A Pathobiological Role of the Insulin Receptor in Chronic Lymphocytic Leukemia

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Abstract

**Purpose:** The chromosomal deletion 11q affects biology and clinical outcome in chronic lymphocytic leukemia (CLL) but del11q-deregulated genes remain incompletely characterized.

**Experimental Design:** We have employed integrated genomic profiling approaches on CLL cases with and without del11q to identify 11q-relevant genes.

**Results:** We have identified differential expression of the insulin receptor (INSR) in CLL, including high-level INSR expression in the majority of CLL with del11q. High INSR mRNA expression in 11q CLL (~10-fold higher mean levels than other genomic categories) was confirmed by quantitative PCR in 247 CLL cases. INSR protein measurements in 257 CLL cases through flow cytometry, compared with measurements in normal CD19+ B cells and monocytes, confirmed that a subset of CLL aberrantly expresses high INSR levels. INSR stimulation by insulin in CLL cells ex vivo resulted in the activation of canonical INSR signaling pathways, including the AKT-mTOR and Ras/Raf/Erk pathways, and INSR activation partially abrogated spontaneous CLL cell apoptosis ex vivo. Higher INSR levels correlated with shorter time to first therapy and shorter overall survival (OS). In bivariate analysis, INSR expression predicted for rapid initial disease progression and shorter OS in ZAP-70–low/negative CLL. Finally, in multivariate analysis (ZAP-70 status, IgVH status, and INSR expression), we detected elevated HRs and trends for short OS for CLL cases with high INSR expression (analyzed inclusive or exclusive of cases with del11q).

**Conclusions:** Our aggregate biochemical and clinical outcome data suggest biologically meaningful elevated INSR expression in a substantial subset of all CLL cases, including many cases with del11q. *Clin Cancer Res; 17(9); 2679–92. ©2011 AACR.*

Introduction

Chronic lymphocytic leukemia (CLL) is the most common form of adult leukemia in the Western world, and is characterized by a highly variable clinical course (1). CLL is heterogeneous, and several disease-related factors have been identified that classify CLL into biologically distinct and clinically relevant subtypes. Lack of mutations in the immunoglobulin heavy chain variable region (IgVH) genes, increased expression of zeta-associated protein of 70 kDa (ZAP-70), presence of del17p/p53 aberrations, del11q, and elevated genomic complexity are some of the molecular parameters which have been associated with an aggressive disease course, shortened disease-free intervals, or compromised survival (2–23). It is likely that additional factors will be identified that have an effect on CLL biology and clinical behavior, particularly in disease subsets.

Interstitial deletions on the long arm of chromosome 11 (del11q) occur in approximately 15% to 20% of all CLL and have been associated with relatively more progressive and aggressive CLL. Given that a comprehensive view of del11q-associated molecular deregulations has not yet emerged, and given the importance of del11q in CLL clinical outcome, studies into the mechanisms of del11q-associated phenotypes in CLL seemed warranted. One of the genes implicated in del11q-associated biology in CLL is ATM, which is monoallelically deleted through all 11q deletions that are defined through the clinically used 11q FISH probe (24–26). Mutations in ATM have been identified on the retained allele in a minority subset of CLL with del11q, and CLL cases with del11q and mutated ATM have been proposed to have a more aggressive disease course than CLL cases with del11q and...
Translational Relevance

Genes with effects on the biology and clinical behavior of CLL in the setting of specific recurrent chromosomal aberrations remain incompletely characterized. Using an integrated genomic profiling approach, we have identified the differential expression of the insulin receptor (INSR) in CLL: a finding that was validated in a large CLL cohort. Intriguingly, high INSR expression was detected in the majority of CLL with 11q deletions: a disease group characterized by relatively rapid initial disease progression and shortened overall survival. We provide biochemical, ex vivo culture, and clinical outcome data supporting a role of the INSR in CLL cell survival and CLL disease progression. These data therefore provide a preclinical rationale to validate the INSR as a therapeutic target in CLL and to lay the foundation for the use of INSR inhibitors in the clinical management of CLL disease subsets.

Methods

Patients

Between January 2005 and October 2009, 267 patients evaluated at the University of Michigan Comprehensive Cancer Center were enrolled onto this study. The trial was approved by the University of Michigan Institutional Review Board (IRBMED #2004-0962) and written informed consent was obtained from all patients prior to enrollment. INSR data from 257 of these 267 patients were included in this analysis (5 patients enrolled on the study were excluded from analysis due to a diagnosis that was not CLL, and 5 patients had insufficient cryopreserved cells available).

Regardless of whether the subjects were originally diagnosed at our institution or another, we used the same CLL diagnostic criteria, based on the National Cancer Institute—Working Group Guidelines for CLL (30). Eligible patients needed to have an absolute lymphocytosis (>5,000 mature lymphocytes/μL), and lymphocytes needed to express CD19, CD23, slg (weak), and CD5 in the absence of other pan–T-cell markers.

Time to first therapy (TTFT) and overall survival (OS) were based on the CLL diagnosis date as previously defined (6). CLL treatment was defined as cytotoxic chemotherapy and/or monoclonal antibody therapy for CLL. Clinical information of all patients, including Rai stage, lymph node (LN) size, and serial absolute lymphocyte count (ALC) measurements (at least 3) at the time of enrollment, and all treatments given, was collected. Patient samples were characterized for selected CLL-associated chromosomal aberrations as a routine clinical test at the Mayo Clinic by using FISH, as previously described (6).

Cell isolation: cell purification

Peripheral blood mononuclear cells (PBMC) from CLL patients were isolated by Ficoll gradient centrifugation (GE Healthcare), aliquoted into fetal calf serum (FCS) with 10% penicillin-streptomycin, and cryopreserved in liquid nitrogen. For purification of CD19+ cells by negative selection, cryopreserved PBMCs derived from CLL patients were washed and recovered by centrifugation and then treated with anti-human CD3 (Miltenyi Biotec #130-050-101) and anti-human CD14 microbeads (Miltenyi Biotec #130-050-201), per the manufacturer’s recommendations. Cell suspensions were run through Miltenyi MACS LS separation columns (#130-042-401) to negatively enrich for CD19+ B cells. This resulted in greater than 90% CD19+ cells. For mRNA isolation, CD19 single-positive cells were sorted on a high-speed FACS Aria (Becton Dickinson) sorter as described (31).

Preparation of CLL-derived amplified RNA for hybridization to Affymetrix Human 133 2.0 Plus arrays and data analysis

Nineteen selected CLL samples (see Supplementary Table S1) were sorted to purity by using a high-speed FACS Aria (Becton Dickinson) sorter and RNA extracted by using the Trizol reagent. RNA was further purified by...
the RNeasy kit (Qiagen). Total RNA (50 ng) was amplified by the Ovation RNA Amplification system (NuGen, Inc.), labeled with the FL-Ovation cDNA Biotin module (NuGen, Inc.), and hybridized to the Human 133 2.0 Plus GeneChip (Affymetrix) following the manufacturer’s recommended protocols.

Affymetrix GeneChip data were analyzed as described (31). Raw probe-level data were converted to expression measures by using the robust multiarray average method, which is implemented in the Affymetrix package of Bioconductor (32). Briefly, the raw perfect match probes are first quartile normalized to reduce array-to-array variation. The normalized probe data are then converted to an expression measure (log scale) for each gene on each chip. For differential expression analysis relating to 11q deletion status, we used 2-sample Z tests to compare the mean log-scale expression level between del11q and non-del11q samples. Genes with a false discovery rate (FDR) less than 0.1 were selected (33); then fold changes, FDR values, and Z scores were used to identify genes having a strong association with 11q deletion status. There were 83 probe sets meeting this criterion. These probe sets are listed in Table 1, sorted by significance levels. Expression array data have been deposited with the National Center for Biotechnology Information Gene Expression Omnibus (GEO) web site under accession number GSE26526.

**Measurement of gene expression by using quantitative PCR**

RNA was prepared from 2 × 10⁶ to 4 × 10⁶ FACS-sorted CD19⁺ cells from all CLL cases by using the Trizol reagent and resuspended in 50 μL diethyl pyrocarbonate-treated water. Complementary DNA (20 μL) was made from approximately 50 ng of RNA by using the Superscript III first strand synthesis kit (Invitrogen) and random priming. Primers and TaqMan-based probes were purchased from Applied Biosystems (Primers-on-demand). Primer/probe mixtures included INSR (Hs00169631_m1) and GAPDH (Hs99999905_m1) (Affymetrix). Duplicate amplification reactions included primers/probes, TaqMan 2x Universal PCR Master Mix, No AmpErase UNG, and 1 μL of cDNA in a 20 μL reaction volume. Reactions were done on an ABI 7900HT machine. Normalization of relative copy number estimates for INSR mRNA was done with the Ct values for GAPDH as reference [Ct(mean INSR) – Ct(mean GAPDH) (ACtINSR – ACtGAPDH)]. Comparisons between CLL subgroups were done though subtractions of mean of normalized Ct values.

**Immunoblotting and immunoprecipitation**

Antibodies used in this study were: anti-α-tubulin (DM1A; Santa Cruz Biotech #SC-32293), anti-IRS-1 (C-20; Santa Cruz Biotech #SC-559), anti-insulin receptor-β (C-1; Santa Cruz Biotech #SC-57342), anti-ERK1/2 (Cell Signaling #9106), anti-phospho-S6 ribosomal protein (Ser235/236; Cell Signaling #2211S), anti-phospho-Erk4/42 MAPK (ERK1/2; Thr202/Tyr204; Cell Signaling #9106S), anti-phospho-p70-S6 kinase (Thr389; Cell Signaling #9203S), anti-phospho-AKT (Ser473; Cell Signaling #9271) and #4060, anti-AKT (Cell Signaling #9272), anti-phosphotyrosine antibody 4G10 (Millipore #05-1050), and anti-actin (AC-40; Sigma-Aldrich #A3853).

Immunoblotting to measure INSR levels was carried out on cell lysates from CD19⁺ cells obtained through negative selection from selected CLL cases. Cell pellets containing 10⁷ cells were lysed in 100 μL of lysis buffer [1% Triton X-100 (Sigma-Aldrich #T8523), 50 mmol/L Tris (pH 8.0; Sigma-Aldrich #T6066), 100 mmol/L NaCl (Sigma-Aldrich #S7653), 20 mmol/L NaF (Fisher Scientific #S299), 2 mmol/L EDTA (Sigma-Aldrich #ED28S), and 2 mmol/L EGTA (Sigma #E3889) containing 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich #P8340), phosphatase inhibitor cocktail (Sigma-Aldrich #02850), phenylmethylsulfonylfluoride (Thermo Fisher Scientific #36978), and 1 mmol/L sodium orthovanadate (Alfa Aesar #13721-39-6)]. Lysates were cleared by centrifugation at 14,000 rpm for 10 minutes at 4°C and supernatants brought to 1× SDS-PAGE sample buffer and boiled. Protein was fractionated on SDS-PAGE gels and transferred onto polyvinylidene difluoride membrane, then blocked for 1 hour in 5% nonfat dry milk in TBS-Tween-20 (TBS-T). Anti-phosphotyrosine immunoblotting, blocking was done by using 5% bovine serum albumin (Sigma-Aldrich #A9654). Membranes were incubated in primary antibodies in milk/TBS-T, washed, and finally incubated with appropriate horseradish peroxidase-conjugated secondary antibodies. The membranes were developed by CLC Plus detection (GE Healthcare #RPN-2132) reagents.

Immunoprecipitation of the INSR or IRS-1 out of CD19⁺ CLL cells was done by using the anti-insulin receptor-β antibody (29B4; Santa Cruz Biotech #SC-09), the anti-IRS-1 antibody (Santa Cruz Biotech #SC-559) and protein A/G Plus sepharose (Santa Cruz Biotech #SC-2003). Briefly, cell lysates from selected CLL cells were cleared through centrifugation and incubated with primary antibodies for 2 hours at 4°C, followed by incubations with protein A/G sepharose beads. Beads were collected through centrifugation and washed with 3 × 1 mL of lysis buffer. Bead-bound protein was liberated by boiling in 1× SDS-PAGE sample buffer and protein fractioned by SDS-PAGE. Immunoblotting was done as described earlier.

For analysis of insulin-induced signal transduction, negatively selected CD19⁺ cells from CLL cases with and without INSR expression were incubated for 1.5 hours in RPMI medium without serum and subsequently stimulated with 10 mmol/L insulin or biotinylated goat anti-human IgM F(ab’)² fragments (Southern Biotech #2022-08) together with avidin for 20 minutes. Cells were washed with ice-cold PBS, spun, pellets were snap frozen, and stored at −80°C. Pellets were lysed and supernatants prepared for immunoblotting with various primary and secondary antibodies at appropriate dilutions followed by enhanced chemiluminescence (ECL) as described earlier.

**FACS-based measurements of INSR expression in CLL**

CLL samples were thawed in a 37°C water bath, added dropwise into prewarmed RPMI medium with 10%...
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**Table 1.** List of differentially expressed genes between CLL cases with and without deletion 11q, ranked by significance levels at a FDR of 0.1 or less

(Continued on the following page)
heat-inactivated FCS, centrifuged, and washed with ice-cold HBSS (Hyclone #SH30588.02), supplemented with 1% FCS and 0.05% sodium azide (Sigma-Aldrich #S2002-100G). Cells were pelleted and then resuspended in cold HBSS, 1% FCS, 0.05% azide buffer. Subsequently, 7.5 × 10^5 cells were blocked for 15 minutes on ice with 10 μg unlabeled mouse IgG antibody (Southern Biotech #0107-01) and 10 μg unlabeled goat IgG antibody (Southern Biotech #0109-01). The following isotype controls were used per sample: 0.05 μg goat IgG isotype-APC control (Southern Biotech #0109-11), 0.35 μg mouse IgG isotype-FITC control (eBioscience #11-4714-73), and 0.0875 μg mouse anti-human CD19-PE (eBioscience #12-0199-42), and 0.05 μg goat anti-human CD220-APC (INSR; R&D Systems #FAB1544A). After 1 hour incubation and washings, cells were resuspended in 300 μL of 1% paraformaldehyde solution and analyzed by using a BD FACSCalibur flow cytometer and BD CellQuest Pro software. For each sample, isotype controls were run first, and thresholds for positivity were set that allowed 2% of gated events to be considered positive. Viable lymphocytes (based on light-scatter characteristics) positive for both CD19 and CD5 were then interrogated for INSR expression. INSR expression data in this manuscript are net INSR staining after subtraction of individual background staining.

**Measurements of radiation-induced ATM autophosphorylation in CLL**

Measurements of radiation-induced ATM autophosphorylation in CLL were done as previously reported (34).

**SNP 6.0 array-based genomic copy number analysis**

FACS-sorted CD19^+ and CD3^+ cells were digested overnight in 100 mmol/L Tris (pH 8.0), 50 mmol/L EDTA, 50
mmol/L NaCl, 0.5% SDS, and 100 μg/mL of Proteinase K (Invitrogen) at 56°C. DNA was extracted by using phenol-chloroform and precipitated by using ammonium acetate, ethanol, and glycogen. DNA was prepared for hybridization to SNP 6.0 arrays according to the manufacturers’ recommendations. Sample copy number heat map displays were obtained from CEL files through use of the freely available software dChip adapted to operate in a 64-bit environment (35) as described (36).

Methods used for CLL outcome analysis
Event times (TTFT, OS) were recorded in months, along with an indicator of whether the event was reached, or the time was censored. Ninety-eight percent of censoring reflects the current status at the date of analysis rather than loss to follow-up. To explore the relationship between INSR expression and outcomes, we considered various thresholds that can be used to partition the sample into 2 groups (high/low INSR expression). The HR for an outcome (e.g., TTFT) can then be estimated by using proportional hazards models. The HR and the P value for the null hypothesis that the HR differs from one (indicating equal hazards in the 2 groups) are displayed as functions of the threshold.

Results

Patient characteristics
Data from 257 unselected CLL patients [of which 79% were untreated and 21% previously treated (median number of prior treatments = 1) at study enrollment] that were consecutively enrolled at the University of Michigan between January 2005 and October 2009 were included in this analysis. Outcome analysis was carried out as reported by using the date of CLL diagnosis as the reference date (6).

The insulin receptor is expressed in a subset of CLL cases, with preferential expression in CLL with deletion 11q

Comparative array-based transcriptome analysis between 10 CLL cases with del11q and 9 cases without del11q (all centered on 11q22 and always inclusive of ATM) resulted in 83 probe sets measuring differential gene expression between the 2 groups at FDR < 0.1 (33). The majority of the associated genes physically mapped to 11q21-23 and all indicated lower expression in CLL with del11q21-23 than CLL without del11q21-23, providing confidence in our assay conditions (Table 1). Twenty-five probe sets representing 22 differentially expressed genes were not located within del11q21-23; among these, multiple sets identified substantially higher INSR expression (log2-fold differences of 2.52, 3, and 3.15) in CLL with del11q21-23 as opposed to the reference cases (Table 1).

To validate the array-based discovery of differential INSR expression in CLL, we measured normalized INSR mRNA expression in mRNA from FACS-sorted CD19+ CLL-derived cells from 247 CLL patients. We grouped CLL by clinically important FISH categories (with all 11q cases clustered as one group, irrespective of other abnormalities) and plotted normalized INSR expression for each case according to genomic CLL subtype. As seen in Figure 1A, INSR mRNA expression in CLL covered a wide range and INSR mRNA was expressed at detectable levels in approximately 50% to 60% of all CLL cases. INSR expression was
absent in approximately 40% of all CLL. Importantly, CLL cases that carried del11q as measured through FISH (using a probe centered on ATM) had significantly and many fold (~10-fold) higher INSR mRNA levels than all other FISH-based CLL subgroups (the mRNA data in Fig. 1A are approximately on a log2-fold scale) and many non-del11q CLL cases did not express the INSR mRNA (with INSR measurements for trisomy 12 patients possibly modestly overestimated through use of the chromosome 12-resident housekeeping gene GAPDH).

To support these INSR mRNA expression findings, we proceeded with INSR immunoblotting on detergent cell lysates from highly enriched CD19+ cells from CLL cases with and without INSR mRNA expression. The INSR protein was readily detected in lysates from Chinese hamster ovary (CHO) cells transfected with an INSR cDNA, as well as in 5 CLL cases with del11q and detectable INSR mRNA; INSR expression was not detected in CLL cases with absent INSR mRNA (Fig. 1B). Next, we determined an approximate threshold for INSR detection by immunoblotting as a function of INSR mRNA level. Using CLL cases spanning the INSR mRNA range of ΔCtINSR – GAPD of 8 to 11, we detected INSR protein in 11 of 12 cases; thus, INSR protein is readily detectable by immunoblotting in a substantial portion (~40%) of all CLL (data not shown). Next we used immunoprecipitation of the INSR from CHO-INSR cells or CLL cells with INSR expression followed by INSR immunoblotting. INSR protein was readily detectable in INSR immunoprecipitates but not in parallel precipitates with a control antibody (Fig. 1C).

We proceeded to develop a quantitative FACS assay for INSR expression in CD19+/CD5+ cells (see Methods). Using this assay we quantitated INSR expression in 257 CLL samples procured at study enrollment. Data are summarized in Figure 2A with CLL cases ordered on the basis of the degree of net INSR expression (range, 0.8%–76.8%). Although many cases displayed INSR expression at relatively low levels (<3%, <4%, or <5% net INSR expression was found in 30%, 60%, and 74% of cases, respectively), 26% (n = 69) of cases expressed the INSR at appreciable levels (≥5%) and characteristics of these cases are summarized in Supplementary Table S2 of the cases with INSR expression measurements in a large CLL cohort.

Figure 2. FACS-based INSR expression measurements in a large CLL cohort. A, INSR expression was measured in duplicate in gated CD19+/CD5+ cells from PBMCs from 257 patients. Background staining was quantified, based on isotype controls (see Methods). Displayed are net INSR expression values for individual cases ranked by the magnitude of INSR expression. CLL cases with del11q or del17p present in 25% or more of nuclei are marked in red and blue. B, INSR expression in normal B cells and monocytes.
≥5%, 69% (48 of 69) were ZAP-70+ and 31% (21 of 69) ZAP-70−; furthermore, 73% (49 of 67) and 27% (18 of 67) were IgVH status unmutated and mutated, respectively, with the IgVH status of 2 cases not known. A subset of CLL cases expressed very high INSR levels and many of these cases carried del11q (coded in red; see also Fig. 3 and Table 2). CLL cases with del17p (coded in blue), in contrast, did not have higher INSR expression than all other CLL. Normal peripheral blood–derived B cells expressed the INSR at moderate levels (Fig. 2B; mean, 7.7%; range, 3.2%–10.1%), whereas monocytes were strongly INSR-positive (mean, 80.2%; range, 61.7%–90.1%).

Next, we obtained estimates for the longitudinal stability/instability of INSR expression in individual cases through measurements of INSR expression in paired follow-up samples. Two serial follow-up measurements were available in 89 CLL cases with a range of follow-up of 15 to 52 months (median 32 months). One follow-up measurement was available in an additional 54 CLL cases with a range of follow-up of 5 to 45 months (median 12 months). As seen in Supplementary Figure S1, INSR expression was rather stable over time, with few cases showing substantial changes from their baseline values.

In summary, INSR is expressed at the transcript and protein levels in a substantial subset of CLL, prompting investigations into a functional role of the INSR in CLL cells.

The anatomy of monoallelic 11q deletions in CLL does not provide guidance to differential INSR expression in CLL with del11q

Attempting to explain why some cases of CLL with 11q deletions did not express the INSR at high levels, we mapped 11q deletions in this cohort by using SNP 6.0 ultra–high-resolution arrays and correlated the del11q anatomy with INSR mRNA and protein levels. As seen in Figure 3 and Table 2, the position or length of 11q deletions alone is insufficient to explain the observed INSR expression differences. Furthermore, the INSR gene was not amplified.

Table 2. Summary of quantitative PCR–based normalized INSR mRNA levels (ΔC_TINSR - ΔC_TGAPDH values) and FACS-based INSR protein expression levels for the CLL cases with 11q deletions marked in Figure 3

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<td>Absent</td>
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Figure 3. The anatomy of 11q deletions in CLL does not explain differences in INSR expression. Heat map display of copy number estimates for parts of chromosome 11 (blue: copy loss; red: copy gain), based on SNP 6.0 array profiling. The location of the ATM-centric FISH probe used clinically is indicated. Cases are ordered by degree of INSR expression.
ATM activity does not correlate with INSR expression

Given the strong enrichment of CLL cases with del11q among the CLL with high INSR expression, it appeared important to test the hypothesis that ATM may be involved in the control of INSR expression. Using a large and unique dataset \( (n = 171) \) of radiation-induced ATM autophosphorylation measurements (expressed as normalized p-ATM/ATM and p-ATM/actin ratios) we were unable to detect evidence for a dominant role of the ATM activity state in the control of INSR expression (Supplementary Fig. S2A–D; ref. 34).

The insulin receptor activates canonical insulin signal transduction pathways in CLL cells

In an effort at deriving an initial understanding of INSR-regulated signal transduction in CLL cells, we focused on the AKT-mTOR and Ras-Raf-Erk pathways as known canonical INSR-activated pathways in insulin-responsive cell types (37).

Highly enriched CD19\(^+\) cells from CLL cases with and without INSR expression were stimulated with low-dose insulin at 10 nmol/L and cells prepared for immunoblotting with various antibodies (Fig. 4A–C). Using an antibody to measure the phosphorylation state at AKT-Ser-473, we detected insulin-induced AKT-Ser-473 stimulation in 11 of 14 CLL with INSR expression (with more pronounced increases seen in cases with high INSR expression), with the remaining 3 of 14 cases showing already elevated phospho-AKT-Ser-473 at baseline. The degree of AKT-Ser-473 phosphorylation was comparable to the phosphorylation induced by B-cell receptor multimerization (Fig. 4B). Consistent with insulin-induced mTOR activation, we detected increased phosphorylation of p70-S6 kinase at Ser-389 and S6-ribosomal protein at residues 235/236. Finally, in 7 of 14 cases, we detected increased insulin-induced Erk1/2 phosphorylation at residues Thr202/Tyr204, whereas the remaining cases showed elevated baseline Erk1/2 phosphorylation (activation) states, as previously described (38).

Given that IRS-1 is a central INSR signaling node, we investigated insulin-induced IRS-1 phosphorylation on tyrosine in enriched CD19\(^+\) CLL cells stimulated with 10 nmol/L insulin. IRS-1 was immunoprecipitated from stimulated and unstimulated lysates; protein was fractioned by SDS-PAGE and then transferred to membrane. Tyrosine phosphorylation was detected by using the anti-phosphotyrosine–specific antibody 4G10. As seen in Figure 4C, insulin stimulation resulted in increased tyrosine phosphorylation on IRS-1 in CHO-INSR cells as well as primary CLL cells.

Insulin treatment partially abrogates CLL cell apoptosis in short term cultures

Given the observed insulin-induced activation of the AKT-mTOR pathway in CLL, we proceeded with measurements of CLL apoptosis \( \text{ex vivo} \) in short term cultures by using CLL cells with and without INSR expression and cultured in the presence or absence of 10 nmol/L exogenously added insulin. Data were normalized to measured spontaneous apoptosis rates in untreated CLL cells.

As seen in Supplementary Figure S3A and B, insulin consistently abrogated some of the spontaneous apoptosis in CLL cases with INSR expression \( (n = 16; \text{in red}) \) whereas most CLL cases without INSR expression \( (n = 16; \text{in black}) \) proceeded with apoptosis at the same rate as untreated parallel cultured cells. These findings were highly statistically significant \( (P < 0.001) \) and provided clear evidence that nanomolar concentrations of insulin can provide prosurvival signals to CLL cells. These data also support...
a role for the INSR as the primary insulin receptor responsible for the observed insulin effects in CLL.

**Expression of the insulin receptor identifies progressive CLL**

We wished to obtain evidence for a role of the INSR in CLL clinical behavior. Initially, we correlated the size of the largest LN or the number of positive LN stations by physical exam with various INSR thresholds. As summarized in Supplementary Figure S4A and B, both measures correlated with elevated INSR expression (a correlation between ALC-doubling time and INSR expression was not found). Next, we analyzed 2 well-defined clinical endpoints, TTFT (a measure of natural disease progression) and OS, as a function of the degree of INSR expression as measured through FACS. Given that INSR expression was observed in all CLL, albeit at very different absolute levels, we chose to analyze INSR as a continuous variable and calculated HRs for TTFT and OS across a wide range of INSR expression cutoffs. This type of outcome analysis seems more suitable for the observed continuous expression levels of the INSR, as it avoids the use of optimized but nonetheless arbitrary cutoffs for INSR expression and instead provides data for a broad range of INSR expression levels. Using proportional univariate hazard modeling, we found that the HRs for shorter TTFT were correlated to higher INSR protein levels across a broad range of absolute INSR levels (Fig. 5A–C). For instance, elevated HRs for a short TTFT emerged at INSR levels of approximately 4% and remained significantly elevated for all higher cutoffs. Removal of all CLL cases with 11q substantially weakened the power of the analysis but still uncovered effects of the INSR on short TTFT (Fig. 5D–F), suggesting that the INSR effect on CLL is independent of 11q but likely contributes to 11q biology given the high INSR expression in this disease subset. The parallel analysis by using OS as the endpoint uncovered univariate associations of high INSR expression on survival in CLL (Fig. 5G–L).

**INSR expression is associated with rapid initial disease progression in CLL with less than 20% ZAP-70 expression (bivariate analyses)**

ZAP-70 expression identifies a CLL subgroup that progresses to need of first therapy (TTFT) within 3 to 4 years, and thus ZAP-70 expression constitutes one of the strongest predictors of progressive CLL (5). We therefore carried out bivariate analyses to detect effects of INSR expression (at various thresholds) on TTFT and OS in the setting of known ZAP-70 status. As seen in Figure 6A–H, INSR expression in CLL with high ZAP-70 expression showed no additional effects on disease progression. However, INSR expression in CLL with ZAP-70 expression of less than 20% was associated with rapid disease progression and shortened OS similar to what was observed in ZAP-70+ CLL. This finding remained significant for some thresholds, despite substantially low case numbers, after the exclusion of all 11q cases (data not shown). Conversely, CLL cases with low ZAP-70 and low INSR expression showed substantial disease stability. A parallel bivariate analysis of the effect of INSR expression on CLL with unmutated or mutated IgVH genes (IgVH UM or M) did not find significant effects for TTFT, but did reveal significant, threshold-dependent effects on OS (Supplementary Fig. S5A–H).

**Results of multivariate analyses**

Finally, we incorporated INSR expression at various thresholds together with ZAP-70 status and IgVH status into multivariate modeling (despite power limitations due to study size). INSR emerged as a risk factor with HRs more than 1 for short OS across multiple thresholds with INSR expression of 10% or more, showing a trend toward independent significance [INSR HR = 1.91, P = 0.07; IgVH11 HR = 3.13, P < 0.01; and ZAP-70 HR = 2.07, P = 0.04 for all cases (n = 246) and INSR HR = 2.35, P = 0.07; IgVH11 HR = 3.34, P < 0.01; and ZAP-70 HR = 1.91, P = 0.1 for all cases without del11q (n = 222)].

**Discussion**

In this report, we provide novel data that support a role for the INSR in the pathobiology of CLL. Both the identification of differential INSR expression in CLL and preferential aberrant high-level expression in CLL with del11q resulted from a comparative array-based screen of transcriptome differences comparing CLL cases with del11q versus cases without del11q and subsequent validation of these findings in more than 250 CLL cases. The INSR expressed on CLL cells subsequent to low-dose insulin stimulation activated prosurvival and progrowth pathways and abrogated CLL apoptosis ex vivo, suggesting that under appropriate conditions the INSR may regulate CLL cells in vivo. Such in vivo effects may be particularly relevant in the LN environment, as elevated INSR levels were associated with increased LN size and numbers (39). Furthermore, INSR expression was associated with CLL disease progression and shortened OS, although definitive data on the independent effects of elevated INSR levels on CLL outcome (or possible effects on CLL subsets like diabetics) requires assessments in larger patient cohorts than available to us for this study (37). A substantially larger study size of 500 to 1,000 patients would allow for a comprehensive multivariate analysis of the effects of CLL-FISH categories, specific gene mutations, ZAP-70 status, IgVH11 status, and INSR status in CLL clinical behavior (5).

Furthermore, we detected rapid initial disease progression as measured through short TTFT and shorter OS in CLL cases with less than 20% ZAP-70 expression, thus identifying a subgroup of CLL within the ZAP-70–low population that may be driven by the insulin/INSR axis. Such effects may be due to the activation of signal transduction pathways shared by the INSR and the B-cell antigen receptor, most notably the AKT and proproliferative pathways.

The novel finding of high aberrant INSR expression in many cases with del11q is intriguing and suggests a contributory role of the INSR to del11q biology. In contrast,
Figure 5. Higher INSR expression is associated with elevated HRs for CLL disease progression or short OS over a broad range of actual INSR expression values. Displayed are HRs (yellow) and corresponding P values (blue) as a function of various INSR protein thresholds measured through FACS. A–C, TTFT and various INSR thresholds (all cases). D–F, TTFT and various INSR thresholds (all cases minus 11q cases). G–I, OS and various INSR thresholds (all cases). J–L, OS and various INSR thresholds (all cases minus 11q cases).
Figure 6. INSR expression is associated with rapid disease progression and shortened OS in CLL with low ZAP-70 expression (bivariate analysis, Kaplan-Meier plots). Displayed are Kaplan-Meier plots as a function of various INSR protein thresholds as measured through FACS. A–H, TTFT or OS and various INSR thresholds in the setting of ZAP-70 expression of >20% or <20%.
elevated INSR expression was not found in CLL with del17p, providing strong evidence against simple epiphenomenal upregulation of INSR expression in proliferative CLL. Nonetheless, the mechanism of preferential and often very high expression of the INSR in CLL with del11q remains unidentified. The high incidence of INSR expression in CLL with del11q implies a regulatory role of a gene(s), located within the boundaries of 11q deletions, on INSR expression. Such a gene(s) may affect INSR transcription or mRNA stability. Nonetheless, a review of the anatomy of more than 20 CLL cases with 11q deletions based on high-resolution SNP-arrays, including data of del11q cases with low/absent INSR expression, did not allow for the formulation of a specific hypothesis based on a candidate gene or minimal deleted region. Further, ATM activation (as measured through irradiation-induced ATM phosphorylation) did not correlate with INSR expression; this is based on analysis of more than 170 CLL cases. One potential model that may explain all experimental observations makes use of multiple discrete INSR regulators, one of which is located within 11q deletions. Such a putative 11q-resident INSR regulator may require additional gene or gene product modifications, as monoallelic deletions seem insufficient to activate INSR expression. Finally, such an 11q-resident INSR regulator may act in concert with non-11q-resident genes (possibly located on chromosome 13, given sporadic high INSR expression observed in this CLL subset) or may be altered or mutated in cases without del11q.

Our findings of INSR expression in CLL add novel information on the role of the INSR in cancer biology and provide the most complete assessment yet of expression and function of this receptor in hematologic malignancies (40, 41). Given that various compounds, based on multiple strategies targeting the IGF1 receptors, have entered clinical investigation, we anticipate increased interest in targeting the INSR or insulin levels in cancer as well.

In summary, the novel identification of INSR expression in CLL subtypes and the association of INSR expression with progressive CLL should allow for the development of assays based on INSR expression that could ultimately be useful in CLL clinical management. Further, identification of the insulin/INSR axis as a pathophysiologic principle in CLL may be the first step toward targeted intervention (40). Here, control of blood sugar levels and postprandial insulin levels could be envisioned, as could be therapeutic interventions based on the knowledge of INSR-activated signaling pathways in CLL. Finally, multiple compounds in early stage clinical development targeting IGF1-R also inhibit the INSR at low micromolar concentrations, and thus could be tested for clinical efficacy in CLL with INSR expression (42).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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