Identification of signaling pathways associated with cancer protection in Laron syndrome

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Abstract

The growth hormone (GH)–insulin-like growth factor-1 (IGF1) pathway emerged in recent years as a critical player in cancer biology. Enhanced expression or activation of specific components of the GH–IGF1 axis, including the IGF1 receptor (IGF1R), is consistently associated with a transformed phenotype. Recent epidemiological studies have shown that patients with Laron syndrome (LS), the best-characterized entity among the congenital IGF1 deficiencies, seem to be protected from cancer development. To identify IGF1-dependent genes and signaling pathways associated with cancer protection in LS, we conducted a genome-wide analysis using immortalized lymphoblastoid cells derived from LS patients and healthy controls of the same gender, age range, and ethnic origin. Our analyses identified a collection of genes that are either over- or under-represented in LS-derived lymphoblastoids. Gene differential expression occurs in several gene families, including cell cycle, metabolic control, cytokine–cytokine receptor interaction, Jak-STAT signaling, and PI3K-AKT signaling. Major differences between LS and healthy controls were also noticed in pathways associated with cell cycle distribution, apoptosis, and autophagy. Our results highlight the key role of the GH–IGF1 axis in the initiation and progression of cancer. Furthermore, data are consistent with the concept that homozygous congenital IGF1 deficiency may confer protection against future tumor development.

Introduction

The growth hormone (GH)–insulin-like growth factor-1 (IGF1) axis has a fundamental role in growth and development throughout life (Rosenfeld 2005). As originally postulated by Salmon et al. in the mid-1950s, anabolic actions of GH are mediated by a liver-produced peptide initially termed somatomedin and, subsequently, IGF1 (Salmon et al. 1957, LeRoith et al. 2001). IGF1 is also produced by extra-hepatic tissues and circulates as a ternary complex with IGF-binding protein-3 (IGFBP3) and an acid-labile subunit (ALS) (LeRoith et al. 2007). The IGF1 receptor (IGF1R), which mediates the biological actions of IGF1 and IGF2, is structurally and functionally related to the insulin receptor. IGF1 displays potent proliferative and antiapoptotic activities and is regarded as a key player...

Growth retardation in infants is multifactorial, albeit a large portion of cases remains idiopathic because no specific (genetic or other) defect can be identified (Klammt et al. 2008). Prenatal IGF1 expression is GH-independent and becomes GH-dependent before birth. IGF1 biosynthesis remains dependent on GH secretion during postnatal life. Congenital IGF1 deficiency is characterized by low to undetectable serum IGF1 levels, but normal to elevated GH production. These conditions may result from the following: (1) GH-releasing hormone-receptor (GHRH-R) gene defect, (2) GH gene deletion (isolated GH deficiency, IGHD), (3) GH receptor (GH-R) gene defects (Laron syndrome, LS), and (4) IGF1 gene deletion. Additional conditions leading to congenital IGF1 deficiency are defects of post-GH-R signaling (e.g. STATS defects) and ALS mutations (Woods et al. 1996, Kofoed et al. 2003, Domene et al. 2004, Cohen et al. 2008).

Laron syndrome (OMIM # 262500), or primary GH insensitivity, is a recessively transmitted genetic form of dwarfism caused by deletion or mutation of the GH-R gene, leading to congenital IGF1 deficiency (Laron 1999, 2004). This genetic entity was identified in the late 1950s and reported by Laron et al. (1966), and subsequently in families with a high incidence of consanguinity (Laron et al. 1966, 2011). The typical features of LS are short stature (~4 to –10 SDS), typical face, obesity, high basal serum GH, and low IGF1, unresponsive to administration of exogenous GH. The recognition that an inherited defect of the GH-R gene, including exon deletions and point mutations, is the etiological factor behind LS that was reported in 1989 (Amselem et al. 1989, Godowski et al. 1989). Sixteen different molecular defects were found in the Israeli cohort, whereas the majority of LS patients in the Ecuadorian cohort described below are homozygous for an A to G splice site mutation at position 180 in exon 6 of the GH-R gene. This finding reflects the fact that the Israeli cohort originated from different areas and ethnic groups (Laron et al. 2011). All of these molecular defects lead to GH insensitivity and, consequently, impaired IGF1 biosynthesis.

Epidemiologic studies carried out over the past 20 years have shown a correlation between circulating IGF1 levels and the risk to develop colon, breast, prostate, and other type of cancers (Chan et al. 1998, Hankinson et al. 1998, Renehan et al. 2004). However, the mechanisms by which IGF1 plays a role in cancer initiation and/or progression are not yet entirely clear. Recent studies have evaluated whether patients with congenital IGF1 deficiency have a reduced risk of cancer (Shevah et al. 2007, Steuerman et al. 2011).

These studies included 538 patients, including 230 patients with LS and 308 patients with congenital IGHD (cIGHD), GHRH-R defects, and congenital multiple pituitary hormone deficiency (cMPHD). The age range of LS patients was 1–75 years. The studies also included 752 first-degree relatives and further family members. Results of these studies revealed that none of the 230 homozygous LS patients, including patients who were treated with IGF1 or GH, had a history of cancer. Only one boy with xeroderma pigmentosum, out of 116 patients with IGHD, presented a basal cell carcinoma. However, 30 patients with cancer were reported among 752 first-degree heterozygote family members (4%) and 31 malignancies (mainly lung, prostate, breast, and colon tumors) were reported among 131 further relatives (23.7%). Despite the small size of this cohort, differences in cancer incidence between LS patients and relatives were regarded as statistically significant. Furthermore, this cohort comprises more than half of the estimated total number of LS patients worldwide. This finding is supported by animal experiments using the Gh-/-Gh-Bp KO mouse ('Laron mouse') (Laron et al. 2011). Similar to humans, these animals had reduced incidence of prostate and mammary tumors in experimental models of cancer (Wang et al. 2005).

The finding that the rate of cancer incidence in LS patients is very low is consistent with the notion that homozygous congenital IGF1 deficiency may confer protection against future development of a tumor. The present genome-wide analysis was designed to identify IGF1-dependent genes and signaling pathways associated with cancer protection in LS. Our results identified a number of genes that are either over- or under-represented in LS-derived lymphoblastoid cells. Gene differential expression occurs in a number of gene families, including cell cycle, metabolic control, cytokine–cytokine receptor interaction, Jak-STAT signaling, and PI3K-AKT signaling. Major differences between LS and healthy controls were also noticed in pathways associated with cell cycle distribution, apoptosis, and autophagy. Our analyses highlight the key role of the GH–IGF1 axis in cancer development and progression.

**Materials and methods**

**Lymphoblastoid cultures**

Epstein–Barr virus (EBV)-immortalized lymphoblastoid cell lines from LS patients and healthy controls were obtained from the National Laboratory for the Genetics of Israeli Populations, Tel Aviv University. At the time
of enrollment, all participants signed written informed consent. The research protocol followed the principles of the Declaration of Helsinki and was approved by the local Ethics Committees. Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and 100 units/mL penicillin + 100 μg/mL streptomycin. Reagents were purchased from Biological Industries Ltd (Bet-Haemek, Israel). Cultures were grown upright in tissue culture flasks and maintained under a humidified 5% CO₂ atmosphere at 37°C.

**Genomic analyses**

RNA was isolated from lymphoblastoid cell lines using the Trizol Reagent (Life Technologies). RNA quality was assessed by the absorbance ratio at 260/280 nm and gel electrophoresis before further analyses. Affymetrix GeneChip Human Gene 1.0 ST Arrays (Affymetrix), which offer whole-transcript coverage, were used. Arrays interrogate 28,869 well-annotated genes and noncoding RNAs with 764,885 probes. Human Gene 1.0 ST Array has greater than 99% coverage of sequences present in the RefSeq database.

**Bioinformatic analyses**

Data analysis was performed on CEL files using Partek Genomics Suite (Partek, St Louis, MO, USA). Data were normalized and summarized with the robust multiaverage method followed by one-way analysis of variance (ANOVA) (Irizarry et al. 2003). Genes of interest that were differentially expressed with \( P < 0.05 \) and fold-change difference cutoff >2 were obtained. Hierarchical cluster analysis was performed using Partek Genomics Suite software with Pearson’s dissimilarity correlation and average linkage methods. Functional analysis was performed using David and WebGestalt analysis platforms. Principal component analysis (PCA) was performed using Partek Genomics Suite v. 6.5 using all the genes in the array.

**Real-time quantitative polymerase chain reactions (RQ-PCRs)**

Total RNA was prepared from lymphoblastoids using the Trizol Reagent. Single-stranded cDNA was synthesized from total RNA samples using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). IGF1R, cyclin A1, UGT2B17, serpinB2, ORSH2, versican, and AKT3 mRNA levels were measured by RQ-PCR, using appropriate primers (Table 1). For control purpose, actin mRNA levels were measured.

**Western blot analyses**

Cells were grown to confluence, centrifuged at 300g for 10 min, washed twice with phosphate-buffered saline, and incubated with a lysis buffer for 20 min. The suspension was centrifuged at 16,200g for 10 min, diluted with sample buffer, and boiled for 5 min. Samples were electrophoresed through 10% SDS–PAGE, followed by blotting of the proteins onto nitrocellulose membranes. After blocking, the blots were incubated overnight with the indicated antibodies, washed, and incubated with a horseradish peroxidase-conjugated secondary antibody.

**Cell cycle analyses**

Cells were grown to confluence, after which medium was changed to starvation medium and grown for an

**Table 1** Sequences of primers employed in RQ-PCR analyses.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Annealing (°C)</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERPNB2-F</td>
<td>CACCCAGAACCTCTTCCCTCC</td>
<td>67.3</td>
<td>134</td>
</tr>
<tr>
<td>SERPNB2-R</td>
<td>TAACTGCAATGTGCTCCCTCC</td>
<td>67.2</td>
<td>134</td>
</tr>
<tr>
<td>ORSH2-F</td>
<td>CAGAGCACTTACATATCAGCAG</td>
<td>60.2</td>
<td>93</td>
</tr>
<tr>
<td>ORSH2-R</td>
<td>AGACCAAGCTTCCACACAAATGT</td>
<td>62.5</td>
<td>103</td>
</tr>
<tr>
<td>CyclinA1-F</td>
<td>TCAGTACTTTAGGAAGCTGAAA</td>
<td>62.8</td>
<td>103</td>
</tr>
<tr>
<td>CyclinA1-R</td>
<td>CCAATCCACCAAGATGGT</td>
<td>64.4</td>
<td>70</td>
</tr>
<tr>
<td>Versican-F</td>
<td>GCACCTGTGGCAGGATA</td>
<td>64.9</td>
<td>70</td>
</tr>
<tr>
<td>Versican-R</td>
<td>CAGGGATTAGATGACATCATCACA</td>
<td>63.9</td>
<td>103</td>
</tr>
<tr>
<td>AKT3-F</td>
<td>TTTGCAAAGGAGGGATACACA</td>
<td>59</td>
<td>103</td>
</tr>
<tr>
<td>AKT3-R</td>
<td>ACTGCTGGCCGATGCTATT</td>
<td>59</td>
<td>103</td>
</tr>
<tr>
<td>UGT2B17-F</td>
<td>GCATATGCTGTAAGAGGTGGGA</td>
<td>64.1</td>
<td>99</td>
</tr>
<tr>
<td>UGT2B17-R</td>
<td>AATGAGCCACATTTCCAGGTTC</td>
<td>64.2</td>
<td>103</td>
</tr>
<tr>
<td>Actin-F</td>
<td>CCTGTCACCCACACCAAT</td>
<td>67.4</td>
<td>144</td>
</tr>
<tr>
<td>Actin-R</td>
<td>GGGCCGACTCGTCATCT</td>
<td>66.3</td>
<td>103</td>
</tr>
</tbody>
</table>
additional 24 h. Cell suspensions were digested with 0.003% trypsin for 10 min. Cells were stained by adding propidium iodide (PI) in a spermine tetrahydrochloride solution. The samples were incubated on ice in the dark for an additional 10 min before analysis. Samples were analyzed using a FACSCalibur system (Becton Dickinson, Franklin Lakes, NJ, USA). Cell cycle analysis on histograms from PI-stained cells was performed using Flowing Software (http://www.flowingsoftware.com).

**Apoptosis measurements**

Basal levels of apoptosis were determined after cells were grown to confluence. Etoposide-induced level of apoptosis was determined 24 h after treatment. Cells were stained for 15 min with an Annexin V-FITC antibody and PI using an MEBCYTO apoptosis kit (MBL International, Woburn, MA, USA). Flow cytometry was performed with a FACSCalibur system and results were analyzed using the Flowing Software. Necrotic cells were stained with PI as well as Annexin V, whereas apoptotic cells were stained only with Annexin V.

**Cell proliferation assays**

Proliferation of lymphoblastoid cell lines was examined using an XTT–cell proliferation kit (Biological Industries Ltd, Israel). Cells were counted using a Nexcelom Bioscience Cellometer (Lawrence, MA, USA), diluted to a concentration of 250,000 cells/mL, and incubated in 96-well plates in a volume of 100 μL for 3 days in RPMI-1640 medium supplemented with 4% of albumin-free serum replacement (Biogro-2, Biological Industries Ltd, Israel) in triplicate. On the third day, activated XTT reagent in a volume of 50 μL was added to each well. After 5 h, absorption was measured using an ELISA reader (Bio-Rad microplate reader model 680; Bio-Rad Laboratories) at a wavelength of 450 nm and a reference absorbance of 655 nm.

**Oxidative damage response and autophagy measurements**

Cells were grown to confluence, after which medium was changed to fresh full medium in the presence of increasing doses of paraquat dichloride (Sigma-Aldrich). Paraquat is a quaternary nitrogen herbicide. The mechanism of paraquat toxicity involves the generation of superoxide anion, which leads to the formation of toxic reactive oxygen species, such as hydrogen peroxide and hydroxyl radical, and the oxidation of cellular NADPH. Proliferation in response to oxidative damage was measured using an XTT kit, as described above. Levels of p62 and LC3β as

[Table 2](#) Age and ethnic origin of patients and controls analyzed by genome-wide assays.

<table>
<thead>
<tr>
<th>Laron syndrome</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chip code</td>
<td>Age</td>
</tr>
<tr>
<td>LS-4551</td>
<td>37</td>
</tr>
<tr>
<td>LS-4490</td>
<td>42</td>
</tr>
<tr>
<td>LS-5300</td>
<td>47</td>
</tr>
<tr>
<td>LS-5859</td>
<td>51</td>
</tr>
<tr>
<td>Mean age</td>
<td>44.25 (±6.1)</td>
</tr>
</tbody>
</table>

LS, Laron syndrome; C, controls.
markers of autophagy were measured by western blot analyses. Antibodies were purchased from Cell Signaling Technology.

Results

Genome-wide analysis of differentially expressed genes in LS

In order to identify differentially expressed genes in LS patients in comparison with healthy controls, a genome-wide analysis was conducted. For this purpose, RNA obtained from EBV-immortalized lymphoblastoids derived from four female LS patients and four controls of the same age range (LS, 44.25 ± 6.08 years; controls, 51.75 ± 11.3 years; mean ± sd; P-value = 0.29) and same ethnic origin was used. Ages and ethnic origin of patients and controls are summarized in Table 2. One-way ANOVA was performed using Partek Genomics Suite to create a list of differentially expressed genes. A cluster analysis of differentially expressed genes is depicted in Fig. 1A. Thirty-nine annotated genes that were differentially expressed in LS compared with controls were identified (with a P-value of <0.05 and fold-change difference cutoff >2]). To summarize multivariate attributes by three dimensions that can be displayed graphically with minimal loss of information, PCA analysis was conducted. As shown in Fig. 1B, PCA revealed a good discrimination between experimental groups. The top 17 up- and down-regulated genes (ranked by fold-change) are given in Table 3.

Functional enrichment analysis

Functional analyses were conducted to find co-expressed genes sharing the same pathway. Analyses provide evidence for a number of shared pathways, including cell adhesion, G-protein signaling pathway, cell migration and motility, immune response, Jak-STAT signaling, apoptosis, and metabolic pathways. For the most part, genes involved in the control of cell cycle, motility, growth, and differentiation were downregulated in LS-derived lymphoblastoid cell lines compared with controls (Table 4).

Table 3 Top 17 differentially expressed genes.

<table>
<thead>
<tr>
<th>RefSeq</th>
<th>Gene assignment</th>
<th>Gene symbol</th>
<th>P-value (LS vs C)</th>
<th>Fold-change (LS vs C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AK299419</td>
<td>UDP glucuronosyltransferase 2 family, polypeptide B15</td>
<td>UGT2B15</td>
<td>0.0194396</td>
<td>11.0898</td>
</tr>
<tr>
<td>ENST00000551239</td>
<td>UDP glucuronosyltransferase 2 family, polypeptide B15</td>
<td>UGT2B15</td>
<td>0.0384672</td>
<td>7.13829</td>
</tr>
<tr>
<td>U59209</td>
<td>UDP glucuronosyltransferase 2 family, polypeptide B17</td>
<td>UGT2B17</td>
<td>0.0158576</td>
<td>7.16028</td>
</tr>
<tr>
<td>BC000181</td>
<td>G protein-coupled receptor 160</td>
<td>GPR160</td>
<td>0.0232108</td>
<td>3.7593</td>
</tr>
<tr>
<td>ENST00000371532</td>
<td>Zyg-11 homolog A (C. elegans)</td>
<td>ZYG11A</td>
<td>0.0219404</td>
<td>3.03514</td>
</tr>
<tr>
<td>AK302847</td>
<td>Ribosomal modification protein rimK-like family member B</td>
<td>RIMKL B</td>
<td>0.0378665</td>
<td>3.11611</td>
</tr>
<tr>
<td>BC152321</td>
<td>// Insulin-like growth factor 1 (somatomedin C)</td>
<td>IGF1</td>
<td>0.046416</td>
<td>1.99144</td>
</tr>
<tr>
<td>AK290029</td>
<td>Nephronectin</td>
<td>NPNT</td>
<td>0.0147893</td>
<td>−3.12657</td>
</tr>
<tr>
<td>AK301897</td>
<td>Cyclin A1</td>
<td>CCNA1</td>
<td>0.0286311</td>
<td>−3.25083</td>
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<tr>
<td>ENST00000343200</td>
<td>Versican</td>
<td>VCAN</td>
<td>0.0433405</td>
<td>−3.37884</td>
</tr>
<tr>
<td>ENST00000383695</td>
<td>Olfactory receptor, family 5, subfamily K, member 3</td>
<td>OR5K3</td>
<td>0.000944527</td>
<td>−4.04477</td>
</tr>
<tr>
<td>BC012609</td>
<td>Serpin peptidase inhibitor, clade B (ovalbumin), member 2</td>
<td>SERPINB2</td>
<td>0.0239913</td>
<td>−4.35871</td>
</tr>
<tr>
<td>ENST00000514084</td>
<td>Olfactory receptor, family 5, subfamily H, member 7</td>
<td>OR5H7P</td>
<td>0.000637513</td>
<td>−4.40605</td>
</tr>
<tr>
<td>BC136984</td>
<td>Olfactory receptor, family 5, subfamily H, member 14</td>
<td>OR5H14</td>
<td>0.0040872</td>
<td>−5.11991</td>
</tr>
<tr>
<td>ENST00000354924</td>
<td>Olfactory receptor, family 5, subfamily K, member 4</td>
<td>OR5K4</td>
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<td>−5.22628</td>
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<tr>
<td>BC137523</td>
<td>Olfactory receptor, family 5, subfamily H, member 6</td>
<td>OR5H6</td>
<td>0.00105229</td>
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<tr>
<td>ENST00000355273</td>
<td>Olfactory receptor, family 5, subfamily H, member 2</td>
<td>OR5H2</td>
<td>0.00184389</td>
<td>−5.79858</td>
</tr>
</tbody>
</table>
Dot-plot and RQ-PCR validation

To validate the differences in specific gene expression between LS patients and controls identified in gene arrays, RQ-PCR was performed. Genes that display fold-change difference higher than three and are potentially relevant in cancer biology were selected. Selected genes included: UGT2B17 (UDP-glycosyltransferase 2B17), VCAN (versican), OR5H2 (olfactory receptor 5H2), SERPINB2 (plasminogen activator inhibitor), CCNA1 (cyclin A1), and AKT3. RQ-PCR validations corroborated genomic data for all six genes.

Log2 expression values are shown using dot-plot, which was performed using Partek GS. Dot-plots are shown in Fig. 2 (insets). Levels of UGT2B17 mRNA, a member of the UDP-glycosyltransferase (UDPGT) family, were markedly elevated in LS-derived cells in comparison with controls. The UDPGTs are of major importance in the conjugation and subsequent elimination of potentially toxic xenobiotics and endogenous compounds, including steroid hormones. However, mRNA levels of VCAN, OR5H2, SERPINB2, CCNA1, and AKT3 were significantly lower in LS patients.

Figure 2
Dot plot and validation analyses of selected genes differentially expressed in LS. Selected genes that display fold-changes higher than three between LS patients and controls were selected for further validation using RQ-PCR. The selected genes include: UGT2B17 (UDP-glycosyltransferase-2B17), VCAN (versican), OR5H2 (olfactory receptor 5H2), SERPINB2 (plasminogen activator inhibitor), CCNA1 (cyclin A1), and AKT3. The insets denote the gene expression values (dot plots) in four individual patients and four controls as measured in gene arrays. Gray bars and dots, LS patients; black bars and dots, controls.
Analysis of transcription factors involved in IGF1R gene regulation

To gain further insight into specific genes whose deregulated expression in LS might impinge upon specific IGF axis components, we measured the basal levels of transcription factors SP1 and pTEN in four LS-derived lymphoblastoid lines and four normal cultures. The rationale for this choice of genes was the fact that transcription factor SP1 was identified as a critical regulator of the IGF1R gene. PTEN was selected because it is a well-characterized target for IGF1 action (Beitner-Johnson et al. 1995, Werner et al. 1996, Werner 2012). Western blot analysis revealed that LS-derived lymphoblastoids express higher levels of tumor suppressor pTEN than controls. However, levels of oncogenic protein SP1 were markedly reduced in LS cells (Fig. 3). Consistent with a reduced rate of cancer incidence, LS-derived cells contain abundant quantities of transcription factors associated with both IGF1R gene suppression and cell cycle inhibition (i.e. pTEN). Conversely, LS cells express reduced levels of potentially oncogenic proteins linked to IGF1R gene transcription and cell cycle progression (i.e. SP1).

Analysis of IGF1R downstream cellular mediators in LS

To examine the expression levels of cellular proteins downstream of IGF1R, we next measured the levels of Akt and ERK, two prototypical components of the PI3K-Akt and ras-raf-MAPK pathways, respectively. In accordance with a reduced expression and activation of the IGF1R in LS patients, levels of total and phospho-Akt were reduced in patients compared with controls (Fig. 4). Interestingly, and despite the fact that no major differences in total ERK expression levels were noticed between LS and controls, a significant reduction in ERK phosphorylation was observed in LS cells.

Cell cycle analyses

Following identification of differentially expressed genes involved in cell cycle regulation, we next investigated potential differences in cell cycle dynamics. To evaluate the hypothesis that protection from cancer in LS patients is associated with altered cell cycle dynamics, lymphoblastoids (n=3 patients and 3 controls) were exposed to etoposide (20µM) for 24h, followed by cell cycle profiling (Fig. 5A). Etoposide is an anticancer agent that inhibits DNA topoisomerase II. The majority of LS
cells (69%) were in G0–G1 phase, compared with 61% of control cells. The proportion of cells in G2–M phases in control cells was increased by 50% compared with LS (4.4% in controls compared with 2.2% in LS; \( P = 0.02 \)), while the proportion of cells in S phase was increased by 120% compared with LS (12.3% in controls compared with 5.6% in LS; \( P = 0.003 \)). Cell cycle analyses are consistent with reduced proliferation of LS-derived cells upon exposure to etoposide in comparison with controls. In agreement with cell cycle distribution data, cyclin A1 (Fig. 2) and cyclin D1 (not shown) levels were markedly reduced in LS in comparison with healthy lymphoblastoids. Notably, no differences in cell cycle distribution between control and LS cells were observed under basal, nonstimulated conditions (not shown).

**Apoptosis measurements**

To evaluate the sensitivity of LS cells to DNA damage in comparison with control cells, lymphoblastoids were exposed to etoposide (20 µM) for 24 h, followed by apoptosis analysis. Results of flow cytometry indicate that the percentage of apoptotic cells under basal conditions was 40% higher in LS compared with controls (\( P = 0.0005 \)) (Fig. 5B). The percentage of necrotic cells under basal conditions was increased by 27% in LS compared with controls (\( P = 0.0002 \)) (Fig. 5B). The percentages of apoptotic and necrotic cells were increased by 20 and 17%, respectively, after treatment with etoposide in LS in comparison with controls (not shown).

**Proliferation analyses**

To explore the effect of IGF1-free medium on proliferation rate in LS cells compared with controls, XTT assays were performed. Cells were grown in RPMI-1640 medium supplemented with 4% of albumin-free serum replacement for 3 days, after which proliferation tests were conducted. As shown in Fig. 5C, significant differences in proliferation rates were seen between LS and controls. Specifically, the proliferation rate of LS cells was reduced by 50% compared with controls (\( P = 0.002 \)).

**Oxidative damage response**

To examine the hypothesis that cancer protection in LS might be associated with enhanced resistance to oxidative damage, lymphoblastoids were treated with increasing doses of the oxidative agent paraquat for different periods of time, after which cell survival was assessed by XTT assays. Results obtained indicate that LS-derived lymphoblastoids displayed an enhanced survivability compared with control cells over a broad
range of paraquat concentrations (0.01–10 mM) (Fig. 6). Of interest, differences in response to oxidative insults were seen in both the presence (A) and the absence (B) of serum.

Evaluation of autophagy markers

Finally, to investigate the potential involvement of the autophagic machinery in the differential response to oxidative damage, LC3β (Fig. 7A and C) and P62 (Fig. 7B and D) levels were measured by western blots. LC3β and P62 constituted to be validated markers of autophagy, being usually expressed in a diametrically opposite manner (Mizushima et al. 2007, Moscat et al. 2009, Rosenfeldt et al. 2011). Results obtained indicate that, under basal conditions, LC3β levels were significantly higher in control lymphoblastoids than in LS-derived cells. However, basal levels of P62 were three-fold higher in LS cells. Following paraquat treatment for 24 h, P62 levels increased approximately two-fold in LS cells while only a marginal increase was seen in control cells.

Discussion

Laron syndrome is a genetic disorder caused by molecular defects (deletion or mutation) of the GH-R gene, or postreceptor pathways, leading to congenital IGF1 deficiency (Laron 2004). This type of dwarfism was first identified in 1958 in a Jewish family of Yemenite origin. Three family members had dwarfism, whereas other five siblings had a normal stature. Studies reported in 1966 established that this condition was associated with high serum GH values (Laron et al. 1966). The typical features of classic LS are short stature, typical face, obesity, and low serum IGF1 levels, unresponsive to the administration of exogenous GH. Several molecular defects of the GH-R gene have been identified, depending on the ethnic group and geographic origin of the patients (Shevah et al. 2006, Laron et al. 2011).
The role of the GH–IGF1 axis in cancer biology has been well established (LeRoith et al. 2003, Pollak 2012). IGF1 has been identified as an important progression factor during the cell cycle. The IGF1R, which mediates the biological actions of IGF1, exhibits potent antiapoptotic, cell survival activities and is usually expressed by most primary tumors and cancer-derived cell lines (Baserga et al. 2003, Werner 2009, Werner & Bruchim 2009). Importantly, the IGF1R emerged in recent years as a promising therapeutic target in oncology, and specific antibodies as well as small molecular weight tyrosine kinase inhibitors have been developed targeting tumors with IGF1R overexpression. Controversy emerged, however, regarding the role of endocrine IGF1 as a cancer risk factor. While early studies suggested that high levels of circulating IGF1 were associated with an elevated risk of premenopausal breast and prostate cancers (relative risk > 4), most recent epidemiological studies are in agreement that the increased risk conferred by high endocrine IGF1 is only ~1.5–2 (Renehan et al. 2004). Furthermore, taller people have higher plasma IGF1 values and being taller was associated with cancer risk in a cohort of 1,297,124 women followed for a total of 11.7 million person-years (Green et al. 2011). The biochemical and cellular mechanisms associated with elevated tumor incidence in an IGF1-rich environment, however, have not yet been totally dissected.

The finding that congenital IGF1 deficiencies and, in particular, LS are associated with a reduced cancer incidence offers the opportunity to study previously unrecognized aspects of IGF1 biology in cancer etiology. Notably, the epidemiological study by Steuerman and coworkers (2011) included, by most estimates, more than 50% of all LS patients worldwide (not including the Ecuadorian cohort, see next). None of the 230 LS patients included had developed a malignancy, despite the fact that 66 of them had been treated with IGF1 for several years. The difference between the prevalence of malignancies in all the first-degree relatives (4%), further family members (23.7%), and siblings only (3.3%), with that in patients (0%), was highly significant. Similar protection from cancer was reported in an Ecuadorian family members (23.7%), and siblings only (3.3%), with malignancies in all the first-degree relatives (4%), further family members (23.7%), and siblings only (3.3%), with that in patients (0%), was highly significant. Similar protection from cancer was reported in an Ecuadorian cohort of LS patients (Guevara-Aguirre et al. 2011). This cohort has been followed since 1988 and mortality data were collected for 53 LS subjects who died before 1988. Cancer was not a cause of death among the individuals who died before 1988 and there was no evidence of cancer among 99 LS patients since 1988. Among relatives, cancer prevalence was similar to the general population. The lack of cancer among LS patients deserves extra attention considering that tumor spread seems to reflect immune escape of circulating malignant cells. Immune deficiency has been reported in association with congenital IGF1 deficiencies (Green et al. 2011), suggesting that cancer protection in LS is most probably not related to improved immune surveillance but rather to a drastic reduction in the primary events leading to cancer development.

Our study identified a number of genes that are differentially expressed in lymphoblastoid cells of LS patients compared with healthy controls of the same gender, age, and ethnic group. This differential expression may, potentially, explain the evasion of LS patients from cancer. Bioinformatic analyses revealed that the differences in gene expression occur in several pathways, including apoptosis, metabolic control, cytokine–cytokine receptor interaction, Jak-STAT signaling, and PI3K-AKT signaling. Of relevance, our analyses detected markedly reduced levels of gene transcripts associated with oncogenic transformation and cell cycle progression. These genes include, among others, cyclin A1, cyclin D1, serpin B2, versican, and zinc finger protein Sp1. Hence, data are consistent with the notion that life-long lack of exposure to endocrine IGF1 in LS might lead to downregulation of genes with a positive impact on proliferation and mitogenesis. While the mechanism/s responsible for IGF1 action has/have not been addressed in the present study, it is reasonable to assume that IGF1 exposure activates epigenetic and transcription pathways critical for gene expression. Lack of exposure to normal IGF1 levels in LS patients abrogates these signaling pathways, with important consequences in terms of cancer avoidance. In this context, we provide evidence that downregulation of cell cycle genes such as cyclin D1 and cyclin A1 was associated with altered cell cycle dynamics and apoptosis. Finally, and given the fact that lymphoblastoid cultures are maintained in serum-containing media, data regarding IGF1 expression are sometimes difficult to interpret. Specifically, while IGF1 mRNA levels are very low in normal lymphoid cells of LS patients, our data indicate a 1.99-fold change in LS-derived lymphoblastoids compared with healthy cells (P = 0.045) (Table 3). We assume that these unexpected results are due to the fact that bovine serum contains high levels of IGF1 or, alternatively, to the immortalized status of these cells.

Conversely, genomic analyses revealed enhanced expression of genes associated with protection from toxic xenobiotic substances and metabolites in LS-derived lymphoblastoid cells. Specifically, the UGT genes, which code for enzymes that convert xenobiotic and endobiotic substances into lipophilic compounds, thereby facilitating clearance from the body as part of a liver detoxification system, were several-fold higher in LS than in healthy
controls. Of special interest is the elevated expression of both UGT2B15 and UGT2B17 (11- and 7-fold, respectively) in LS cells. For UGT2B17, the increased expression was validated by RQ-PCR. The UGT enzymes encoded by both genes facilitate the catabolism of steroid hormones with key roles in certain cancers, in particular breast and prostate tumors. These data points toward an enhanced capacity of LS cells to respond to a variety of cellular insults. Consistent with these genomic findings, we demonstrate that the ability of LS cells to respond to oxidative damage was higher than that of control cells over a broad range of paraquat doses.

Autophagy is a major housekeeping mechanism, crucially involved in the maintenance of normal cellular homeostasis. The morphological hallmark of autophagy is the formation of double-membrane vesicles called autophagosomes, which sequester and transport integral regions of the cytoplasm to the lysosomal system. This mechanism enables the clearance of damaged or superfluous proteins and organelles. The role of autophagy, however, extends beyond the removal of damaged cell components to many physiological and pathological processes such as development, oxidative stress, and tumorigenesis. Proteins LC3β and P62 are involved in different aspects of autophagosome biology, and are regarded as valid autophagy markers. P62 is specifically involved in the activation of the mTORC1 pathway, which negatively regulates autophagy in response to nutrient availability. The finding that basal LC3β levels were reduced in LS cells while P62 values were elevated, in comparison with healthy controls, along with a major paraquat-induced increase in P62 levels in patient cells, suggests the existence of a differentially regulated autophagy machinery in LS. These autophagic adaptations correlate with enhanced survival of LS cells in response to oxidative stress.

In the context of IGF1R gene regulation, we provide evidence that genes associated with transcriptional activation (i.e. transcription factor SP1) were markedly reduced in LS cells (Beitner-Johnson et al. 1995). In addition to its cardinal role in IGF1R gene activation, zinc finger protein SP1 is a major player in cellular transformation and oncogenesis. We suggest that reduced levels of SP1 in LS cells may provide protection from cancer development via suppression of IGF1R activity. Cancer protection associated with reduced circulating IGF1 was also noticed in animal models of IGF1 deficiency. Thus, the ‘Laron mouse’ (Gh-t/Gh-Bp KO) was shown to exhibit reduced estrogen-independent mammary carcinogenesis when crossed with the C3(1)Tag mouse, which develops estrogen receptor α negative mammary cancers (Zhang et al. 2007). A similar crossing led to a significant decrease in prostate epithelial cell proliferation and an increase in basal apoptotic indices (Wang et al. 2005).

In summary, our analyses identified genes, pathways, and biological processes that are impaired in congenital IGF1-deficient patients and are associated with reduced cancer prevalence. Future studies will address the transcriptional and epigenetic mechanisms that underlie the IGF1-dependent pathways associated with cancer protection at the organism level.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
L Lapkina, Z Laron, and H Werner conceived of and designed the experiments. The experimental procedures were performed by L Lapkina, I Rotem, and R Sarfstein, and were analyzed by L Lapkina, R Sarfstein, D Gurwitz, M Pasmanik-Chor, Z Laron, and H Werner. L Lapkina and H Werner prepared the manuscript. M Pasmanik-Chor contributed analysis tools.

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