Leptin-R and its association with PI3K/AKT signaling pathway in papillary thyroid carcinoma

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Abstract

The putative role of leptin and its receptor (Ob-R) in the pathogenesis of various primary human malignancies has been reported; however, their role in papillary thyroid cancer (PTC) has not yet been evaluated. We investigated the role of Ob-R in a large tissue microarray cohort of PTC followed by *in vitro* studies using a panel of PTC cell lines. Ob-R overexpression was seen in 80% PTCs and was significantly associated with poor disease-free survival ($P=0.0235$). PTCs that overexpressed Ob-R showed an aggressive phenotype characterized by older age, extrathyroid extension, larger tumor size, nodal metastasis, advanced stage, tall cell variant histological subtype, and a poor disease-free survival ($P=0.0005$, $P=0.0006$, $P=0.0398$, $P=0.0004$, $P=0.0111$, $P=0.0003$, and $P=0.0235$ respectively). However, Ob-R expression was not an independent prognostic marker to predict disease-free survival in multivariate analysis. PTCs with overexpression of Ob-R showed a significant direct association with overexpression of XIAP ($P<0.0001$) and Bcl-XL ($P<0.0001$). *In vitro* analysis showed that leptin stimulated cell proliferation and inhibited apoptosis via activation of phosphatidylinositol 3' kinase (PI3K)/protein kinase B (AKT) signaling pathway. Inhibition of PI3K activity by its inhibitor LY294002 abrogated leptin-mediated PI3K/AKT signaling. Gene silencing of Ob-R in PTC cells resulted in downregulation of phospho-AKT, Bcl-XL, and XIAP expression suggesting that leptin-mediated pathogenesis of PTC occurs via involvement of these downstream targets. Altogether, these data show that leptin plays an important role in PTC pathogenesis through PI3K/AKT pathway via Ob-R and is a potential prognostic marker associated with an aggressive phenotype and poor disease-free survival.

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Introduction

Papillary thyroid carcinoma (PTC) is the most common malignant thyroid tumor, representing 80–90% of all thyroid malignancies (Hundahl *et al.* 1998). The prognosis for PTC is often favorable; however, ~20% of PTC tumors recur and some reach advanced stages (Loh *et al.* 1997). Several clinicopathological variables including stage, cancer invasion, and distant

metastasis are used for prognostication and treatment selection for PTC (Hay 1990, Siironen *et al.* 2005). However, the factors and mechanisms determining the aggressive behavior of some papillary carcinomas are not yet completely understood.

Similar to the increasing incidence of thyroid cancer in the United States (Davies & Welch 2006), incidence of thyroid cancer is also gradually increasing in Saudi

Arabia (Al-Eid 2004, Khoja 2008). A recent report indicates that there are a fairly high number of Saudi patients with differentiated thyroid cancer who had locally advanced disease at presentation and/or persistent disease after adequate standard treatment (Raef *et al.* 2008). It is highly improbable that genetic factors alone can explain increase in PTC incidence rate over the past 2 decades, suggesting the role of other environmental factors, including obesity as an etiological factor. So, we believe that population like Saudis, in which dramatic changes in cancer incidence have occurred, may offer a unique opportunity to identify gene environmental interaction. There is a known association between obesity and cancers such as colon cancer (Pan *et al.* 2004). Many factors including those related to lifestyle may underlie this association, but factors produced by the increased fat mass may be involved. Prevalence of obesity is high in Saudi Arabia among both genders, and the reported incidence is 33.9% (Al-Othaimen *et al.* 2007).

Leptin, the product of obesity gene (Ob), is a 16 kDa adipokine produced predominantly by adipocytes, which regulates food intake and energy expenditure (Janeckova 2001). The adipokine leptin is elevated in the serum of obese people in direct correlation to fat mass (Considine *et al.* 1996), and may be an important biological link between obesity and various types of cancers. Leptin exerts its activity through specific membrane receptor, the obesity receptor (Ob-R), which is assigned to class I cytokine receptor family (Zabeau *et al.* 2003). Six splice variants of Ob-R have been identified up to now; a long isoform, four short isoforms discriminated by the different lengths of intracellular domain, and the secreted isoform, which modulates blood leptin (Zabeau *et al.* 2003, Hegyi *et al.* 2004). According to the current knowledge, leptin signaling pathway is mainly transduced by Janus activated kinase/signal transducers and activators of transcription (JAK/STAT), mitogen-activated protein kinase (MAPK), and phosphatidylinositol-3'-kinase (PI3K) signaling pathways (Hegyi *et al.* 2004). Previously, we have shown that aberrant PI3K/AKT signaling activation plays a role in Middle Eastern PTC pathogenesis (Abubaker *et al.* 2008b). However, the expression of leptin receptors and the potential correlation between leptin receptors and PI3K/AKT activation have yet to be studied in the Middle Eastern population.

Therefore, in the present study, we assessed the prevalence of leptin receptor (Ob-R), expression, and its correlation with expression patterns of PI3K/AKT pathway proteins as well as other clinicopathological variables in large cohort of Saudi PTC. We further

conducted *in vitro* analysis to examine the expression of leptin receptors using PTC cell lines, and then we investigated the effect of leptin on malignant properties of PTC including proliferation and inhibition of apoptosis.

Materials and methods

Patient selection and tissue microarray construction

A total of 536 patients with PTC diagnosed between 1988 and 2004 were selected from King Faisal Specialist Hospital and Research Centre. All PTC and 304 adjacent non neoplastic thyroid tissue were analyzed in a tissue microarray format. Clinical and histopathological data were available for all these patients. Long-term follow-up data were available for most of the patients. Tissue microarrays were constructed from formalin-fixed, paraffin-embedded PTC specimens as previously described (Bavi *et al.* 2006). Tumor regions were mapped by a pathologist for coring. The tissue microarray was constructed with 0.6-mm diameter cores spaced 0.8 mm apart using a modified tissue microarrayer (Beecher Instruments, Sun Prairie, WI, USA). The tissue microarray block was cut into 5 mm sections, adhered to the slide by an adhesive tape-transfer method (Instrumedics Inc., Hackensack, NJ, USA) and u.v. cross-linked. The Institutional Review Board of the King Faisal Specialist Hospital and Research Centre approved the study.

Immunohistochemistry

Tissue microarray (TMA) slides were processed and stained manually. The streptavidin–biotin peroxidase technique with diaminobenzidine as chromogen was applied. For antigen retrieval, Dako Target Retrieval Solution, pH 9.0 (catalog number S2368) was used, and the slides were microwaved at 750 W for 5 min and then at 250 W for 30 min. Primary antibodies used, their dilutions, and incidences are listed in Table 1. Endogenous peroxidase activity was quenched using 3% hydrogen peroxidase. Endogenous biotin was blocked, and all slides were counterstained with hematoxylin, dehydrated, cleared, and cover slipped with premount. Only fresh cut slides were stained simultaneously to minimize the influence of slide aging and maximize repeatability and reproducibility of the experiment.

Table 1 Antibodies used for tissue microarray immunohistochemical analysis

Antibody	Positive cases (%)	Clone	Company	Source	Dilution O/N ^a	Retrieval	Subcellular localization	Detection system
Leptin-R	410/512 (80.1%)	B-3	SCBT	Mouse monoclonal	1:20 O/N	pH 9, pressure cooker	Cytoplasmic	EnVision+
Leptin	252/513 (49.1%)	Y20	SCBT	Rabbit monoclonal	1:1000 O/N	pH 6, pressure cooker	Nuclear and cytoplasmic	EnVision+
pAKT	255/463 (55.1%)	Ser473	Cell signaling	Rabbit polyclonal	Predilute O/N	pH 9, microwave	Nuclear and cytoplasmic	Survival marker ^b
XIAP	126/505 (24.9%)	48	BD transduction	Mouse monoclonal	1:300 O/N	pH 9, microwave	Cytoplasmic	EnVision+
BCLXL	112/514 (21.8%)	54H6	Cell signaling	Rabbit polyclonal	1:800 O/N	pH 9, microwave	Cytoplasmic	EnVision+
PIK3CA	344/503 (68.4%)	p110 α	Cell signaling	Rabbit polyclonal	1:100 O/N	pH 9, pressure cooker	Cytoplasmic	EnVision+

^aOvernight incubation of primary antibody.^bSignal stain IMC detection kit.

Immunohistochemistry assessment

Ob-R expression was categorized by doing an *H* score as previously described (Uddin *et al.* 2008c). Each TMA spot was assigned an intensity score from 0 to 3, and proportion of the tumor staining for that intensity was recorded as 5% increments from a range of 0 to 100 (P0, P1–3). A final *H* score (range 0–300) was obtained by adding the sum of scores obtained for each intensity and proportion of area stained ($H \text{ score} = I1XP1 + I2XP2 + I3XP3$). PTCs were grouped into two groups based on X-tile plots: one with complete absence or reduced staining ($H \text{ score} = 0–25$), and the other group showed over expression ($H \text{ score} > 25$) depending on the *H* score. Similarly, X-tile plots were used to stratify the PTC cases into two groups for leptin (Ob), XIAP, and Bcl-XL. p-AKT scoring was done as described earlier (Uddin *et al.* 2006, Abubaker *et al.* 2008a). X-tile plots were constructed for assessment of biomarker and optimization of cutoff points based on outcome as has been described earlier (Camp *et al.* 2004, McCarthy *et al.* 2005, Badve *et al.* 2007, Grossman *et al.* 2007). X-tile plots are created by dividing marker data into three populations: low, middle, and high (i.e. two divisions). We used X-tile version 3.6.1 to define the optimal cutoff point for the overexpression and reduced/absent expression scores for Ob-R. Using this approach, scores of 0–25 were defined as reduced/absent Ob-R expression and 26–300 as Ob-R overexpression.

Statistical analysis

The JMP7 (SAS Institute, Inc., Cary, NC, USA) software package was used for data analyses. Survival curves were generated using the Kaplan–Meier method, with significance evaluated using the Mantel–Cox log-rank test. Risk ratio was calculated using the Cox proportional hazard model in both univariate and multivariate analyses. The χ^2 tests were used to examine relationship between nominal variables. The limit of significance for all analyses was defined as a *P* value of 0.05.

Cell culture

PTC cell lines B-CPAP and 8505C were grown, and maintained as described earlier (Uddin *et al.* 2008c). Briefly, PTC cell lines were cultured in RPMI 1640 medium supplemented with 10% (vol/vol) fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin at 37°C in humidified atmosphere containing 5% CO₂.

Reagents and antibodies

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays (MTT) was purchased from Sigma. Ob-R antibody was purchased from Abcam (Cambridge, UK). Antibodies against phospho-AKT, phospho-FKHR/FoxO1 antibodies were purchased from Cell Signaling Technologies (Beverly, MA, USA). β -Actin antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Annexin V kit was purchased from Molecular Probes (Eugene, OR, USA). Apoptotic DNA-ladder kit was obtained from Roche.

MTT assays

Cells (10^4) were incubated in triplicate in a 96-well plate in the presence or absence of indicated test doses of leptin in a final volume of 0.20 ml for 48 h. The ability of leptin to induce cell growth was determined by MTT cell proliferation assays, as previously described (Uddin *et al.* 2008a). Replicates of six wells for each dosage including vehicle control were analyzed for each experiment.

Annexin V/propidium iodide dual staining

PTC cell lines were treated with the indicated concentrations of leptin in conditions treated as indicated in figure legends. The cells were harvested, and the percentage of cells undergoing apoptosis was measured by flow cytometry after staining with fluorescein-conjugated annexin V/propidium iodide as previously described (Hussain *et al.* 2008).

Cell lysis and immunoblotting

Cells were treated either with leptin or leptin in combination with PI3K inhibitor LY294002 described in the legends and lysed as previously described (Hussain *et al.* 2007). Proteins (15–20 µg) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (Immobilon, Millipore, Billerica, MA, USA). Immunoblotting was done with different antibodies and visualized by enhanced chemiluminescence (Amersham) method.

Gene silencing using small interfering RNA

Leptin receptor small interfering RNA (siRNA) and scrambled control siRNA were purchased from Qiagen. Cells were transfected using Lipofectamine 2000 (Invitrogen) and siRNA as described earlier (Uddin *et al.* 2008b). After transfection for 6 h, the lipid and siRNA complex was removed, and fresh growth medium was added and incubated for 48 h. Cells were then treated with leptin as indicated, and after lysis, protein levels were determined by western blot analysis with specific antibodies.

Real-time quantitative PCR

Total RNA was extracted from PCPAP thyroid cell line after transfection with Ob-R siRNA using TRIzol reagent (Invitrogen). Five microgram of total RNA for each sample was reverse-transcribed in a 20-µl reaction

volume with SuperScript First-strand Synthesis System (Invitrogen) according to the manufacturer's protocol. The quantitative PCR reaction was performed in glass capillary (Roche) with a final reaction volume of 10 µl of $1 \times$ LightCycler-FastStart DNA Master SYBR Green I reaction mixture (Roche) containing FastStart *Taq*, reaction buffer, and deoxynucleoside triphosphate, 1 mM $MgCl_2$, and final concentrations of 0.5 µM for each primer. For each sample, PCR amplification was performed in duplicate with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as an endogenous control. The primer sequences for PCR were as follows: OBR-S (sense): 5'-TGATTCTGTGG-TGAAGCCACTGC-3', OBR-S (anti-sense): 5'-TTCC-ATTGTACTTCTTTTCCACTT-3' for OBR-S; OBR-L (sense): 5'-AATCACTTCTCCAGAAACGTTTGA-GC-3', OBR-L (anti-sense): 5'-AGACCACAGT-TGTTGGCATC-3' for OBR-L; Bcl-XL (sense): 5'-TTACCTGAATGACCACCTA-3', Bcl-XL (anti-sense): 5'-ATTTCCGACTGAAGAGTGA-3' for Bcl-XL; XIAP (sense): 5'-GATGCTGTGAGTTCT-GATACG-3', XIAP (anti-sense): 3'-CCTAATG-TCGAAACTGAAC-3' for XIAP; GAPDH (sense): 5'-CCATGGAGAAGGCTGGGG-3', GAPDH (anti-sense): 5'-CAAAGTTGTCATGGATGACC-3' for GAPDH. The relative expression ratio of a target gene is calculated based on efficiency (E) and crossing point (CP) deviation of BCPAP transfected cell line (OBR siRNA) versus control (scrambled siRNA), and expressed in comparison to a reference gene, GAPDH (Pfaffl *et al.* 2002, Siraj *et al.* 2007).

Results

Immunohistochemical detection of Ob-R expression

Levels of Ob-R were examined by immunohistochemistry (IHC) in a series of 536 PTC. High levels of Ob-R expression were seen in 80.0% (410/512) PTC, and in 37.0% (97/262) adjacent non neoplastic thyroid tissue (Fig. 1). The incidence as well as mean expression levels of Ob-R was higher in PTC (mean 148.73 ± 107.41) as compared to adjacent non neoplastic thyroid tissue (41.45 ± 64.14 ; $P < 0.0001$). Ob-R expression was significantly higher in the PTC as compared to non neoplastic thyroid tissue (Fig. 2a).

Association of Ob-R with PI3K/AKT pathway proteins

As shown in Table 2, PTC with Ob-R overexpression showed a significant direct association with PIK3CA 110 α protein (p110 α) expression ($P < 0.0001$).

Figure 1 Leptin-R expression in PTC and its association with leptin and downstream anti-apoptotic markers XIAP and Bcl-XL. A PTC tissue microarray spot showing overexpression of (a) leptin-R, (c) leptin, (e) XIAP, and (f) Bcl-XL. Another PTC spot showing low expression of (b) leptin-R, (d) leptin, (f) XIAP, and (g) Bcl-XL. Original magnification $\times 200$ with the inset showing a higher magnification $\times 400$ (immunoperoxidase stain).

Figure 2 (a) Box plot charts indicate the mean and s.d. of leptin-R expression in two groups – adjacent non neoplastic thyroid tissue and papillary thyroid carcinoma. The expression of Leptin-R in PTC (148.73 ± 107.41) was significantly higher than adjacent non neoplastic thyroid tissue (41.45 ± 64.14 ; $P < 0.0001$). (b) Patients with overexpression of Ob-R had a reduced disease-free survival of 68.9% at 5 years as compared to 79.3% with reduced Ob-R expression ($P = 0.0235$).

In addition, PTCs with Ob-R overexpression showed a significant association with downstream targets of PI3K signaling pathway: anti-apoptosis markers XIAP ($P < 0.0001$) and Bcl-XL ($P < 0.0001$).

Ob-R correlation with clinicopathological parameters

Ob-R expression was correlated with various clinicopathological parameters, and the results are presented in Table 2. PTCs that overexpressed Ob-R showed a aggressive phenotype characterized by older age, extrathyroid extension, larger tumor size, nodal metastasis, advanced stage, and tall cell variant histologic subtype ($P = 0.0005$, $P = 0.0006$, $P = 0.0398$, $P = 0.0004$, $P = 0.0111$, and $P = 0.0003$ respectively).

Patients with reduced Ob-R expression had a better disease-free survival of 79.3% at 5 years as compared to 68.9% with overexpression of Ob-R ($P = 0.0235$; Fig. 2b). In the multivariate analysis using Cox proportional hazard model for multiple factors like age, gender, extrathyroidal extension, AJCC stage, and Ob-R expression, the relative risk was 1.28 for Ob-R overexpression (95% CI 0.83–1.97; $P = 0.2487$) and 10.08 for advanced AJCC stage (95% CI 5.29–19.22; $P \leq 0.0001$). Thus, Ob-R was not an independent prognostic marker in multivariate analysis (Table 3).

Leptin increase proliferation of PTC cells

The effects of leptin on growth rate of PTC cell lines were determined using MTT assay. 8505C and B-CPAP cells were initially serum starved for 24 h and then stimulated with various doses of recombinant leptin (10–200 ng/ml) for 48 h and compared to cell serum-free control cultures. As shown in Fig. 3a, leptin induced significant cell growth of both PTC cell lines in a dose-dependent manner.

Leptin prevent serum-starved apoptosis in PTC cells

PTC cell lines were seeded in six-well plates, and after 24 h, serum-free medium alone or 100 ng/ml leptin was added. After 48 h, apoptosis was measured by annexin/PI staining. As shown in Fig. 3b, serum starvation resulted in apoptosis. Treatment of PTC cell line with leptin significantly prevented serum-starved apoptosis demonstrating that leptin counteracts apoptosis in PTC cells ($P = 0.0005$).

Leptin activates the PI3K/AKT signaling pathway

PI3K pathways have been implicated in playing crucial roles in regulating cell growth, cell proliferation, and prevention of apoptosis, which altogether attribute tumorigenesis (Osaki et al. 2004). In view of these findings, we sought to determine whether PI3K signaling is activated during leptin stimulated PTC cell line proliferation. B-CPAP cells were stimulated with 100 ng/ml leptin for various time periods. Cells were lysed, and proteins were separated on SDS-PAGE and immunoblotted with p-AKT (activated AKT) and p-FOXO1 antibodies. As shown in Fig. 4a, leptin treatment of B-CPAP phosphorylated AKT and FOXO1 as early as 15 min and remained phosphorylated till 3 h. Similar results were obtained with other PTC cell lines (data not shown). These results show that leptin-mediated cell proliferation occurs via PI3K/AKT signaling pathway.

Table 2 Clinical characteristics and Ob-R in papillary thyroid cancer (PTC)

	Total		Ob-R overexpression		Low Ob-R expression		P value
	No.	%	No.	%	No.	%	
No. of patients	512		410	80.1	102	19.9	
Age (years)							
≤45	306	59.8	230	75.2	73	24.8	0.0005
>45	206	40.2	180	87.4	26	12.6	
Sex							
Female	371	72.5	292	78.7	79	21.3	0.2003
Male	141	27.5	118	83.7	23	16.3	
Extrathyroidal extension							
Absent	254	49.6	188	74.0	66	26.0	0.0006
Present	258	50.4	222	86.0	36	14.0	
pT							
pT1	141	28.2	104	73.8	37	26.2	0.0398
pT2	90	18.0	71	78.9	19	21.1	
pT3	254	50.8	214	84.2	40	15.8	
pT4	15	3.0	14	93.3	1	6.7	
pN							
pN0	176	37.1	126	71.6	50	28.4	0.0004
pN1	298	62.9	254	85.2	44	14.8	
pM							
pM0	445	89.5	357	80.2	88	19.8	0.5799
pM1	52	10.5	40	76.9	12	23.1	
Stage							
I	288	60.4	220	76.4	68	23.6	0.0111
II	34	7.1	25	73.5	9	26.5	
III	35	7.3	30	85.7	5	14.3	
IV	120	25.2	107	89.2	13	10.8	
Histology type							
Follicular variant	65	12.7	39	60.0	26	40.0	0.0003
Papillary–classical	428	83.6	355	82.9	73	17.1	
Tall cell variant	19	3.7	16	84.2	3	15.8	
Surgical margins							
Positive	73	38.6	64	87.7	9	12.3	0.4407
Negative	116	61.4	97	83.6	19	16.4	
OB-Y20							
Any one high	250	49.6	218	87.2	32	12.8	0.0004
Both low	254	50.4	190	74.8	64	25.2	
p-AKT							
High (2–3)	245	54.3	202	82.4	43	17.6	0.5232
Low (0–1)	206	45.7	165	80.1	41	19.9	
XIAP							
Above 120	126	25.4	124	98.4	2	1.6	<0.0001
Below = 120	370	74.6	278	75.1	92	24.9	
BCLXL							
Above 270	112	22.2	109	97.3	3	2.7	<0.0001
Below = 270	392	77.8	300	76.5	92	23.5	
PIK3CA							
Above 170	340	69.0	301	88.5	39	11.5	<0.0001
Below = 170	153	31.0	97	63.4	56	36.6	

Table 3 Distribution of Leptin-R in papillary thyroid carcinoma by Cox model analysis

Clinical parameters	Univariate		Multivariate	
	Risk ratio (95% CI)	P value	Risk ratio (95% CI)	P value
Age above=45	2.60 (1.93–3.49)	<0.0001	0.34 (0.18–0.65)	0.0014
Sex male	1.36 (0.98–1.87)	0.0624	1.21 (0.86–1.70)	0.2719
ET: present ^a	2.07 (1.53–2.82)	<0.0001	1.25 (0.88–1.77)	0.2081
Advanced stage	4.41 (3.23–6.06)	<0.0001	10.11 (5.19–18.84)	<0.0001
Histology	1.59 (0.75–2.93)	0.2065	1.38 (0.62–2.63)	0.3994
Leptin-R high	1.58 (1.08–2.40)	0.0184	1.28 (0.84–2.01)	0.2494

^aPresence of extrathyroidal extension.

Inhibition of PI3K prevents leptin-mediated AKT activation and its downstream effector FOXO1

Since our study has demonstrated that leptin stimulated PI3K signaling plays a role in PTC proliferation and promotes its anti-apoptotic effects, we sought to determine whether the inhibition of PI3K by its specific inhibitor, LY294002, abrogated leptin-mediated PI3K/AKT signaling in PTC cell lines. Cells were seeded on culture plates for 24 h. Starved PTC cell were pretreated with 20 μ M LY294002 for 2 h, and subsequently treated with and without 100 ng/ml leptin for 3 h. Cells were

lysed, and proteins were separated on SDS-PAGE and immunoblotted by antibodies against p-AKT and p-FOXO1. As shown in Fig. 4b, leptin phosphorylated AKT and FOXO1 in B-CPAP PTC cell line and pretreatment with LY294002, prevented AKT and FOXO1 phosphorylation suggesting that PI3K/AKT pathway plays a critical role in leptin-induced growth and proliferation of PTC cells. These data clearly demonstrate that leptin is acting upstream of PI3K/AKT pathway in modulating its anti-apoptotic response in PTC cells.

PTC cell lines express leptin receptors that mediate the PI3K/AKT signaling pathways

To investigate whether leptin receptors are functional and linked to coordinate with PI3K/AKT signaling pathway to regulate cell growth and proliferation of PTC cell lines, we utilized siRNA strategies to transfect Ob-R specific siRNA as well as scrambled non specific siRNA in B-CPAP cell line. After 48 h transfection, cell were starved and then treated with and without 100 ng/ml leptin for 3 h. As shown in Fig. 5a, B-CPAP expressed functional leptin receptors, as previously shown (Hardwick et al. 2001). Treatment of scrambled siRNA harboring B-CPAP cells with leptin showed activation of AKT, FOXO1, and elevated level of XIAP and Bcl-XL proteins that are involved in PI3K/AKT pathway and play a critical role in cell survival. On the other hand, expression of Ob-R specific siRNA in B-CPAP cells knocked down the expression of leptin receptor and prevented leptin-induced activation of AKT, FOXO1 as well as abrogated the expression of XIAP and Bcl-XL proteins. Gene silencing of Ob-R in B-CPAP cells downregulated Bcl-XL and XIAP at the transcription level (Fig. 5b). These data suggest that leptin utilizes PI3K/AKT signal transduction pathway in mediation of PTC cell proliferation, further confirming that leptin is acting upstream of PI3K/AKT pathway.

Figure 3 Leptin increases proliferation of PTC cells and prevents serum-starved apoptosis in PTC cells. (a) PTC cell lines were seeded in 96-well plates, and after 24 h, serum-free medium alone or with various doses of leptin as indicated was added. After 48 h, cell proliferation was measured MTT as described in Materials and methods. The graph displays the mean $\pm$ s.d. of six independent experiments. (b) PTC cell lines were seeded in six-well plates, and after 24 h, serum-free medium alone or 100 ng/ml was added. After 48 h, apoptosis was measured by annexin/PI staining.

Figure 4 Leptin activates PI3-kinase/AKT signaling pathway. (a) BCPAP cells were serum starved for 24 h in serum-free medium, followed by stimulation with recombinant leptin (100 ng/ml) for various time periods as indicated. After cell lysis, 20 μg proteins were separated by SDS-PAGE, transferred to immobilon membrane, and immunoblotted with antibodies against p-AKT-Ser 473, FOXO1, and β-actin. (b) LY294002 inhibitor abrogated leptin-induced anti-apoptosis PI3-kinase/AKT signaling in PTC. B-CPAP cells were serum starved for 24 h, and then cells were pretreated with 20 μM LY294002 for 2 h and subsequently stimulated with recombinant leptin (100 ng/ml) for 15 min; proteins were separated by SDS-PAGE, transferred to immobilon membrane, and immunoblotted with antibodies against p-AKT-Ser 473, FOXO1, and β-actin.

Discussion

Recent reports show that leptin is overexpressed in various cancer cells and plays a role in the development and/or progression of variety of malignancies including colon, gastric, endometrial, and breast cancers (Hu *et al.* 2002, Ishikawa *et al.* 2004, Hong *et al.* 2006, Koda *et al.* 2007a,b). These findings were further supported by experimental evidence that leptin can stimulate growth and prevent apoptosis in different cellular cancer models (Garofalo & Surmacz 2006, Hoda *et al.* 2007, Ogunwobi & Beales 2007). Earlier reports have highlighted that leptin might have an important role in thyroid pathophysiology due to

thyroid hormone involvement in thermogenesis, regulation of uncoupling proteins, and leptin levels are significantly higher in hypothyroid patients (Leonhardt *et al.* 1998, Zimmermann-Belsing *et al.* 2003).

However, the potential role of leptin/Ob-R in the pathogenesis of PTC has not been elucidated. In the current study, therefore, we examined the prevalence of leptin and its receptor Ob-R expression in 536 Saudi PTC samples. Our data show that Ob-R protein expression was detected in 80% PTC. Interestingly, Ob-R was significantly associated with older age, extrathyroid extension, larger tumor size, nodal metastasis, advanced stage, and tall cell variant histologic subtype, thereby indicating that Ob-R overexpression identifies an aggressive phenotype of Middle Eastern PTC. Furthermore, patients with high Ob-R-expressing tumors showed a significant poor disease-free survival ($P < 0.0235$) as compared with

Figure 5 Leptin required functional leptin receptor (Ob-R) for activation of PI3-kinase/AKT signaling in PTC cell lines. (a) B-CPAP cells were transfected with scrambled siRNA and leptin receptor siRNA (50 and 100 nM) with Lipofectamine. After 48 h, cells were starved and treated with 100 ng/ml for 3 h, and proteins were immunoblotted with antibodies against leptin receptor, p-AKT-Ser473, FOXO1, Bcl-XL, XIAP, and β-actin. (b) B-CPAP cells were transfected with scrambled siRNA and leptin receptor siRNA (100 nM) with Lipofectamine in six-well plates. After 48 h, total RNA was isolated. RT-PCR analysis of ObR-L, ObR-S, XIAP, and Bcl-XL was performed. The relative expression (fold of inhibition) of Ob-R, XIAP, and Bcl-XL analyzed by real-time PCR was normalized to GAPDH. $P < 0.01$ versus scrambled control; mean $\pm$ s.e.m.; $n = 6$.

reduced Ob-R expression. Raef *et al.* (2008) have reported a fairly high number of patients with differentiated thyroid cancer (DTC) in Saudi Arabia who had locally advanced disease at presentation and/or persistent disease after standard treatment (Raef *et al.* 2008). It is tempting to speculate that high Ob-R expression levels seen in around 80% of the Saudi Arabian PTCs could be one of the putative factors for this disease persistence and/or recurrence. Leptin-R along with a panel of other markers could be used to develop an algorithm to prognosticate PTC patients on fine needle aspiration biopsy (FNAB) or other presurgical screening assays. This would be quite similar to the recently reported use of leptin along with IL6 as key early markers of the metabolic alterations and symptoms associated with disease progression in advanced stage ovarian cancer (Maccio *et al.* 2008, Kim *et al.* 2009). The use of Ob-R as a panel of prognostic marker in thyroid cancer is yet only an attractive hypothesis and needs to be confirmed by similar such studies in different cohorts.

Interaction between leptin/Ob-R and other signaling pathway such as PI3K/AKT in oncogenesis has been reported in other solid tumors previously (Chen *et al.* 2006, Ogunwobi *et al.* 2006, Sharma *et al.* 2006, Hoda *et al.* 2007, Saxena *et al.* 2007). Therefore, we examined the relationship between the expression of Ob-R and the PI3K/AKT pathway protein targets in PTC. Activated AKT (pAKT) protein expression was seen in 55% of our PTC examined. However, no correlation was observed with Ob-R expression and AKT activation by IHC staining, which might suggest the presence of other upstream signaling pathways can also be involved in the activation of AKT. However, Ob-R overexpression was correlated with upstream marker PIK3CA 110 α protein expression ($P < 0.0001$) and other downstream targets of PI3K/AKT, the anti-apoptotic markers Bcl-XL and XIAP ($P < 0.0001$ and < 0.0001 respectively).

After documenting the high prevalence of Ob-R in Saudi PTC, we sought to conduct *in vitro* analysis to elucidate the molecular mechanism by which leptin exerts its oncogenic action on PTC and its relation with PI3K/AKT signaling pathway. Using PTC cell lines, we investigated the oncogenic effect of leptin on PTC cells, and this oncogenic effect is due to a combination of cell proliferation and inhibition of apoptosis by leptin. Our *in vitro* experiments clearly showed that leptin rapidly stimulates the PI3K pathway and induced the phosphorylation of AKT, thus activating of this key signal transduction pathway associated with cell growth. In addition, prevention of leptin-induced activation of PI3K with chemical

inhibitors in turn significantly reduced the activation of AKT pathway. AKT provides a survival signal protecting cells from apoptosis induced by various stresses by multiple mechanisms such as phosphorylation of Bad, GSK3, FOXO transcription factors, and caspase 9 (Datta *et al.* 1997, Brunet *et al.* 2001). Phosphorylation of these proteins results in inactivation of their apoptotic functions. As shown in our study, AKT phosphorylation was increased in leptin-treated human PTC cells, and inhibition of PI3K with LY294002 abolished leptin-induced proliferation. Thus, we can hypothesize that leptin mediates its actions upstream of PI3K.

In summary, our data for the first time deciphered the molecular mechanisms responsible for the leptin-mediated PTC cell proliferation, establishing direct link between leptin and PTC carcinogenesis through PI3K/AKT signaling pathway. Ob-R expression is seen in majority of the PTC, and OB-R could be a panel of biomarkers with potential prognostic utility.

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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