

The Role of Autophagia in Non-Small Cell Lung Cancer

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ABSTRACT

Lung cancer is the most common type of cancer all over the world and first rank in cancer related deaths. Autophagy was first defined in yeast at the 1970's, programmed cell death mechanism. In recent years, many studies have been made regarding the role of the autophagy in carcinogenesis. In these studies, autophagy both initiate the carcinogenesis and providing survival of cancer cells as well as launching their apoptosis, as opposed to this two role as a mechanism to destroy cancer cells in the direction of that evidence to the task. Especially in the treatment manipulation of autophagy may have some future treatment opportunities (inhibition or activation). But we have not yet to clarify these questions so the data are inadequate. One of the greatest obstacles in treatment of lung cancer is dysregulation of cancer cell death signalization. In this study we evaluate the autophagy genes expression mechanisms in relation with the expressions of genes in different pathological types in different clinical stages of lung cancer. But due to some technical data on gene expression in yield of negativity did not reach significant information.

Keywords: Autophagy, Cell death, NSCLC, Prognosis

ÖZET

Küçük Hücre Dışı Akciğer Kanseri Otofajinin Rolü

Akciğer kanseri tüm dünyada en yaygın kanser türüdür ve kansere bağlı ölümlerde birinci sıradadır. Otofaji 1970'lerde ilk olarak mayalarda tanımlanmış bir programlı hücre ölüm mekanizmasıdır. Otofajinin karsinogenezdeki rolü son yıllarda popüler araştırma konularından birisidir. Bu çalışmalarda ofofajinin hem karsinogenezin başlatılması hem kanser hücrelerinin sağkalımı hem de bunlara zıt olarak apoptozdan kaçan kanser hücrelerinin yok edilmesinde bir mekanizma olarak görev yapabileceği yönünde kanıtlar bulunmaya başlanmıştır. Özellikle tedavide ofofajinin manüplasyonu (inhibisyonu veya aktivasyonu) ile başarılı olunabileceği fikri doğmuştur. Ancak elimizdeki veriler bu soruların aydınlatılması için henüz çok yetersizdir. Akciğer kanserinde de tedavi önündeki en büyük engellerden birinin kanser hücresinin ölüm sinyalizasyonundaki disregülasyon olduğu bilinmektedir. Çalışmamızda ofofaji yolağında rol alan genlerin ekspresyonlarının akciğer kanserinin farklı klinik evrelerinde, farklı patolojik tiplerinde nasıl değişkenlik gösterdiği araştırılmıştır. Ancak gen ekspresyon verilerinin eldesinde bazı teknik olumsuzluklar nedeniyle istatistiksel olarak anlamlı bilgilere ulaşamadık.

Anahtar Kelimeler: Otofaji, Hücre ölümü, KHDAK, Prognoz

INTRODUCTION

Lung cancer is the most common cancer type worldwide.¹ Despite developments in radiotherapy, introduction of target-specific biological agents and optimization of therapeutic regimes, the desired improvement in prognosis has not been achieved.² One of the most important obstacles in curative treatment of lung cancer is dysregulation in cell death signaling.^{3,4,5}

Autophagic cell death was defined in the 1970's.⁶ Autophagia is a process characterized by the formation of double membrane autophagic vacuoles (autophagosome) that have been maintained evolutionarily.^{7,8} These autophagosomes are transformed into autolysosomes by combining with lysosomes and provide reuse of proteins and cellular organelles by breaking them up.⁹

Autophagia has multiple roles in induction of carcinogenesis. In states of malnutrition it provides time for the adaptive changes required for gene expression and metabolic activities by allowing the reuse of intracellular nutritional sources.¹⁰ Additionally, it may decrease the escape from chemotherapy-induced and radiotherapy-induced apoptosis.¹⁰⁻¹³ It is also thought that suppression of carcinogenesis is provided by autophagia. Another mechanism in eliminating cancer cells that have not been exposed to apoptosis may be autophagia.¹³ In addition, autophagia should be prevented in order to maintain malign phenotypes of some cancer cells.¹⁴

Currently, there are various chemotherapeutical agents that induce autophagia. These include temozolomide, histone deacetylase inhibitors, arsenic trioxide, tamoxifen, rapamycin, and radiation.¹⁵⁻²¹

In this study, the relationship between the clinicopathologic properties of lung cancer and autophagia was investigated.

PATIENTS AND METHODS

Patient Group

Patients with a diagnosis of NSCLC who were operated on by the department of chest surgery of our hospital between 2008 and 2012 were screened retrospectively. Informed consent was obtained from

the patients whose clinical data could be accessed. Afterwards, the glass blocks belonging to these patients were examined in the department of pathology. The tissue blocks containing a tumor were considered tumor tissue and the tissue blocks in which dysplasia was not observed were considered normal tissue on microscopic examination. These tissues were obtained from the paraffin block archive. Tissue sample sections were prepared with microtome.

Specification of Gene Expression

RNA was obtained from the tissue samples embedded in paraffin using Qiagen FFPE RNA isolation kits. Isolated RNA was kept at -85°C to be used in further processes.

cDNA extract: cDNAs were synthesized using Qiagen miScript Reverse Transcription Kits. cDNA synthesis was performed using the AB Applied Biosystems 96-Well Thermal Cycler device.

A gene expression study was performed with BioMark™ HD System using Nano technology-based high-capacity Real Time PCR. cDNAs were pre-amplified for gene expression analysis.

The required PCR conditions were applied using PreAmp Primers. Plates were pipetted by preparing the pre-amplification mix, cDNA was added and appropriate PCR conditions were applied using the AB Applied Biosystems Veriti 96-Well Thermal Cycler device. The primers belonging to 175 regions of the autophagia family were designed specifically and real-time PCR was performed using the IFC Controller HX and BioMark™ HD System. 175 primers designed for autophagia gene expression analysis were studied in different chips. Two different mixes were prepared for the Samples and Assays.

The Primers were Removed from the Autophagia Gene Expression Primer

Plate and loaded to the chip. They were placed in the BioMark IFC Controller HX (S/N: HX 10132) device for Chip assay, for the samples to enter and mix in the wells found in the chip and for being prepared for RT PCR. After the procedure in the

Table 1. Gene primers

GENE NAME	GENE NAME	GENE NAME
ACTB	ATG4C	CTSS
AKT1	ATG4D	CXCR4
AMBRA1	ATG5	DAPK1
APP	ATG7	DRAM1
ARSA	ATG9A	DRAM2
ATG10	ATG9B	EIF2AK3
ATG11	BAD	EIF4G1
ATG12	BAX	ESR1
ATG13B	BECN1	FADD
ATG14	BID	FAS
ATG16L1	BLC2	GAA
ATG16L2	BLC3L1	GABARAPL1
ATG18	BNIP3	GABARAPL2
ATG2B	CAP3	GAPDH
ATG3	CASP3	HDAC1
ATG4A	CASP8	HGS
ATG4B	CLN3	HPRT1
ACTB	CTSB	HTT

BioMark IFC Controller HX was completed, the chip was placed in the BioMark™ HD System (S/N:104) and the appropriate PCR conditions were applied. Each assay was analyzed separately for each sample. The peaks, Ct values and copy numbers of the samples in each assay were analyzed separately and statistical calculations were performed. The gene primers, which were planned to be tested, are shown in the Table 1.

RESULTS

The clinical and pathological data of 92 patients who were operated on with a diagnosis of lung cancer in the department of chest surgery in our university between 2008 and 2012 were obtained.

The mean age of the patients at the time of diagnosis was 60.19 (44-78) years. 4 (4.35%) of the patients were female and 88 (95.65%) were male. When the distribution of the patients by staging was examined, it was found that 11 (11.95%) were in stage 1, 58 (63.04%) patients were in stage 2, 19 (20.65%) patients were in stage 3 and 4 (4.34%) patients were in stage 4. The distribution by histological subtypes was as follows: 67 (72.82%) patients SCC, 18 (19.56%) patients adenoca, 7 (7.60%) patients LCC. At the end of data collec-

tion for the study, 30 (32.60%) of the patients had lost their lives and 62 (67.39%) were living. The number of non-smoker patients was 4 (4.34%) and one of these (1.08%) was a female case of adenocarcinoma. The data related with smoking could not be obtained in 7 (7.60%) patients.

Ninety-two patients were grouped by their pathological data and sample properties, and gene expression analysis was performed on the samples obtained from tumor and non-carcinoma tissues. Since obtaining nucleic acid in FFPE tissues is a substantially problematic approach, the ACTB gene was used in order to normalize experimental errors occurring during the process of obtaining samples.

Our experimental approach in comparison of gene expression data is the approach of comparison with relative quantification. The data were expressed only as fold change in the gene expression level between the groups.

In statistical analysis, the Mann-Whitney test, which we used to compare the groups, also examined the distributions for making a decision if there was a significant difference in mean values. In the tests, which examine the distributions in mean comparison states, 20 data scores have been recommended and at least 12 data scores are required.

ACTB gene amplification for normalization could be found in 61 control samples and 51 tumor samples. Therefore, the gene expression data in the carcinoma tissue in the study sample were limited to these 51 cases.

Test Parameters

Real-time PCR measurements for gene expression performed with the Fluidigm Dynamic Array in BioMARK HD System were evaluated with the Biogazelle qBasePLUS real-time PCR data-analysis program. Among target genes, ACTB was used as the housekeeping gene in the normalization of the targets.

Test for Gene Comparison: Control (non-carcinoma) / Tumor

In the test in which the gene expression data were compared between the samples obtained from the

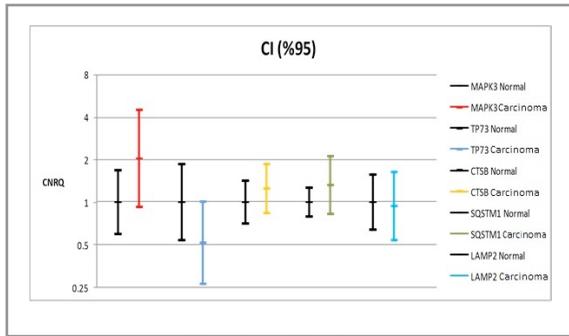


Figure 1. Comparison of gene expression

carcinoma tissues of 51 individuals and the noncarcinoma control samples of 61 individuals, it was investigated if a statistically significant difference was present using the Mann-Whitney test which considers the distributions as well as mean values.

A statistically significant difference at a significance level of 95% ($p < 0.05$) could not be found in terms of the genes examined between the non-carcinoma and tumor tissues.

A score of 12 and above recommended statistically was obtained for the genes MAPK3, TP73, CTSB, SQSTM1, and LAMP2. The amplification level in the other genes was not statistically appropriate for analysis of the results. Since all data in the analysis were scaled to the mean value of the synthesis level in the non-carcinoma tissue, the mean value of non-carcinoma tissues appears to be 1. The CNRQ (calibrated normalized relative quantity) fold change is shown in the Figure 1.

The details related with the comparison of the normal and tumor tissues belonging to these genes for which a score of 12 and above was obtained are shown in the following Table 2.

When examined for MAPK3, the lowest expression ratio was found to be 0.594 and the highest expression ratio was found to be 1.684 in the normal tissue. These ratios were exposed to normalization according to the ACTB gene, which was selected as the control gene, and were expressed as 1. In the tumor tissue, the lowest expression ratio was found to be 0.928 and the highest expression ratio was found to be 4.488 for MAPK3. When the expression ratios were compared, it was observed that a 2.041-fold increase in MAPK3 expression was

Table 2. Comparison of normal and tumor tissues

	MAPK3	TP73	CTSB	SQSTM1	LAMP2
Normal	0,594	0,54	0,703	0,787	0,64
Normal 95% value ci high	1	1	1	1	1
Tumor 95% value ci low	0.928	0.263	0.839	0.823	0.534
Tumor	2.041	0.515	1.251	1.32	0.932
Tumor 95% value ci high	4.488	1.009	1.865	2.119	1.626

present in the tumor tissue compared to the normal tissue. This is not statistically significant.

While the expression ratios in the normal tissue for CTSB ranged between 0.703 and 1.423, they ranged between 0.839 and 1.865 in the tumor tissue. When compared with normalization, a 1.251-fold increase in CTSB gene expression was observed in the tumor tissue, though it was statistically insignificant.

While the SQSTM1 gene was expressed with a rate raging between 0.787 and 1.27 in the normal tissue, it was expressed with a rate raging between 0.823 and 2.119 in the tumor tissue. When these rates were compared, a 1.32-fold increase was observed in the tumor tissue, though it was statistically insignificant.

While the TP73 gene expression ratio ranged between 0.54 and 1.85 in the normal tissue, it ranged between 0.263 and 1.009 in the tumor tissue. When these ratios were compared, it was observed that the TP73 expression was decreased 0.515-fold in the tumor tissue.

The lowest expression ratio was found to be 0.64 and the highest expression ratio was found to be 1.562 for the LAMP2 gene in the normal tissue. In the tumor tissue, the lowest LAMP2 expression ratio was found to be 0.534 and the highest LAMP2 expression ratio was found to be 1.626. It was found that the LAMP2 expression was decreased 0.932-fold in the tumor tissue, though it was statistically insignificant.

In conclusion, 5 genes that allowed comparison with the Mann-Whitney test were examined and it was found that the MAPK3, CTSB, and SQSTM1

Table 3. Gene expression of SCC Subtype

	TP73	CTSB	LAMP2
Normal 95% value ci low	0.461	0.709	0.495
Normal	1.063	1.086	0.934
Normal 95% value ci high	2.451	1.663	1.761
Tumor 95% value ci low	0.307	0.791	0.404
Tumor	0.688	1.322	0.915
Tumor 95% value ci high	1.538	2.212	2.069

gene expressions were increased and TP73 and LAMP2 gene expressions were decreased in the tumor tissue.

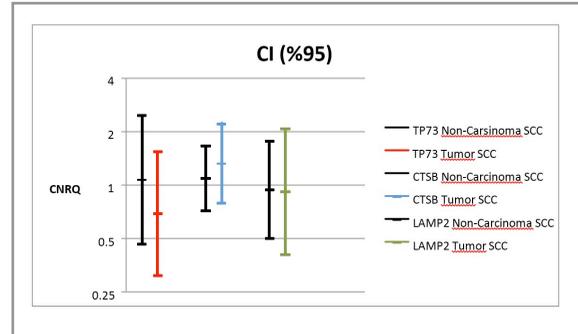
A statistically significant difference was not found between the stages in terms of the genes examined at a significance level of 95% ($p < 0.05$). Although a statistically sufficient number of individuals were present only in stage 2 and stage 3, a sufficient number of data scores could not be obtained for both groups in this test.

A statistically significant difference at a significance level of 95% ($p < 0.05$) could not be found between the histological phenotypes in terms of the genes examined.

Although a statistically sufficient number of individuals were present only in the SCC phenotype, the numbers of individuals possessing the other histological phenotypes were not sufficient for a statistical analysis and a sufficient number of data scores could not be obtained.

As a result of the comparison of the gene expression data of the patients who had a histological phenotype of SCC (42 individuals) with the gene expression data obtained from the normal tissues (43 individuals), a sufficient data score could be obtained for 3 genes for statistical comparison. The Table 2 shows the comparison between the normal tissues and the tumor tissues with the histological type of SCC for these 3 genes.

When comparison was made for the P73 gene expression, the lowest expression ratio was found to be 0.61 and the highest expression ratio was found to be 2.451 in the normal tissue, while the lowest expression ratio was found to be 0.307 and

**Figure 2.** Gene expression of SOC subtype

the highest expression ratio was found to be 1.538 in the tumor tissue with SCC histology. It was observed that the expression of TP73 was decreased 1.545-fold, when the normal tissue and the tumor tissue with SCC histology were compared.

While LAMP2 was expressed with a ratio ranging between 0.495 and 1.761 in the normal tissue, it was expressed with a ratio ranging between 0.404 and 2.069 in the tumor tissue with SCC histology. It was observed that LAMP2 expression was decreased by 1.020-fold in the tumor tissue with SCC histology.

When CTSB expression was examined, it was found that the lowest expression ratio was 0.709 and the highest expression ratio was 1.663 in the normal tissue and the lowest expression ratio was 0.791 and the highest expression ratio was 2.212 in the tumor tissue with SCC histology. When these ratios were compared, it was observed that CTSB expression was increased by 1.217-fold in the tumor tissue.

A statistically significant difference at a significance level of 95% ($p < 0.05$) was not found in comparison of the expression ratios between the normal tissue and the tumor tissue with SCC histology for the genes TP73, CTSB and LAMP2. The graphic version of this table is Figure 2.

DISCUSSION

Genetic research suggest that autophagia mostly operates as a tumor suppressor mechanism, but these mechanisms have been elucidated very little so far.²² White et al. have two hypotheses in relation with how the loss of autophagia stimulates on-

cogenesis. The first one is as follows: tumor cells that cannot be eliminated by apoptosis under metabolic stress activate autophagia in order to be protected from necrosis, local inflammation occurs in this case and the tumor growth rate is increased.²³ The second one is as follows: genomic instability occurs in the cells that are under metabolic stress as a result of ATG5 deletion and this leads to oncogene activation and tumor progression.²²

MAPK3 is a protein kinase like PAK-1 and AR. In a study conducted by Fu et al. in patients with breast cancer, it was shown that these 3 protein kinases operated as a caspase/ATG key in autophagia and apoptosis. In this study, 3 miRNAs were defined for MAPK3 (miR-132, miR-212 and miR-613) and some drug molecules (lansaprazol, flubendazole, Miconazole, bicalutamide, carvedilol, baclofen and rizatriptan), which target these, were also defined. In the conclusion part of the study, the investigators stated that MAPK3 had a key role in programmed cell death (autophagia and apoptosis), drugs which affected this enzyme could be efficient in cancer treatment, and new and more effective drugs were needed.²⁴ In our study, an increase in MAPK3 expression was found in carcinoma tissues, though it was statistically insignificant.

In our study, increased SQSTM1 expression was observed in cancer cells compared to normal cells. Sequestosome-1 (SQSTM1/p62) is a multifunctional protein, which is efficient in signal transduction, protein degradation and cell transformation. It has been observed that SQSTM1 is downregulated in cancer cells in case of hypoxia and is rapidly re-expressed in case of re-oxygenation. Downregulation of SQSTM1 is required for ERK-1/2 phosphorylation. In the state of normoxia, SQSTM1 is activated and in case of hypoxia, increased expression of SQSTM1 blocks activation of ERK-1/2.

In conclusion, hypoxic activation of autophagia induces clearance of SQSTM1. This shows the importance of SQSTM1 in the regulation of cancer cell survival under hypoxic conditions.²⁵

LAMP1 and LAMP2 are mainly found in the lysosomal membrane and rarely on the normal cell surface. Literature data show that LAMP1 and 2 are continuously expressed on the surface of some colon cancer cells and this is related with metas-

tasis. In this study, it was stated that E-selectin found on the surface of cancer cells were related with upregulation of LAMP1 and LAMP2 and this was thought to lead to metastasis.²⁶ In our study, LAMP2 expression was found to be decreased in the tumor tissue.

Cathepsin B (CTSB) is a cysteine protease involved in autophagia and mainly found in lysosomes and lysosome like organelles. It acts to process and destroy lysosomal proteins. The role of CTSB in the pathogenesis of breast cancer has been demonstrated. In the study conducted by Capparelli et al., it was shown that tumor growth increased approximately 2-fold in the fibroblasts containing CTSB. Conclusively, the investigators stated that autophagia was effective in tumor growth independent of angiogenesis.²⁷ It was observed that CTSB expression was increased in tumor tissues compared to normal tissues in our cases in accordance with the literature.

TP73 is a member of TP53 tumor suppressor gene family. TP73 has a significant role in the protection of the genome. In addition, it has a key role in cellular response in cisplatin treatment. Resistance to cisplatin treatment has been observed in cases of SCC in which the expression of TP73 is decreased.²⁸ In our study, it was observed that TP73 expression was decreased in tumor tissues, though statistically insignificantly, similar to the literature. This may be related with resistance encountered in cisplatin treatment used in the treatment of NSCLC. However, a comparison between treatment resistance and TP73 expression could not be made because of the insufficient number of data in our study.

In conclusion, autophagia is thought to be an efficient factor in both the promotion and prevention of cancer. The role of autophagia in cancer probably varies by tumor progression. Inhibition of autophagia may lead to continuous growth in precancerous cells and autophagia acts as a tumor suppressor in this case.^{10,29}

When the tumor is grown sufficiently later, cancer cells need autophagia to survive under conditions poor in nutrition and oxygen. This may especially be prominent in the less vascularized internal parts of the tumor.³⁰

It is clear that autophagia has a very important role in the development, progression, and treatment of cancer, like apoptosis. It is very important to continue to investigate this important pathway in further studies.

We think that the problem experienced in our study was the inability to access sufficient data in terms of gene expression. These problems may be overcome with use of fresh tissue and larger tissue samples in future studies.

In addition, studies should compare autophagia data obtained from different regions of the same tumor tissue and even from the primary tumor and tumor metastasis assuming that autophagia operates differently in different parts of the same tumor.

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