Expression of the C1orf31 Gene in Human Embryonic Stem Cells and Cancer Cells

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ABSTRACT

Human embryonic stem (ES) cells retain the capacity for self-renewal, are pluripotent and differentiate into the three embryonic germ layer cells. The regulatory transcription factors Oct4, Nanog and Sox2 play an important role in maintaining the pluripotency of human ES cells. The aim of this research was to identify unknown genes upregulated in human ES cells along with Oct4, Nanog, and Sox2. This study characterizes an unknown gene, named chromosome 1 open reading frame 31 (C1orf31) mapping to chromosome 1q42.2. The product of C1orf31 is the hypothetical protein LOC388753 having a cytochrome c oxidase subunit VIb (COX6b) motif. In order to compare expression levels of C1orf-31 in human ES cells, human embryoid body cells, vascular angiogenic progenitor cells (VAPCs), cord-blood endothelial progenitor cells (CB-EPCs) and somatic cell lines, we performed RT-PCR analysis. Interestingly, C1orf31 was highly expressed in human ES cells, cancer cell lines and SV40-immortalized cells. It has a similar expression pattern to the Oct4 gene in human ES cells and cancer cells. Also, the expression level of C1orf31 was shown to be upregulated in the S phase and early G2 phase of synchronized HeLa cells, leading us to purpose that it may be involved in the S/G2 transition process. For these reasons, we assume that C1orf31 may play a role in on differentiation of human ES cells and carcinogenesis.

(Key words : C1orf31, Human ES cells, Differentiation, Carcinogenesis)

INTRODUCTION

Populations of pluripotent human embryonic stem (ES) cells can be derived from the inner cell mass of blastocysts (Thomson *et al.*, 1998). Human ES cells differentiate into multiple phenotypes including endoderm, mesoderm and ectoderm, as well as trophoblast and germ cells (Odorico *et al.*, 2001). The ability for self-renewal and pluripotency of human ES cells are maintained by the intrinsic and extrinsic factors or several signal pathways.

Undifferentiated human ES cell surface expressed markers, such as Oct4, Nanog and Sox2 plays in maintaining the pluoripotency of ES cells (Hyslop *et al.*, 2005). Among them, Nanog is a homeobox-containing transcription factor with an essential function in the derivation of embryonic stem cells (Misui *et al.*, 2003). Oct4 and Sox2 bind to the Nanog promoter in mouse and human embryonic stem cells and are involved in the transcriptional regulation process (Rodda *et al.*, 2005). In this manner, Oct4, Nanog and Sox2 have an effect on the pluripotency of human ES cells, but not all the genes related to human ES cells pluripotency have been discovered. Recently, a novel human ES cell related gene (HESRG) was shown to be expressed in hES cells but downregulated in differentiated EB cells. Also, it was not detected in somatic or non-pluripotent cell types (Zhao *et al.*, 2007). HESRG is expected to play an important role in maintaining the pluripotency of human ES cells. The aim of our research is to identify and characterize unknown genes associated with the differentiation process of human embryonic stem cells.

In previous studies, our team identified specific genes involved in the immortalization process using the suppression subtractive hybridization method (Choi *et al.*, 2006; Jung *et al.*, 2008). We screened for specific genes in undifferentiated human ES cells. Among them, we found an unknown gene, chromosome 1 open

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reading frame 31 (C1orf31), mapping to chromosome 1 in the region 1q42.2. The C1orf31 gene consisted of 641 base pairs and 125 amino acids, and generates a hypothetical protein LOC388753 (NCBI Accession No. 338753). Recently, our team successfully isolated endothelial like cells derived from human ES cells. Sorted differentiated vascular angiogenic progenitor cells (VAPCs) showed a cobble-stone morphology similar as Cord-blood endothelial progenitor cells (CB-EPCs) (Cho *et al.*, 2007).

In this study, we investigated the mRNA expression pattern of the unknown gene, C1orf31, in undifferentiated and differentiated human ES cells. The C1orf31 was highly expression in undifferentiated human ES cells. Also, it was expressed in cancer and SV40-immortalized cell lines. These results suggest that C1orf31 may play a role in the differentiation process of human ES cells and in carcinogenesis.

MATERIALS AND METHODS

Culture of Cell Lines

The cell lines WI-38 VA13, DU-145, KM1214, NCI-H596, HeLa, A172 and WI-26 VA4 were obtained from the Korean Cell Line Bank (Seoul, Korea), and WI-38, RWPE-1, CCD-18Co, WM-266-4, K562 and SVG p12 were purchased from the American Type Culture Collection (Manassas, VA, USA). WI38, CCD-18Co, WM-266-4 and SVG p12 were cultured in Minimal Essential Medium Eagle (EMEM, Lonza, USA), supplemented with 1 mM sodium pyruvate. WI-38 VA13, DU-145, NCI-H596, HeLa and A172 in RPMI-1640 (Lonza, USA); K562, KM1214, WI-26 VA4 in Dulbecco's Modified Eagle's Medium (DMEM, Cambrex, USA); and RWPE-1 in keratinocyte serum-free medium. All media were supplemented with 10% fetal bovine serum, streptomycin (100 μg/ml) and penicillin (100 U/ml). Cell lines were grown at 37℃ in a humidified atmosphere with 5% CO_2 incubator. Human embryonic cells (CHA-3hESCs), human embryonic body (EB), vascular angiogenic progenitor cells (VAPCs) and cord blood endothelial cells (CB-EPCs) were provide by Dr. Chung at CHA Stem Cell Institute (Cho *et al.*, 2007; Kim *et al.*, 2008).

RNA Isolation and cDNA Synthesis

Total RNA was prepared from human ES cells, VAPCs, CB-EPCs and various somatic cell lines using the TRI reagent (Molecular Research Center, Ohio, USA) according to the manufacturer's recommendations. The quantity and quality of each sample were determined spectrophotometrically by A260 and the A260/280 ratio. The integrity of the RNA was examined by electrophoresis of 1 μg RNA on a 1.2% agarose gel containing 0.1% of ethidium bromide. Total RNA was reverse transcribed into cDNA using the Superscript first strand synthesis system for RT-PCR kit (Invitrogen, Carlsbad, CA, USA). For cDNA synthesis, 1 μg of each sample RNA was treated with DNase I and incubated sequentially at 37℃ for 20 min and 65℃ for 10 min. Reverse transcription reactions were prepared with: 1 μl of random hexamers, 1 μl of dNTP mix (10 mM of each dNTP), 1 μl of RNase out, 2 μl of 10 X RT buffer, 2 μl of 0.1 M DDT (20 mM), 4 μl of $MgCl_2$ and BD PowerScript II Reverse Transcriptase (200 units) and incubated sequentially at 25℃ (10 min), 42 ℃ (50 min) and 70℃ (15 min). RNase H was added to the cDNA samples followed by incubation at 37℃ for 10 min. The samples were placed at 4℃ to terminate first-strand cDNA synthesis.

Reverse Transcription PCR Anlalysis

All cDNA was tested for genomic DNA contamination by performing PCR with primers recognizing the housekeeping gene 18S rRNA. Also, each cDNA was used as a template for PCR amplification with C1orf31 gene specific primers designed in 20 mers (Table 1). We performed RT-PCR analysis using Ex Taq polymerase (TaKaRa, Otsu, Shiga, Japan). PCR conditions were as follows: i) a denaturation step at 94℃ (5 min), ii) 35 cycles of: 94℃ (30 s), 55℃ (30 s) and 72℃ (30 s), and iii) an elongation step at 72 ℃ for 5 min. The PCR products were separated in 1.2% agarose gels containing 0.1% ethidium bromide.

Quantitative Real-Time PCR Analysis

Quantitative real-time PCR was performed using a SYBR Green I kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quantitative real-time PCR conditions consisted of 45 cycles of: 94℃ (10

Table 1. Specific primers for C1orf31 gene

Gene name	Sense primer	Anti-sense primer	Size(bp)
C1orf31(RT-PCR)	AGAGGATGCTTCTCAATGCAAG	TTCATTTCTTCACAGATCTTCTGG	323
C1orf31(qRT-PCR)	TTCATCGCAGTAGGAATGGC	GGGGACAACTTGATTCGAAAG	151
18SrRAN	TACCTACCTGGTTGATCCTG	GGGTTGGTTTTGATCTGATA	243

RT: reverse transcription; qRT: quantitative real time; bp: base pairs.

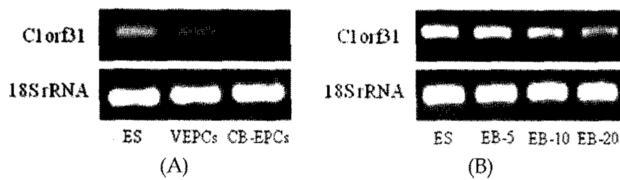

Fig. 1. RT-PCR analysis of the C1orf31 gene expression in human ES cells and differentiated cells. (A) ES: CHA3hESCs, VAPCs: Vascular angiogenic progenitor cells, CB-EPCs: cord-blood endothelial progenitor cells (B) Expression of C1orf31 in the undifferentiated human ES cells, EBs at different time points. ES: CHA3h-ESCs, EB-5: EB day 5 (derived endothelial cells), EB-10: EB day 10 (derived endothelial cells), EB-20: EB day 20 (derived endothelial cells). Human 18S rRNA was used as an internal standard.

s), 55℃ (20 s) and 72℃ (30 s). PCR were performed in a Rotor-gene 3000 (Corbett Research, Sydney, Aus tralia) and all samples were normalized to 18S rRNA. Primers used for PCR are list in Table 1.

Synchronization of HeLa Cell

HeLa cells were cultured in RPMI 1640 supplemented with 2 mM thymidine (Sigma, St. Louis, MO, USA) for 18 hr, washed with PBS and then cultured in fresh media without thymidine for 8 hr. Thymidine (2 mM) was added again to block cells at G1/S and after 18 hr, cells were transferred to fresh medium, and samples were harvested at one hour intervals from 0 through 15 hours (Fang *et al.*, 1998; Stewart and Fang, 2005).

RESULTS

We have screened for specific expressed gene in undifferentiated human ES cells compared to differentiated cells. We performed RT-PCR in undifferentiated human ES cells, differentiated human EB cells, differentiated VPACs, and CB-EPCs. Among them, C1orf31 has higher expression level in human ES cells compared to VAPCs and CB-EPCs (Fig. 1A). And, the m-RNA expression level gradually decreased in the embryoid body developmental stage of endothelial cells, EB-5, EB-10 and EB-20 (Fig. 1B).

In addition, we used RT-PCR to determine the expression pattern of C1orf31 in normal, cancer and immortalized cell lines. Expression data were generated from thirteen cell lines and the RT-PCR assay was repeated a minimum of three times. We used three normal cell lines: WI-38, RWPE-1 and CCD-18Co, seven cancer cell lines: DU-145, NCI-H596, KM1214, K562, WM-266-4, A172 and HeLa cells, and three immortalized cell lines: SVG p12, WI-38 VA13 and WI-26 VA4. Among these cell lines, C1orf31 expression was comparably higher in the cancer cell lines: DU-145, NCI-H596 and KM1214 than normal cell lines: RWPE-1,

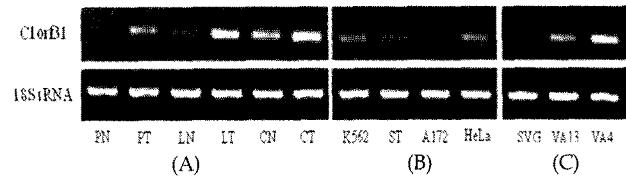

Fig. 2. RT-PCR analysis of the C1orf31 gene expression in various human somatic cell lines. (A) Normal and cancer cell lines. PN: Normal prostate cells (RWPE-1), PT: Prostate adenocarcinoma cells (DU-145), LN: Normal lung cells (WI38), LT: Lung cancer carcinoma (NCI), CN: Normal colon cells (CCD-18Co), CT: Colon carcinoma cells (KM1214), (B) Cancer cell lines. K562: Erythroleukemia cells (K562), ST: skin tumor cells (WM-266-4), A172: Brain glioblastoma cells (A172), HeLa: Cervix adenocarcinoma cells (HeLa), (C) Immortalized cell lines. SVG: SV40-immortalized brain cells (SVG p12), VA13: SV40-immortalized lung cells (WI-38 VA13), VA4: SV40-immortalized lung cells (WI-26 VA4). Human 18S rRNA was used as an internal standard.

WI-38 and CCD-18Co (Fig. 2A)., C1orf31 also was showed expression in other cancer cell lines (K562, ST and HeLa) and SV40-immortalized cell lines (SVG12p, WI-38 VA13 and WI-26 VA4) (Fig. 2B, C).

To observe the relevance of C1orf31 in cell cycle, we analyzed the expression pattern in synchronized HeLa cells. HeLa cells were synchronized using double thymidine blocks and released from their G1/S arrest. The cells were released into fresh medium and harvested at one hour intervals from 0 to 15 hours. We profiled the expression pattern of C1orf31 in cell cycle stages from 0 hours to 15 hours using quantitative real-time PCR. We analyzed the expression level throughout the cell cycle using the $2^{-\Delta\Delta Ct}$ method (Fig. 3) (Jung *et al.*, 2008; Livak and Schmittgen, 2001). Interestingly, C1orf31 was highly expressed from 1 hour to 4 hours and its levels gradually decreased after 9 hours.

DISCUSSION

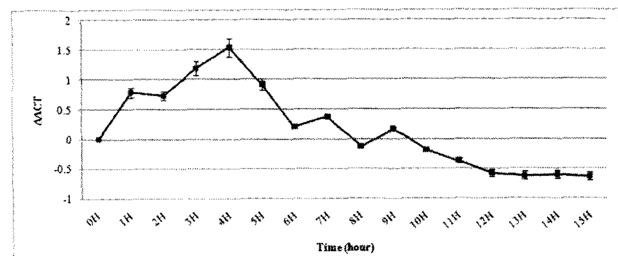

Fig. 3. Expression of the C1orf31 gene in synchronized HeLa cells. We determined the expression pattern of C1orf31 in synchronized HeLa cells using quantitative real-time PCR. Data was analyzed using the comparative $2^{-\Delta\Delta Ct}$ method. 0~3 H: S phase, 4~7 H: G2 phase, 8~9 H: M phase, 10~15 H: G1 phase. Human 18S rRNA was used as an internal standard.

In previous study, our team found specific genes expressed in immortalized cells using the suppression subtractive hybridization (SSH) method (Choi *et al.*, 2006). We screened the expression levels of those genes in undifferentiated human ES cells and differentiated vascular angiogenic progenitor cells (VAPCs), as well as cord-blood endothelial cells (CB-EPCs). Among them, we chose to more closely investigate the unknown C1-orf31 gene. Clorf31 encodes a hypothetical protein consisting of 125 amino acids, and its biological functions remain unknown at present.

Clorf31 has a cytochrome c oxidase subunit VIb (COX6b) motif according to a search using the national center for biotechnology information (http://www.ncbi.nlm.nih.gov/). Cytochrome c oxidase (COX) is the terminal enzyme complex of the mitochondrial electron transport chain (Barrientos *et al.*, 2002). Eukaryotic COX is a complex enzyme, which consists of 11 (*S. cerevisiae*) to 13 (mammals) depending on the organism (Khalimonchuk and Rödel, 2005). Among them, the COX6, encoding COX subunit VI, is mediated by at least four transcriptional factors (Hap complex, Snflp, Ssn6p and Abflp). The HAP complex is a heteromeric transcriptional regulator, Snflp and Ssn6p are involved in COX6 transcriptional regulation and the action of Ssn-6p is epistatic to Snflp-mediated regulation. Abflp has at least four different phosphorylation states, correlating with the cellular growth conditions (Fontanesi *et al.*, 2006). COX6b also function as a transcript regulator of nuclear genes. It was distinct increases of transcript levels are seen between fetal and adult state in heart and skeletal muscle. (Grossman *et al*,. 1998; Lenka *et al.*, 1998; Bonne *et al.*, 1993). However, it still has not been well studied in human ES cells.

Our team determined that Clorf31 was highly expressed in undifferentiated human ES cells. But it was downregulated in the VAPCs and CB-EPCs. Moreover, this gene's expression level decreased during the progression of EB differentiation (Fig. 1). This suggests that Clorf31 may be involved in differentiation. Undifferentiated human ES cells have the capacity for self-renewal and pluripotency, and various genes such as Oct4, Nanog and Sox2 are associated with the mechanisms of previously identified "stemness" genes, these are upregulated in undifferentiated hESC lines and downregulated in differentiated cells (Ramalho-Santos *et al.*, 2002). Among them, Oct4 gene was expressed in human tumours but not expressed in normal somatic tissues, and known to be expressed in immortal human breast, weakly tumorigenic and highly tumorigenic cell lines (Monk and Holding, 2001; Tai *et al.*, 2005). Clorf31 expression levels follow this pattern of expression in human ES cells like the aforementioned "stemness" genes. In addition, Clorf31 was highly expressed in cancer cells compared to normal cells (Fig. 2(A)). And, it was expressed in other cancer cell lines and Immortalized cell lines (Fig. 2(B), (C)). But, it was not expressed in brain glioblastoma cell line.

Our results indicate that Clorf31 has a similar expression pattern to the Oct4 gene in undifferentiated cells and cancer cells. Also, it was highly expressed in immortalized cells. Immortalization has an important role in carcinogenesis, most common genetic changes in cancer are known to be inactivation of the p53 and Rb/p16^{INK4a} pathways, Which has a key roles in the immortalization process (Reddel, 2000). Therefore, we assume that Clorf31 may be involved in carcinogenesis.

The pattern of Clorf31 expression in cell cycle was determined using the HeLa cell synchronization method. Clorf31 was most highly expressed during the S phase and gradually decreased until the G1 phase (Fig. 3). We observed an association of Clorf31 in S phase and early G2 phase. Based on this expression pattern, we purposed that Clorf31 may be involved in the regulation of the S/G2 phase transition.

In conclusion, we have identified the Clorf31 expressed in undifferentiated human ES cells and continuously dividing cells. And it was particularly expressed during the S phase of the cell cycle. Our study suggests that Clorf31 might be involved in differentiation process of ES cells as well as in carcinogenesis. Clorf31 has a COX6b motif, which is associated with transcription of nuclear genes. However, it still has not been well studied in human ES cells. We will further investigate the relationship between Clofr31 genes and transcription process in human ES cells. Also we will determine the function of Clorf31 in undifferentiated human ES cells, cancer cells and its connection to the cell cycle.

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