DEVELOPMENT OF A TOOL KIT FOR DNA METHYLATION AND EXPRESSION ANALYSIS OF THE
HUMAN LEPTIN RECEPTOR LOCUS IN CANCER

A Thesis in

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by

Pooja Nadkarni

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The thesis of Pooja Nadkarni was reviewed and *approved by the following:

Sagarika Kanjilal
Associate Professor of Molecular Medicine Graduate Program
Thesis Adviser

Surojit Sarkar
Assistant Professor of Veterinary and Biomedical Sciences

Adam Glick
Associate Professor of Veterinary and Biomedical Sciences
Chair of Molecular Medicine Graduate Program

* Signatures are on file in the Graduate School.
ABSTRACT

The leptin receptor (LEPR) is dysregulated in several cancer cell lines, wherein it is known to promote cellular growth, survival and migration. In addition, the protein product of a leptin receptor-overlapping transcript (LEPROT) has been shown to negatively regulate the cell surface expression of LEPR and LEPR mediated signaling. However, a comprehensive understanding of the patterns of epigenetic modification and expression of the LEPR, and LEPROT gene transcripts in cancer cells, as well as the molecular tools required for performing such analyses, have been lacking. In order to begin to address these knowledge gaps, we have developed methods and tools to determine the DNA methylation and expression patterns at the LEPR/LEPROT locus. The methodology and assays developed provide a molecular biological toolkit that will significantly contribute to the comprehensive analysis of the epigenetic modification and expression patterns of the LEPR and LEPROT transcripts and provide a foundation for follow-on investigations of the role of these genes in cancer development and progression.
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RESEARCH OBJECTIVES

After the Human genome project began, nearly 35,000 genes have been identified and sequenced and some of these genes exist as multiple variants. Subtle differences in the structure and function of these various transcripts dictate health and disease. Leptin receptor is a gene which has several alternatively spliced transcript variants derived from and related to the LEPR locus on chromosome 1 and the functions of some of these variants in normal physiology as well as in cancer remain unexplored.

HYPOTHESIS

Through our investigations, we have attempted to test the hypothesis that the relative expression of various transcripts derived from and related to the LEPR locus is epigenetically modulated by processes such as methylation of critical CpG islands/sites in cancer.

SPECIFIC AIMS

1) Develop reagents for the relative quantification of various transcripts derived from and related to the LEPR locus.

2) Map the CpG islands in the LEPR locus through bioinformatic analysis and develop tools for preliminary screening of their methylation status.
Leptin

Leptin is a 16 kDa cytokine and master hormone that was identified after a mouse colony with an obese phenotype spontaneously developed at the Jackson Laboratory in Maine, in 1950 [1]. Based on further studies, Colemann, D. et al. suggested that these mice were deficient in a blood borne nutrient regulating factor eventually named leptin that controlled appetite, energy expenditure and metabolism [2]. In 1994, Zhang, Y. et al. mapped the murine leptin gene locus (named \textit{ob} due to the associated obese phenotype) to mouse chromosome 6 by positional cloning and confirmed the expression of the gene transcript in adipose tissue by northern blot analysis [3]. The human leptin gene (\textit{LEP}) is located on chromosome 7 and its protein product (\textit{LEP}) is primarily expressed and secreted by the adipocytes with the concentration of leptin in the blood depending on the amount of adipose fat tissue present [4]. The plasma concentrations of LEP in the normal individuals is $< 10 \text{ ng/ml}$, whereas in obese individuals ranges between 30 – 100 ng/ml [5].

Leptin is transported across the blood brain barrier and binds to the leptin receptors in the arcuate nucleus of the hypothalamus to regulate various neuropeptides controlling food intake and energy expenditure. This, in turn, controls body weight and adipose tissue mass, thereby completing a feedback loop [6]. Besides its primary physiological role in appetite regulation,
leptin has been shown to be a pleiotropic hormone with a diverse range of biological functions in bone formation, reproduction, immune system regulation and angiogenesis [6-9].

**Leptin Receptor (LEPR)**

In 1973, parabiotic studies conducted by Coleman suggested that a defect in *db/db* mice led to decreased responsiveness to leptin signaling in the brain, indicating that the hormone may act via its receptor encoded by the *db* gene [2]. The mouse leptin receptor (*db*, denoted as *LEPR* in humans) was first identified in the choroid plexus by screening an expression library for leptin binding sites using a leptin – alkaline phosphatase fusion protein [10]. In humans, the *LEPR* gene is located on chromosome 1p31 and contains 20 exons. The receptor is a member of the Class I cytokine receptor family, to which most cytokine receptors such as IL-6 receptor and granulocyte monocyte colony stimulating factor (GMCSF) receptor belong. The extracellular domain of the leptin receptor contains several cytokine receptor homology domains (CRH), of which the CRH2 domain has been shown to bind to the hydrophobic amino acids Leu 13 and Leu 86 in leptin (Figure 1).
Figure 1: Structure of the human leptin receptor. Adapted from Hannes, I. et al. [11]. The extracellular region of the receptor consists of several domains. There is a cytokine receptor homology (CRH) module, termed CRH1, at the amino terminal, which contains two sub domains that have a fibronectin type III (FNIII) fold. The CRH2 domain on the leptin receptor has been found to exhibit high-affinity binding for leptin [12-14]. The exact role of CRH1 is unknown, but the Ig-like and the FN-III domains are important in leptin receptor activation [12, 14]. The WSXWS motif in the FNIII domain is believed to be important for the receptor protein folding and effective binding of leptin to the receptor [15, 16].
**Isoforms of Leptin Receptor**

The leptin receptor is expressed as alternatively spliced isoforms both in humans and mice (Figure 2). The extracellular domain of the leptin receptor consists of 818 amino acids in both human and mice and is 78% homologous [10]. The intracellular region contains a 29 amino acids long region that is common to all the receptor isoforms and contains a “Box 1” domain that binds Janus Kinase (JAK). This region is conserved across all species [10]. The different isoforms vary at the carboxy-terminal end in both human and mouse with alternate splicing occurring in intron 19-20 in humans. The “long form” of the receptor also contains a “Box 2” domain and a “Box 3” domain, which are absent in the other short forms of the receptors. “Box 3” interacts with signal transducer and activator of transcription 3 (STAT3), in the leptin-signaling pathway [10]. In the mouse, a soluble leptin receptor (SLR) is formed by skipping of the 3’ splice site in exon 14 and introduction of a premature stop codon and polyadenylation signal [17] whereas, in humans, the SLR protein is generated by proteolytic cleavage of cell surface leptin receptors by matrix metalloproteases (MMP’s) [18, 19].
**Structure of the Human LEPR Protein Isoforms**

**Figure 2: Structure of the human LEPR protein isoforms.** Adapted from Hannes, I. et al. [11]

The extracellular domain of the human LEPR consists of 818 a.a. The intracellular region contains a 29 a.a. long region that is common to all the receptor isoforms and contains a “Box 1” domain that binds Janus Kinase (JAK) and is conserved across all species. The “long form” of the receptor also contains “Box 2” and “Box 3” domains, with “Box 3” interacting with the transcription factor STAT3, in the leptin-signaling pathway. SLR protein is generated by proteolytic cleavage of cell surface leptin receptors by matrix metalloproteases (MMP’s) [17, 18, 19].
Table 1: Nomenclature of the Mouse and Human Leptin Receptor Isoforms

<table>
<thead>
<tr>
<th>Transcript/Isoform</th>
<th>h LEPR</th>
<th>Murine Leptin Receptor</th>
<th>m Obr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of unique amino acids at C-terminal</td>
<td>No. of unique amino acids at C-terminal</td>
<td></td>
</tr>
<tr>
<td>219</td>
<td>274 ( (p\text{Tyr}^{1141} \text{ required for Stat3 binding}) )</td>
<td>( Obrb )</td>
<td>273 ( (p\text{Tyr}^{1138} \text{ required for Stat3 binding}) )</td>
</tr>
<tr>
<td>219.1</td>
<td>67</td>
<td>( Obrc )</td>
<td>11</td>
</tr>
<tr>
<td>219.2</td>
<td>15</td>
<td>( Obrd )</td>
<td>5</td>
</tr>
<tr>
<td>219.3</td>
<td>5</td>
<td>( Obra/Obrs )</td>
<td>3</td>
</tr>
<tr>
<td>SLR</td>
<td>0 *</td>
<td>( Obre )</td>
<td>0 **</td>
</tr>
</tbody>
</table>

*In humans, soluble leptin receptor is generated by cleavage of cell surface leptin receptors by matrix metalloproteases. No hSLR transcript is known to exist.

**In mice a short transcript is generated for a soluble leptin receptor through skipping of the 3’ splice site of exon 14 which leads to a premature stop codon and polyadenylation.
Expression and Physiological Role of Carboxy Terminal Isoforms of Leptin Receptor

The long form of the human leptin receptor (LEPR219) is 1165 amino acids in length, comprising of an extracellular domain of 816 amino acids that binds to leptin, a transmembrane region of 34 amino acids and a cytoplasmic domain that activates downstream kinases. The four isoforms of LEPR (219, 219.1, 219.2, and 219.3) share the same extracellular domain at the amino terminal, transmembrane domain, as well as a part of the cytoplasmic domain, but vary partially in the intracellular carboxy terminal (19). Leptin binds to its long form receptor LEPR and activates Janus Kinase 2 (JAK2) and STAT3 and other downstream signaling components thereby regulating the transcription of several genes controlling cell proliferation and survival such as cJUN, cFOS and EGR1 [20]. While the physiological function of leptin and signaling mediated by its long form receptor has been extensively investigated in obesity, reproduction, immune system as well as in cancer, the quantitative expression levels and the physiological relevance of the short form receptors remain unknown. Three human leptin receptor short forms do not contain the JAK binding “Box 2” domains, Stat 3 binding “Box 3” and SHP-2 binding sites, but retain the “Box 1” [21]. A report describes the occurrence of a repetitive human endogenous retroviral sequence (HERVK) inserted at the 3’ end of the leptin receptor isoform 219.1 [22]. HERVK’s promote malignant transformation in melanoma cells [23], and are expressed in some other cancer cell lines like breast cancer [24], making their association to leptin receptor signaling in cancer, worth exploring. The role of the receptor isoform 219.2 is still enigmatic, although it is known to be expressed in various tissues like human stomach, fibroblasts and adrenal glands [25-27]. Contrary to the earlier belief that the short form LEPR
219.3 (ObRs) is not capable of signaling, it has been proved to exhibit minimal signaling capacity through the JAK-MAPK pathway without activating the STAT pathway owing to the lack of STAT 3 binding site [28]. The short form of the receptor in rodents (denoted as ObRa/ObRs) has been shown to be expressed in rat choroid plexus and is known to function as an intracellular transporter of leptin across the blood brain barrier [29]. In human plasma, the soluble form of the receptor (SLR) sequesters leptin, blocking the leptin receptor signaling. This effect has also been shown both in vitro as well as in vivo in rodents by evaluating stat3 inhibition following injection of SLR into the central nervous system of adult rats [30]. A recent study has reported an association between increasing plasma soluble leptin receptor levels in plasma and advanced tumor stage in colorectal cancer patients [31].

Among the various alternatively spliced isoforms in the mouse, the long form, ObRb, is highly expressed in different regions of the hypothalamus [32], whereas lower levels of the receptor are reported in many peripheral organs like murine placenta (where it may aid in growth and development of fetus) as well as in testis [33, 34]. Not much is known about the expression and function of human leptin receptor isoform 219.1, although the murine form of the receptor obRc is shown to enhance the binding and internalization of leptin, when the receptor is expressed in Xenopus oocytes [35]. In mice, ObRa is found to be ubiquitously expressed in all tissues with maximum level of expression observed in lung, whereas relatively lower levels are reported in kidney, spleen, heart and brain [36]. Through experiments conducted in mice and cultured cells overexpressing leptin receptors, the murine soluble leptin receptor has been
shown to inhibit the leptin receptor mediated endocytosis of leptin across the blood brain barrier [37].

### Table 2: Expression of Leptin Receptors in Cancer Cell Lines and Tumor Samples Relative to Normal Tissues

<table>
<thead>
<tr>
<th>Cancer site</th>
<th>Type of Samples</th>
<th>Isoforms Analyzed</th>
<th>Methods (references)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain Cancer</td>
<td>Glioblastoma cell lines (LN229 and LN18), and brain tumor specimen</td>
<td>LEPR 219 long form</td>
<td>WB and IHC [38]</td>
</tr>
<tr>
<td>Breast</td>
<td>Cell Lines (MCF-7 and MDA-MB-231), and breast cancer specimen</td>
<td>Common domain to all LEPR isoforms targeted</td>
<td>IHC &amp; RTPCR [20]</td>
</tr>
<tr>
<td>Colon and Rectum</td>
<td>Specimen from patients</td>
<td>LEPR 219 long form analyzed</td>
<td>IHC [39]</td>
</tr>
</tbody>
</table>
Alternate Transcripts of LEPR

In addition to the four 3’ variants, the LEPR locus has two other sets of variants: The B219/R variants that provide an alternate 5’UTR and transcriptional start site for the four 3’ variants of LEPR; and the leptin receptor overlapping transcripts (LEPROT) which encode a completely different set of protein isoforms (Figure 3).

Figure 3: Alternative splicing of LEPR, LEPROT and B219/R. Adapted from Bailleul, B et al. [41].

LEPROT transcript shares the first two exons (Exon 1 and Exon 2 in red color) with LEPR. The 5’ variant of LEPR, B219/R varies from LEPR at the promoter and first two exons (yellow color).
The **B219/R** 5’ UTR Variant of **LEPR**

The **huB219/R** variants share a unique 5’ UTR and promoter and differ from the **LEPR219** series of transcripts in the sequence of the first two exons (Figure 4). This variant has been cloned from human fetal liver and leads to four different 3’ isoforms **huB219**, and **B219.1 – B219.3** corresponding to **LEPR219**, and **LEPR219.1-219.3** [32].

![Figure 4: Schematic representation of human LEPROT, LEPR 219 and B219/R transcripts.](image)

Adapted from Bailleul, B *et al.* [41]. The AUG initiation codons are indicated by vertical arrows and the open reading frames (ORFs) are indicated by horizontal arrows that end at stop codons. Similar colors represent identical sequence. The red colored region represents the first two exons that are common between **LEPROT & LEPR 219** transcripts. Green color represents exon 3-20 which are common to **LEPR 219** and **B219/R** transcripts. The blue color represents exons 3 & 4 of **LEPROT**, and the yellow color represents the unique 5’ UTR region of **B219/R**.
In humans, B219/R has been shown to be expressed in fetal liver, hematopoietic cell lines and reproductive organs such as ovary and prostate. It is also expressed in the choroid plexus in the brain although at low levels [32]. It is also highly expressed in the early primitive precursor populations of hematopoietic stem cells and has been suggested to be a marker of early hematopoietic development in fetal liver [32]. It is of note that, B219/R is expressed at significant levels in several hematopoietic cell lines such as K562 human granulocytic erythroleukemia, KG1a, human myelogenous leukemia, BB88, murine leukemia, and in lymphoid cells like RAJI, human B cell, CTLL 2, and murine T cell [32]. The significance of the expression of B219/R isoforms has not been elucidated as of yet [32].

**Leptin Receptor Overlapping Transcript (LEPROT)**

Direct sequence homology searching of EST databases for human leptin receptor cDNA led to the discovery of human leptin receptor overlapping transcript (LEPROT) which shares the 5’ end of the leptin receptor, but expresses a distinct protein due to the usage of an alternate translation start site (Figure 5) [41].
Figure 5: Genomic organization of human LEPR and LEPROT. As indicated in the figure, LEPROT and LEPR share the promoter and the first two exons (red color). Exon 1-4 of LEPROT encodes a protein LEPROT which is 131 amino acids in length and completely different in sequence from the 1165 amino acids long LEPR protein encoded by the exons 3-20 of LEPR. ❄️ denotes slippage of initiation codon.

The LEPROT gene encodes for a tetraspanning membrane protein, containing 131 amino acids that is associated with the Golgi complex and endosomes [42]. The protein product of this gene, LEPROT is abundantly expressed in various human tissues with high levels of expression observed in the heart and placenta, whereas low levels are seen in the brain and kidney [41]. Human LEPROT is 29% homologous to a vacuolar protein, Vps55p, in S.cerevisiae. The protein is likely involved in late endosome to vacuole trafficking and subcellular localization of proteins since phenotypic defects observed in vacuolar protein sorting in Vps55p deletion mutants, were
corrected by over expression of human LEPROT [42]. Both *in vitro* and *in vivo* experiments have shown that silencing of LEPROT negatively regulates the presence of LEPR at the cell surface and leptin receptor mediated signaling. However, the down-regulation of LEPROT using siRNA in cells as well as in the arcuate nuclei in the brains of mice resulted in reduced expression of the protein and prevented diet induced obesity in mice fed with a high fat diet, by increasing leptin receptor sensitivity to leptin [43].

In a large-scale human genome-sequencing project, another gene located on chromosome 8 called leptin receptor overlapping transcript-like1 (*LEPROTL1*) has been cloned and characterized. It is 67 % homologous to LEPROT in its DNA sequence, but the protein derived from it has a distinct amino acid sequence [44]. However, a recent investigation of the mechanisms of inhibition of cell surface expression of leptin receptor showed that both LEPROT and LEPROTL1 (also called endospannin 1 and endospannin 2) regulate leptin receptor endocytosis and degradation at a post internalization step [45]. LEPROT and LEPROTL1 also cooperatively play a role in the cell surface expression of growth hormone receptor in the liver, thereby modulating the liver resistance to growth hormone during periods of reduced nutrient availability [46]. Both LEPROT and LEPROTL1 contain the box 1 (Pro<sup>46</sup>–Ile–Pro<sup>48</sup>) motif, which also occurs in all the LEPR isoforms. It has been suggested that LEPROT and LEPROTL1 may serve as accessory proteins that heterodimerize with LEPR isoforms involved in signal transduction [41, 44]. Genomic studies indicate that LEPR and LEPROT have been conserved through evolution [47, 48].
Leptin Receptor Signaling Pathway in Cancer

Since leptin impacts various physiological pathways and controls many cellular functions including proliferation, angiogenesis, invasion, metastasis, and inhibition of apoptosis, it has recently gained considerable attention from researchers interested in exploring dysregulation in these signaling networks [20]. However, evaluation of the tumorigenic effect of leptin is challenging due to lack of clarity regarding the status and function of the numerous leptin receptor isoforms.
Figure 6: A schematic representation of leptin receptor signaling in cancer. Adapted from Garofalio, C. et al. [20]. Binding of leptin to its receptor recruits cytoplasmic JAK which binds to the “Box 1” motif present in all human leptin receptor isoforms. The activation of JAK 2 by the long form of the receptor recruits STAT3, which undergoes phosphorylation and dimerization (48). Phosphorylated STAT3 also increases the expression of SOCS-3 which inhibits the JAK activation, thus causing a negative feedback inhibition on leptin pathway [49]. Several reports indicate that leptin contributes to activation of JAK–STAT and ERK pathways thereby increasing cellular proliferation in human prostate and endometrial cancer [50, 51]. MAPK/ERK Pathway; Phosphorylation of Tyr 985 on the leptin receptor recruits the proteins SHP2, which in turn binds GRB2 and activates the ERK1/2 cascade which provides mitogenic signals through phosphorylation and inactivation of the retinoblastoma (RB) tumor suppressor and stimulation of early immediate response genes such as cFOS, EGR1 and c-JUN [20, 49]. PI3/AKT Pathway; Induction of the leptin receptor with exogenous leptin in breast cancer cell lines such as T47D and MCF-7 also activates phosphatidylinositol 3-kinase (PI3K), which can enhance growth and survival and inhibit apoptosis via AKT phosphorylation [20]. A recent study shows a similar activation of PI3K/AKT signaling pathway in papillary thyroid carcinoma [52]. Cell Migration, Invasiveness and Angiogenesis; Leptin has been reported to be an inducer of angiogenesis and vascular modeling by itself as well as synergistically with vascular endothelial factor (VEGF) and fibroblast growth factor (FGF) through up- regulation of Matrix Metalloproteinases (MMPs) 2 and 9 [20]. In addition, recent studies have demonstrated the effect of leptin on promotion of cell motility in metastatic human colon cancer cell lines through Rac and Rho dependent pathways [53].
**Epigenetics**

Epigenetics refers to inheritable changes in gene expression occurring without any alterations in the genomic DNA sequence. The primary epigenetic mechanisms regulating gene expression include DNA methylation, histone modification, and expression of miRNA.

**DNA Methylation**

DNA methylation of cytosine bases occurs through a biochemical process which covalently attaches a methyl group to the 5’ carbon atom, resulting in 5-methylcytosine [54]. In adult somatic tissues, DNA methylation typically occurs on the C residue of a CpG dinucleotide sequence and is inherited and maintained through cell division [55]. In mammals, CpG sites are less frequent across the entire genome, except in regions called CpG islands where CpG sites are highly clustered [54]. These islands are typically found in the regulatory regions in or around promoter elements of genes. Methylation in the CpG islands can inhibit transcription, either by directly interfering with the binding of transcription factors such as cyclic AMP responsive element binding protein or AP-2, or by recruitment of transcription repressive methylated DNA binding proteins (MBPs) which specifically bind to methylated DNA, forming chromatin complexes that repress transcription [56].
**Histone Modifications**

Acetylation and methylation of histone components of chromatin determine expression levels of genes. Histone Acetylation and methylation of H3K4 lysine is associated with active chromatin, whereas deacetylation and di- and tri-methylation of H3K9 lysine is characteristic of heterochromatin and silencing of genes [57].

**Epigenetics and Cancer**

While epigenetic changes play a critical role in controlling normal physiological processes and health, aberration in the epigenetic system can lead to several disease states. Epigenetic alterations can lead to abnormal activation or inactivation of genes often associated with diseases like cancer, mental retardation and syndromes involving chromosomal aberrations [58]. Cancer was the first human disease to be linked to abnormal epigenetics, 1983 [59]. Since then several characteristic differences in DNA methylation and histone modification have been observed between normal and cancer cells (Figure 7).
Figure 7: Epigenetic modifications in normal and cancer cells. Adapted from Gal-Yam, E. et al. [57].

A) DNA methylation. In normal cells most of the internally located CpG nucleotides are methylated whereas, the CpG islands in 5’ regulatory regions of genes are generally found to be unmethylated. However, in cancer cells, many CpG islands undergo methylation causing silencing of their respective genes.

B) Chromatin and histone modifications. Modifications in the histone proteins such as acetylation of histone tails, methylation of lysine 4 on the histones H3 (H3K4) and depletion of the nucleosomes at promoters often lead to activation of genes. Loss of histone acetylation and changes in the methylation marks cause silencing of genes, which recruit inhibitory complexes, often observed in cancer cells.
Methylation and Demethylation

DNA methylation is catalyzed by a family of enzymes called DNA methyltransferase enzymes (DNMT’s) which include two general classes; maintenance methylation and de novo methylation. DNMT1 plays a major role in preserving methylation of the newly synthesized strand of DNA after every replication cycle. DNMT3a and DNMT3b are the de novo methyltransferases which form the initial DNA methylation patterns early in development (Figure 8) [60].

![DNA methylation reactions and enzymes. Adapted from Szyf, M. et al. [60]. DNMT’s catalyze the transfer of methyl groups onto DNA. De novo methyltransferases incorporate methyl groups (CH3) on the CpG sites (represented by black circles), which are unmethylated (represented by white circles) on the original parental strands of DNA. Demethylases cleave the](image_url)
methyl groups to form unmethylated CpG sites. After a methylation pattern is formed by the methylases and demethylases, the maintenance methyltransferases (like DNMT1) maintain the methylation, during DNA replication [60].

Loss of DNA methylation occurs by two consecutive rounds of DNA replication without maintenance methylation. Besides the replication dependent demethylation, there is evidence of replication independent enzymatic demethylation by demethylases (Figure 8). Vairapandi, M et al. have reported 5-methylcytosine glycosylase enzymatic activity in HeLA nuclear extracts [61].

**Changes in DNA Methylation Patterns of Cancer Cells**

Cancer progression and metastasis involve several changes in gene expression characterized by aberrant modifications in their methylation patterns [62] and overall methylation levels.

Hypermethylation and silencing of several genes expressing cell adhesion molecules, growth regulating proteins and tumor suppressors is often observed in cancers. Examples of some of these genes are p16/INK4A, p15/INK4B, p14/ARF, BRCA1, TIMP3, GSTP1, mismatch repair gene MLH1 in colorectal and breast cancers and the VHL (von Hippel Lindau) gene in renal cancer [63].

Reduced global methylation has been reported in a gamut of malignancies in the past few years. [62, 64-67]. Gama Sosa, M. et al. have found that in various tumors, hypomethylation not
only correlated with transformation, but also with tumor progression [67]. Hypomethylation of gene promoters has also been implicated in the development and progression of cancer through enhanced expression of oncogenes. Examples of oncogenes activated by hypomethylation of promoter CpG elements are WNT5A, CRIP1 and S100P in prostate cancer [68], and ras oncogenes in carcinomas [59], and c-JUN and c-MYC in liver tumors [69]. A strong correlation was observed between hypomethylation at a specific CpG dinucleotide in the third exon of the c-MYC gene and development of human hepatocellular carcinoma (HCC) [70]. Activation of transcription of LINE1 retrotransposable elements due to hypomethylation has also been reported in chronic myeloid leukemia [71].

The ability of demethylating agents such as 5-azacytidine (azacitidine) and 5-azadeoxycytidine (decitabine) which can reactivate silenced genes, has prompted interest in the development of demethylating agents for epigenetic cancer therapy [72].

**Epigenetics and Alternative Splicing**

Alternative splicing plays a crucial role in physiological processes such as differentiation and development, as well as in disease and is a primary source of protein diversity in eukaryotes [73]. While alternative splicing has been observed to be affected by RNA elements and splicing factors, recent evidence shows the role of histone modifications and other epigenetic factors in the regulation of alternative splicing patterns [73]. Recruitment of splicing factors to the target RNA is not only determined by the RNA motifs, the tissue or developmental specific pattern of
splicing factors, or their post translational modifications as traditionally believed, but is also
influenced strongly by chromatin structure and histone modifications [73].

In the last few years, researchers have explored the effect of DNA methylation of CpG sites
affecting tissue specific or cell line specific expression of alternatively spliced transcripts. A
report has shown an inverse correlation between DNA methylation levels and mRNA expression
of an alternatively spliced transcript of chicken Brain-derived neurotrophic factor 1 (cBDNF1) in
inbred chicken cell lines 63 and 72, suggesting that DNA methylation may affect the gene
expression of this particular transcript [74]. They also observed high expression of this
transcript in cell line 72, which shows higher susceptibility to Marek’s disease tumor compared
to the other cell line 63. Diniz, S. et al. have shown that DNA methylation in the promoter region
of the IL15RA gene may influence differential expression of its variants in peripheral blood
mononuclear cell (PBMC), probably by affecting binding of transcription factors [75]. They have
also confirmed the effect of DNA methylation on gene expression, by treatment of PBMC with
the demethylating agent 5-azacitidine (AZA), which led to a significant increase in the
expression of variant 1 of IL15RA [75]. Recent evidence shows that DNA methylation of CpG
sites in exon 5 of CD45R inversely affects binding of a DNA binding insulator protein CTCF to the
exon and gives rise to the longer transcript of CD45R by alternative splicing [76].

**DNA Methylation in Leptin Receptor Gene**

Although the human leptin receptor is expressed and retains signaling activity in malignancies
of breast, colon, brain and other tissues, there are no known reports on the methylation status
of 5’ upstream region regulating the expression of LEPR and LEPROT. A recent study observed that promoter methylation may not be related to the expression of leptin receptor and other genes in diet induced obesity [77]. However, the methylation status of the human leptin receptor in cancer as well as the effect of such epigenetic modifications on alternate splicing have not been elucidated till date and requires further analysis. Hence, we initiated the development of tools and methods necessary for analysis of the methylation status of the LEPR/LEPROT locus and examination of any correlation with alternatively spliced transcripts.
CHAPTER 2

MATERIALS & METHODS

Cell Culture

CCD-1070Sk (CRL 2091) and BJ (CRL 2522), fibroblast cell lines isolated from normal foreskin, HS913T (HTB152), fibrosarcoma derived from metastatic site of lung, CCL-121 (HT-1080), epithelial fibrosarcoma, were obtained from American Type Culture Collection (Manassas, VA). All normal and cancer cell lines were maintained in high-glucose DMEM containing heat-inactivated fetal bovine serum (10%), penicillin (100U/ml), streptomycin (100µg/ml), and amphotericin B (250mg/ml) (Invitrogen, Life Technologies, Grand Island, NY) at 37°C in a humidified atmosphere with 5% CO₂.

Analysis of Expression of LEPR, LEPROT, & B219OBR Variants

Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from the cultured cell lines (10⁷ cells) using PureLink™ RNA Mini Kit (Ambion, Life Technologies) according to the manufacturer’s instructions. DNase I, Amp Grade (Invitrogen, Life Technologies) was used to treat the RNA samples at room temperature for 15 min in order to remove the contaminating genomic DNA, and was subsequently heat-inactivated for 11 min at 72°C. First-strand cDNA synthesis was performed using SuperScript™ III First-Strand Synthesis Super Mix for qRT-PCR (Invitrogen, Life Technologies) according to the
manufacturer’s instructions. The cDNA was used fresh or stored at -20°C for a subsequent analysis.

**Quantitative Real Time PCR**

Quantitative reverse transcriptase - PCR assays were carried out in triplicate on Applied Biosystems StepOnePlus™ Real-Time PCR System (Life Technologies). Fifteen µl of reaction mix contained 48ng of cDNA, TaqMan® Gene Expression Master Mix (Life Technologies), 0.5 µM of each sequence-specific primer, and 0.2 µM of 5’ FAM (5’JOE for GAPDH) and 3’ IBQ labeled probes with ZEN internal quencher (IDT, Coralville, IA). A human control cDNA (Clontech Laboratories, Inc) was run as a positive control, whereas nuclease free water (Invitrogen, Life Technologies) was run as a no template control for standardizing all primer sets. The cycling conditions were: 2 min at 50°C, 10 min polymerase activation at 95°C followed by 45 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, and extension at 72°C for 7 min. The threshold was set above the non-template control background and within the linear phase of target gene amplification to determine the number of cycles at which the transcript was detected (denoted as $C_T$). Relative quantification of genes was calculated using the formula $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = (C_T_{Target} - C_T_{GAPDH})_{reference} - (C_T_{Target} - C_T_{GAPDH})_{sample}$. CRL 2091 Fibroblast cell line was considered as the reference gene to calculate fold change in expression for the other three sample cell lines. Standard Error was determined from triplicate Ct values for each sample.
Primer and Probe Designing for RTPCR

Primer Design for LEPR Variants

Primers were designed using Oligo 7.51 software (Molecular Biology Insights Inc., Cascade, CO) targeting the unique region of each isoform and transcript variant and were designed to span the exon-exon junction in order to minimize amplification of residual genomic DNA. Secondary structure formations with $\Delta G$ values $> -5$ kcal/mol were avoided, and the primer set was checked for specificity using NCBI primer blast tool.

Primers were designed specific to the Leptin receptor variants spanning 19-20 exon-exons Junction.

Figure 9: Primer design for LEPR variants. Primers were designed using Oligo 7.51 software and were verified for specificity using NCBI primer blast tool. Red color represents cDNA sequence containing exons 1-19 common to all variants and other colored regions indicate regions unique to the isoforms. Black arrow indicates the forward primer and red indicates the reverse primer.
Primer Design for *LEPROT* Variants

**Figure 10: Primer design for *LEPROT* variants.** Primers were designed for the three transcript variants of *LEPROT* in the NCBI database using Oligo 7.51 software and were verified for specificity using NCBI primer blast tool.
Primer Design for B219/R and LEPROT L1 Variants

**B219R Variant**

**LEPROTL1 Variants**

![Diagram of primer and probe design](image)

Figure 11: Primer design for B219/R and LEPROT L1. Primers were designed for the B219/R transcript (in the region common to all its four 3’ variants) and the two transcript variants of LEPROT L1, using Oligo 7.51 software and were verified for specificity using NCBI primer blast tool.

Dual labeled probes were designed using Oligo 7.51, following the general probe designing guidelines.

1. Tm of probe should be 8 – 10° higher than the primers.

2. Probe should have more C’s than G’s.

3. Avoid G at 5’ end of the probe, since it causes quenching of the reporter dye.

4. Avoid repeats of nucleotides especially G’s in the probe.

5. Amplicon size should be in the range of 50 – 150 bp’s.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward 5’ – 3’</th>
<th>Reverse 5’ – 3’</th>
<th>Probe 5’ – 3’</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LEPR 219</strong></td>
<td>GGACTTAATTTTCA</td>
<td>AGAACCCTTTTC</td>
<td>FAM-</td>
<td>201</td>
</tr>
<tr>
<td></td>
<td>GAAGCC</td>
<td>AAGATC</td>
<td>TGTGGTCTCTACTTTCAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CAAC</td>
<td></td>
</tr>
<tr>
<td><strong>LEPR 219.1</strong></td>
<td>GGACTTAATTTTCA</td>
<td>CTATGCAGAGG</td>
<td>FAM-</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td>GAAGATGC</td>
<td>ACCCTGTG</td>
<td>CCACTCCCTAATCTCAAGT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ACCCAGG</td>
<td></td>
</tr>
<tr>
<td><strong>LEPR 219.2</strong></td>
<td>GCTATTTTTGGAA</td>
<td>CTACAAGCATTTC</td>
<td>FAM-</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>GATGTTC</td>
<td>CCTAAGTC</td>
<td>CCAACCTCCACCCAGTAG</td>
<td></td>
</tr>
<tr>
<td><strong>LEPR 219.3</strong></td>
<td>GGACTTAATTTTCA</td>
<td>GTTCTAAATCAA</td>
<td>FAM-</td>
<td>197</td>
</tr>
<tr>
<td></td>
<td>GAAGAG</td>
<td>CATATCCAC</td>
<td>AAATCAGTTCTGAGAG</td>
<td></td>
</tr>
<tr>
<td><strong>LEPROT</strong></td>
<td>CCCAGTTCGGAGAC</td>
<td>GCACTACTGGTT</td>
<td>FAM-</td>
<td>209</td>
</tr>
<tr>
<td>(var1)</td>
<td></td>
<td>GCATC</td>
<td>TTTACTGGCCCTTATTCTGTC</td>
<td></td>
</tr>
<tr>
<td><strong>LEPROT</strong></td>
<td>GGCCTTAAGAGAGA</td>
<td>GGATAATGCCCA</td>
<td>FAM-</td>
<td>159</td>
</tr>
<tr>
<td>(var2)</td>
<td>AGAAAC</td>
<td>CGAGAG</td>
<td>CTTCAGAGCAATCGAGA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Forward Primer</td>
<td>Reverse Primer</td>
<td>FAM Probe</td>
<td>Methylation Status</td>
</tr>
<tr>
<td>----------</td>
<td>----------------</td>
<td>----------------</td>
<td>-----------</td>
<td>--------------------</td>
</tr>
<tr>
<td><strong>LEPROTL1 (VAR1)</strong></td>
<td>TGTGCCCTTCAAT ATAC</td>
<td>CAAAGATGACT GTGTTCC</td>
<td>FAM-AGTGCACAAGCTCCCC</td>
<td>259</td>
</tr>
<tr>
<td><strong>LEPROTL1 (VAR2)</strong></td>
<td>TGTGCCCTTCAAT ATAC</td>
<td>AGCTGTTCCCAT CTTG</td>
<td>FAM-TGGGCGCCTACCCTTC</td>
<td>246</td>
</tr>
<tr>
<td><strong>B219R/ObR</strong></td>
<td>CGGATCAAGGTTG ACTTC</td>
<td>CAAGTAAACCG CAGTTATCAC</td>
<td>FAM-CCCAATGTA ACAAAAACCACACAG</td>
<td>101</td>
</tr>
<tr>
<td><strong>GAPDH</strong></td>
<td>AGGTGAAGGTCGG AGTCA</td>
<td>GGTCATTGATG GCAACAA</td>
<td>JOE-CAGGCTGCTTTTACTCT GG</td>
<td>99</td>
</tr>
</tbody>
</table>

The sequences of all PCR products were confirmed by sequencing at the Genomic core facility at Pennsylvania State University.

**Analysis of Methylation of Leptin Receptor Gene**

Analysis of CpG methylation can be achieved by acid catalyzed conversion of genomic DNA with bisulfite, which selectively converts unmethylated cytosines into uracils. This methodology was described by Hayatsu in 1970 [78]. Methylation usually occurs in the CpG islands, which are genomic regions with clustered CpG’s. The criteria proposed by Gardiner-Garden & Frommer
for identifying a CpG island is: a sequence having C+G content > 0.5, observed to expected CpG dinucleotide ratio > 0.6, and length of DNA sequence > 200bp.

Bioinformatic Analysis

The LEPR genomic sequence (NCBI acc # NG_015831.1) was analyzed using CpG plot software EMBOSS (http://emboss.bioinformatics.nl/cgi-bin/emboss/cpgplot) and Web Promoter Scan Service (http://www-bimas.cit.nih.gov/molbio/proscan/) to scan for CpG islands and promoters. Primer sets for the bisulfite modified DNA were selected using Meth Primer software (http://www.urogene.org/methprimer/index1.html) and checked for specificity using online Bisearch tool. (http://biseach.enzim.hu/)

Bisulfite Sequencing

Bisulfite sequencing involves the use of bisulfite treatment of DNA to determine its pattern of methylation. Treatment of DNA with bisulfite converts cytosine residues to uracil, but leaves 5-methylcytosine residues unaffected (Figure 12). The bisulfite converted DNA sequence is then amplified using primers specific to bisulfite modified DNA and compared to the respective unmodified genomic DNA sequence to determine the methylation of CpG’s.
Genomic DNA was isolated using QIAmp DNA mini kit (QIAGEN, Valencia, CA) according to the manufacture’s protocol. Bisulfite conversion of genomic DNA was performed using MethylCode™ Bisulfite Conversion Kit (Invitrogen, Life Technologies) according to the manufacture’s protocol. Converted DNA was used fresh or stored at -20°C.
**Primer Designing Guidelines for Bisulfite Modified DNA**

Primers for Bisulfite modified DNA were designed using Meth Primer and, Oligo 7. Bisearch was used to confirm specificity of these primer pairs. Following criteria and guidelines were considered for the primer design [81].

1) Primers should not contain any CpG sites within their sequence to avoid discrimination against methylated and unmethylated DNA. If CpG sites are unavoidable in a primer, degenerate bases should be used at the CpG sites to represent the methylated and unmethylated cytosines. For e.g. using a 'Y' to represent 'C' and 'T' in a forward primer, and using a 'R' to represent 'G' and 'A' in a reverse primer.

2) A minimum number of non CpG 'C's should be present in the primers, so that they recognize and amplify only bisulfite modified DNA. Primers with more non-CpG 'C's are preferred.

3) In order to screen as many CpG sites as possible, a primer pair should preferably span a CpG-rich region.
### Table 4: List of Primers for Bisulfite Sequencing

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward 5’ – 3’</th>
<th>Reverse 5’ – 3’</th>
<th>Ta</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primers for bisulfite modified DNA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Region A</strong></td>
<td>METHLEPRCPGa bsF1</td>
<td>TTTGTGGGATT AGGTGGGAT</td>
<td>TAACAATACAAAA CTCCTCCCCCTAC</td>
<td>62°</td>
</tr>
<tr>
<td></td>
<td>METLEPRCPGAbs mR1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Region B</strong></td>
<td>LEPRMPBbsmF</td>
<td>GGAGGAGTTT TGTATTGTTTG</td>
<td>AAAATAACAACCC YACCCACAC</td>
<td>62°</td>
</tr>
<tr>
<td></td>
<td>LEPRMPBbsmR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Region C</strong></td>
<td>LEPRMPCbsmF1</td>
<td>GTTTGTTTTGG GTAGGTTTGT</td>
<td>R1: TCTTTCTAAATCT TTCCATTAACAC</td>
<td>60°</td>
</tr>
<tr>
<td></td>
<td>LEPRMPCbsmR1</td>
<td></td>
<td>R2: CAAAAATAAAAA ACCTTTTTAAAA AC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LEPRMPCbsmR2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Region D</strong></td>
<td>LEPRMPDbsm1F</td>
<td>TTAGGGAATAT</td>
<td>TAAATCCTAAACC</td>
<td>62°</td>
</tr>
<tr>
<td>Region</td>
<td>Forward Primer</td>
<td>Reverse Primer 1</td>
<td>Reverse Primer 2</td>
<td>Tm</td>
</tr>
<tr>
<td>------------</td>
<td>----------------</td>
<td>------------------</td>
<td>------------------</td>
<td>----</td>
</tr>
<tr>
<td>Region A</td>
<td>LEPRCPGAF</td>
<td>GGCTTGAGGA</td>
<td>TACAGAGCTCCTC</td>
<td>55°</td>
</tr>
<tr>
<td></td>
<td>LEPRCPGAR</td>
<td>TTACTTGG</td>
<td>CCCTAC</td>
<td></td>
</tr>
<tr>
<td>Region B</td>
<td>LEPRCPGBF</td>
<td>GGACGGCCTCT</td>
<td>GGCAACCCCAACCA</td>
<td>55°</td>
</tr>
<tr>
<td></td>
<td>LEPRCPGBR</td>
<td>GAGAGT</td>
<td>CA</td>
<td></td>
</tr>
<tr>
<td>Region C</td>
<td>LEPRCPGCF</td>
<td>GTCGTGTGGT</td>
<td>GTCTTTCTGGGTG</td>
<td>55°</td>
</tr>
<tr>
<td></td>
<td>LEPRCPGCR</td>
<td>GGGGTG</td>
<td>CTTC</td>
<td></td>
</tr>
<tr>
<td>Region D</td>
<td>LEPRCPGDF</td>
<td>GCTCAGGGAA</td>
<td>GAGCCCTCAAAGT</td>
<td>58°</td>
</tr>
<tr>
<td>Putative</td>
<td>LEPRCPGDR</td>
<td>CATAACAC</td>
<td>AATC</td>
<td></td>
</tr>
<tr>
<td>promoter</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Putative</td>
<td>LEPRMPDbsm1R</td>
<td>AATATTGGTTA</td>
<td>CTCCAAATAATC</td>
<td></td>
</tr>
<tr>
<td>promoter)</td>
<td></td>
<td>GTGAGT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Amplification of Bisulfite Modified DNA by PCR

PCR reaction was performed using 4 μl of bisulfite modified DNA, 200 nM each of forward and reverse primers, 100μM dNTPs, 1.5mM MgCl₂, 0.75 U AmpliTaq Gold® 360 DNA Polymerase, and AmpliTaq Gold® 360 buffer (Applied Biosystems, Life Technologies) at the total volume of 25μl with the following cycling conditions: 95°C for 10 min and 40 cycles of 95°C for 30 sec, 55-62°C for 30 sec and 72°C for 40 sec followed by 7 min at 72°C. One μl of the PCR product was further used as a template for the second round PCR with the same reactions and cycling conditions. The PCR product was run on 1% agarose gel and purified using QIAquick gel extraction kit (QIAGEN, Valencia, CA) according to the manufacturer’s instructions. Alternatively, 50 ng of genomic DNA was used for the PCR reaction with 200 nM each of forward and reverse primers, 100μM dNTPs, 1.5mM MgCl₂, 10% (v/w) GC enhancer, 0.75U AmpliTaq Gold® 360 DNA Polymerase, and AmpliTaq Gold® 360 buffer at the total volume of 25μl with the following conditions; 95 °C for 10 min and 35 cycles of 95 °C for 30 sec, 55-62°C for 30 sec and 72 °C for 30 sec followed by 7 min at 72 °C. PCR product was purified using YM-30 (Millipore, Billerica, MA) and sent to the core facility at the Pennsylvania State University for sequencing.

Cloning and Plasmid Isolation

Gel purified PCR fragments were cloned into pGEM-T vector from Promega (Madison, WI) according to the manufacture’s protocol. White colonies containing an insert were selected
from LB plates supplemented with 100μg/ml ampicillin, 50uM isopropyl beta-D-1-thiogalactopyranoside, and 80ug/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactoside (SIGMA, St. Louis, MO), grown in LB broth with ampicillin for 6 hours at 37°C on shaker at 200 rpm, and then further inoculated into 1.5 ml of LB broth with 100mg/ml ampicillin for 16 hours at 37 °C on shaker at 200 rpm. After the 16-hr incubation, plasmid was extracted using QIAprep Turbo Miniprep kit (QIAGEN, Valencia, CA) according to the manufacturer’s instructions. The presence of an insert was verified using Eagl restriction enzyme digestion (New England Biolabs, Ipswich, MA) at 37 °C for 4 hours and gel electrophoresis. The plasmids with an insert were selected and sent for sequencing. 2μl of the plasmid (200 – 300ng/μl) samples were sent in a 96 well plate, for sequencing to the Genomics Core Facility, University Park, Pennsylvania State University. Sequencing reactions were performed in the DNA sequencer Applied Biosystems 3730 XL using Sanger’s method of sequencing and T7 or SP6 primers. The chromatogram outputs generated by the sequencer were further analyzed for methylation.

**Data Analysis**

Lasegene® SeqMan (DNASTAR, Madison, WI) was used to trim pGEM-5Zf (+) vector, to eliminate low quality sequencing ends, and to align sequences. Using Bisulfite Sequencing DNA Methylation Analysis software (http://biochem.jacobs-university.de/BDPC/BISMA/), methylation patterns were analyzed at an individual CpG level and overall methylation percentage across the region analyzed.
**Combined Bisulfite Restriction Analysis (COBRA); Taq I Restriction Enzyme for Determination of Methylation at a CpG site.**

COBRA is a quantitative technique used to determine DNA methylation levels at specific loci in small amounts of genomic DNA and consists of a standard sodium bisulfite PCR treatment followed by restriction digestion and quantification step (Figure 13).

![Figure 13: Outline of the Combined Bisulfite Restriction Analysis (COBRA) procedure; TaqI restriction enzyme for determination of methylation at a CpG site. Adapted from Xiong, Z. et al. [82] COBRA consists of a standard sodium bisulfite PCR treatment followed by restriction analysis.](image-url)

COBRA consists of a standard sodium bisulfite PCR treatment followed by restriction analysis.
digestion and agarose gel electrophoresis. During the first step, unmethylated cytosine residues are converted to thymine, whereas methylated cytosine residues are retained as cytosine. The consequences for the restriction site TaqI are illustrated.

In brief, Methylated (M) and Unmethylated (UM) clones for CpG site 2 in the predicted promoter region D (-622 bp to -800 bp), were mixed in variable proportions (total 50ng) to be utilized as a control. These mixed fractions of M and UM clones and PCR products of bisulfite modified DNA from various cancer cell lines and normal fibroblast cells were digested with TaqI enzyme (0.05 U) (New England Biolabs) at 67 °C for 2.5 hrs, which cuts methylated DNA at CpG site 2. Products of digestion were run on a 1.8 % agarose gel for electrophoresis.
CHAPTER 3

RESULTS

Analysis of Expression of LEPR, LEPROT, & B219/R Variants

Standardization of Primers and Probes for Quantification of Expression of LEPR, LEPROT, LEPRTL1 & B219/R Variants

Leptin receptor has been shown to be upregulated in several cancer types [20, 38-40] and the leptin-leptin receptor mediated signaling has been shown to promote cellular growth, survival, and migration of a variety of cancer cell lines [20]. While only the extracellular domain of the isoforms were analyzed in some studies [20], in others, the expression of either the long form of the receptor or the shortest form, or LEPROT, or LEPRTL1, or B219/R was examined [32, 39, 40, 41, 44]. However, a comprehensive analysis of the relative expression of all the transcripts is lacking. Hence we sought to develop molecular tools to quantify these individual variants. This is the first study wherein dual labeled probes for quantitative measurement of the transcripts (along with all known splice variants) is being addressed in cancer.

Primer pairs and Taqman probes were successfully developed to measure and distinguish between LEPR219 and its variants LEPR219.1-3 as well as variants 1 and 2 of each of LEPROT and LEPRTL1 (Figure 14). Reagents were also successfully generated for analysis of the expression of B219/R (region common to all its four 3’ variants). Using a commercially available control cDNA as template and amplification of GAPDH as a control for normalization of Ct values, the cycling conditions were optimized for all the primer and probe sets, such that all
reactions could be performed at the same annealing temperature with a view to multiplexing the reactions at a future date. The specificity of each primer and probe set was further confirmed by sequencing the respective amplified products. Although the specificity was achieved for these primer sets, the sensitivity of these assays is yet to be verified. Also, primers and probes for \textit{LEPROT} (var3) and \textit{B219R} (219, 219.1, 219.2, 219.3) are yet to be designed.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure14.png}
\caption{Standardization of isoform specific primers & Taqman probe assays for \textit{LEPR} isoforms. Taqman probes and primers for \textit{LEPR} and \textit{LEPROT} transcripts were optimized using genomic DNA (CRL 2091) \& control cDNA (Clonetech Laboratories). A no template control was run for every primer pair. \textbf{NT}: No template control \textbf{C}: control cDNA (Clonetech Laboratories, Inc) \textbf{G}: genomic DNA. GAPDH was used as the control for normalization of Ct values.}
\end{figure}

42
Expression of LEPROT (var 1) in Fibrosarcoma and Fibroblast Cell Lines

LEPROT negatively regulates the cell surface expression of the leptin receptor and inhibits the leptin signaling pathway [43, 45]. Since exogenous leptin has been implicated in enhancing the growth and migration of several cancer cell lines and tumors, we have evaluated the mRNA expression levels of LEPROT in fibrosarcoma cells relative to the fibroblast cells.

Relative Quantification of LEPROT(var1) in Fibroblast and Fibrosarcoma cell lines

For evaluation of LEPROT expression, three experimental replicates were performed and results indicated significant differences in the relative quantification of LEPROT in HT1080 fibrosarcoma cell line. Results of the triplicate experiments are shown in 15 A and 15 B.
A)

I)

II)
III)

![Graph showing relative quantification (F/R) for different samples.]

- P < 0.05
- CRL2091
- CRL2522
- HT1080
- HT18352
Figure 15: Relative quantification of LEPROT(var1) in fibroblast and fibrosarcoma cell lines. A) mRNA levels of LEPROT(var1) were analyzed by quantitative Real time PCR with three experimental repetitions. Relative quantification of genes was calculated using the formula $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = (C_{T\text{, Target}} - C_{T\text{, GAPDH}})_{\text{reference}} - (C_{T\text{, Target}} - C_{T\text{, GAPDH}})_{\text{sample}}$. The fibroblast cell culture, CRL 2091 was considered as the reference for calculation of fold change in expression for the other three cell lines. Standard Error was determined from quadruplet CT values for each sample. LEPROT (var1) was significantly reduced in HT1080 verses CRL2091, $p < 0.05$, t-test. B) Real Time PCR products were run on 1.8 % agarose gel at 120V and 100bp ladder from Promega was run for reference. No Transcriptase Control (NRT) were run in quadruplet for all samples.

This finding needs to be validated at the protein level and the status of LEPROT (var1) needs to be examined in other cancer cell lines.
Analysis of Methylation of the Leptin Receptor Gene

Epigenetic modifications control transcription as well as alternate splicing of variants of several genes [73-76]. The LEPR locus is known to have a number of transcripts which are also spliced differentially leading to a number of variants. However there are no reports on the methylation status of human LEPR. Hence the human LEPR gene was scanned for CpG islands which were analyzed for methylation of CpG sites.

Bioinformatic Analysis

The LEPR genomic sequence (NCBI acc # NG_015831.1) was analyzed using the CpG plot software EMBOSS (http://emboss.bioinformatics.nl/cgi-bin/emboss/cpgplot) and the CpG islands (CGIs) detected as shown in Figure 18. CGI 1 was located between -478 to +470 nucleotides with respect to the transcription start site (TSS), CGI 2 and CGI 3 were located in intron 2-3, CGI 4 was located in intron 4-5, and CGI 5 was located in intron 14-15. CGI 1 was divided into three sub regions A, B and C for the purpose of PCR amplification for analysis of methylation status. The putative promoter region (named D) was detected by Web Promoter Scan Service (http://www-bimas.cit.nih.gov/molbio/proscan/). Two small islands (islets) CGI 59 and CGI 85 were detected in the intron 19-20 using the criteria: observed/Expected ratio > 0.60, %C+%G > 50.00, length > 50bp. CGI 1 – 5, promoter region D, and the islets CGI 59 and CGI 85 regions were subjected to bisulfite sequencing and methylation analysis.
**Figure 16: CpG islands detected in the human LEPR locus.** The LEPR genomic sequence (NCBI acc # NG_015831.1) was analyzed using CpG plot software EMBOSS (http://emboss.bioinformatics.nl/cgi-bin/emboss/cpgplot) and five CpG islands were detected. CpG islands 1 – 5, promoter region D, and the islets CGI 59 and CGI 85 regions were subjected to methylation analysis by bisulfite sequencing
Methylation Analysis of *LEPR* 5’ Regulatory Region

Overall percentage methylation of CGI 1 and putative promoter D region was analyzed by subcloning of PCR products (amplified from bisulfite treated DNA) in plasmids by TA cloning method followed by isolation of plasmids and sequencing of the DNA inserts (Table 5).

**Table 5: Methylation Analysis of CGI 1 and Putative Promoter D Analyzed by Sub Cloning and Bisulfite Sequencing**

<table>
<thead>
<tr>
<th>Region</th>
<th>&lt;-------- CGI 1 --------&gt;</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Total No. of CpG sites</td>
<td>20</td>
<td>47</td>
</tr>
<tr>
<td>Cell line</td>
<td>Overall % Methylation (No. of Clones analyzed)</td>
<td></td>
</tr>
<tr>
<td>CRL2091</td>
<td>1.3 (81)</td>
<td>1.33 (27)</td>
</tr>
<tr>
<td>CRL2522</td>
<td>2.0 (77)</td>
<td>4.2 (23)</td>
</tr>
<tr>
<td>HT1080</td>
<td>1.6 (81)</td>
<td>3.5 (12)</td>
</tr>
<tr>
<td>HTB152</td>
<td>2.3 (81)</td>
<td>1.9 (12)</td>
</tr>
</tbody>
</table>
Low levels of overall methylation were detected in CGI 1. Hence transcription of LEPR and LEPROT is likely not down-regulated by methylation in the 5’ CGI. These results need to be confirmed with more repetitions and analyses in more cancer cell lines.

Table 6: Percent Methylation in Cpg site 2 of Putative Promoter Region D

<table>
<thead>
<tr>
<th>Cell line analyzed</th>
<th>Methylation at Cpg Site 2 in Region D (% Methylation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRL2091</td>
<td>1.6</td>
</tr>
<tr>
<td>CRL2522</td>
<td>52</td>
</tr>
<tr>
<td>HT1080</td>
<td>78.3</td>
</tr>
<tr>
<td>HTB152</td>
<td>0</td>
</tr>
</tbody>
</table>

High level of methylation was observed at Cpg site 2 in the putative promoter region D (78.3 %). Methylation at specific Cpg sites affecting transcription and invasive potential of cancers have been reported in the literature [70, 83, 84]. A COBRA assay was designed in order to verify the methylation status at this spot.
The TaqI Digestion Assay for Detecting Methylation in CpG site 2 in the Putative Promoter

In our preliminary sequencing analysis, 78% methylation was detected at the CpG site 2 in the putative promoter region D in HT1080 fibrosarcoma cell line. To confirm the methylation of this site, we developed the COBRA assay (Figure 19).

Figure 17: COBRA assay using TaqI enzyme to detect methylation of CpG site 2 in the leptin receptor promoter region. Methylated (M) and Unmethylated (UM) clones for CpG site 2 in the predicted promoter region D (-622bp to -800bp) obtained from sub cloning of PCR products, were mixed in variable proportions (total 50ng). Amplified products prepared by using these mixed fractions as template and PCR products of the corresponding segment prepared from
bisulfite modified DNA from various cancer cell lines and normal fibroblast cells were digested with Taq I enzyme (0.05 U) (which cuts the sequence TCGA that is retained when CpG site 2 is methylated). Restriction digestion was performed at 67° for 2 ½ hours as per manufacturer’s recommendations. Products of digestion were electrophoresed on a 1.8 % agarose gel for analysis.

Although we were able to standardize this assay using clones with inserts representing methylated and unmethylated CpG site 2, we were not able to confirm methylation at this CpG site in cell lines. The low sensitivity of COBRA technique has been reported in the literature [85, 86], however, our initial observation of methylation in 78% of clones for HT1080 would be well within the detectable range of the assay that was standardized. A more sensitive technique based on the design of methylation-specific primers for PCR amplification (MSP) could better confirm the methylation at this spot. Alternatively, the methylation observed at this CpG site could be an artifact that got amplified under the extended cycling conditions required for PCR amplification of bisulphite treated DNA. Further analyses are needed to resolve the possibility that site 2 is highly methyated in cancers.
Analysis of Methylation in Internal CpG Islands in Human *LEPR* Locus

Although methylation at the CpG islands in the promoter region affecting transcription are widely known, recent reports have shown that methylation in internal CpG islands located far away from the promoter, also controls transcription [84, 87]. Hence the internal CpG islands detected in human *LEPR* were screened for methylation. For initial screening purposes, the less expensive and far less laborious method of direct sequencing was employed. The incomplete results of these screening tests are summarized in Table 7, even though some of these regions are yet to be completely analyzed.
Table 7: Methylation Analysis of Internal CpG Islands Analyzed By Bisulfite Modification & Direct Sequencing

<table>
<thead>
<tr>
<th>Cell line</th>
<th>CGI 2 (# of CpG sites)</th>
<th>CGI 3 (# of CpG sites)</th>
<th>CGI 4 (# of CpG sites)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRL2091</td>
<td>(20)</td>
<td>(47)</td>
<td>(35)</td>
</tr>
<tr>
<td>CRL2522</td>
<td>Primer design and PCR was successful.</td>
<td>Primers design incomplete</td>
<td>PCR not successful</td>
</tr>
<tr>
<td>HT1080</td>
<td>Incomplete sequencing results.</td>
<td></td>
<td>Incomplete sequencing results</td>
</tr>
<tr>
<td>HTB152</td>
<td></td>
<td></td>
<td>PCR not successful</td>
</tr>
</tbody>
</table>
Screening of Methylation of CGI 5, Islet 59 and Islet 85 by Direct Sequencing

The CGI 5, CpG islet 59 and CpG islet 85 were analyzed in the fibroblast and fibrosarcoma cell lines by direct sequencing of PCR amplified segments of bisulfite treated genomic DNA. (Figure 20).

**CGI 5**

**CpG islet 59**

**CpG islet 85**
Figure 18: Methylation analysis of CGI 5 and CpG islets 59 and 85 by direct sequencing of PCR products of bisulfite modified DNA. Black boxes indicate ~ 100 % methylated CpG sites. Grey boxes represent partially methylated CpG site (dark grey: ~ 70% M, 30 % UM; light grey : ~ 50 % M, 50 % UM); and white boxes represent ~ 100 % unmethylated CpG sites.

Our preliminary results indicate that CGI 5 and CGI 85 may be differentially methylated in normal and tumor cells. This observation needs to be followed up both with thorough repetitions as well as analyses of additional normal and cancer cell lines.
CHAPTER 4

DISCUSSION

Our goal was to establish a sensitive and specific tool kit to investigate all the \textit{LEPR} and \textit{LEPROT} transcript variants quantitatively in individual samples. After these tools are completely assembled, they could be applied towards testing of methylation of the \textit{LEPR} promoter and the expression of the human \textit{LEPR} locus in various cancer cell lines and clinical samples. They could also be evaluated and employed as sensitive diagnostic markers in clinical samples of human cancer patients to establish correlations between the different stages of cancer progression and expression levels of these isoforms. These tools could be used to study any human physiological phenomenon or pathological condition which is affected by leptin mediated signaling.

This is the first study of its kind to initiate methylation analysis of the human leptin receptor gene. A more detailed analysis of methylation of the regulatory region of \textit{LEPR}/\textit{LEPROT} and its correlation with expression of these transcripts in more cell lines would provide clues to the importance of this locus in cancer and normal cells. If the DNA methylation pattern in \textit{LEPR}/\textit{LEPROT} regulatory region is shown to differentially affect the transcription of these genes in cancer and normal cells, this difference in methylation can perhaps be manipulated using methylating or demethylating agents. These findings could additionally be explored as targets to control progression of diseases like cancer and obesity that are affected by leptin receptor signaling.
The LEPROT protein negatively regulates cell surface leptin receptor expression and hence inhibits the leptin signaling pathway [45, 46]. Couturier, C et al. have described the interaction between LEPR and LEPROT proteins through bioluminescence resonance energy transfer (BRET) and coimmunoprecipitation assays [43]. It would be interesting to study the inhibitory effect of LEPROT on LEPR and the mechanism of interaction between the two proteins in cancer cells. Molecular docking and simulation studies can be conducted to determine the specific docking site of LEPROT in leptin receptor protein structure and study the manipulation of these interactions. Elucidation of these interactions in cancer cells could be of possible therapeutic potential. We expected cancer cells to downregulate the expression of LEPROT, a negative regulator of cell surface LEPR and the leptin signaling. We observed a significant decrease in the LEPROT expression in HT1080 fibrosarcoma cell line. If it can be confirmed at the protein level and in other cancer cell lines, this finding could be of considerable importance.

Overall the methods developed for comprehensive analyses of the LEPR locus and may serve as a good starting point for further investigations of LEPR variants in cancer.
REFERENCES


