BHLHE41/DEC2 in Non-Small Lung Cancer Development

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The circadian rhythm-related genes *BHLHE40/DEC1* and *BHLHE41/DEC2* have various functions under different cell and tissue conditions. *BHLHE41/DEC2* has been reported to be both a cancer-suppressive and an oncogenic gene during cancer development. The effects of *BHLHE41/DEC2* on differentiation have been examined using *Bhlhe41/Dec2* knockout mice and/or in vitro differentiation models, and research has been conducted using genetic analysis of tumor cells, in vitro analysis of cancer cell lines, and immunohistochemical studies of the clinical samples.

Keywords: BHLHE41/DEC2 ; cancer ; circadian rhythm ; differentiation

1. Introduction

BHLHE40/DEC1/STRA13/SHARP2/BHLHB2 and BHLHE41/DEC2/SHARP1/BHLHB3, which belong to the basic-helix loop helix (BHLH) protein family, function as suppressive transcription factors and are involved in circadian rhythm regulation. Both are induced by the principal circadian rhythm-related genes *CLOCK* and *BMAL1* and suppress *PER* and *CRY* expression [1]. Individuals with a variant of BHLHE41/DEC2, in which arginine replaces proline at amino acid position 384 or histidine substitutes tyrosine at position 362, exhibit the human short-sleep phenotype [2][3]. BHLHE41/DEC2 can suppress orexin, a molecule to maintain mammalian arousal, but P384R-mutated BHLHE41/DEC2 has less binding activity to the prepro-orexin promoter region and decreases the expression of orexin [4].

2. BHLHE41/DEC2 as a Tumor Suppressor Protein in NSCLC Development

According to recent statistical data, cancer-related mortality rates in the USA have been declining due to a steady decrease in incidence, likely due to the decreasing number of smokers, and the progression of molecular targeted therapies and immune checkpoint inhibitors. Nevertheless, lung cancer remains the leading cause of cancer-related deaths (21%) [5]. Several oncogenes and tumor suppressor genes have been identified as molecules associated with the development of non-small cell lung cancer (NSCLC). However, similar to the multistep model of colorectal cancer, the stages of cancer development in NSCLC remain unknown. Therefore, it is critical to understand the developmental processes in lung cancer to identify further therapeutic targets. Previously, BHLHE41/DEC2 has been reported to function as a tumor suppressor by downregulation of cyclin D in NSCLS [6]. Researchers found that BHLHE41/DEC2 plays a crucial role in NSCLC development and hypothesized that the loss of BHLHE41/DEC2 expression may be an early step in the development of NSCLC. BHLHE41/DEC2 expression is associated with better prognosis in patients with lung adenocarcinoma (LUAD). Induction of BHLHE41/DEC2 expression resulted in autophagic cell death in *huma* lung cancer cells [7]. The Cancer Genome Atlas data, cBioPortal, provides information on genetic changes containing gene amplification, truncated mutation of BHLHE41/DEC2 in lung squamous cancer (LUSC), and data on amplification, point mutation, and *SHROOM2-BHLHE41* gene fusion in LUAD; however, there are no data on mutation in small cell lung cancer (SCLC). This might reflect the difference in cancer development background between NSCLC and SCLC, although the meaning of these genetic changes of BHLHE41/DEC2 is still unclear. Immunohistochemical studies showed that BHLHE41/DEC2 expression is almost exclusively limited to the lepidic growth part of LUAD, in situ adenocarcinoma, very early LUSC cells, and normal lung epithelial cells. The observations indicated that most surgically resected LUSC samples lost BHLHE41/DEC2 expression. In addition, early LUSC can be effectively removed using radiofrequency ablation. Therefore, it is difficult to obtain information regarding BHLHE41/DEC2 function in LUSC. BHLHE41/DEC2 is expected to be an early inactivated molecule in NSCLC, possibly because BHLHE41/DEC2 is vulnerable to protein stability and epigenetic regulation of mRNA expression. Identifying partner molecules is expected to be an important step in understanding the functions of BHLHE41/DEC2 in NSCLC development. Clearly, reproducible models of cancer development are required.

3. Post-Translational Modifications Regulate the Functions of BHLHE40/DEC1 and BHLHE41/DEC2

SUMOylation is a post-translational modification that regulates several important cellular functions. In the SUMOylation process, a small ubiquitin-like modifier (SUMO) protein is covalently attached to a lysine residue in a consensus sequence, by enzymes consistent with E1-activating enzyme (AOS1/UBA2), E2-conjugating enzyme (UBC9), and sometimes E3 ligases, RAnBP2 and PIAS. In contrast, SUMOylation is negatively regulated by deSUMOylation with sentrin-specific protease (SENP) proteins, which are SUMO-specific isopeptidases comprising six cysteine proteases. Under hypoxic conditions, the activities of SENP1 and SENP3 were fully and reversibly suppressed, and SUMOylation was enhanced. SUMOylation can be recognized as another mechanism of adaptation to hypoxic conditions, rather than HIF-1 stabilization. From searches of hypoxia-induced SUMO1 targeting proteins using comparative mass spectrometry of HeLa cell extract, 48 SUMOylation proteins were defined, with more than twice as many in hypoxia than in normoxia. These proteins include SUMO ligases, RanBP2 and PIAS2, glucose transporter 1, several transcriptional regulators, and chromatin regulators. BHLHE40/DEC1 was identified as one of the more than five-fold SUMOylated target proteins belonging to a subgroup, which is composed of the other transcriptional repressors, *FSBP*, *NAB1*, *KCTD1*, *KCTD15*, or *ETV6*. Expression of *PGC-1α*, a master regulator of metabolism, was more strongly suppressed in wt BHLHE40/DEC1 than in the SUMOylated lysine-deficient mutant BHLHE40 under hypoxia [8].

Starvation conditions increased SUMOylation of BHLHE40/DEC1 at two major SUMOylation sites, K159 and K279, in MCF-7 cells, and SENP1 reduced SUMOylation. SUMOylation of BHLHE40/DEC1 promotes the repression of CLOCK/BMAL1-heterodimer-mediated transcriptional activity by interacting with HDAC1. The authors' results also suggested that SUMOylation of BHLHE40/DEC1 inhibits ubiquitination and ubiquitin-proteasome degradation [9]. BHLHE40/DEC1 overexpression suppresses the proliferation of NIH3T3 *mouse* fibroblast cells and embryonic fibroblasts from *Bhlhe40/Dec1* knockout mice via SUMOylation of BHLHE40/DEC1. SUMOylation of BHLHE40/DEC1 enhances its interaction with HDAC1. In turn, HDAC1 decreases the SUMOylation of BHLHE40/DEC1 and attenuates the cyclin D1 suppressive effect of BHLHE40/DEC1 [10]. One observation, that HDAC1 expression suppressed the attenuation effect of BHLHE40/DEC1 on cyclin D1, is inconsistent with results from other studies. Further studies are needed to clarify the biological effects of SUMOylation of Bhlhe40/Dec1 and exogenous HDAC1.

The *mouse* Bhlhe41/Dec2 protein has two SUMOylation consensus sequences, OQKLE and IKQE, containing SUMOylation sites K240 and K255, as does Bhlhe40/Dec1. In the C2C12 myogenesis model, Bhlhe41/Dec2 suppressed terminal differentiation. SUMOylation of Bhlhe41/Dec2 enhances the recruitment of the corepressor G9a and histone H3 lysine 9 demethylations (H3K9me2) to the *MyoD* promoter. Mutant Bhlhe41/Dec2, with arginine instead of lysine at positions 240 and 255, decreased the suppressive function, and SENP1 almost abolished the suppression of myogenesis by Bhlhe41/Dec2 [11]. Also, in 3T3L1 cells, induction of adipose cell differentiation could increase SENP1 expression and coincide with attenuation of SUMOylation of Bhleh41/Dec2. This observation is consistent with the deSUMOylation of Bhleh41/Dec2 upon Senp1 expression. Compared to Senp1-expressing *mouse* embryonic fibroblasts, embryonic fibroblasts derived from *Senp1* knockout mice with adipose cell induction had lower *RRARγ* promoter activity, with low expression of its target genes including adipocyte Protein 2 *(aP2),* adiponectin, and lipoprotein lipase *(Lpl)*, which increase in the differentiated adipocyte. Mutant Bhleh41/Dec2, without the main SUMOylation lysine residues had lower suppressor activity of *RRARγ* promoter [12]. These observations of SUMOylation and deSUMoylation demonstrate how interacting with other proteins has crucial effects on the functions of BHLHE40/DEC1 and BHLHE41/DEC2.

BHLHE40/DEC1 stability is controlled through SCFβTrCP, which mediates the ubiquitin-proteasome system dependent on the phosphorylation of BHLHE40/DEC1 by casein kinase I. BHLHE40/DEC1 protein increases by suppressing ubiquitination in an ATM/ATR-dependent manner by USP17 ubiquitin protease, after exposure to anticancer agents, etoposide or doxorubicin, in *huma* osteosarcoma U2OS cells and *huma* colon cancer HCT116 cells, both of which have wt *TP53* [13]. BHLHE41/DEC2 may be similarly regulated because they have similar casein kinase 1 consensus sequences. Therefore, the stability of BHLHE41/DEC2 may be regulated by the ubiquitin-proteasome system.

To understand the precise role of BHLHE41/DEC2 in a specific context, it is necessary to interpret its effects carefully. Therefore, it is necessary to identify the interacting proteins that suppress gene expression to suppress cancer development.

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