의학 박사 학위논문

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교모세포종의 치료내성에서의
HOXA11의 역할

2017 년 2 월

서울대학교 대학원
의 학 과 신경외과
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# Abstract

## The role of HOXA11 gene in the treatment resistance of glioblastomas

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**Purpose.** Homeobox (HOX) genes are essential developmental regulators and should normally be in the silenced state in the adult brain.Aberrant expression of HOX genes has been associated with prognosis in many cancer types including glioblastoma (GBM). In this study, the identity and role of HOX genes affecting GBM prognosis and treatment resistance were investigated.

**Materials and Methods.** By performing microarray analysis of 5 pairs of initial and recurrent human GBM samples, we screened the full series of HOX genes for the most plausible candidate responsible for GBM prognosis. We used another 20 newly diagnosed GBM samples for prognostic validation. *In vitro* experiments were performed to confirm the HOX role in treatment resistance. Mediators involved in HOX gene regulation were searched by using differentially expressed gene analysis, gene set enrichment tests, and network analysis.

**Results.** Underexpression of HOXA11 was identified as a consistent signature for poor prognosis among HOX genes. Overall survival of GBM pa-

tients indicated significantly favorable prognoses in patients with high HOXA11 expression (31±15.3 months) compared to the prognoses in those with low HOXA11 expression (18±7.3 months, $p$=0.03). When HOXA11 was suppressed in GBM cell lines, anticancer effect of radiotherapy and/or temozolomide was declined. In addition, we identified five candidate mediators (*TGFBR2, CRIM1, TXNIP*, *DPYSL2*, and *CRMP1*) which may confer oncologic effect after HOXA11 suppression.

**Conclusion.** Treatment resistance induced by underexpression of HOXA11 can contribute to a poor prognosis in GBM. Further investigation is needed to confirm the value of HOXA11 as a potential target for overcoming treatment resistance by developing chemo- or radio-sensitizers.

**keywords** : homeobox genes, HOXA11, glioblastoma, treatment resistance
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# INTRODUCTION

Homeobox (HOX) genes are essential developmental regulators controlling numerous processes including apoptosis, differentiation, motility, and angiogenesis [1]. In humans, there are four HOX clusters (A, B, C, and D), and 39 HOX genes have been identified [1]. The HOX genes are normally active during embryogenesis, but most are not expressed or are expressed at very low levels in adult brain [2]. However, several reports have indicated that there is aberrant expression of HOX genes in brain tumors as well in as other cancers from various organs [3-6]. Moreover, growing clinical evidence has indicated a prognostic effect of HOX gene expression in several cancers [7-10].

Aberrantly expressed HOX genes in cancer cells have multicapacity functions including metastasis, tumor growth, anti-apoptosis, and differentiation suppression [1]. Overexpression of multiple HOX genes have been shown in glioblastoma (GBM) cell lines and primary astrocytoma [4]. Additionally, some studies have shown that HOX genes are important in treatment resistance in glioblastoma [11-13]. However, the exact mechanism showing the role of HOX genes and their functional relevance in glioma cells remains unclear. Glioblastomas, similar to other cancers, harbor a cell subpopulation that has a stem cell-like capacity that is associated with the development of tumor progeny and treatment resistance [9, 14, 15]. Given the roles of HOX genes in development and organogenesis, it has been postulated that a portion of the relative expression of HOX genes is integral to stem cell activity, specifically self-renewal, tissue specificity, and quiescence [1]. Among the HOX genes, the HOXA cluster is important in

human embryonic stem cell differentiation [16].

Collectively, these previous results suggest that HOX genes are plausible candidates as biomarkers for use in GBM prognosis and a credible target for overcoming treatment resistance in GBM.

We have previously shown that HOX genes are genes of interest related to GBM recurrence and treatment resistance [17]. Moreover, we have reported a mechanism through which the HOXA10 gene affects temozolomide (TMZ) resistance in GBM cell lines [12]. HOXA10 induces transcription of early growth response 1, which sequentially results in phosphatase and tensin homolong (PTEN) and Rad51 paralogs. As a result, the homologous recombination DNA repair system with Rad51 genes can protect cancer cells from temozolomide-induced cytotoxicity [12].

In the present study, we extend our research to another HOX gene, HOXA11, and investigate its role in GBM prognosis.

# MATERIALS AND METHODS

## 1. Patient samples and cell lines

Fresh frozen tumor tissue samples of 5 GBM patients who were available of pairs of initial and recurrent samples for screening, and of another 20 newly diagnosed GBM patients for validation were used in this study. All patients were managed with a standard GBM treatment protocol of concurrent radiotherapy and TMZ treatment, followed by adjuvant TMZ as a primary treatment. Tumor tissues were obtained during surgery, snap-frozen in liquid nitrogen, and stored at -80° C before use. The study was approved by an institutional review committee.

Human glioma U251, U373, and LN18 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA), and cultured in DMEM containing 10% fetal bovine serum and 5% antibiotics (streptomycin) in a humidified atmosphere of 5% $CO_2$ and 95% air at 37° C.

## 2. Reverse transcription-polymerase chain reaction

Cell lines were lysed with TRIzol®(Life Technologies, Carlsbad, CA, USA), and RNA isolation was performed by using an RNeasy Mini Kit (#74104, Qiagen, Valencia, CA, USA). Total RNA was treated with DNase and then quantified by using spectrophotometry. Additionally, cDNA was synthesized from 1 μg of total RNA by using a reverse transcription kit (#205311, Qiagen) according to the manufacturer's protocol. The primers used were designed by using an online primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primer sequences for HOXA11 were: 5'-GATTTCTCCAGCCTCCC TTC-3'(forward) and 5'-AGAAATTGGACGAGACTGCG-3'(reverse).

Using these primers, reverse transcription-polymerase chain reaction (RT-PCR) was performed for 35 cycles. Each cycle comprising 95℃ for 30seconds, 62℃ for 30 seconds, and 72℃ for 45 seconds with each primer set. The RT-PCR products were resolved by using 2% agarose gel electrophoresis.

## 3. Western blot

Whole protein extracts from the tissue samples were prepared by using PRO-PREP lysis buffer (#17081, iNtRon Biotechnology, Sungnam, Korea), and protein concentrations were determined by using a BCA protein assay (#23227, Thermo Fisher Scientific, Waltham, MA, USA). Proteins were separated by using 10% SDS-PAGE, followed by blotting onto nitrocellulose membranes, and probing with antibodies against HOXA11 (#SC-48542, 1:500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blotted membranes were then incubated with a goat anti-rabbit IgG secondary antibody for 1h. Subsequently, the membranes were incubated in Amersham ECL-prime solution (#RPN2232, GE Healthcare Life Sciences, Pittsburgh, PA, USA) in the dark for 1 min and then exposed under Fluor ChemHD2 (Cell Biosciences, Santa Clara, CA, USA) for visualization. Only the samples with consistent result from repeated experiments are selected for analysis. The densities of bands were measured using free image analyzer software (ImageJ V1.8x; National Institutes of Health, USA, http://rsb.info.nih.gov/ij/). Results are presented as the mean ± SEM calculated from independent samples.

## 4. RNA interference

To knock down HOXA11 expression in cells, small interfering RNA (siRNA) experiments were performed with commercially available sequences tar-

geting HOXA11 (#SASI-Hs01-00110410, #SASI-Hs01-00110413, and #SASI-Hs01-00110417, Sigma Aldrich, St. Louis, MO, USA) as well as with non-targeting control siRNA (#D-001610-01-05, Dharmacon, Lafayette, CO, USA). At 70-80% confluence, the cells were transfected with siRNA at the most efficient transfection condition as determined by the NEON® Transfection system(#MPK5000, Life Technologies, Carlsbad, CA, USA). The cells were cultured in media without antibiotics to increase the siRNA transfection efficiency for 24 hours.

## 5. Cell viability analysis after drug and radiation treatment (RT)

Control and transfected cells were grown on 96-well plates at a density of $4 \times 10^3$cells per well for 24 hours. Subsequently, cells were either treated with TMZ (#ALX-420-044-M100, Enzo Life Sciences, Farmingdale, NY, USA) in a final concentration of 300 μg/mL for 24 h or irradiated with 4 MV X-rays from a linear accelerator (Clinac 4/100, Varian Medical Systems, Palo Alto, CA, USA) at a dose rate of 10.0 Gy/min. For combination treatment, cells were irradiated first and then treated with TMZ.

Cell viability analysis was performed by using Colorimetric Cell Counting Kit-8(CCK; Dojindo Molecular Technologies, Kumamoto, Japan). Quantification of viable cells was performed according to the manufacturer's instructions by reading the ultraviolet absorption spectra at 450 nm on a microplate 2 h after adding 10 μL of CCK solution per well. All experiments were conducted in triplicate.

## 6. Microarray

For tissue sample analysis, total RNA extracted from tissue samples by using the mirVana™miRNA Isolation Kit (#AM1560, Ambion, Austin, TX, USA) was prepared for microarray analysis after quantification and qualification.

Total RNA quality was determined by using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The cut off RNA integrity number (RIN) for RNA used in RNA amplification was 7.0 or above. The cRNA was produced by using the Illumina TotalPrep RNA Amplification Kit (#IL1791, Ambion) according to the provided protocol. The cRNA was used for hybridization to a human HT12-v4 Illumina Beadchip gene expression array (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. The arrays were scanned and fluorescence signals obtained by using an Illumina BeadArray Reader (Bead Station 500GXDW; Illumina). The signal obtained from the scanned beadchip was transformed to intensity raw data by using Genome Sortudio software (version 2009.1, Illumina) and was used for further data analysis. Raw data were normalized by applying $\log_2$transformation, quantile normalization, and gene and array centering. All data processing was performed by using the R/Bioconductor packages (version 2.14; www.bioconductor.org).

To determine gene expression changes before and after HOXA11 knock-down, extracted total RNA from LN18 cells transduced with siHOXA11 or control siRNA were analyzed by using Affymetrix GeneChip Human Gene 1.0ST Arrays (Affymetrix, Santa Clara, CA, USA). The RNA was amplified and labeled with the GeneChip® WT Sense Target Labeling and Control Reagents Kit (Affymetrix). The cDNA was synthesized, labeled, and hybridized to the GeneChip array according to the manufacturer's protocol. The GeneChips were washed and stained by using the GeneChip Fluidics Station 450 (Affymetrix) and then scanned with the GeneChip Scanner 3000 7G (Affymetrix). Expression data were normalized by using the robust multi-array average method. Affymetrix Expression Console Version 1.1 (Affymetrix) was used to compare the group signals, and data were log-transformed (base 2) for parametric analysis. Differentially expressed

genes (DEGs) were identified by using the significance analysis of microarrays method in the R package 'samr' (R 2.11.1).

## 7. Statistical analysis

Data from experiments were tested for significance by using the unpaired two-tailed Student's *t* test.ANOVA and Student's *t* test were used to identify significant differences in cell death rates. The Kaplan-Meier curve analysis was used for generating the overall survival curves. The differences between the survival curves were analyzed using the log-rank test. The results were analyzed by using IBM SPSS Statistics software (version 19.0; SPSS, Armonk, NY, USA).

For microarray analyses, false discovery rates (FDRs) were calculated by using three GenePattern software modules (www.broadinstitute.org /cancer/software/genepattern; Comparative Marker Selection version 10, Hierarchical Clustering version 6, and HeatMap Viewer version 13) [18]. Cutoff value for FDR significance was <0.05. Significantly regulated genes were subjected to functional gene classification by using the DAVID Bioinformatics Resources annotation tool (version 6.7; http://david. abcc.ncifcrf.gov/) [19]. The selected gene IDs of the identified DEGs were entered into Gene MANIA software (http://www.genemania.org, version 3.1.2.8) for network analysis [20].

# RESULTS

## 1. Down-regulation of HOXA11 is associated with poor prognosis in glioblastoma patients

Relative HOX gene expression changes between five pairs of primary and recurrent GBM samples were assessed by using microarray analysis (Figure 1). Among the 39 HOX genes, HOXA11 was the only gene that consistently showed significant down-regulation in the recurrent samples ($p$=0.046). Overall survival of a separate set of 20 GBM patients indicated significantly favorable prognoses in patients with high HOXA11 expression compared to the prognoses in those with low HOXA11 protein expression (Figure 2; survival 31 ± 15.3 months with high HOXA11 expression vs. 18 ± 7.3 months with low HOXA11 expression, $p$ = 0.03; expression status determined by western blot analysis). Based on results from the clinical samples, down-regulation of HOXA11 is associated with poor prognosis in GBM patients.

## 2. Suppression of HOXA11 mediates treatment resistance in vitro

To confirm the effect of HOXA11 expression on resistance to the current standard treatment protocol for GBM, three malignant glioma cell lines (U251, U373, and LN18) were transduced with HOXA11 siRNA to assess cell viability. Viable cell fractions were analyzed 72 hours after treatment with either single or combination applications of RT and TMZ. There were significant increment in cells with HOXA11 suppression after either RT or TMZ. (Table 1, Figure 3). When HOXA11 was suppressed, the treatment resistance effect was more pronounced following treatment with a combination of RT and TMZ (p=0.022 for RT vs RT/TMZ, and p=0.053 for TMZ

vs RT/TMZ).

## 3. Mediators of HOXA11-related oncologic effect

To identify genes under the regulation of HOXA11, we compared the microarray expression profiling data of control LN18 cells with that of HOXA11-silenced cells with HOXA11 siRNA. LN18 was chosen because it showed the most significant reduction of HOXA11 expression after siRNA transduction among the 3 cell lines tested.(Figure 4) After normalization of values and DEG analysis, we identified 11 up-regulated and 51 down-regulated genes that exhibited more than two-fold changes after HOXA11 suppression (Figure 5, Supplementary dataset 1).

We then used the functional annotation tools within DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov/home.jsp) to perform gene annotation enrichment analysis for the identified DEGs [19, 21]. Three gene ontology categories (GO:0040008 regulation of growth, GO:0005739 mitochondrion, and GO:0004157 dihydropyrimidinase activity) were significantly enriched (EASE score <0.05) in 11 genes (*EI24, CRIM1, MUL1, TGFBR2, TXNIP, STXBP1, ELK3, COX7A2, NRAS, DPYSL2, CRMP1*) among the DEG set (Table 2). For a deeper insight into the HOXA11 regulatory mechanism, a network analysis was performed by using GeneMANIA software (version 3.1.2.8) [20].The network was constructed with coexpression relationships, which revealed *TGFBR2* (transforming growth factor, beta receptor 2), *CRIM1* (cysteine rich transmembrane BMP regulator 1), *TXNIP* (thioredoxin interacting protein), *DPYSL2* (dihydropyrimidinase-like 2), and *CRMP1* (collapsin response mediator protein 1) genes as key hub regulators associated with HOXA11 suppression (Figure 6).

# DISCUSSION

Homeobox genes, a cluster of master regulators of embryogenesis, are expressed temporarily during the developmental phase in vertebrates, and they should be silenced in the adult central nervous system [2, 21, 22]. Accumulating evidence of aberrant expression of HOX genes in cancers imply that these genes have diverse roles in oncogenesis [2, 3, 21-26]. In addition, a relationship between HOX genes and treatment resistance or prognosis in cancer has been frequently proposed [9, 11, 12, 27, 28]. There are reports that have shown the tumor suppressor roles of HOX genes in many cancers [1]. Moreover, there are evidences that restoration of expression of tumor suppressor HOX genes can attenuate the cancer progression in vitro and in vivo [29-31]. We previously presented experimental evidence of an association between HOXA10 and TMZ resistance in GBM [12]. Subsequent reports into the oncogenic role of HOXA10 in various cancers have been published by other study groups [32-40]. However, based on the previous experimental data we suspected that HOXA11 acts as a tumor suppressor in opposition to HOXA10, which encouraged us to study further the function of HOXA11 in GBM.

In this study, we propose a tumor suppressor function of HOXA11 in GBM based on results from both *in vitro* experiments and human samples. There are reports on the role HOXA11 in diverse cancers. In gastric cancer, epigeneticdown-regulation of HOXA11 has been related to carcinogenesis, proliferation, migration, and invasion [41, 42]. Similarly, in lung and ovarian cancers down-regulation of HOXA11 was shown to be a poor prognostic factor [43, 44]. In GBM, the epigenetic down-regulation rate of HOXA11

was reported to be 51–75%, and HOXA11 was one of the most frequently methylated genes in GBM [45–47]. The methylation of HOXA11 was significantly associated with older patient age and poor survival in GBM [47]. Beyond that evidence of epigenetic characteristics of HOXA11 as a prognostic marker, we provide direct evidence of the prognostic value of HOXA11 expression in GBM samples (Figures 1 and 2). Based on results in previous reports and those in the present study, it is obvious that HOXA11 is a tumor suppressor in GBM and other cancers.

Treatment resistance induced by HOXA11 down-regulation, as detected by *in vitro* experiments in this study, is a mechanism contributing to a poor prognosis. In addition, we detected candidate mediators (*TGFBR2, CRIM1, TXNIP*, *DPYSL2*, and *CRMP1*) that may impart treatment resistance after HOXA11 suppression. Reportedly, HOXA11 is one of the tumor suppressor genes that can sensitize a chemotherapeutic agent in ovarian cancer [48, 49]. However, there is little direct evidence of treatment resistance induced by HOXA11 suppression in other cancer types. Regardless, our results provide a promising basis for developing HOXA11 applications that target chemo- or radio-sensitizers in GBM.

Among the five candidate mediators (*TGFBR2, CRIM1, TXNIP*, *DPYSL2*, and *CRMP1*) of HOXA11 suppression-induced oncologic effect, none have been reported to be associated with a HOXA11 regulatory mechanism.

There are several previous reports that TGF-$\beta$ signaling plays an important role in the regulation of proliferation, migration, and apoptosis regarding glioblastona [50]. High activity of TGF-$\beta$-SMAD signaling was observed in aggressive glioblastomas and conferred poor prognosis in the patients [51]. Only HOXA13, SMAD2/p-SMAD2 and SMAD3/p-SMAD3 primarily co-localize in the nucleus in GBM cells and that HOXA13 may stabilize phosphorylated R-SMAD by interacting with the MH2 domain in order to

activate the TGF-$\beta$ signaling pathway [52] .

However, *CRMP1* is of note as it is an invasion-suppressor gene in cancer cells [53]. Recently, it was suggested that decreased CRMP1 expression in GBMs harboring EGFRvIII positivity is responsible for promoting invasion [54] Moreover, those authors proposed counter-activation of Rac-1 after CRMP1 suppression as a mechanistic hypothesis for the invasive phenotype [54]. Other evidence regarding to the role of HOXA11 in oncogenesis includes a report on the regulation of MMP-2 expression, which can affect cancer cell migration and invasion [55]. Based on these results and those in the present study, we postulate an oncogenic pathway involving EGFRvIII, HOXA11, CRMP1, and Rac-1 in GBM; a pathway worthy of investigation in future studies.

# CONCLUSION

In summary, treatment resistance induced by underexpression of HOXA11 can contribute to a poor prognosis in GBM. Further investigation is needed to confirm the value of HOXA11 as a potential target for overcoming treatment resistance by developing chemo- or radio-sensitizers.

**Table 1.** Differences in survival fraction after direct inhibition of HOXA11 by siRNA compared with control in cells 72 hours after treatment with single or combination applications of radiation (RT) and temozolomide (TMZ) treatments.

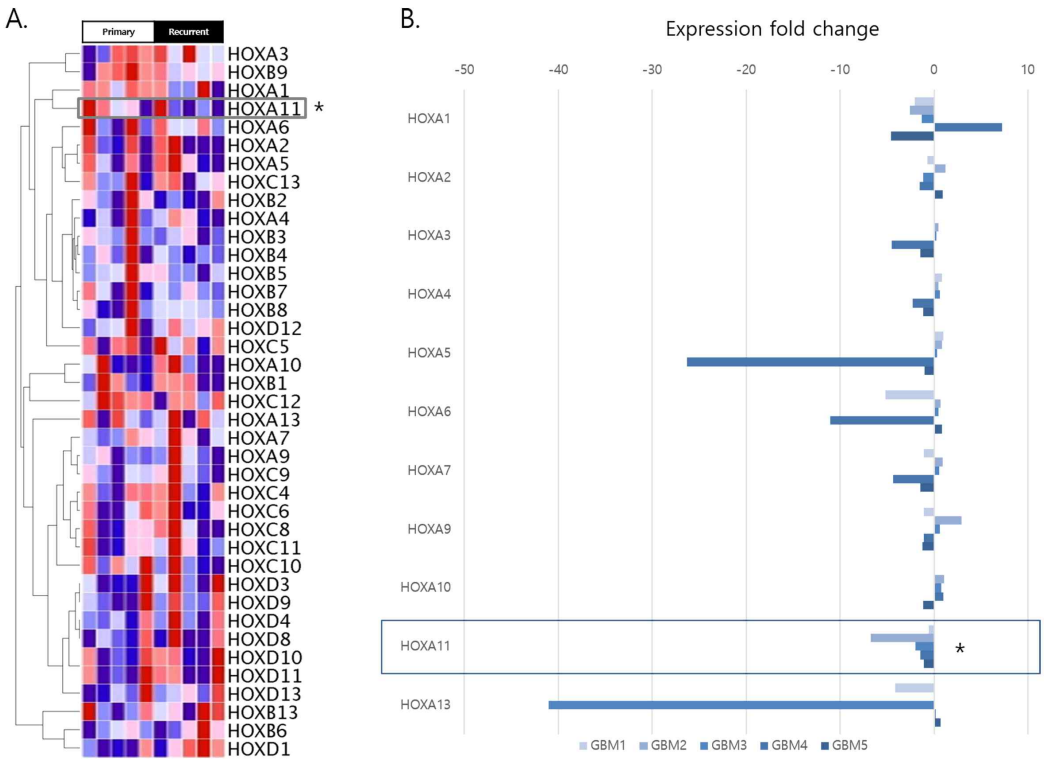
| | RT | TMZ | RT/TMZ |
|---|---|---|---|
| U251 | 10.6% | 19.7% | 45.5% |
| U373 | 36.8% | 34.6% | 55.8% |
| LN18 | 7.0% | 24.6% | 29.1% |

**Table 2.** Enriched gene ontology categories in genes expressed differently after HOXA11 suppression in LN18 cells.

| Gene ontology category | EASE Score | iHOXA11/Control | | Gene Accession | Gene Symbol | Gene Description | Cytoband |
|---|---|---|---|---|---|---|---|
| | | log2 ratio | Absolute fold change | | | | |
| GO:0040008 Regulation of growth | 0.0390089 | -1.01254 | 2.0174599 | NM_004879 | *EI24* | etoposide induced 2.4 mRNA | 11q24 |
| | | -1.0791 | 2.1127177 | NM_016441 | *CRIM1* | cysteine rich transmembrane BMP regulator 1 (chordin-like) | 2p21 |
| | | -1.105641 | 2.1519447 | NM_024544 | *MUL1* | mitochondrial E3 ubiquitin protein ligase 1 | 1p36.12 |
| | | -1.645323 | 3.1281789 | NM_001024847 | *TGFBR2* | "transforming growth factor, beta receptor II (70/80kDa) " | 3p22 |
| GO:0005739 Mitochondrion | 0.0436296 | 1.06418 | 2.0909811 | NM_006472 | *TXNIP* | thioredoxin interacting protein | 1q21.1 |
| | | -1.10251 | 2.1472795 | NM_003165 | *STXBP1* | syntaxin binding protein 1 | 9q34.1 |
| | | -1.105641 | 2.1519447 | NM_024544 | *MUL1* | mitochondrial E3 ubiquitin protein ligase 1 | 1p36.12 |
| | | -1.191529 | 2.2839467 | NM_005230 | *ELK3* | "ELK3, ETS-domain protein (SRF accessory protein 2) " | 12q23 |
| | | -1.229528 | 2.3449026 | NM_001865 | *COX7A2* | cytochrome c oxidase subunit VIIa polypeptide 2 (liver) | 6q12 |
| | | -1.43432 | 2.7025475 | NM_002524 | *NRAS* | neuroblastoma RAS viral (v-ras) oncogene homolog | 1p13.2 |
| | | -1.627447 | 3.0896577 | NM_001386 | *DPYSL2* | dihydropyrimidinase -like 2 | 8p22-p21 |
| GO:0004157 Dihydropyrimidinase activity | 0.0101296 | -1.017135 | 2.0238958 | NM_001313 | *CRMP1* | collapsin response mediator protein 1 | 4p16.1 |
| | | -1.627447 | 3.0896577 | NM_001386 | *DPYSL2* | dihydropyrimidinase -like 2 | 8p22-p21 |

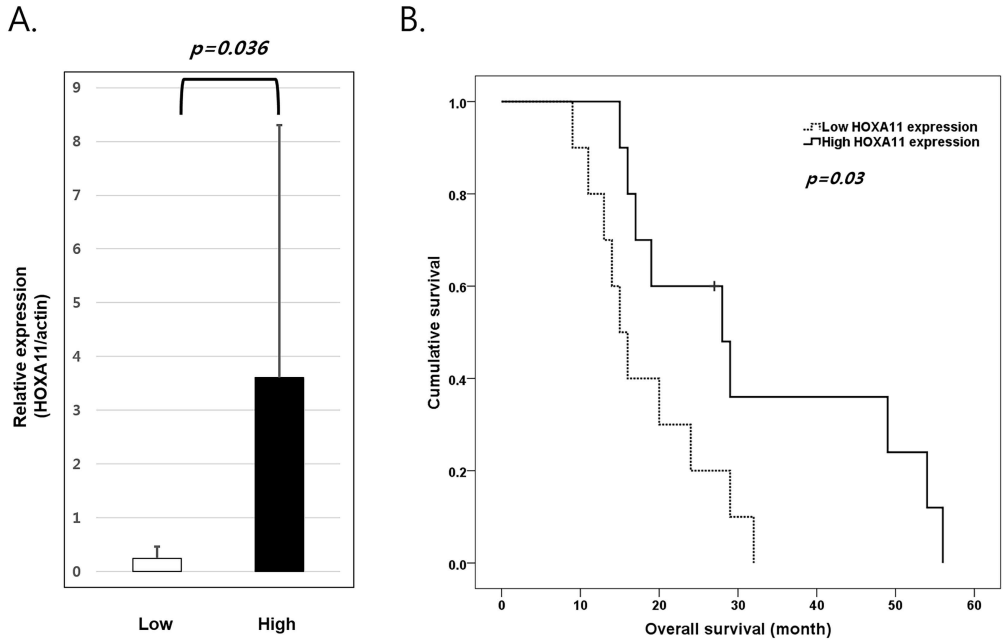
# Figure legends

## Figure 1



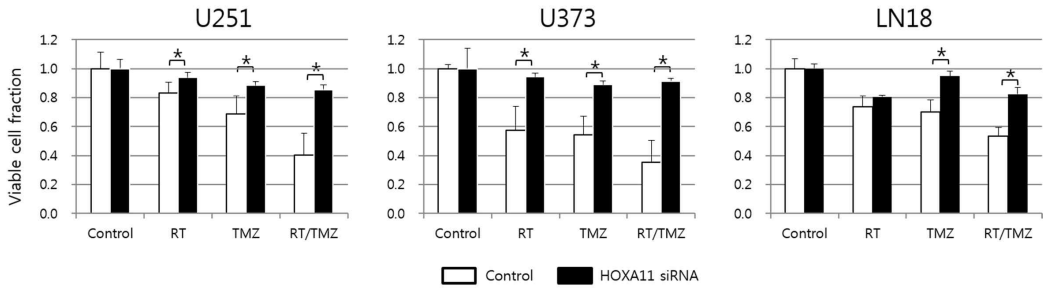

Relative expression changes in homeobox (HOX) genes among five pairs of primary and recurrent glioblastoma (GBM) samples as determined by using microarray analysis **A.** Heatmap and hierarchical clustering analysis shows inconsistent results in sequential HOX gene expression changes between primary and recurrent samples, except for HOXA11. **B.** HOXA11 gene is the only HOX gene that consistently down-regulated in recurrent GBM samples compared to primary samples for all five sample pairs.

Figure 2



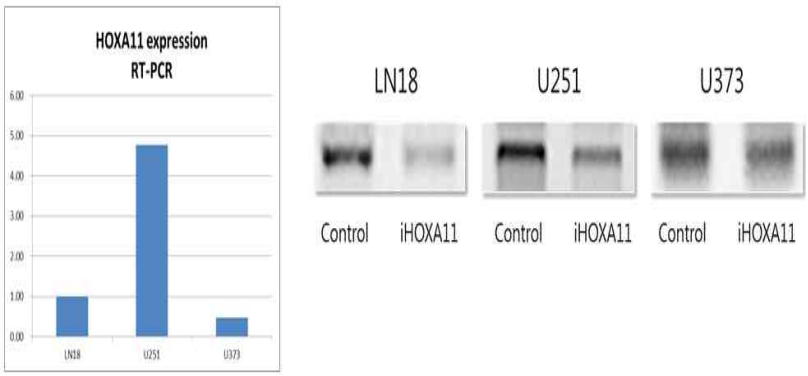

**A.** Result of western blot analysis for normalized HOXA10 expression. Patients were grouped according to the HOXA10 expression macroscopically, and confirmed the difference in expression level by intensity measurement of the bands. **B.** Overall survival of a separate cohort of GBM patients by HOXA11 expression. Survival was significantly longer in patients with high HOXA11 expression (31 ± 15.3 months) than in those with low HOXA11 expression (18 ± 7.3 months, $p$ = 0.037).

Figure 3




Treatment resistance of glioma cell lines (U251, U373, and LN18) assessed by cell viability tests after inhibition of HOXA11 and presented as relative viable cell fractions. Direct inhibition of HOXA11 by siRNA resulted in a significant increase in cell survival, 72 hours after single or combination applications of radiation (RT) and temozolomide (TMZ) treatments (* indicates $p<0.05$).

**Figure 4**. LN18 was chosen because it showed the most significant reduction of HOXA11 expression after siRNA transduction among the 3 cell lines tested.



## Figure 5.

Identification of HOXA11-regulated genes in LN18 cells. **A.** RT-PCR results from LN18 cells after HOXA11 knockdown by siRNA. **B.** HOXA11-silencing siRNA versus control siRNA transduced cells compared by using Affymetrix GeneChip Human Gene 1.0ST Arrays. Probe sets with fold changes greater than 2-fold are shown. After HOXA11 suppression, 11 up-regulated and 51 down-regulated genes were identified. **C.** MA plots showing the distribution of regulated genes.

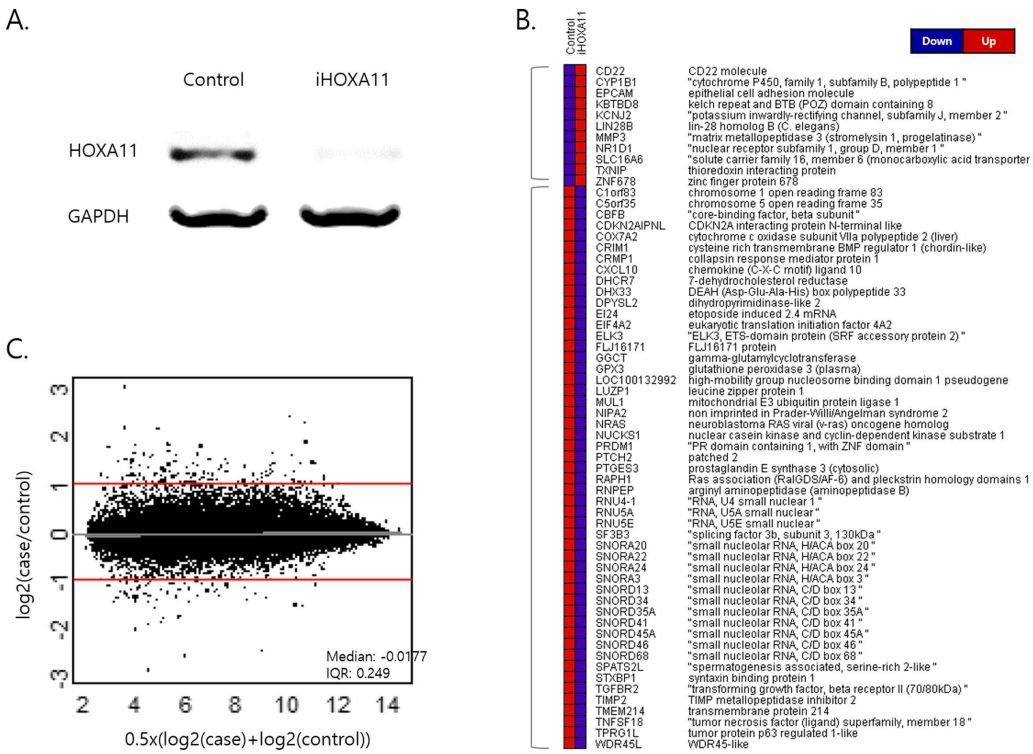
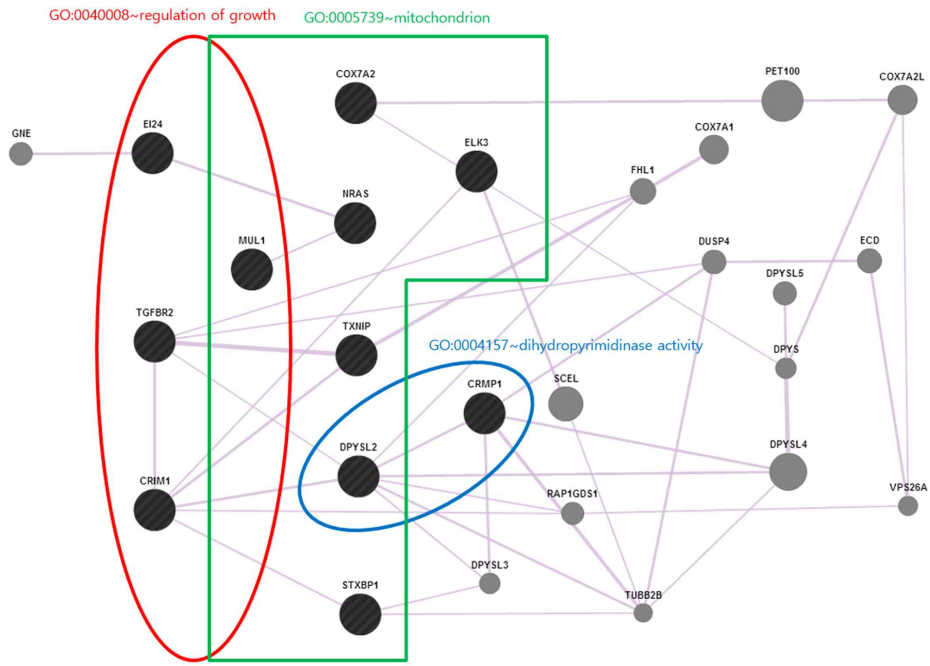


**Figure 6**. Coexpression network incorporating selected gene sets drawn from the functional gene annotation enrichment analysis of differentially expressed genes after HOXA11 suppression. Network was constructed based on coexpression interactions by using GeneMANIA software (version 3.1.2.8). The key hub regulators associated with HOXA11 suppression are expressed as black solid circles.



# REFERENCES


1. Shah N, Sukumar S. The Hox genes and their roles in oncogenesis. Nature reviews Cancer. 2010;10(5):361-71. Epub 2010/04/02. doi: 10.1038/nrc2826. PubMed PMID: 20357775.
2. Takahashi Y, Hamada J, Murakawa K, Takada M, Tada M, Nogami I, et al. Expression profiles of 39 HOX genes in normal human adult organs and anaplastic thyroid cancer cell lines by quantitative real-time RT-PCR system. Exp Cell Res. 2004;293(1):144-53. Epub 2004/01/20. doi: S001448270300510X [pii]. PubMed PMID: 14729064.
3. Abate-Shen C. Deregulated homeobox gene expression in cancer: cause or consequence? Nature reviews Cancer. 2002;2(10):777-85. Epub 2002/10/03. doi: 10.1038/nrc907nrc907 [pii]. PubMed PMID: 12360280.
4. Abdel-Fattah R, Xiao A, Bomgardner D, Pease CS, Lopes MB, Hussaini IM. Differential expression of HOX genes in neoplastic and non-neoplastic human astrocytes. J Pathol. 2006;209(1):15-24. Epub 2006/02/08. doi: 10.1002/path.1939. PubMed PMID: 16463268.
5. Bodey B, Bodey B, Jr., Siegel SE, Kaiser HE. Immunocytochemical detection of the homeobox B3, B4, and C6 gene products in childhood medulloblastomas/primitive neuroectodermal tumors. Anticancer Res. 2000;20(3A):1769-80. Epub 2000/08/06. PubMed PMID: 10928106.
6. Buccoliero AM, Castiglione F, Rossi Degl'Innocenti D, Ammanati F, Giordano F, Sanzo M, et al. Hox-D genes expression in pediatric low-grade gliomas: real-time-PCR study. Cellular and molecular neurobiology. 2009;29(1):1-6. doi: 10.1007/s10571-008-9282-1. PubMed PMID: 18404365.
7. Ferrando AA, Armstrong SA, Neuberg DS, Sallan SE, Silverman LB, Korsmeyer SJ, et al. Gene expression signatures in MLL-rearranged T-line-

age and B-precursor acute leukemias: dominance of HOX dysregulation. Blood. 2003;102(1):262-8. Epub 2003/03/15. doi: 10.1182/blood-2002-10-3221. PubMed PMID: 12637319.

8. Miao J, Wang Z, Provencher H, Muir B, Dahiya S, Carney E, et al. HOXB13 promotes ovarian cancer progression. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(43):17093-8. Epub 2007/10/19. doi: 10.1073/pnas.0707938104. PubMed PMID: 17942676; PubMed Central PMCID: PMC2040435.
9. Murat A, Migliavacca E, Gorlia T, Lambiv WL, Shay T, Hamou MF, et al. Stem cell-related "self-renewal" signature and high epidermal growth factor receptor expression associated with resistance to concomitant chemoradiotherapy in glioblastoma. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2008;26(18):3015-24. Epub 2008/06/21. doi: 10.1200/JCO.2007.15.7164. PubMed PMID: 18565887.
10. Wang Z, Dahiya S, Provencher H, Muir B, Carney E, Coser K, et al. The prognostic biomarkers HOXB13, IL17BR, and CHDH are regulated by estrogen in breast cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2007;13(21):6327-34. Epub 2007/11/03. doi: 10.1158/1078-0432.CCR-07-0310. PubMed PMID: 17975144.
11. Gaspar N, Marshall L, Perryman L, Bax DA, Little SE, Viana-Pereira M, et al. MGMT-independent temozolomide resistance in pediatric glioblastoma cells associated with a PI3-kinase-mediated HOX/stem cell gene signature. Cancer Res. 2010;70(22):9243-52. Epub 2010/10/12. doi: 0008-5472.CAN-10-1250 [pii] 10.1158/0008-5472.CAN-10-1250. PubMed PMID: 20935218.
12. Kim JW, Kim JY, Kim JE, Kim SK, Chung HT, Park CK. HOXA10 is associated with temozolomide resistance through regulation of the

homologous recombinant DNA repair pathway in glioblastoma cell lines. Genes & cancer. 2014;5(5-6):165-74. Epub 2014/07/26. PubMed PMID: 25061500; PubMed Central PMCID: PMC4104759.

13. Kim KJ, Moon SM, Kim SA, Kang KW, Yoon JH, Ahn SG. Transcriptional regulation of MDR-1 by HOXC6 in multidrug-resistant cells. Oncogene. 2013;32(28):3339-49. Epub 2012/08/22. doi: 10.1038/onc.2012.354. PubMed PMID: 22907429.
14. Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature. 2006;444(7120):756-60. Epub 2006/10/20. doi: 10.1038/nature05236. PubMed PMID: 17051156.
15. Chen J, Li Y, Yu TS, McKay RM, Burns DK, Kernie SG, et al. A restricted cell population propagates glioblastoma growth after chemotherapy. Nature. 2012;488(7412):522-6. Epub 2012/08/03. doi: 10.1038/nature11287. PubMed PMID: 22854781; PubMed Central PMCID: PMC3427400.
16. Atkinson SP, Koch CM, Clelland GK, Willcox S, Fowler JC, Stewart R, et al. Epigenetic marking prepares the human HOXA cluster for activation during differentiation of pluripotent cells. Stem Cells. 2008;26(5): 1174-85. Epub 2008/02/23. doi: 10.1634/stemcells.2007-0497. PubMed PMID: 18292213.
17. Kwon SM, Kang SH, Park CK, Jung S, Park ES, Lee JS, et al. Recurrent Glioblastomas Reveal Molecular Subtypes Associated with Mechanistic Implications of Drug-Resistance. PloS one. 2015;10(10):e0140528. Epub 2015/10/16. doi: 10.1371/journal.pone. 0140528. PubMed PMID: 26466313; PubMed Central PMCID: PMC4605710.
18. Reich M, Liefeld T, Gould J, Lerner J, Tamayo P, Mesirov JP. GenePattern 2.0. Nature genetics. 2006;38(5):500-1. Epub 2006/04/28. doi: 10.1038/ng0506-500. PubMed PMID: 16642009.

19. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature protocols. 2009;4(1):44-57. Epub 2009/01/10. doi: 10.1038/ nprot.2008.211. PubMed PMID: 19131956.

20. Warde-Farley D, Donaldson SL, Comes O, Zuberi K, Badrawi R, Chao P, et al. The GeneMANIA prediction server: biological network integration for gene prioritization and predicting gene function. Nucleic acids research. 2010;38(Web Server issue):W214-20. doi: 10.1093/nar/gkq537. PubMed PMID: 20576703; PubMed Central PMCID: PMC2896186.

21. Hueber SD, Lohmann I. Shaping segments: Hox gene function in the genomic age. BioEssays : news and reviews in molecular, cellular and developmental biology. 2008;30(10):965-79. doi: 10.1002/bies.20823. PubMed PMID: 18798525.

22. Ohgo S, Itoh A, Suzuki M, Satoh A, Yokoyama H, Tamura K. Analysis of hoxa11 and hoxa13 expression during patternless limb regeneration in Xenopus. Developmental biology. 2010;338(2):148-57. doi: 10.1016/j.ydbio.2009.11.026. PubMed PMID: 19958756.

23. Argiropoulos B, Humphries RK. Hox genes in hematopoiesis and leukemogenesis. Oncogene. 2007;26(47):6766-76. Epub 2007/10/16. doi: 1210760 [pii]10.1038/sj.onc.1210760. PubMed PMID: 17934484.

24. Bahrani-Mostafavi Z, Tickle TL, Zhang J, Bennett KE, Vachris JC, Spencer MD, et al. Correlation analysis of HOX, ErbB and IGFBP family gene expression in ovarian cancer. Cancer Invest. 2008;26(10):990-8. Epub 2008/12/19. doi: 906929018 [pii] 10.1080/07357900802074349. PubMed PMID: 19093257.

25. Cillo C, Barba P, Freschi G, Bucciarelli G, Magli MC, Boncinelli E. HOX gene expression in normal and neoplastic human kidney. International journal of cancer Journal international du cancer. 1992;51(6):892-7.

PubMed PMID: 1379214.

26. De Vita G, Barba P, Odartchenko N, Givel JC, Freschi G, Bucciarelli G, et al. Expression of homeobox-containing genes in primary and metastatic colorectal cancer. European journal of cancer. 1993;29A(6):887-93. PubMed PMID: 8097920.
27. Costa BM, Smith JS, Chen Y, Chen J, Phillips HS, Aldape KD, et al. Reversing HOXA9 oncogene activation by PI3K inhibition: epigenetic mechanism and prognostic significance in human glioblastoma. Cancer Res. 2010;70(2):453-62. Epub 2010/01/14. doi: 0008-5472.CAN-09-2189 [pii] 10.1158/0008-5472.CAN-09-2189. PubMed PMID: 20068170; PubMed Central PMCID: PMC2849935.
28. Lim JY, Yoon SO, Seol SY, Hong SW, Kim JW, Choi SH, et al. Overexpression of miR-196b and HOXA10 characterize a poor-prognosis gastric cancer subtype. World journal of gastroenterology : WJG. 2013;19(41):7078-88. Epub 2013/11/14. doi: 10.3748/wjg.v 19.i41.7078. PubMed PMID: 24222951; PubMed Central PMCID: PMC3819543.
29. Carrio M, Arderiu G, Myers C, Boudreau NJ. Homeobox D10 induces phenotypic reversion of breast tumor cells in a three-dimensional culture model. Cancer Res. 2005;65(16):7177-85. Epub 2005/08/17. doi: 10.1158/0008-5472.CAN-04-1717. PubMed PMID: 16103068.
30. Petrini M, Felicetti F, Bottero L, Errico MC, Morsilli O, Boe A, et al. HOXB1 restored expression promotes apoptosis and differentiation in the HL60 leukemic cell line. Cancer cell international. 2013;13(1):101. Epub 2013/10/24. doi: 10.1186/1475-2867-13-101. PubMed PMID: 24148231; PubMed Central PMCID: PMC3874656.
31. Rhoads K, Arderiu G, Charboneau A, Hansen SL, Hoffman W, Boudreau N. A role for Hox A5 in regulating angiogenesis and vascular patterning. Lymphatic research and biology. 2005;3(4):240-52.

Epub 2005/12/29. doi: 10.1089/lrb.2005.3.240. PubMed PMID: 16379594.

32. Carrera M, Bitu CC, de Oliveira CE, Cervigne NK, Graner E, Manninen A, et al. HOXA10 controls proliferation, migration and invasion in oral squamous cell carcinoma. International journal of clinical and experimental pathology. 2015;8(4):3613-23. Epub 2015/06/23. PubMed PMID: 26097543; PubMed Central PMCID: PMC4466930.
33. Cui XP, Qin CK, Zhang ZH, Su ZX, Liu X, Wang SK, et al. HOXA10 promotes cell invasion and MMP-3 expression via TGFbeta2-mediated activation of the p38 MAPK pathway in pancreatic cancer cells. Digestive diseases and sciences. 2014;59(7):1442-51. Epub 2014/01/28. doi: 10.1007/s10620-014-3033-6. PubMed PMID: 24464212.
34. Kurscheid S, Bady P, Sciuscio D, Samarzija I, Shay T, Vassallo I, et al. Chromosome 7 gain and DNA hypermethylation at the HOXA10 locus are associated with expression of a stem cell related HOX-signature in glioblastoma. Genome biology. 2015;16:16. Epub 2015/01/28. doi: 10.1186/s13059-015-0583-7. PubMed PMID: 25622821; PubMed Central PMCID: PMC4342872.
35. Li B, Cao X, Weng C, Wu Y, Fang X, Zhang X, et al. HoxA10 induces proliferation in human prostate carcinoma PC-3 cell line. Cell biochemistry and biophysics. 2014;70(2):1363-8. Epub 2014/06/22. doi: 10.1007/s12013-014-0065-7. PubMed PMID: 24951955.
36. Liborio-Kimura TN, Jung HM, Chan EK. miR-494 represses HOXA10 expression and inhibits cell proliferation in oral cancer. Oral oncology. 2015;51(2):151-7. Epub 2014/12/17. doi: 10.1016/j.oraloncology. 2014.11.019. PubMed PMID: 25500095.
37. Oue N, Sentani K, Sakamoto N, Yasui W. Clinicopathologic and molecular characteristics of gastric cancer showing gastric and intestinal mucin phenotype. Cancer science. 2015. Epub 2015/06/03. doi:

10.1111/cas.12706. PubMed PMID: 26033320.

38. Xiao ZD, Jiao CY, Huang HT, He LJ, Zhao JJ, Lu ZY, et al. miR-218 modulate hepatocellular carcinoma cell proliferation through PTEN/AKT/PI3K pathway and HoxA10. International journal of clinical and experimental pathology. 2014;7(7):4039-44. Epub 2014/08/15. PubMed PMID: 25120782; PubMed Central PMCID: PMC4129017.
39. Zhang HY, Li JH, Li G, Wang SR. Activation of ARK5/miR-1181/HOXA10 axis promotes epithelial-mesenchymal transition in ovarian cancer. Oncology reports. 2015. Epub 2015/07/08. doi: 10.3892/or.2015.4113. PubMed PMID: 26151663.
40. Zhang L, Wan Y, Jiang Y, Ma J, Liu J, Tang W, et al. Upregulation HOXA10 homeobox gene in endometrial cancer: role in cell cycle regulation. Med Oncol. 2014;31(7):52. Epub 2014/06/20. doi: 10.1007/s12032-014-0052-2. PubMed PMID: 24943991.
41. Bai YG, Fang N, Gu TX, Kang YH, Wu J, Yang DS, et al. HOXA11 gene is hypermethylation and aberrant expression in gastric cancer. Cancer cell international. 2014;14. doi: ARTN 79 DOI 10.1186/s12935-014-0079-7. PubMedPMID:WOS:000340964700001.
42. Cui Y, Gao D, Linghu E, Zhan Q, Chen R, Brock MV, et al. Epigenetic changes and functional study of HOXA11 in human gastric cancer. Epigenomics. 2015;7(2):201-13. Epub 2015/01/16. doi: 10.2217/epi.14.92. PubMed PMID: 25590359.
43. Fiegl H, Windbichler G, Mueller-Holzner E, Goebel G, Lechner M, Jacobs IJ, et al. HOXA11 DNA methylation - A novel prognostic biomarker in ovarian cancer. International Journal of Cancer. 2008;123(3):725-9. doi: DOI 10.1002/ijc.23563. PubMed PMID: WOS:000257171900032.
44. Hwang JA, Lee BB, Kim YJ, Park SE, Heo K, Hong SH, et al. HOXA11 hypermethylation is associated with progression of non-small cell lung cancer.

Oncotarget. 2013;4(12):2317-25. PubMed PMID: WOS:000328936800015.

45. Laffaire J, Everhard S, Idbaih A, Criniere E, Marie Y, de Reynies A, et al. Methylation profiling identifies 2 groups of gliomas according to their tumorigenesis. Neuro-oncology. 2011;13(1):84-98. Epub 2010/10/12. doi: 10.1093/neuonc/noq110. PubMed PMID: 20926426; PubMed Central PMCID: PMC3018904.

46. Martinez R, Martin-Subero JI, Rohde V, Kirsch M, Alaminos M, Fernandez AF, et al. A microarray-based DNA methylation study of glioblastoma multiforme. Epigenetics : official journal of the DNA Methylation Society. 2009;4(4):255-64. Epub 2009/06/25. PubMed PMID: 19550145.

47. Skiriute D, Vaitkiene P, Asmoniene V, Steponaitis G, Deltuva VP, Tamasauskas A. Promoter methylation of AREG, HOXA11, hMLH1, NDRG2, NPTX2 and Tes genes in glioblastoma. Journal of neuro-oncology. 2013;113(3):441-9. Epub 2013/04/30. doi: 10.1007/s11060-013-1133-3. PubMed PMID: 23624749.

48. Fang F, Munck J, Tang J, Taverna P, Wang Y, Miller DF, et al. The novel, small-molecule DNA methylation inhibitor SGI-110 as an ovarian cancer chemosensitizer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2014;20(24):6504-16. Epub 2014/10/16. doi: 10.1158/1078-0432.CCR-14-1553. PubMed PMID: 25316809; PubMed Central PMCID: PMC4268357.

49. Matei D, Fang F, Shen C, Schilder J, Arnold A, Zeng Y, et al. Epigenetic re-sensitization to platinum in ovarian cancer. Cancer Res. 2012;72(9):2197-205. Epub 2012/05/03. doi: 10.1158/0008-5472.CAN-11-3909. PubMed PMID: 22549947; PubMed Central PMCID: PMC3700422.

50. Bruna ,A., Darken ,R.S.,Rojo, F., Ocana, A., Penuelas, S., Arias, A., et al. High TGF- beta Smad activity confer poor prognosis in glioma patients and priomotes cell proliferation depending on the methylation of the PDGF-B gene. Cance Cell 2007 ;11: 147-160.

51. Caja,L., Bellomo C., and Moustakas ,A. Transforming growth factor β and bone morphogenetic protein actions in brain tumors. FEBS Lett.2015; 589, 1588-1597

52. Ran Duan, Lei Han, Qixue Wang, Jianwei Wei, Luyue Chen, Jianning Zhang, Chunsheng Kang, et al. HOXA13 is a potential GBM diagnostic marker and promotes glioma invasion by activating the Wnt and TGF-β pathways. Oncotarget 2015 sep 29:6(29):27778-93

53. Shih JY, Lee YC, Yang SC, Hong TM, Huang CY, Yang PC. Collapsin response mediator protein-1: a novel invasion-suppressor gene. Clinical & experimental metastasis. 2003;20(1):69-76. Epub 2003/03/26. PubMed PMID: 12650609.

54. Mukherjee J, DeSouza LV, Micallef J, Karim Z, Croul S, Siu KW, et al. Loss of collapsin response mediator Protein1, as detected by iTRAQ analysis, promotes invasion of human gliomas expressing mutant EGFRvIII. Cancer Res. 2009;69(22):8545-54. Epub 2009/11/12. doi: 10.1158/0008-5472.CAN-09-1778. PubMed PMID: 19903856.

55. Yilmaz N, Ozaksit G, Terzi YK, Yilmaz S, Budak B, Aksakal O, et al. HOXA11 and MMP2 gene expression in uterosacral ligaments of women with pelvic organ prolapse. Journal of the Turkish German Gynecological Association. 2014;15(2):104-8. Epub 2014/07/01. doi: 10.5152/jtgga.2014.0088. PubMed PMID: 24976777; PubMed Central PMCID: PMC4072547.

# 국문초록

## 교모세포종의 치료내성에서의 HOXA11의 역할

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**연구 배경**: Hox(homeobox) 유전자의 이상발현이 교모세포종(Glioblastoma)을 포함한 다양한 유형의 암의 예후와 연관되어 있음이 밝혀지고 있다. 우리는 교모세포종의 예후와 치료내성(treatment resistance)과 연관된 Hox 유전자의 역할을 연구하고자 하였다.

**연구 방법**: 본 연구는 인간 교모세포종 조직표본의 미세정렬분석(microarry analysis)을 이용하여 치료내성과 예후에 영향을 미치는 가장 타당한 후보(candidate)를 찾기 위해 HOX 유전자 전체를 검색하였다. 또한 치료내성에 대한 역할을 입증하기 위한 생체외 실험을 수행하였으며 유전자 분석(expressed gene analysis)과 유전자 집단 농축시험(gene set enrichment test) 과 회로 분석(network analysis)를 별도로 수행하여 Hox 유전자의 조절을 포함한 매개체(mediators)들을 검색하였다.

**연구 결과**: HOX 유전자 중 HOXA11의 저발현이 나쁜 예후를 예측할 수 있는 일관된 특성으로 확인되었다. 교모세포종 환자의 전체 생존률은 높은 HOXA11 발현률을 보인 환자군(31 ± 15.3 months)에서 낮은 HOXA11 발현률을 보인 환자군((18 ± 7.3 months)보다 통계학적으로 의미있는($p$=0.03)좋은 예후를 보였다. 교모세포종 세포주 (GBM cell lines)에 HOXA11을 억제

했을때, 방사선 치료 및 temozolomide의 항종양효과가 감소했다. 더욱이 우리는 HOXA11 억제 이후 발암 효과를 매개하는 5개의 후보 중개자 (candidate mediators ; *TGFBR2, CRIM1, TXNIP ,DPYSL2, CRMP1*)를 발견하였다.

**결론**: HOXA11의 저발현으로 인한 치료 내성은 교모세포종의 나쁜 예후의 한 원인이 될 수 있다. 항암 및 방사선치료에 대한 치료 내성을 극복하기 위한 가능성 있는 지표로서의 HOXA11의 가치를 확립하는 추가적인 연구가 더 필요할 것으로 생각된다.

**주요단어**: Hox(homeobox) 유전자, HOXA11, 교모세포종, 치료 내성
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