Eskisehir Medical Journal
Eskişehir Tıp Dergisi

Eskisehir Med J. 2025; 6(1): 61-66
doi: 10.48176/esmj.2025.182

Original Article / Araştırma Makalesi

A LOOK TO THE RELATIONSHIP BETWEEN AUTOPHAGY AND EMBRYONAL CARCINOMA FROM A DIFFERENT PERSPECTIVE

OTOFAJİ VE EMBRİYONEL KARSİNOM ARASINDAKİ İLİŞKİYE FARKLI BİR PERSPEKTİFTEN BAKIŞ

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ABSTRACT

Introduction: Testicular embryonal carcinoma is a type of testicular cancer that affects the germ cells which are the precursors of sperm cells that will eventually develop into sperm. These carcinoma cells are often aggressive. In such cells, autophagy may promote the survival and proliferation of cancer cells, as autophagy can help cells survive under stress. Autophagy may also allow cancer cells to develop resistance to their environment and to metastasize. ATG4 (Autophagy related 4) is a protein that plays a role in the initiation of autophagy. JAB1 (Jun activation domain binding protein 1) is a protein involved in cellular signaling and regulates many biological processes such as cell cycle, apoptosis, and gene expression. JAB1 is also involved in the regulation of the p27 protein, and its high expression can be observed in cancer cells. NEDL2 (Neuroepithelial differentiation marker like 2) is involved in cellular growth and developmental processes. It is also a protein that is thought to play a role in cancer progression. The aim of the study was to demonstrate expression and localization of ATG4, JAB1 and NEDL2 in testicular embryonal carcinoma cells.

Methods: The morphological examination of testis embryonal carcinoma cells were performed by using hematoxylin eosin (H-E) staining. Then, using the immunohistochemical technique, cellular expression and location of ATG4, JAB1 and NEDL2 in testis embryonal carcinoma cells were examined.

Results: According to immunohistochemistry results, ATG4, JAB1 and NEDL2 expression was detected in human testicular embryonal carcinoma cells.

Conclusions: By determining the expression levels of these three proteins, more information can be obtained about how important processes such as autophagy, cell cycle regulation and cellular development are affected in testicular embryonal carcinoma. This information could be an important step in understanding the biology of cancer and developing treatment strategies.

Keywords: Testis embryonal carcinoma, autophagy, ATG4, JAB1, NEDL2.

ÖZET

Giriş: Testis embriyonal karsinom, sperme dönüşecek olan sperm hücrelerinin öncüleri olan germ hücrelerini etkileyen bir testis kanseri türüdür. Bu karsinom hücreleri genellikle agresiftir. Bu hücrelerde otofaji, hücrelerin stres altında hayatta kalmasına yardımcı olabileceğinden, kanser hücrelerinin hayatta kalmasını ve çoğalmasını destekleyebilir. Otofaji ayrıca kanser hücrelerinin çevrelerine karşı direnç geliştirmesine ve metastaz yapmasına izin verebilir. ATG4 (Autophagy related 4), otofajinin başlatılmasında rol oynayan bir proteindir. JAB1 (Jun activation domain binding protein 1), hücresel sinyalizasyon yer alan ve hücre döngüsü, apoptoz ve gen ifadesi gibi birçok biyolojik süreci düzenleyen bir proteindir. JAB1 ayrıca p27 proteininin düzenlenmesinde de yer alır ve yüksek ifadesi kanser hücrelerinde görülebilir. NEDL2 (Neuroepithelial differentiation marker like 2), hücresel büyüme ve gelişim süreçlerinde yer alır. Ayrıca kanser ilerlemesinde rol oynadığı düşünülen bir proteindir. Çalışmanın amacı, testis embriyonal karsinom hücrelerinde ATG4, JAB1 ve NEDL2'nin ekspresyonunu ve lokalizasyonunu göstermektir.

Yöntemler: Testis embriyonal karsinom hücrelerinin morfolojik incelemesi hematoksilen eozin (H-E) boyama kullanılarak yapıldı. Daha sonra, immünohistokimyasal teknik kullanılarak, testis embriyonal karsinom hücrelerinde ATG4, JAB1 ve NEDL2'nin hücresel ekspresyonu ve lokalizasyonu incelendi.

Bulgular: İmmünohistokimya sonuçlarına göre, insan testis embriyonal karsinom hücrelerinde ATG4, JAB1 ve NEDL2 ekspresyonu tespit edildi.

Sonuç: Bu üç proteinin ekspresyon seviyelerinin belirlenmesiyle, otofaji, hücre döngüsü regülasyonu ve hücresel gelişim gibi önemli süreçlerin testis embriyonal karsinomunda nasıl etkilendiği hakkında daha fazla bilgi elde edilebilir. Bu bilgi, kanser biyolojisinin anlaşılması ve tedavi stratejilerinin geliştirilmesinde önemli bir adım olabilir.

Anahtar Kelimeler: Testis embriyonal karsinomu, otofaji, ATG4, JAB1, NEDL2.

INTRODUCTION

Human testicular embryonal carcinoma is a rare type of cancer found in the testicles and usually occurs in young men. Embryonic carcinoma is a type of germ cell tumor and is one of the most aggressive and fast-growing types of testicular cancer (1). Embryonal carcinoma cells can divide and grow rapidly, similar to cells seen during embryonic development. These cells have characteristics that accelerate the proliferation of the cancer (2).

ATG4 plays important roles in the initiation of autophagy. This protein is involved in a series of modifications required for the functionalization of autophagic vesicles. In particular, ATG4 is involved in the activation of autophagy-related

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Submission Date: 18.05.2024 **Acception Date:** 25.07.2024
Cite as: Kipel S, Nakkas H. A Look To The Relationship Between Autophagy And Embryonal Carcinoma From A Different Perspective. Eskisehir Med J. 2025; 6(1): 61-66. doi: 10.48176/ esmj.2025.182

proteins such as LC3 (Microtubule-associated protein 1A/1B-light chain 3) and contributes to the progression of the autophagy process (3). In addition, it has been suggested that the expression levels of ATG4 in cancer cells, and especially in testicular embryonal carcinoma cells, may affect the sensitivity of cells to autophagy, thus determining tumor growth and response to treatment. The role of ATG4 in testicular embryonal carcinoma cells may be important in understanding the effects of autophagy on tumor progression and resistance to treatment. Some studies have suggested that inhibiting ATG4 may support cancer treatment because autophagy may help cells survive and develop resistance to cancer treatments (4,5). Therefore, ATG4 may be a potential target for cancer treatment. In testicular embryonal carcinoma cells, ATG4 may play an important role in the modulation of autophagy processes and this protein may be a molecule that should be evaluated as a target for cancer treatment strategies.

JAB1 (Jun activating binding protein 1), also known as CSN5 (COP9 Signalosome Subunit 5), is a protein that plays important roles in various cellular processes. JAB1 is part of a protein complex known as the COP9 (Constitutive Photomorphogenic 9) signaling complex (CSN) and has multifaceted effects on cellular functions (6). Jun family proteins are important proteins that regulate processes such as cellular growth and differentiation (7). JAB1 also plays a role in protein degradation by the proteasome. As part of the COP9 signaling complex, it helps the function of the proteasome to degrade and properly control cellular proteins. JAB1 interacts with some proteins that control the cell cycle. In particular, it can accelerate the cell cycle by reducing the stability of the cell cycle regulator called p27. This feature may be related to cancer because the irregular progression of the cell cycle can lead to cancer development (8). The relationship between JAB1 and cancer has been widely studied. In many types of cancer, overexpression of JAB1 and uncontrolled reduction of cell cycle regulators such as p27 can lead to excessive proliferation of cancer cells and tumor formation (9,10). JAB1 also has an effect on the migration and metastasis potential of cells in some types of cancer. Therefore, JAB1 is thought to be directly related to cancer progression (11). JAB1 can also affect cell differentiation. This may be especially important in processes such as embryonal development or tissue regeneration. JAB1 is a versatile protein that plays a role in a number of important biological processes, such as cellular growth, differentiation, apoptosis, cell cycle regulation, and protein degradation. Due to its association with cancer, a better understanding of JAB1's functions is of great importance for cancer treatment and modulation of cellular processes.

NEDL2 (Neuroepithelial differentiation marker-like 2) is a protein discovered in humans and plays a role in neural development and cellular processes. This protein may play an important role in various biological processes associated with cellular signaling, differentiation, and some diseases (12). NEDL2 can function as an E3 ubiquitin ligase. Ubiquitin ligases are enzymes that facilitate the recognition and degradation of cellular proteins by proteasomes via marking them with ubiquitin (13). This is important in maintaining cellular control and homeostasis. NEDL2 regulates the degradation of proteins through ubiquitination. This plays a critical role in processes such as the cell cycle, gene expression, and cellular signaling. Ubiquitination ensures that proteins are degraded at the right time at the end of their life cycle. NEDL2 function as an E3 ubiquitin ligase may be critical in ensuring proper transitions in the cell cycle. However, some studies suggest that NEDL2 regulates the cell cycle while ensuring that certain proteins are destroyed. The relationship between E3 ubiquitin ligases and cancer is quite common, because they directly affect the growth and survival processes of cells (14). NEDL2 may be involved in processes such as neural tube development and neural cell differentiation. This role of NEDL2 may be important in embryonal development and some aspects of nervous system diseases (15). Abnormal expression of NEDL2 in some types of cancer may cause uncontrolled cell growth.

Demonstrating the expression of ATG4, JAB1, and NEDL2 proteins in testicular embryonal carcinoma cells is an important step towards a deeper understanding of how these proteins play a role in cancer cells and contribute to the molecular biology of cancer.

MATERIALS AND METHODS

In this study, human testicular embryonal carcinoma cell line CRL-2073 was used (NCCIT/ATCC). This cell line consists of adherent germ cell tumors isolated from a male patient with pluripotent embryonal carcinoma in adult testicular tissue. The pluripotent cell line has the ability for somatic and extraembryonic differentiation. The undifferentiated cells were equivalent to the intermediate stage between seminoma and embryonal carcinoma.

Cell Culture:

The CRL-2073 (NCCIT / ATCC) cell lines were obtained. Embryonal carcinoma cells were cultured in RPMI-1640 (with L-glutamine, HEPES, Gibco Capricorn, 21875-034) supplemented with 1% antibiotics (penicillin/streptomycin, Gibco, 15140-122) and 5% fetal bovine serum (FBS, Capricorn, 10270-106) under 5% CO2 in a 37 °C humidified incubator.

Histologic Evaluation:

Hematoxylin and eosin staining was performed for morphological analyses of CRL-2073 cells. Cells were suspended in 2 ml of cell medium on round coverslips placed in 6-well cell plates. The media was removed from the cells adhering to the coverslips and washed with Phosphate Buffered Salt Solution (PBS). 4% Paraformaldehyde (PFA) was added to the cells and waited for 10 minutes to fix the cells. After fixation, the cells were washed with PBS. Then, the round coverslips were placed on the slide. The cells were kept in 100%, 96% and 75% alcohol series for 2 minutes, respectively, and washed with distilled water. It was kept in Hematoxylin (Sigma-Aldrich, 105174) for 5 minutes. After washing with distilled water, it was kept in Eosin (Sigma Aldrich, 109844) for 1 minute. It was quickly passed through 75%, 96% and 100% alcohol series. It was kept in Xylene for 30 minutes and covered with entellan. Imagery was performed with a light microscope (Nicon, Eclipse E100).

Immunocytochemistry:

It is a microscopic method based on examining the localization and expression of proteins within the cell. We also examined the localization and expression of ATG4, JAB1 and NEDL2 proteins with this technique. For immunocytochemistry staining, cells were cultured on round coverslips (12x12mm, Nest) placed in 6 well cell plates.

When the cells reached a certain density, the medium of the cells was withdrawn and the cells were washed with PBS. Then, the cells were fixed with 4% paraformaldehyde (PFA) for 15 minutes. The coverslips in the wells were placed on the slide and then were drawn with Pap pen. They were incubated with blocking solution (Abcam) for 1 hour to prevent nonspecific binding. After incubation, they were washed again with PBS. Primary antibodies ATG4 (Abcam, ab108322), JAB1 (Abcam, ab12323) and NEDL-2 (Abcam, ab236784) prepared at a certain dilution (1:100) were added to cover the coverslips and incubated overnight at +4°C. The next day, the primary antibodies were withdrawn and washed with PBS. Then, they were incubated with Biotin-containing secondary antibody for 1 hour. After washing with PBS, they were incubated with Streptavidin solution for 30 minutes. In order for the reaction to become visible, AEC solution (Patholab, PL-125-HA) was prepared and added to them and the incubation time was adjusted by checking under a microscope. After washing, they were incubated with Mayer's Hematoxylin for 30 seconds to stain the nucleus and covered with mounting medium. Evaluations were made with a light microscope (Nicon, Eclipse E100).

RESULTS

Histomorphological result:

The morphological appearance of human testicular embryonal carcinoma cells under the light microscope typically has certain characteristics that can help in the identification of this tumor. Embryonic carcinoma is included in the category of germ cell tumors and usually shows fast-growing, aggressive and heterogeneous cellular structures. Embryonic carcinoma cells were usually large, round or oval in shape. Nuclei were usually dark, large and irregularly shaped. Hyperchromatic nuclei had a denser chromatin structure and can be clearly seen under the light microscope. The nuclei of embryonal carcinoma cells were usually irregular. The nuclei were large and can be of various shapes; some are round, while others were more oval or elliptical. Testicular embryonal carcinoma cells tend to be invasive and metastatic potential of the tumor. These cells were often found in large, heterogeneous clusters. The clusters can be regular or irregular in shape (Figure 1).

Immunocytohemistry results:

ATG4, JAB1 and NEDL2 protein expression has not been examined in human testicular embryonal carcinoma cell lines before. In this study, firstly, in order to understand whether there is protein expression, the presence of ATG4 (Figure 2), JAB1 (Figure 3) and NEDL2 (Figure 4) was demonstrated in CRL-2073 cells by immunocytochemistry method. For ATG4; high expression is observed in testicular embryonal carcinoma cells (Figure 2). With JAB1 immunostaining high expression is observed (Figure 3). At NEDL2 stained preparations high expression is observed (Figure 4).

DISCUSSION

Testicular embryonal carcinoma is a malignant type of cancer that originates from germ cells in the testis. Embryonal carcinoma contains cancer cells that can grow and metastasize rapidly. (16). This type of cancer can use autophagy as a survival mechanism. Autophagy provides proteins that support cancer cell proliferation, while also clearing toxic substances and damaged organelles within

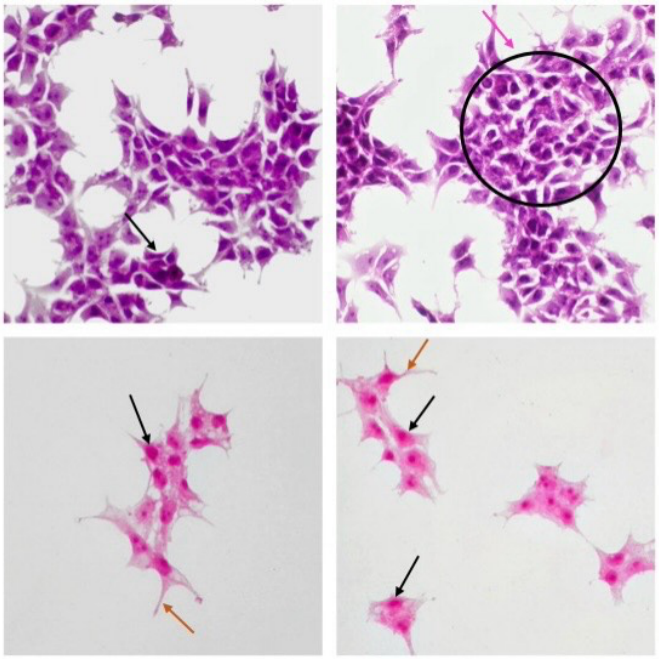

Figure 1. Light microscopic image of human testicular embryonal carcinoma cells (CRL-2073) (Stain: Hematoxylin Eosin, Magnification: 400X). Black arrows show the nuclei of cells. Orange arrows indicate the extension of the cells. Pink arrow and circle show the clusters of cells.

the cell, allowing cancer cells to survive (17). This process can support the rapid division and growth of cells in rapidly growing cancers such as testicular embryonal carcinoma. Autophagy can enable cancer cells to develop resistance to chemotherapy and radiotherapy. When cancer treatments damage cells, cells can repair this damage and survive thanks to autophagy. Testicular embryonal carcinoma cells can also develop resistance to treatment, and autophagy may form the basis of these resistance mechanisms.

Demonstrating the expression of ATG4, JAB1 and NEDL2 proteins is an important step towards understanding the biological and molecular basis of testicular embryonal carcinoma. All three proteins are involved in cellular process, especially cell cycle, cell survival, apoptosis and protein degradation. ATG4 is a protease that plays an important role in the autophagy process. Autophagy is a process that helps cells survive under stress and destroys damaged or unnecessary organelles and proteins within the cell. ATG4 enables the conversion of LC3 and ATG8 proteins into their active forms during autophagy. Since testicular embryonal carcinoma cells usually exhibit high proliferation and aggressive growth characteristics. ATG4 expression may help these cells survive and cope better under stress. In this case, ATG4 expression is expected to be high because autophagy contributes to the survival of cancer cells and balances metabolic imbalances. In addition, it has been shown that ATG4 may be associated with resistance to chemotherapy and cell death.

JAB1 is an E3 ubiquitin ligase involved in the regulation of the cell cycle, control of transcriptional activity, and differentiation of cells. It leads to cell cycle progression and acceleration of cell proliferation by increasing the destruction of cell cycle regulators such as p27. JAB1 can be highly expressed in testicular embryonal carcinoma cells as a factor that accelerates the cell cycle and increases the proliferation of cancer cells. High JAB1 expression may

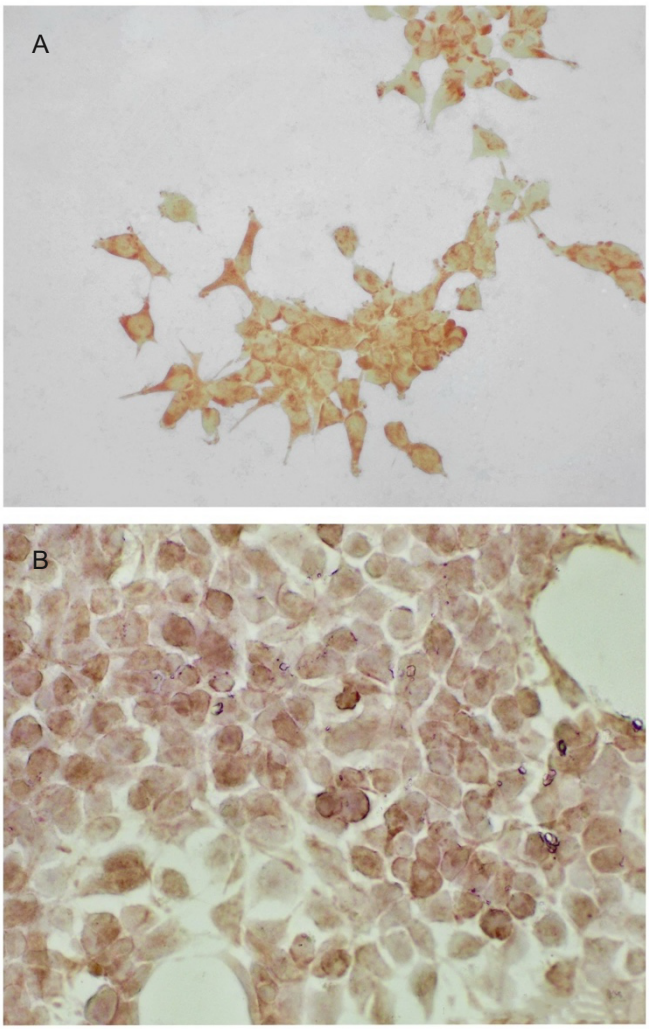

Figure 2. Immunocytochemical demonstration of ATG4 protein in human embryonal carcinoma cells (Magnification A; 200X B; 400X and 1:100 dilution). A higher expression was observed in the cytoplasm.

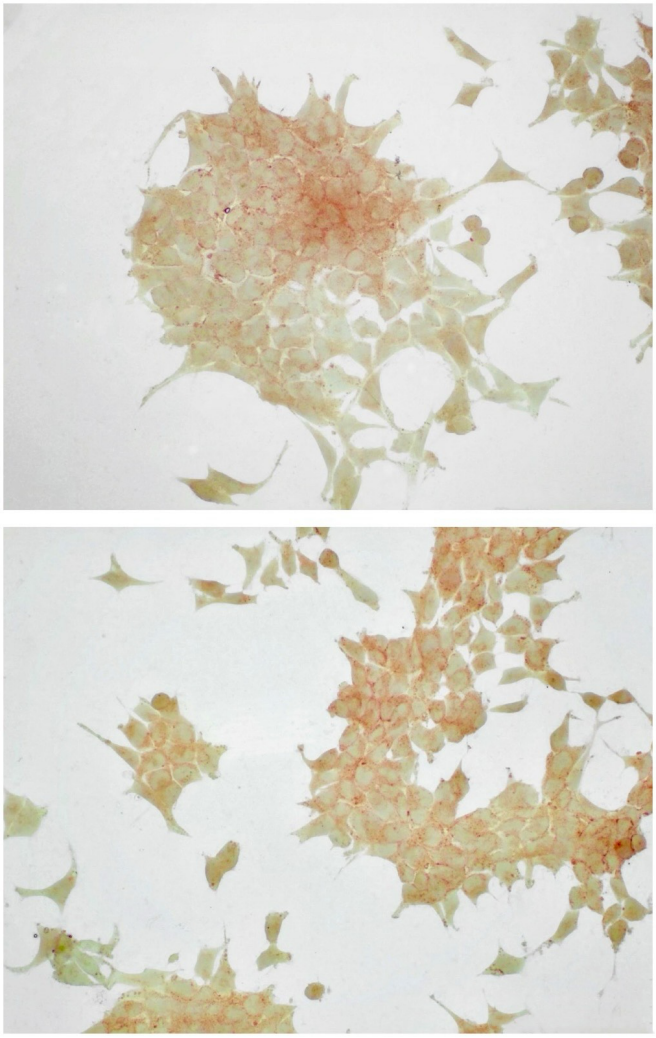

Figure 3. Immunocytochemical demonstration of JAB1 protein in human embryonal carcinoma cells (Magnification: 400X and 1:100 dilution). A higher expression was observed in the cytoplasm.

support the growth and metastasis potential of cancer cells, because JAB1 has an important role in regulating the cell cycle. JAB1's promotion of p27 degradation accelerates the G1/S transition of the cell cycle, which allows cancer cells to divide rapidly. In our previous study on human testicular cancers (18), the highest JAB1 protein was seen in the embryonal carcinoma group among testicular cancers. In this study, the high JAB1 expression in CRL-2073 cells is consistent with the literature. Consequently, high JAB1 expression is expected, because this contributes to faster division of testicular embryonal carcinoma cells and tumor growth.

NEDL2 acts as an E3 ubiquitin ligase and is associated with the cell cycle. It also plays a role in the modulation of some signaling pathways. NEDL2 is important for the correct timing of cell differentiation and survival. NEDL2 is likely to be highly expressed in testicular embryonal carcinoma cells because this protein may play a role in promoting cell proliferation. In cancer cells, overexpression of E3 ligases generally regulates factors that control the cell cycle and prevent cell death. This may contribute to tumor growth.

Abnormal differentiation of cancer cells can lead to the cell becoming malignant and acquiring metastatic properties. So, the expression of these three proteins can cause cancer cells to survive, grow rapidly, and develop resistance to treatment. Even it is so important to understand the relation between these proteins and cancer, until now ATG4, JAB1 and NEDL2 protein expression has not been examined in human testicular embryonal carcinoma specifically.

Although it is not possible to perform control staining on the cancer cell line, the data found with the relevant antibodies in previous studies on the testis are as follows. In our previous study (18), JAB1 antibody expression in human testicular tissue was generally detected in the cytoplasm of testicular cells. In the embryonal carcinoma group, JAB1 expression was seen in the cell nucleus and cytoplasm. According to HScore analysis, the number of JAB1 immunopositive cells increased significantly in the embryonal carcinoma group. In the literature (19), the expression of different isoforms of NEDL2 was shown in pig oocytes, embryos, spermatozoa and somatic cells. Expression of NEDL2 protein was shown in both the nucleus and cytoplasm. According to this study, NEDL2 has an important role in oocyte fertilization, especially in the decondensation of sperm DNA and in the early stages of pronuclear development. According to the information in The Human Protein Atlas (20), ATG4 expression is seen in the testis, especially in the seminiferous tubules and Leydig cells.

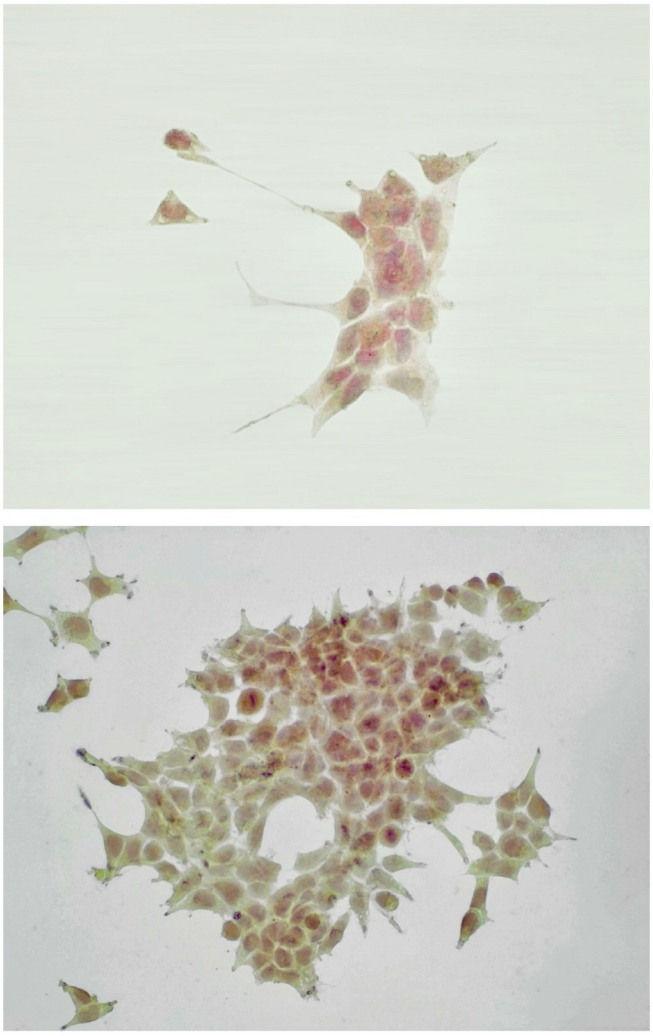

Figure 4. Immunocytochemical demonstration of NEDL2 protein in human embryonal carcinoma cells (Magnification: 400X and 1:100 dilution). A higher expression was observed in the nucleus.

Targeting these proteins can potentially help develop cancer treatment strategies. Autophagy may be an important potential target in the treatment of testicular embryonal carcinoma. Inhibiting autophagy may make it harder for cancer cells to survive and reduce resistance to treatment. In addition, activation of autophagy may lead to more aggressive cell growth. Various strategies are being investigated to inhibit autophagy in cancer treatment. Autophagy inhibitors may be an effective adjunctive therapy in cancer treatment. Such treatment may allow more effective destruction of cancer cells when combined with chemotherapy or radiotherapy. For example, inhibition of ATG4 can make it difficult for cancer cells to survive by inhibiting autophagy. In particular, inhibiting ATG4 and other autophagy-related proteins may be an important strategy to reduce resistance to treatment and more effectively destroy cancer cells. This research could open new ways to treat cancers such as testicular embryonal carcinoma. Inhibition of JAB1 can prevent tumor growth by slowing down the cell cycle. Inhibition of NEDL2 can reduce the aggressiveness of tumors by making cancer cells more differentiated. In this study, we aimed to show the expression of these three proteins firstly because of their possible roles in autophagy, cell cycle and differentiation mechanisms. In further studies, it can be examined how these mechanisms can be affected by inhibiting these proteins.

This study, which shows the expression of ATG4, JAB1 and NEDL 2 in testicular embryonal carcinoma cells for the first time, is a descriptive study. In further studies, numerical data can be obtained that compared by using inhibitors of these proteins or gene silencing. In this context, the effects of the expression of ATG4, JAB1, and NEDL2 on cancer biology can be investigated in more depth, and targeting these proteins can be an important strategy for developing cancer treatment. The relationship between testicular embryonal carcinoma and autophagy constitutes an important area in understanding the biological functioning of cancer. The relationship of autophagy with cancer survival and resistance mechanisms will shed light on future research on how these processes can be used in cancer treatment.

CONCLUSION

In summary, the expression of these proteins may contribute to the aggressive nature of testicular embryonal carcinoma and the rapid proliferation of cells. And the observation of these proteins in testicular embryonal carcinoma offers important opportunities for understanding the molecular mechanism of cancer, early diagnosis, generating therapeutic targets and biomarker discovery.

Ethics Committee Approval: Ethics committee approval was not required for this study because of there was no study on animals or humans. Commercial cell lines were used in this study and therefore ethical approval was not required.

Informed Consent: Informed consent was not required for this study because of there was no study on animals or humans.

Conflict of Interest: No conflict of interest was declared by the authors.

Authorship Contributions: Idea/Concept:ŞK, Design:ŞK, Supervision:ŞK, HN, Data Collection and Processing:ŞK, Analysis or Interpretation:ŞK, HN, Literature Search:ŞK, Writing:ŞK, HN, Critical Review:ŞK, HN, References and Fundings: -, Materials: -.

Financial Disclosure: The authors declare that they have no relevant financial.

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