




# The Impact of ALX Genes Expression Difference on Cancer

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Cancer is a disease that emerged as a result of uncontrolled division and growth in the tissue or organ. It also causes gene expression changes because it disrupts the life cycle and mechanism of the cell or tissue in which it occurs. Cancer can be classified as benign and malign and also classified into six classes according to the cell type. These classifications are; carcinomas, sarcomas, myomas, leukemias, lymphomas, and mixed types. Carcinomas are the kind of cancer seen in epithelial cells and form the big majority of cancers. Sarcomas-consisting of mesenchymal cells in the body- are the type of cancer that occurs in soft and bone tissues. Myomas are cancer formed by plasma cells i.e. immune system cells. Leukemias occur in bone marrow because they are blood cell cancer. Lymphomas; are the cancerous growth of lymphocytes which are the defense cells of our body.<sup>[1-3]</sup> In addition, since the mechanism of cancer occurs includes various types, how cancer occurs also differs. For example, it has been known that cancer can occur with mutations in cancer cells, proto-oncogenes, or carcinogens.<sup>[4]</sup> Besides this classification, it is been thought that there are also still some mechanisms and cancer types that don't understand yet. On the genetic pathway to cancer, mutation of tumor suppressors and oncogenes occurs

## ABSTRACT

Cancer is a disease that became well-known in the twentieth century. Some factors cause cancer such as environmental factors or mutations formed in the genome and differentiation in gene expression. Differentiation in gene expression happens because of the hypomethylation or hypermethylation in some parts of the genome. Early diagnosis and treatment of cancer are very important due to its mechanism and occurring for various reasons. Therefore the science world aimed to gather more information about cancer and its early diagnosis methods. The estimate of the prognosis was made possible by the information and new tests revealed by the development of science and technology. According to the information obtained about epigenetics and its mechanism, DNA methylation has emerged. Some tests identify the Aristaless-like homeobox family (ALX) gene with methylation differences and detect them in cancer tissues or cells. Furthermore, it has been found that there are no methylation differences in some cancer types. The ALX1, ALX3, and ALX4 genes from the homeobox gene family were mentioned in this review, and methylation differences in these genes can be biomarkers in many cancer types. Also, the types of cancers associated with ALX genes that are hypermethylated, hypomethylated, or do not perform any expression alterations were discussed in this review.

**Keywords:** ALX1, ALX3, ALX4, cancer, DNA methylation, homeobox

in abnormal gene expression caused by loss/gain of function. The epigenetic pathway in cancer is not so simple and it is determined by chromatin structure, including DNA methylation, histone variants and modifications, nucleosome remodeling, and small non-coding regulatory RNAs.<sup>[5]</sup>

## EPIGENETICS

Epigenetics is a variation in gene expression through environmental factors not caused by changes in DNA sequence or by factors such as chromosomal imbalance, genomic imprinting, and methylation differentiation in DNA.<sup>[6]</sup> Although most scientists

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define this term differently, it can be defined as the interaction of genetic variables in general and the process by which genotype is expressed into phenotype.<sup>[7]</sup> DNA methylation is an epigenetic factor that appears in many examples, including the X chromosome inactivation mechanism. The difference in methylation of DNA is associated with important formations for human life, such as cancer and aging.<sup>[8]</sup>

## DNA METHYLATION

DNA methylation; occurs in the promoter regions at the 5th end of half of the mammalian genes, in areas called CpG island. These regions are formed by binding methyl to the 5th carbon of cytosine nucleotide, which comes after guanine nucleotide. This is executed by DNA methyltransferase (DNMT). This is the most studied epigenetic event in mammals because of its micro and macro effects on the genome. It causes gene expression differences with stable gene silencing and gene expression arrangement. It provides vital functions to the organism such as silencing at the retroviral elements, regulating tissue-specific gene expression, genomic imprinting, and regulating X chromosome inactivation.<sup>[9]</sup> The methylation mechanism can be regulated by post-translational mechanisms such as microRNAs (miRNA) called epi-miRNAs.<sup>[10]</sup> miRNAs are small non-coding RNAs that play an important role in regulating gene expression by connecting specific messenger RNAs (mRNAs) to the 3' UTR (untranslated region) region that lead to mRNA division or translation.<sup>[11,12]</sup> Mature miRNA controls gene expression by blocking the translation of connected mRNA or activating target mRNA's degradation. After revealed studies about miRNAs, it has been seen that DNA methylation regulates miRNA expression. It has been shown that miRNA can regulate histone modifications and DNMT expression but also regulate DNA methylation too.<sup>[9]</sup> The expression of miRNAs differs substantially from the alteration caused by DNA hypomethylation or hypermethylation, according to a study in cancer cells. Abnormal methylation in CpG islands has also been identified as an important distinguishing feature of cancer. Thus, DNA methylation regulatory genes have shown that they can increase or suppress the development of cancer in any specific type of cancer.<sup>[13,14]</sup> The expression of the *Aristaless*-like homeobox family (ALX) gene was discovered to be an important variable in the occurrence of this difference.

## ALX HOMEBOX GENE FAMILY

In the human genome, there are more than two hundred homeobox families categorized as eleven classes and approximately a hundred gene families.<sup>[15]</sup> There are two subclasses of this homeobox family. The first one is *antennapedia* (*Antp*) and the other is the paired (*PRD*) class. ALX genes are included in the *PRD* subclass. The ALX gene family plays a role in the face, skull, and orbit development and contains two to four homeobox genes in each vertebrate genome analyzed.<sup>[16]</sup> Humans and mice have three ALX homeobox genes; ALX1, ALX3, and ALX4 genes. The functions of these genes are to ensure normal development of the head and face. They are important transcription factors for the formation of the eyes, nose, and mouth starting from the fourth week of development. These proteins control the activity of genes that regulate events in the form of a specific time and cycle such as the growth, division, and movement of the cell. As a result, they're crucial for the cell since they ensure cells grow at specified times, stop growing at specific times, and make positioning correctly during development.<sup>[17]</sup> In addition, it has been thought that the ALX4 gene also plays a role in skin layer formation but the mechanism of it still has not been understood properly yet. The link between ALX genes and cancer has also been discovered as a result of recent research. Since methylation differences in ALX genes may affect the expression of the gene and its response to the cell, the mechanism of causing cancer has started to be investigated. Further, it is seen on the table that ALX genes cause different cancers according to their type.

## ALX AND CANCER

It has been known that epigenetic differences play a key role in human carcinogenesis.<sup>[18,19]</sup> It has been found that the methylation differences occurring in ALX genes can be biomarkers for cancer. Recent experiments have shown that different transcription factors are also present. It was investigated whether these transcription factors caused any differences in cancer samples. The ALX homeobox gene family became the standout among these variables.<sup>[18]</sup> The differences in the ALX1, ALX3, and ALX4 genes found in humans and mice vary depending on the types of cancer they are present in. For this reason, the association between cancer and ALX is investigated in this review based on ALX.

### ALX1

Overexpression that occurred in the ALX1 gene has been detected in some cancer types such as lung cancer, osteosarcoma, and ovarian cancer.<sup>[20-22]</sup> Also, it has been seen that there is more expressed ALX1 gene in metastatic lung cancer samples than non-metastatic lung cancer samples. So, it has been accepted that overexpression of ALX1 in lung cancer is associated directly with metastasis and early diagnosis.<sup>[20]</sup> In vivo and in vitro studies with osteosarcoma, it is aimed to see if ALX1 can be used as a new therapeutic target, as in lung cancer. This study strongly demonstrated that specific short-hairpin RNA (shRNA) mediated silencing of ALX1 leads to the elimination of osteosarcoma cells through the inactivation of p21/cyclin B1 mediated mitotic cell cycle. For this reason, it has been observed that ALX1 can lead us to conclusions as a potential target in the treatment of human osteosarcoma.<sup>[21]</sup> Epithelial-mesenchymal transition (EMT), a morphological change is known as the transition from epithelium to mesenchyme in ovarian cancer cells, is linked to the development of malign features. EMT is thought to be controlled by regulatory factors. There has been a search for specific factors involved in this mechanism. ALX1 gene revealed by siRNA (small interfering RNA) scanning which searches for new regulatory as well as HOXB7 (homeobox B7), SIX1 (SIX Homeobox 1), FOXQ1 (Forkhead Box Q1), FOXM1 (Forkhead Box M1) transcription factors.<sup>[23-27]</sup> ALX1 was found by this study that induce morphologic changes in ovarian cancer cells. ALX1 mRNA expression levels in ovarian cancer cells were evaluated by using quantitative RT-PCR (reverse transcription-polymerase chain reaction) analysis. Interestingly after this analysis, it has been observed that the expression level of ALX1s mRNA is associated with malign features of ovarian cancer cells.<sup>[22]</sup> The results of the research were found that transcription factors associated with EMT have been shown to cause malignant properties such as invasion, metastasis, and resistance to chemotherapy to the cancer cells.<sup>[28]</sup> Additionally, it has been analyzed 13 abnormal methylated genes from the tissue samples taken from early-stage non-small cell lung cancer (NSCLC). When compared to the control group, it has been seen that the ALX1 gene in these 13 genes did not change significantly.<sup>[29]</sup> Tumor samples were taken from patients in other experiments with NSCLC. Along with many genes analyzed for DNA methylation, it has been observed that hypermethylation of the ALX1 gene may be a marker in early-stage non-small cell lung cancer.<sup>[30]</sup>

Likewise, methylation analyses to predict survival in Glioblastoma showed that ALX1 could lead to prognosis prediction as a result of hypermethylation, thus helping to make an early diagnosis.<sup>[31]</sup>

### ALX3

Until 20 years ago, methylation alterations in human tumor cells were mostly unknown.<sup>[29,30]</sup> CpG islands -occurring in promoter regions of genes- are protected from abnormal methylation but because of some activation of genes or inactivation of tumor suppressors, abnormal methylation is also seen there too.<sup>[31]</sup> The abnormally methylated CpG islands were identified using a genome scanning technology called RLGS, which allowed for a simultaneous assessment of thousands of CpG islands. An experiment was designed using methylation-sensitive PCR to assess the methylation status of ALX3 in primary neuroblastoma tumors. The designed experiment was successful and provided further information about ALX genes. Decreased ALX3 expression in neuroblastoma cell lines has shown a correlation. ALX3 expression was seen to be re-inducing in the sequences of neuroblastoma cells treated with 5-Aza-2'-deoxycytidine and it was thought that methylation of ALX3 is associated with gene silencing. But it is not determined whether methylation of ALX contributes to neuroblastoma phenotype.<sup>[32]</sup> Another ALX-associated cancer example is a soft tissue cancer, seen frequently in children which are called rhabdomyosarcoma. After genome-wide analysis of DNA methylation, such as in neuroblastoma, abnormal methylation has also been detected in this type of cancer on many CpG islands.<sup>[33]</sup> In colorectal cancer (CRC), since the early periods of this type of cancer progress through an asymptomatic process, diagnosed patients have often been late cases.<sup>[34]</sup> Periodic tests will help to speed up the process and diagnose the problem earlier. Hypermethylation of CpG islands has been a determinant factor for colorectal cancer. A direct scanning test based on the microarray of DNA methylation was used to find this relation. The ALX3 gene was significant among the factors observed in the expression change during the tests. When the non-neoplastic colon mucosa samples used in the colorectal cancer study of ALX3 were examined, it was observed that it was significantly hypermethylated. However, in these samples, it was not observed that the difference in methylation in ALX3 had a significant relationship with age or the stage of cancer.<sup>[35]</sup>

## ALX4

According to recent statistics, breast cancer is the most diagnosed type of cancer and the leading cancer death among women worldwide.<sup>[36]</sup> The discovery of ALX genes was driven by the aim of finding early detection of cancer. Breast cancer has the same condition. It is a factor that has been noticed by the search for early diagnosis and reducing the death rate. Breast cancer occurs at the end of a complex process consisting of tumorigenesis, genetic and epigenetic changes. As a result of the change in epigenetic mechanisms, DNA hypermethylation was found to be the most common mechanism of inactivation of the tumor-suppressing gene.<sup>[37,38]</sup> A number of methylation-silencing tumor suppression genes have been reported to be associated with the onset and progression of cancer.<sup>[39]</sup> Colorectal cancer screening has been made using blood and feces in order to detect genetic and epigenetic differences in colorectal cancer proteome. Developed precancerous colorectal lesions have been associated with increased methylated DNA frequency in plasma. The SEPT9 and ALX4 genes were emphasized in the study, and it was discovered that a combined analysis of these genes, which are believed to act as markers, was helpful in diagnosing colorectal polyps with high sensitivity and specificity.<sup>[40]</sup> When searching at the ALX4 transcription factor in lung cancer tissues, hypermethylated DNA with a typical CpG island structure was found. 98 primary lung cancer tissue samples and 20 normal lung tissue have been examined to verify these findings. As a result, ALX4 hypermethylation has been seen frequently in primary lung cancer tissues but not in normal lung tissues. In this way, ALX4 methylation has been proven to occur frequently and in a tumor-specific way. Further, it has also been observed that ALX4 is not affected by conditions such as age, gender, smoking, or pathological stage, but it is only related to poor differentiation of cancer. It has been also observed in the experiments that ALX4 induces apoptosis in lung cancer and affects the cell cycle. Eliminating the expression of ALX4 has been found to promote cell proliferation and decrease apoptosis.<sup>[41]</sup> After analyzing the association between ALX1 and ovarian cancer, researchers looked into the ALX4 relationship in ovarian cancer. The expression of ALX4 has been silenced by hypermethylation in ovarian cancer samples. In this way, it has been observed that ALX4 induces tumors by promoting EMT and invasion in ovarian cancer. The HOXB13 (homeobox B13) and ALX4 genes studied in this experiment have been shown to be associated with EMT as well as the

invasion of ovarian cancer cells.<sup>[42]</sup> Unlike all these studies, hypomethylation of the ALX4 gene examined in hepatocellular carcinoma (HCC) has been observed. ALX4's role as a tumor suppressor in HCC, where the ALX4 gene is seen to be hypomethylated, has attracted attention. It has been found that the EMT of HCC cells was suppressed by overexpression of this gene. These findings showed overexpression of ALX4 plays a role as a suppressor in the migration and invasion of HCC cells.<sup>[43]</sup> Another type of cancer that has an anti-tumor effect by hypomethylated ALX4, is breast cancer. After experiments examining breast cancer cell lines, it has been seen that ALX4 is hypermethylated in 100% of breast cancer cell lines and 69.44% of primary breast tumor tissues. Hypermethylation was not observed in any of the normal breast tissues. In the studies, it has been seen that ectopic expression of ALX4 inhibits cell proliferation and metastasis *in vivo* and *in vitro*. With the understanding of its mechanism, ALX4 has been found to apply anti-tumor function dependent on Glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ), by promoting the degradation of phosphorylation of  $\beta$ -catenin and by suppressing the pathway of the Wnt/ $\beta$ -catenin.<sup>[44, 45]</sup> In pancreatic adenocarcinoma, many genes were analyzed for whether they are hypermethylated or not. ALX4 is available in these genes. In this experiment where samples of pancreatic adenocarcinoma patients were taken at different stages, it has been also observed that hypermethylated gene accumulation is seen more in samples taken from IV. stage than samples taken from I., II., and III. stages.<sup>[46]</sup> It's also been discovered that hypomethylation, or the downregulation of ALX4, can play a key role in the development of *in vitro* and *in vivo* MC-LR-induced liver cancer; Microcystin-LR (MC-LR) is a potent carcinogen. Significant hypermethylation of samples treated with MC-LR was encountered in the 20th and 35th weeks of the experiment. Tumor suppressor function of ALX4 by p53 path has been found in liver cancer caused by MC-LR. In addition, it was discovered that the methylation levels of the ALX4 promoter were strongly linked to the development of lung cancer in a study that ran concurrently with this one.<sup>[47]</sup> Finally, in another experiment related to ALX4, its relationship with thyroid cancer was mentioned. Irregular expression of long intergenic non-protein coding RNA 313 (LINC00313) in some tumors was tried to be explained through the cellular processes of thyroid cancer, including proliferation, migration, and invasion through ALX4 methylation. Long non-coding RNAs (lncRNAs) and genes related to thyroid cancer have been analyzed using microarray analysis. The anti-tumor effect of

LINC00313 has been examined by gain and loss of function experiments. In addition, the binding of the promoter region of LINC00313 and ALX4 and the interaction of LINC00313 with methylation-related proteins were detected. Microarray analysis showed that LINC00313 is overexpressed, ALX4 is downregulated in thyroid cancer and results of these are confirmed in thyroid cancer tissues. Also in these results, it has been found that LINC00313 has been bound promoter region of ALX4 and LINC00313 recruits DNMT1 (DNA methyltransferase 1) and DNMT3b (DNA methyltransferase 3b) proteins to support methylation of ALX4 promoter region and in this way, it suppresses the expression of ALX4. The downregulation of LINC00313 and the up-regulation of ALX4 have been shown to suppress the protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling, preventing the proliferative, migration, invasive abilities, and the mesenchymal passage from the epithelium of thyroid cancer cells.<sup>[48]</sup>

In conclusion, expression differences in the ALX1, ALX3, and ALX4 genes have been reported to play a role in cancer development. In addition, as seen in the cancer samples examined in this review, it was observed that a single ALX gene is not specific to a single type of cancer. With the examined samples and experiments, it was also observed that the ALX gene in some cancer cells/tissues does not affect cancer even if there is a difference in methylation. The cause or mechanism of action may be different, but as has been seen in many experiments, expression differences in ALX genes are an important factor in cancer. On the basis of the ALX gene analyzed, the differences in the samples utilized in the experiment, such as cell line or tissue samples, may yield different results for us. When tissue samples were used, as in the NSCL experiment, no significant changes were observed in the expressed genes after the control group comparison was made. However, when tumor samples of the same type of cancer were taken, hypermethylation of the ALX1 gene exhibited a noticeable change. In line with the materials or samples used during the experiment, it is usual to change the course and outcome of the experiment. Another situation to be considered on the basis of these variables is the stage at which the cancer is isolated from the individual. The most conclusive aspect of the findings is that with more experiments and trials, a previously unknown aspect of the ALX genes will emerge. This is owing to the fact that the process of cancer is complex and currently unclear. Especially the mechanism of the ALX4 gene and hypermethylation in cancer types

in which it is detected as well as hypomethylation, and suppression as well as inducing tumor formation is an interesting result. A recent study suggests that these transcription factors, which appear to perform a variety of functions, may reveal more cell functions in the coming days. With the development of technology, it can be thought that the usual tests can develop and give more accurate results. The lack of certainty in science will always give scientists more research power, so the information they present to us is changing and developing day by day. The relationship between methylation of ALX genes, an epigenetic factor mentioned in this review, and cancer was compiled using information from the literature.

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