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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 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.

1. 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 subunit [1]. 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 the InsR component of IGF signaling is additionally 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].

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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 rational 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 blockading 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 cancer [5]. 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 theses 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 storage [35–37]. 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 cDNA [35]. 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 cDNA [35]. 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 developed [39]. Each assay was validated using established minimum information for publication of quantitative real-time PCR experiments (MIEQ) guidelines [40]. 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.

2. Materials and methods

2.1. 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-CCAACATCGCCAAGGGACCT [39]), 42 base pairs (InsR-B: F-CACTGGTGCCGAGGACCCTA, R-GACCTGCGTTTCCGAGATGG [39]), 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 (404 bp) and -B (440 bp) using Oligo 6 Primer Analysis Software. Isoform specific sequences were synthesized and individually cloned into pUCminusMCS plasmids by Blue Heron Biotechnology (Bothell, WA.) (Fig. 1A–B). Sequence identity and cloning were validated by Blue Heron and resulting plasmids were supplied in purified form. Log_{10} dilutions of each assay standard were made in 0.01 M Tris–HCl pH 8.0 using the molecular weight and OD_{260} readings. Each standard was amplified by PCR across a 5 log_{10} range of input and visually examined by agarose gel electrophoresis to confirm reactions produced a single product of the expected molecular weight (Fig. 1A–B, IGF-1R not shown).

2.2. 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 and 10^6 copies per reaction. InsR-A and -B qPCR assay were additionally tested for isoform crossreactivity using between 10 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 rations 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.

2.3. Breast cancer specimen power analysis and selection

Based on preliminary data with cell lines and data from the literature, it was hypothesized that 75% of breast cancer samples had a predominance ($A/A+B>0.5$) of InsR-A [4,41]. Based on this, a sample size of 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