\pm$ 0.230
R squared Ave $\pm$ SD, (n=10)	0.996 $\pm$ 0.003	0.975 $\pm$ 0.011	0.998 $\pm$ 0.001
LOD copies/reaction (n=48)	5	4	4
Intra-assay variation Ave $\pm$ SD, (n=10)	1.03% $\pm$ 0.86%	0.71% $\pm$ 0.55%	0.83% $\pm$ 0.83%
Overall CV Ave $\pm$ SD, (n=10)	1.073% $\pm$ 0.959%	3.052% $\pm$ 2.6%	0.97% $\pm$ 0.52%
Range of Detection copies/reaction	5–10 ^{6.4}	4–10 ^{6.4}	4–10 ⁷
Average Recovery Ave $\pm$ SEM, (n=45)	94.87% $\pm$ 4.83%	97.27% $\pm$ 5.54%	98.67% $\pm$ 3.24%
Isoform Crossreactivity	0.188% ^I	No	NA
Detection Rate Assay Specific	93.6% (193/206)	91.7% (189/206)	91.8% (56/61)

^IOnly when InsR-B 10^{4.4} copies/reaction.

Table 2

InsR Isoform Expression Analysis

Group	All	ER+	ER-	HR ER+
N	183 ¹	74 ¹	53 ¹	56 ¹ , 54 ²
Detection Rate Multiple Assays	88.8% ¹	89.2% ¹	82.8% ¹	91.8% ¹ , 88.5% ²
InsR-A (median copies/ μ g RNA)	66066	83281	37164 ³	74347 ⁴
InsR-B (median copies/ μ g RNA)	23199	49738	23773	3606 ^{4,5}
IGF-1R (median copies/ μ g RNA)	ND	ND	ND	19031 ⁴
InsR-A > InsR-B	p<0.0001	p<0.05	NS	p<0.0005
InsR-A + InsR-B (median copies/ μ g RNA)	98479	121613 ⁵	81860	79241
InsR-A : InsR-B (median)	2.341	1.682	1.202	20.98 ⁵
Statistics	Mann-Whitney U-test	Kruskal-Wallis one-way ANOVA with Dunn's Multiple Comparison Test		
InsR-A Predominance (% of samples)	83.6%	82.4%	67.9%	100%
Predominance >50% InsR-A (% of samples)	p<0.0001	p<0.0001	p=0.0092	100%
Statistics	Wilcoxon Signed Rank Test			

¹ InsR-A and InsR-B detected.

² InsR-A, InsR-B, and IGF-1R detected.

³ p<0.0005 vs ER+, p<0.05 vs HR ER+

⁴ p<0.0005 vs other assay targets

⁵ p<0.0005 versus other tumor groups