Functional analysis of the -2548G/A leptin gene polymorphism in breast cancer cells

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Leptin is overexpressed in human breast tumors and is produced by breast cancer cells in response to obesity-related stimuli. The leptin promoter polymorphism Lep-2548G/A can be associated with increased leptin secretion by adipocytes and elevated cancer risk. However, molecular mechanisms underlying the link between Lep-2548G/A and breast cancer have never been addressed. Lep-2548G/A is proximal to a binding site for the transcriptional factor Sp1. Furthermore nucleolin, a transcriptional repressor, can bind Sp1 or its consensus site. Consequently, we focused on the impact of Lep-2548G/A on Sp1- and nucleolin-dependent leptin transcription in breast cancer cells. The Lep-2548G/A was identified in a homozygous conformation in BT-474 and SK-BR-3 breast cancer cells, in a heterozygous conformation in MDA-MB-231 cells, and a wild-type Lep-2548G/G sequence was present in MCF-7 and ZR-75-1 cells. The occurrence of Lep-2548A/A and Lep-2548G/A coincided with high and intermediate leptin mRNA expression, respectively, while cells containing Lep-2548G/G expressed low leptin mRNA levels. We demonstrated that the existence of Lep-2548G/A improved efficient recruitment of Sp1 to DNA under insulin treatment, while Sp1 loading on DNA containing Lep-2548G/G was not insulin-dependent. In contrast, nucleolin binding to Lep-2548G/A was downregulated in response to insulin, while it was not regulated on Lep-2548G/G. The presence of Lep-2548G/A was studied in breast cancer epithelial cells by IHC and LCM. Interestingly, all 14 tumors expressing high leptin levels contained Lep-2548A/A. In conclusion, the occurrence of Lep-2548G/A can enhance leptin expression in breast cancer cells *via* Sp1- and nucleolin-dependent mechanisms and possibly contribute to intratumoral leptin overexpression.
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Leptin is an adipose tissue-derived hormone whose main role is to control appetite and energy balance by acting in the hypothalamus. Leptin levels are proportional to fat body mass and are higher in women than in man.[1] In addition, leptin has been shown to modulate several other processes, such as immune response, fertility, hematopoiesis.[1] In peripheral organs, leptin can act as a mitogenic, angiogenic, and survival factor.[2] Recent studies in cellular and animal models provided strong evidence that leptin can be involved in neoplastic processes, for instance, in the development and progression of breast, colorectal, and endometrial cancers.[3–5] In addition, both leptin and its receptor (ObR) have been detected in different neoplastic tissues, usually at levels higher than that in respective normal tissues.[6–8] Interestingly, overexpression of the leptin system in breast cancer appears to be associated with higher tumor grade and size.[9–13] The mechanisms of leptin overexpression in breast cancer cells is partially known, for instance, we have shown that leptin transcription and synthesis can be induced by hypoxia or high levels of insulin.[9,13,14]

Here we investigated whether leptin expression in breast cancer cells could be related to the existence of the Lep-2548G/A polymorphism in the promoter region of leptin gene.

Lep-2548G/A has previously been shown to correlate with variations in serum leptin levels, degree of obesity, as well as cancer.[15–17] In particular, studies of Mammes *et al.* using DNA from peripheral blood cells documented that the Lep-2548G/A polymorphism was associated with higher leptin levels independent of BMI, before and after diet.[15] In addition, male, but not female, subjects with the Lep-2548A/A genotype had higher leptin concentrations adjusted for fat mass.[16] Similar studies suggested that nonobese women with Lep-2548A/A had higher serum leptin levels adjusted for body mass index, and significantly higher adipose tissue leptin mRNA expression than did carriers of the wild-type Lep-2548G/G sequence.[17]

In the context of neoplastic processes, Lep-2548A/A detected in DNA from peripheral blood leucocytes associated with increased risk for non-Hodgkin lymphoma,[18] and both Lep-2548A/A and Lep-2548G/A with non small cell lung cancer as well as prostate and breast carcinoma.[19,20] Furthermore, the existence of Lep-2548A/A has been shown to correlate with significantly increased breast cancer risk, and the presence of Lep-2548G/A with intermediate risk, compared with the wild-type Lep-2548G/G genotype.[21] The Lep-2548G/A polymorphism also correlated with a larger breast tumor size at diagnosis and a shorter disease-free survival of patients.[21] However, the functional significance of the Lep-2548G/A polymorphism and its occurrence in cancer tissues has never been experimentally addressed, and is a subject of this study.

The human leptin gene promoter ($\sim$2,500 bp) contains several consensus binding sites for a variety of transcription factors.[22] The Lep-2548 does not map to any known regulatory site, but is proximal to an Sp1 binding motif (CCCGCCT; -2539/-2534) that is known to be regulated by insulin in breast cancer cells.[23] Nucleolin is another nuclear factor that can bind a Sp1 consensus site in the promoter of cytosolic phospholipase A_2 ($cPLA_2$)[24] and that can interact with Sp1, as reported for human keratinocyte (HaCat) cell extracts.[25]

Nucleolin is a multifunctional phosphoprotein involved in ribosomal RNA transcription, maturation and assembly,[26] chromatin decondensation[27] as well as transcriptional regulation,[28] including modulation of Sp1-dependent gene expression.[24] Furthermore, insulin may induce changes in the phosphorilation of nucleolin.[29]

Consequently, we studied in breast cancer cell models if the occurrence of the Lep-2548G/A polymorphism can enhance basal and insulin-induced leptin expression *via* Sp1- and nucleolin-dependent mechanisms. In addition, the existence of the Lep-2548G/A polymorphism was assessed in breast cancer tissues expressing high leptin levels using laser capture microdissection.

Material and methods

Cell culture

MCF-7, MDA-MB-231, SK-BR-3, BT-474, ZR-75-1 cell lines were purchased from the American Type Culture Collection (Rockville, MD). MCF-7 and MDA-MB-231 cells were grown in Dulbecco's modified Eagle's medium (DMEM:F12) (Cellgro, Herndon, VA) containing 5% calf serum and 1% Penicillin/Streptomycin (P/S) (Cellgro). SK-BR-3 cells were grown in DMEM:F12 containing 10% fetal bovine serum (FBS) and 1% P/S. BT-474 cells were grown in DMEM:F12 containing 10% FBS, 1% P/S and 0.01 μg/ml insulin. ZR-75-1 cells were grown in RPMI-1640 (BioWhittaker, Walkersville, MD) containing 10% FBS, 1% P/S and 1mM sodium pyruvate (Cellgro). SK-BR-3 AND ZR-75-1 cell lines were omitted from experiments requiring serum-free medium (SFM) synchronization because serum depletion induced loss of adhesion and anoikis in these cells.

Detection of the Lep-2548G/A polymorphism

Genomic DNA was extracted from breast cancer cells with DNeasy Tissue Kit (Qiagen, Hilden, Germany). The existence of the Lep-2548G/A polymorphism was studied by PCR using a 2700 thermal cycler (Applied Biosystems, Foster City, CA). The following primers were used: forward 5′-ACTTTATAGACACA GAAATGAAAA-3′ reverse 5′-AAAATTTATGTTCCTCTGC CAC-3′. The PCR conditions were: 25 cycles at 30 sec at 94°C, 30 sec at 53°C, 30 sec at 72°C. The quality of PCR products was evaluated in a 2% agarose gel electrophoresis stained with ethidium bromide. Direct sequencing of the PCR products was performed using a BigDye Terminator reaction chemistry on ABI PRISM 377 Genetic Analyzer (Applied Biosystems). Each genetic variant was confirmed by direct sequencing of at least two independent samples.

Western blotting (WB)

Eighty percent confluent cultures were transferred to phenol red-free SFM for 24 hr (MCF-7 and MDA-MB-231 cells) or 72 hr (BT-474) and then stimulated with 2 μg/ml insulin for 3 h, or left untreated. Because normal growth medium for BT-474 cells contains insulin, this cell line was cultured in SFM for 72 hr to completely eliminate insulin effects on gene transcription, including Sp1-mediated regulation.[23] Then cells were lysed to separate cytoplasmic and nuclear proteins, as described before.[30] The expression of nuclear proteins was analyzed in 200 μg of nuclear cell lysates. The following antibodies (Abs) were used for WB: Sp1 (PEP2) polyclonal (p)Ab (Santa Cruz Biotechnology, CA), nucleolin mAb (Santa Cruz) and c-Jun (N) polyclonal (Santa Cruz). The proteins were separated on a 4–15% polyacrylamide gel, immunoblotted and quantified, as described before.[30]

Chromatin immunoprecipitation (ChIP)

We followed ChIP methodology described in detail previously.[31] Eighty percent confluent cultures were transferred to SFM for 24 hr (MCF-7 and MDA-MB-231 cells) or 72 h (BT-474) and then stimulated with 2 μg/ml insulin for 3 hr, or left untreated. Following treatment, the cells were crosslinked with 1% formaldehyde and chromatin was collected and sonicated. Soluble chromatin was immunoprecipitated with the following Abs: Sp1 PEP₂ (Santa Cruz Biotechnology), nucleolin C23 MS-3 (Santa Cruz). DNA-protein immune complexes were eluted, reverse crosslinked and DNA was extracted. The binding of Sp1, nucleolin to the leptin promoter region -2632/-2453 containing the -2548 site was tested using the following primers: forward 5′ACTTTATAGACA CAGAAATGAAAA and reverse 5′-CTCGGCACCGTCTCCT TG-3′. The PCR conditions were: 30 cycles at 30 sec at 94°C, 30 sec at 53°C, 30 sec at 72°C. In control samples, the primary Abs were replaced with appropriate nonimmune IgG.

DNA affinity precipitation assay (DAPA)

Binding of Sp1 and nucleolin to the leptin promoter region containing an Sp1 binding motif and Lep-2548 site was assessed in vitro using modified DAPA protocol, as described by us before.[32] Briefly, nuclear extracts were obtained from cells stimulated or not with 2 μg/ml insulin for 3 hr. A total of 300 μg of nuclear proteins were mixed with 2 μg of specific biotinylated DNA probes (see below) in 400 μl of Buffer D (20 mM HEPES pH 7.9, 10% glycerol, 50 mM KCl, 0.2 mM EDTA, 1.5 mM MgCl2, 10 mM ZnCl2, 1 mM DTT and 0.25% Triton X100) and then incubated on ice for 45 min. After that, 50 μl of streptavidin beads (Pierce, Rockford, IL) were added and the samples were agitated for 2 hr at 4°C. Next, the agarose beads-protein complexes were collected by brief centrifugation and washed twice in Buffer D. Proteins were uncoupled from DNA probes by addition of 40 μl SDS loading buffer, and heating at 96°C for 10 min. After removal of the beads, the supernatants were analyzed by WB for the presence of Sp1 and nucleolin. The specific DNA probes were prepared by annealing a biotinylated sense oligonucleotide (for -2548G/G: 5′-Bio GGGTTGCGCTGATCCTCCCGCCTCAGTTCCCTA-3′ and for -2548A/A: 5′-Bio GGGTTGCACTGATCCTCCCGCCTCAG TCTCCCTA-3′) with nonbiotinylated antisense oligonucleotide (for -2548G/G: 5′-CCCAACGCGACTAGGAGGGGCGGAGTCA GAGGGAT-3′ and for -2548A/A: 5′-CCCAACGTGACTAGGA GGGCGGAGTCAGAGGGAT3′). Unlabeled probes were used as negative controls. As an additional control, a 10-fold excess of unlabeled probes was added to the nuclear lysates 30 min prior to the addition of the labeled probes to block specific probe-protein interactions.

Real time-quantitative PCR (Q-PCR)

The cells were synchronized in SFM for 24 hr (MCF-7, MDA-MB-231) or 72 hr (BT-474) and then treated for 24 hr with 2 μg/ml insulin. Total cellular RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). 5 μg of total RNA was reverse transcribed using the TaqMan RT kit (Applied Biosystems, Branchburg, CA) according to vendor's instructions. 5 μl of the RT products were used to amplify leptin sequences using the Hs00174877 A1 Lep TaqMan kit (Applied Biosystems). To normalize QRT-PCR reactions, parallel TaqMan β-actin control reagents assays (Applied Biosystems) were run on each sample. Changes in the leptin mRNA content relative to β-actin mRNA were determined using a comparative CT method (ABI User Bulletin no. 2) to calculate changes in CT, and ultimately fold and percent change. An average CT value for each RNA was obtained for triplicate reactions.

Breast cancer patients and samples

The analysis of leptin gene polymorphism was performed in 14 breast cancer tissue samples, previously identified as strongly leptin-positive.[9] Tissue samples were obtained from women who underwent partial or total mastectomy for primary breast cancer. The age of patients ranged from 45 to 71 years. Histopathological examination of sections was based on the WHO and pTN classification of breast tumors. Tumor grading (G) was identified as G2 in 78.5% and G3 in 21.5% of cases; node status (N) was identified as N1 (cancer cells found in one lymph nodes) in 43% and negative (absence of regional metastases, N0) in 57% of cases. Tumor size (pT) was identified as pT1 (0–2 cm) in 40% and pT2 (2–5 cm) in 60% of cases. Immediately after excision, tissue samples were fixed in 10% buffered formaldehyde solution, embedded in paraffin blocks at 56°C.

Laser capture microdissection (LCM) of breast cancer tissue

Sections of 6 μm were cut from breast cancer tissue specimens paraffin-embedded and mounted on PEN membrane glass slides (Arcturus, Mountain View, CA). The expression of leptin was investigated by IHC with specific Abs. Tissue sections were deparaffinized in xylene and rehydrated in graded series of
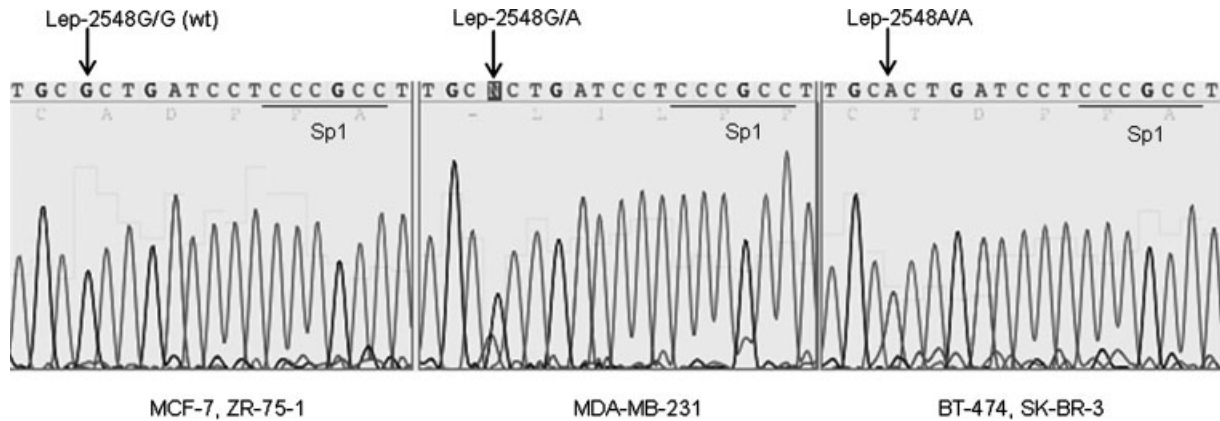
FIGURE 1 – Detection of the Lep-2548G/A polymorphism in breast cancer cells. Presence of the Lep-2548G/A polymorphism was assessed by direct sequencing in five different breast cancer cell lines: MCF-7 and ZR-75-1 cells displayed a wild type (Lep-2548G/G) genotype, MDA-MB-231 cells were heterozygotic (Lep-2548G/A), and SK-BR-3 and BT-474 cells were homozygotic for the polymorphism (Lep-2548A/A).

ethanol. After rinsing in PBS, endogenous peroxidase activity was inhibited by incubation with 30% hydrogen peroxide, diluted in 100% methanol for 30 min at 4°C. After three washes in PBS the sections were incubated with 1.5% blocking serum for 1 hr, then the sections were incubated for 3 hr with primary antibodies. For leptin staining, we used the A20 leptin polyclonal Ab (pAb) (Santa Cruz) at 1:100 dilution, as described before.[9] The leptin antigens were detected with avidin-biotin-peroxidase ABC staining systems (Santa Cruz). All slides were counterstained with Mayer's hematoxylin. Areas of interest were collected using a Veritas (Arcturus, Mountain View, CA) LCM instrument equipped with Veritas image archiving software. The cutting beam diameter was 2.0 μm and the capture beam diameter was 7.0–9.0 μm. The laser intensity control was set at 4.0 mV for cutting and 200 mV for capture. Typically, 6–8 laser pulses were required to cut out the selected area, which was then captured on thermoplastic film coated CapSure[TM] Macro Caps mounted films (Arcturus) according to manufacturer's directions. Portions of the films holding captured areas were placed in microcentrifuge tubes and used for DNA extraction performed with PicoPure DNA Extraction Kit (Arcturus). Detection of the Lep-2548G/A polymorphism was performed as described above.

Results

Detection of the Lep-2548G/A polymorphism
in breast cancer cell lines

Genotypes were determined for different breast cancer cell lines to test if the occurrence of the Lep-2548G/A polymorphism in the leptin promoter can enhance basal and insulin-induced leptin expression. MCF-7 and ZR-75-1 cells contained the wild type (Lep-2548G/G) promoter sequence, MDA-MB-231 cells contained heterozygous (Lep-2548G/A) variant, while BT-474 and SK-BR-3 cells were homozygous for the (Lep-2548A/A) polymorphism (Fig. 1). MCF-7, MDA-MB-231 and BT-474 cells were selected for further experiments.

Interaction between Sp1, nucleolin and the leptin promoter
domain containing the Lep-2548 site

The Lep-2548 site doesn't map to any known regulatory site, but is proximal to an Sp1 binding motif (CCCGCCT; -2539/ -2534). We recently reported that this leptin promoter region is regulated by Sp1 in an insulin-dependent fashion in MDA-MB-231 cells.[23] Interestingly, a Sp1-binding sequences of the cytosolic phospholipase A_2 promoter seem to be also recognized by another transcriptional regulator, nucleolin.[24] It has also been proved a protein-protein interaction between Sp1 and nucleolin in human keratinocyte (HaCat) cell extracts.[25] Here we investigated how the
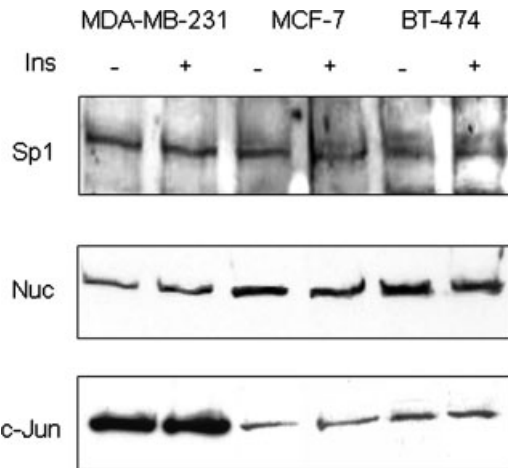

FIGURE 2 – Expression of Sp1 and nucleolin in breast cancer cell lines. The expression of nuclear Sp1 and nucleolin (Nuc) was analyzed by WB, as described in Material and methods section, in 200 μg of nuclear proteins obtained from cells treated with 2 μg/ml insulin (Ins) for 3 hr, or left untreated. Protein loading was controlled by reprobing the WB filter for the expression of a nuclear marker c-Jun.

presence of the Lep-2548G/A polymorphism might affect insulin-dependent leptin transcription.

First, we determined basal and insulin-induced nuclear expression levels of Sp1 and nucleolin in different breast cancer cell lines (Fig. 2). The abundance of Sp1 and nucleolin in the nuclei of MCF-7, MDA-MB-231 and BT-474 cells varied depending on cell line, but in each case was not affected by insulin addition (Fig. 2).

Next, we investigated, by ChIP assays, the binding of Sp1 and nucleolin to the Lep-2548 region using native chromatin of MCF-7, MDA-MB-231 and BT-474 cell lines (Fig. 3a). We found that, under insulin treatment, the presence of the polymorphism improves Sp1 binding to the promoter, while it reduces nucleolin association in MDA-MB-231 (Lep-2548G/A) and BT-474 (Lep-2548A/A) cells. In MCF-7 (Lep-2548G/G) we did not observe any regulation of Sp1 or nucleolin under insulin treatment. Chromatin samples precipitated with control antibodies did not reveal any PCR products for Lep-2548 region (data not shown).

To specifically map Sp1 and nucleolin binding site within the ChiP-tested DNA region, we used DAPA protocol. Short biotinylated DNA probes containing a Sp1 binding motif adjusted to Lep-2548A/A or Lep-2548G/G sequences were hybridized with
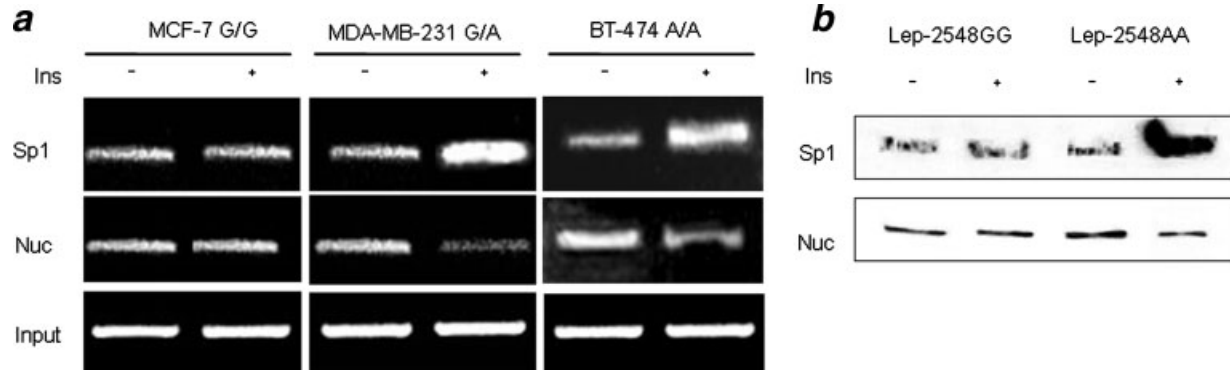
FIGURE 3 – Interaction between Sp1, nucleolin and the leptin promoter domain containing the Lep-2548 site. (*a*) The binding of Sp1 and nucleolin (Nuc) to the leptin promoter region containing an Sp1 binding motif and the Lep-2548 site was tested by ChIP, as described in Material and methods section. Cells were transferred to SFM for 24 hr (MCF-7 and MDA-MB-231 cells) or 72 hr (BT-474) and then stimulated with 2 µg/ml insulin for 3 hr. In control samples, the primary Abs were replaced with nonimmune IgG (not shown). (*b*) The binding of Sp1 and nucleolin (Nuc) to the leptin promoter region containing an Sp1 site and the Lep-2548 sequence was assessed in vitro using modified DAPA, as described in Material and methods section. MDA-MB-231 cells were transferred to SFM for 24 hr and then stimulated with 2 µg/ml insulin for 3 hr. Next, 300 µg of nuclear protein were mixed with 2 µg of specific biotinylated DNA probes for Lep-2548G/G or Lep-2548A/A and nonbiotinylated antisense oligonucleotides, as detailed in Material and methods section. Control nonbiotinylated probes did not produce any Sp1 or nucleolin binding (not shown).

nuclear proteins obtained from MDA-MB-231 cells, stimulated or not with 2 µg/ml insulin. Confirming ChIP results, we found that the Lep-2548A/A genotype improves recruitment of Sp1 to DNA under insulin treatment, while it reduces the recruitment of nucleolin. In contrast, binding of nucleolin was not regulated on the wild type Lep-2548G/G (Fig. 3*b*). Unlabeled probes, used as controls, did not precipitated Sp1 or nucleolin proteins (data not shown).

Differential leptin mRNA expression in breast cancer cells carrying different Lep-2548 genotypes

To assess whether the presence of Lep-2548G/A and differential binding of Sp1 and nucleolin to this site can be associated with differential leptin transcription, we used QRT-PCR to measure basal and insulin-induced leptin mRNA levels in MCF-7, MDA-MB-231 and BT-474 cells. The lowest levels of basal leptin mRNA were found in MCF-7 cells (Lep-2548G/G), intermediate levels in MDA-MB-231 cells (Lep-2548G/A), and the highest levels in BT-474 cells (Lep-2548A/A) (Fig. 4). Treatment with 2 µg/ml insulin did not significantly modulate leptin expression in MCF-7 cells (Lep-2548G/G), relative to basal expression, while enhanced leptin mRNA levels in MDA-MB-231 cells (Lep-2548G/A). In BT-474 cells (Lep-2548A/A) expressing high basal leptin mRNA insulin addition did not significantly modulate leptin expression (Fig. 4).

Detection of the Lep-2548G/A polymorphism in breast cancer tissues

We performed preliminary analysis of leptin promoter polymorphism in human breast cancer biopsies. Leptin expression in the cancer tissues studied was previously identified as very high (+++ score).[9] In this analysis, we confirmed by specific IHC staining high leptin expression in the selected tumors. The leptin-expressing epithelial cells were isolated by laser microdissection (LCM). Analysis of DNA from the leptin overexpressing cancer tissues revealed the presence of the Lep-2548A/A polymorphism in all samples.

Discussion

The common Lep-2548G/A polymorphism in the leptin gene promoter has previously been associated with degree of obesity, variations in serum leptin levels, and leptin synthesis by adipose
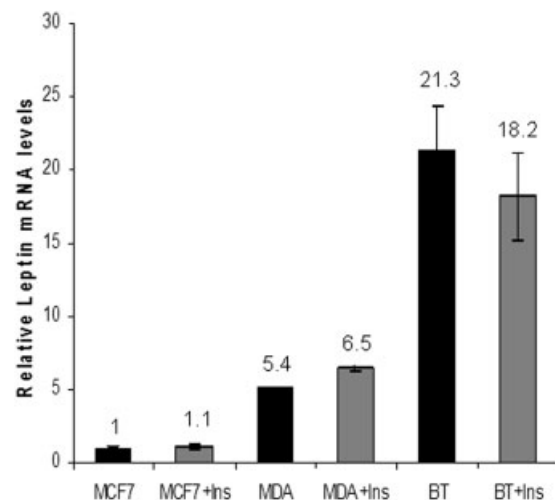

FIGURE 4 – Leptin mRNA expression in breast cancer cell lines with different genotype for the Lep-2548 polymorphism. The abundance of leptin mRNA was studied using QRT-PCR in MCF-7, MDA-MB-231 (MDA) and BT-474 (BT) cells untreated or treated with 2 µg/ml insulin (Ins) for 24 hr, as described in Material and methods section. The graphs represent the increase of leptin mRNA relative to the increase of constitutive mRNA control (β-actin) in the same sample ± SDs. The differences between the two conditions (untreated and treated) were statistically significant ($p < 0.05$).

tissue.[15] New data suggested the link between Lep-2548G/A and risk of neoplastic disease.[18–21]

Studies in cellular and animal models strongly suggested that high levels of leptin might promote breast carcinogenesis.[3] Reports from our and other laboratories demonstrated that both leptin and ObR are overexpressed in breast cancer cells,[4,6,33] which implies that leptin could promote breast cancer not only through endocrine but also through autocrine mechanisms. As reported by us before, leptin synthesis in breast cancer cells can be upregulated by obesity-related stimuli, such as high levels of insulin, insulin-like growth factor I, or estrogens.[9,23,34] However, the possibility that leptin promoter poymorphism might influence leptin transcription in breast cancer cells have never been studied.

Until now, the molecular basis of the link between the occurrence of Lep-2548G/A and increased leptin expression have not been investigated. One report implied, however, that some transcriptional events can be involved: electric mobility shift assay revealed that nuclear extracts from human monocyte U937 cells

form a protein-DNA complex with the polymorphic site Lep-2548G/A, binding with higher affinity to the Lep-2548A allele.[17] However, using nuclear proteins from human subcutaneous adipocytes, two complexes were found, one with the same mobility as the protein complex derived from U937 cells and the other with higher mobility. The molecular composition of these two complexes has not been described.[17]

Here we investigated if human breast cancer cells contain the Lep-2548G/A polymorphism and if the presence of this variant can affect leptin gene expression. Because the Lep-2548G/A site in the leptin promoter is proximal to Sp1 binding motif[23] and nucleolin could interact with Sp1[25] and bind his consensus sites,[24] we focused on the interplay between Lep-2548G/A and the two transcriptional regulators in the process of basal and insulin-induced leptin mRNA synthesis.

We first identified three different Lep-2548 genotypes in breast cancer cell lines. Specifically, MCF-7 and ZR-75-1 cells contained the Lep-2548G/G wild-type genotype, while MDA-MB-231 contained the Lep-2548G/A polymorphism, and BT-474 and SK-BR-3 cells expressed the Lep-2548A/A genotype. ChIP assays suggested that in MCF-7 cells, basal binding of Sp1 or nucleolin to Lep-2548G/G was not affected by insulin treatment. On the other hand, in MDA-MB-231 and BT-474 cells, insulin induced Sp1 binding and reduced nucleolin association with Lep-2548G/A and Lep-2548A/A, respectively (Fig. 5). The differential binding of Sp1 and nucleolin to Lep-2548G/G and Lep-2548A/A was confirmed by DAPA assays (Fig. 3*b*). These results suggested that the presence of Lep-2548A/A and Lep-2548G/ A sequences might increase insulin-dependent activation of the leptin promoter by promoting the exchange between the negative transcriptional regulator nucleolin and the positive regulator Sp1 (Fig. 5). Based on these results, one could speculate that Lep-2548 sequence variations in the leptin gene promoter might contribute to the
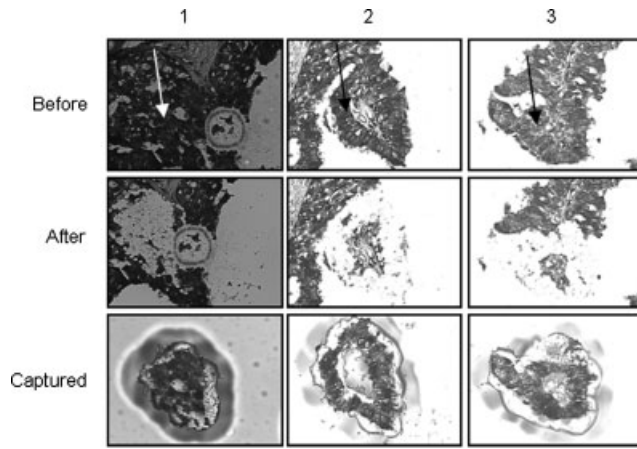

FIGURE 5 – Detection of the Lep-2548G/A polymorphism in breast cancer tissues. Before LCM experiment, the expression of leptin in each tissue sample was visualized by IHC with specific Abs and all slides were counterstained with Mayer's hematoxylin. The areas before and after capture, as well as captured areas were photographed using Veritas image archiving software. Three representative LCM experiments (1, 2, 3) are shown. Arrows indicate leptin-positive areas.
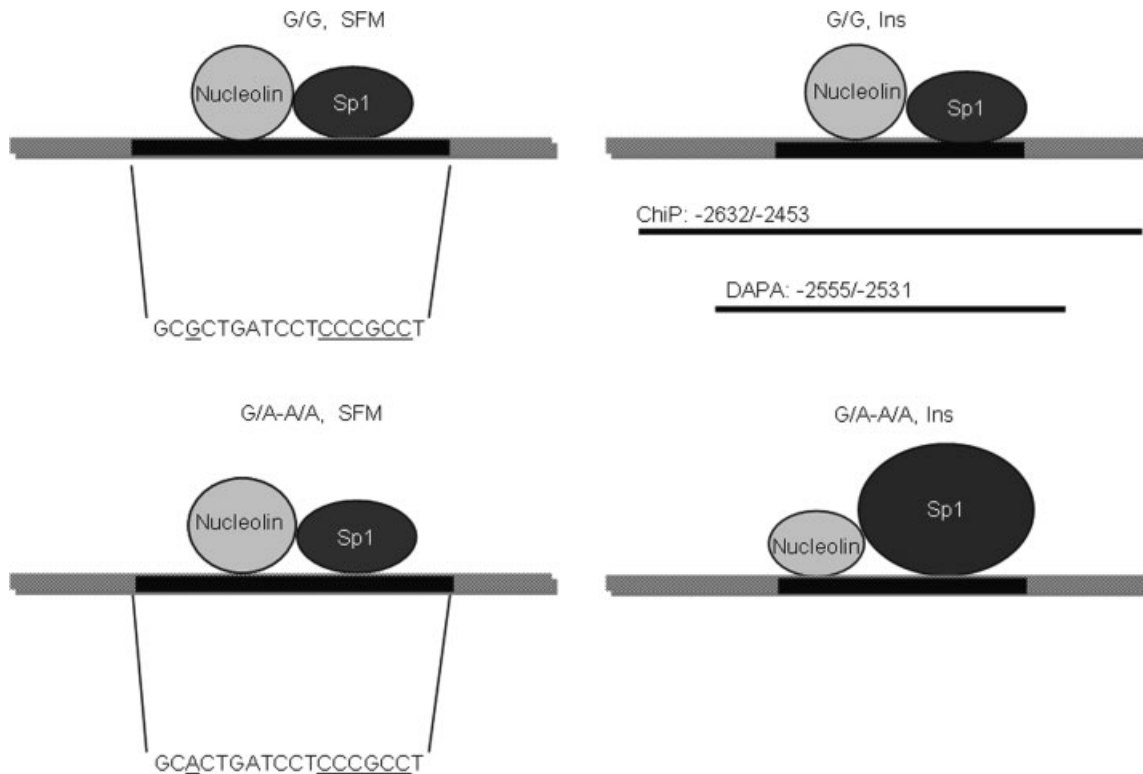

FIGURE 6 – Model of functional interactions at the Lep-2548 site. Hypothetical model of Sp1/nucleolin binding to the Lep-2548 region in MCF-7, MDA-MB-231 and BT-474 cells untreated (SFM) or treated with insulin (Ins) is presented. The presence of an A allele (Lep-2548G/A, Lep-2548A/A) improves efficient recruitment of Sp1 to DNA under insulin treatment. In contrast, binding of nucleolin to Lep-2548G/A or Lep-2548A/A is downregulated in response to insulin, while it is not regulated on Lep-2548G/G.

differences in leptin mRNA expression observed in different breast cancer cell lines. Indeed, the lowest levels of basal leptin mRNA were found in MCF-7 cells (Lep-2548G/G), intermediate levels in MDA-MB-231 cells (Lep-2548G/A), and the highest levels in BT-474 cells (Lep-2548A/A) (Fig. 4). Interestingly, MCF-7 cells are characterized by low aggressive, metastatic phenotype, while BT-474 and SK-BR-3 are of a more aggressive phenotype.[35] Whether or not the expression of leptin in these cells might determine the degree of their malignancy is not yet known.

It is noteworthy, however, that the leptin promoter contains multiple transcription regulatory motifs regulated by insulin. For instance, seven Sp1 sites, and 8 elements binding HIF.[22,35,36] Our previous study demonstrated that in MDA-MB-231 cells treated with insulin, Sp1 acts through two sites, one of which is in Lep-2548 region. On the other hand, functional insulin-regulated Sp1 sites in the leptin promoter have not been mapped in other cells lines. Thus, except for MDA-MB-231 cells, the relative importance of Lep-2548 to basal and insulin-induced leptin expression is difficult to measure in other cells. The possible involvement of multiple transcription mechanisms could explain the very high basal leptin mRNA expression in BT-474 cells and the lack of appreciable insulin-induced stimulation in these cells, despite the apparent activation of the Lep-2548 site. Moreover, it has to be noted that concentrations of insulin used in our study are relatively high (to mimic, at least to some extent) the conditions of hyperinsulinemia. These conditions might lead to activation not only insulin receptors but also receptors for IGF-1 as well as hybrids insulin/IGF-IR.[37,38]

The presence of Lep-2548G/A and A/A polymorphism in breast cancer cells lines prompted us to investigate whether this genotype can be found in breast tumors. Using LCM, we extracted leptin-positive cancer areas from breast tumors and sequenced DNA of cancer cells (Fig. 6). Interestingly, in all cases, cancer cells contained the Lep-2548A/A polymorphism. The predominance of the Lep-2548A/A genotype might be related to the fact only tumors expressing leptin at high levels were studied. It is possible that more genotypic variability would be detected in greater number of cases characterized by various degrees of leptin expression.

In conclusion, our study suggests that the Lep-2548G/A polymorphism, previously detected in PBC and associated with increased leptin synthesis, occurs in breast cancer cell DNA and could contribute to leptin overexpression in this cellular context.

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