DOI: 10.37990/medr.1210613 Med Records 2023;5(1):164-9

MEDICAL RECORDS-International Medical Journal

Research Article

Characterization of Apelin/APJ Axis Expression in Normal Testicular Tissue, Germ Cell Neoplasia in Situ, and Testicular Seminoma

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Abstract

Aim: A testicular germ cell tumour is not observed widely, but its incidence and mortality rates have increased in recent years. One of the most common forms of this tumour is seminoma. Germ cell neoplasia *in situ* (GCNIS) is the precursor of seminoma. The apelin/APJ axis is increased in many cancers and is a pathway that plays an active role in angiogenesis, lymphangiogenesis, tumour growth, and migration. This study investigated the cellular distributions of apelin and APJ protein expressions in normal testicular tissue (TT), GCNIS, and seminoma.

Material and Methods: Tissues from 18 patients who had undergone orchiectomy were used in this study. These tissues include areas of normal TT, GCNIS, and seminoma. Immunolocalisation of apelin and APJ were identified through the immunohistochemical method.

Results: Apelin expression was significantly increased in seminoma and GCNIS compared to normal. Apelin expression were the same in GCNIS and seminoma. APJ expression was significantly increased in seminoma compared to normal and GCNIS. Normal and GCNIS APJ expressions were similar.

Conclusion: Expressions of apelin and APJ proteins were significantly increased in seminoma in our study. Our findings were consistent with the results of relevant studies as increased expression of apelin/APJ has been observed in many different cancers. It can be predicted that the increase of this pathway in seminoma may support angiogenesis, lymphangiogenesis, migration, and metastasis. Therefore, the increase in mortality rates in seminoma patients may be related to apelin/APJ axis. Ultimately, the use of inhibitors of this pathway in these patients may reduce their mortality rate. New studies are needed before these inhibitors can be used clinically.

Keywords: Apelin, APJ, GCNIS, seminoma, testis

INTRODUCTION

Testicular germ cell tumour (TGCT) is a rare tumour seen mostly in developed countries (1). TGCT is common in young men, and 60% of these patients are seminoma. Germ cell neoplasia *in situ* (GCNIS) is the noninvasive precursor of type II TGCT. The pathogenesis of TGCT is an arrest that occurs during the maturation of primordial germ cells (PGCs) or gonocytes in the embryonic period. The reason for the PGCs/gonocytes' arrest remains unclear (2). At puberty, GCNIS transforms into a seminoma with the same gene expression and histology as PGC (2,3) or is remodelled into embryonal carcinoma (EC) (2). In the United States, it is expected that there will be 9910 new testicular cancer diagnoses and 460 deaths from testicular cancer in 2022 (4). Although TGCT is not commonly observed, the incidence rate has increased by 70% in the last 20 years (5). Despite the increase in the incidence of the disease, the mortality rate of the disease has decreased considerably with the new generation of surgical methods accompanied by radiotherapy and chemotherapy (6). Almost all TGCT patients in stage 1 can be treated. However, 30% of TGCT patients are diagnosed in the metastatic stage, which reduces the cure rates of the disease. The 5-year survival rates in patients with metastatic seminoma range from 72% to 86% (7). However, these patients may resist chemotherapy or relapse in the future (8). This situation causes the treatment to fail. One study found that 16.8%

CITATION
Soylu H, Unal B, Aksu K, et al. Characterization of Apelin/APJ Axis Expression in Normal Testicular Tissue, Germ Cell Neoplasia in Situ, and Testicular Seminoma Med Records. 2023;5(1):164-9. DOI: 10.37990/medr. 1210613

Received: 27.11.2022 **Accepted:** 13.12.2022 **Published:** 14.01.2023
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of the more than 1500 unselected TGCT patients had a recurrence (9). Due to the increase in incidence day by day, its resistance to chemotherapy, and the possibility of recurrence of TGCT, the development of new specific therapeutic agents is needed to increase the success of the treatment of this disease. In order to develop these therapeutics, molecular pathways in seminoma cells need to be elucidated in more detail.

Apelin is the ligand for APJ. This endogenous ligand is encoded by the APELIN gene (10). APJ is a G protein-coupled receptor with 7 transmembrane domains, first identified in 1993 (11). Apelin and APJ proteins are very common in the body. Apelin and APJ play a protective role in cells by regulating blood pressure, food intake, endoplasmic reticulum stress, and cardiac metabolism in the body (12,13). The apelin/APJ axis is expressed in many tissues, including testis (14,15). Apelin and APJ play an important role in angiogenesis and vessel morphogenesis (16). In addition, they are expressed in many cancer tissues. Apelin is an important factor for angiogenesis in muscle-invasive bladder cancer tissues (17). The expression of apelin/APJ is significantly increased in brain tumour microvascular proliferation (18). On the APELIN gene in endothelial cells, there is a binding site for HIF-1α, which is released due to hypoxia, and HIF-1α binding to this region enables the expression of apelin (19). Hypoxia is also clearly observed in solid tumours. Hypoxia in solid tumours increases invasion and metastasis and may cause angiogenesis by activating proangiogenic factors (20,21). Apelin stimulates lymph node metastasis and lymphangiogenesis by activating ERK and PI3K pathways (10). Negative correlations were found between high apelin expression and survival in many different cancers and adenocarcinomas (16,17,22-32). The apelin/APJ axis expression is higher in colorectal cancer patients' tissues (30). Significant correlations have also been found between APJ expression level and the aggressiveness of renal cell carcinoma (33). These cancer studies performed in different organs and tissues showed that the apelin/APJ axis can be used as a marker to predict the patient's prognosis.

The cellular expression distributions of apelin and APJ in TGCT and GCNIS have not been investigated before when the literature was searched. It is essential to elucidate the molecular mechanisms of the apelin/APJ pathway in cancer cells to treat TGCT patients, whose incidence has increased in recent years. In this study, we investigated the expression pattern of apelin and APJ in tissues obtained from patients who undergoned orchiectomy.

MATERIAL AND METHOD

Clinical assessment of patients with prostate cancer

Tissues of patients who had undergone orchiectomy were used in this study. Paraffin-embedded tissues of 18 patients diagnosed with seminoma in postoperative pathology were used. These samples contain areas of normal TT, GCNIS, and seminoma tissue. The study was performed in accordance with the Declaration of Helsinki. Ethical approval was obtained from Akdeniz University Clinical Research Ethics Committee (KAEK-511).

Immunohistochemistry

Paraffin blocks were cut into 5 µm thick sections. After deparaffinisation with xylene, rehydration was performed in decreasing-grade alcohols (100% -90% -80% -70% -dH_2O). Antigen retrieval was made by boiling with pH:6.0 citrate buffer and allowed to cool at room temperature. Afterwards, the sections were washed with phosphate-buffered saline (PBS). Endogenous peroxidase activity was inhibited with 3% H_2O_2. Sections were washed with PBS and blocked for 7 minutes at room temperature with UV block solution (TA-125UB; Lab Vision). Then, apelin rabbit polyclonal primary antibodies (1:200 dilution, Novus Biologicals LLC; NBP10730) and APJ rabbit polyclonal primary antibodies (1:400 dilution, Bioss; bs2430R) were incubated overnight at +4°C. The next day, the sections were washed with PBS and incubated with biotin-conjugated anti-rabbit secondary antibodies (1:500 dilution, Vector Lab.; BA-1000) for 40 minutes in a humidified chamber at room temperature. Sections were washed with PBS and incubated with HRP-conjugated streptavidin (TA-125HR; Lab Vision) for 20 min. Signals were developed with diaminobenzidine (DAB) (D4293; Sigma-Aldrich) by washing with PBS and counterstaining with hematoxylin (Merck, MHS32). The sections were dehydrated, taken into xylene, and then covered with entellan (Merck, MHS32).

ImageJ Analysis

Ten photographs were taken from each immunohistochemically stained section with an Olympus CX31 microscope. The staining intensities of the photographs were evaluated with the ImageJ (http://imagej.nih.gov/ij/) program. The immunostaining intensity in each region was calculated as a percentage by proportioning the immunostained area in each photograph to the total area and multiplying it by 100. The same processes were applied in our previous study (34).

Semi-Quantitative Evaluations

Cells were immunostained with apelin and APJ, and the staining intensity was measured semi-quantitatively. The scoring was performed in the following manner: 0 = negative; (+) = weak positive; + = positive; ++ = dense positive; +++= very dense positive.

Statistical Analysis

All data were analysed with GraphPad Prism 9.0 (GraphPad Software) for statistical significance and are reported as mean±s.e.m. A one-way ANOVA and Tukey method were used for multiple comparisons to evaluate differences. Differences with $p<0.05$, compared to the normal seminiferous tubule, were considered statistically significant.

RESULTS

Apelin immunolocalisation on normal TT, GCNIS, and the seminoma of the human testis

In normal TT, GCNIS, and seminoma, apelin immunolocalisation was cytoplasmic. Apelin immunostaining was negative in normal TT, germ, Sertoli,

and endothelial cells. However, apelin immunostaining in Leydig cells was very dense positive (Figure 1A, Table 1). Apelin expression was significantly increased in GCNIS compared to normal TT ($p<0.05$, Figure 1B, E). The apelin expression in GCNIS was densely positive in germ cells, weakly positive in Sertoli and endothelial cells, and very dense positive in Leydig cells (Figure 1B, Table 1). The expression of apelin in seminoma was significantly increased compared to normal seminiferous tubule ($p<0.05$), while it was the same as in GCNIS ($p>0.05$, Figure 1C, E). Apelin expression was the same in seminoma and GCNIS tissues ($p<0.05$, Figure 1B-C, E). Seminoma apelin expression was very dense positive in tumour cells and weakly positive in endothelial cells (Figure 1C, Table 1). Seminoma areas contain tumour cells and infiltrated leukocytes.

APJ immunolocalisation on normal TT, GCNIS, and seminoma of human testis

APJ immunolocalisation was cytoplasmic and nuclear in normal tissue, GCNIS, and seminoma (Figures 2A-C). Expression of APJ in normal tissue was weakly positive in germ cells and very dense positive in Sertoli, Leydig, and endothelial cells (Figure 2A, Table 1). GCNIS APJ expression was very dense positive in germ, Leydig, and endothelial cells and dense positive in Sertoli cells (Figure 2B, Table 1). Normal TT and GCNIS APJ expression were similar ($p>0.05$, Figure 2E). APJ expression was significantly higher in seminoma than in normal TT and GCNIS ($p<0.05$, Figure 2E). Seminoma APJ expression was very dense positive in tumour cells and positive in endothelial cells (Figure 2C, Table 1).

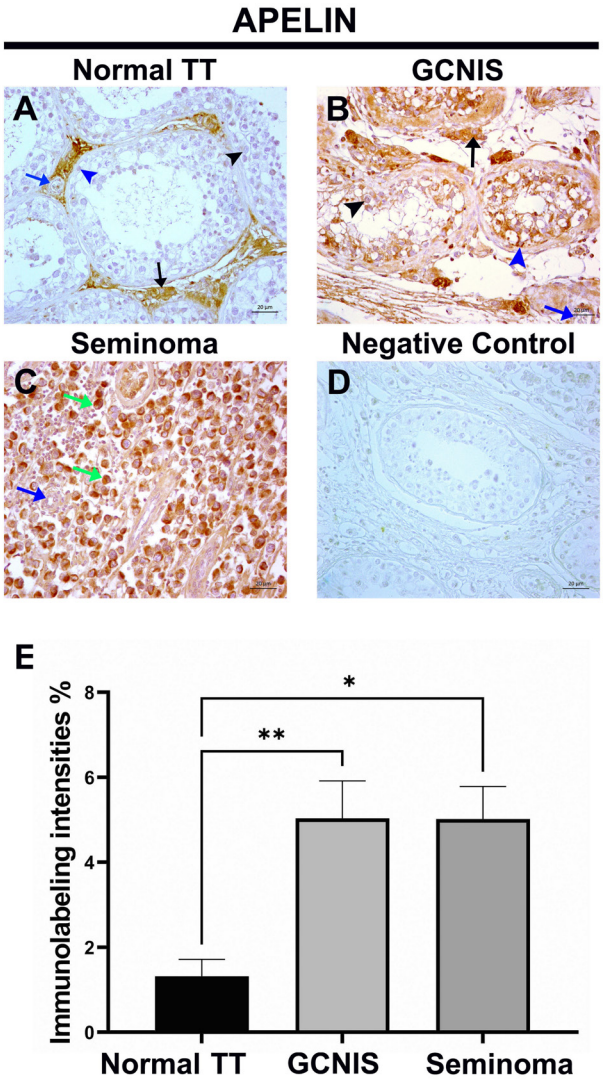

Figure 1. Apelin immunostaining. Apelin's immunohistochemical reaction was significantly increased in GCNIS and seminoma compared to normal TT. A, Normal TT (testicular tissue); B, GCNIS (germ cell neoplasia in situ); C, Seminoma; D, Negative control. Germ cells (blue arrowhead), Sertoli cells (black arrowhead), Leydig cells (black arrow), endothelial cells (blue arrow), and germ cell tumours (green arrow). E, Semi-quantitative analysis of apelin immunolabeling intensity, '*' indicates the significance between normal TT and seminoma ($P<0.05$) '**' denotes the significance of normal TT versus GCNIS ($P<0.05$)

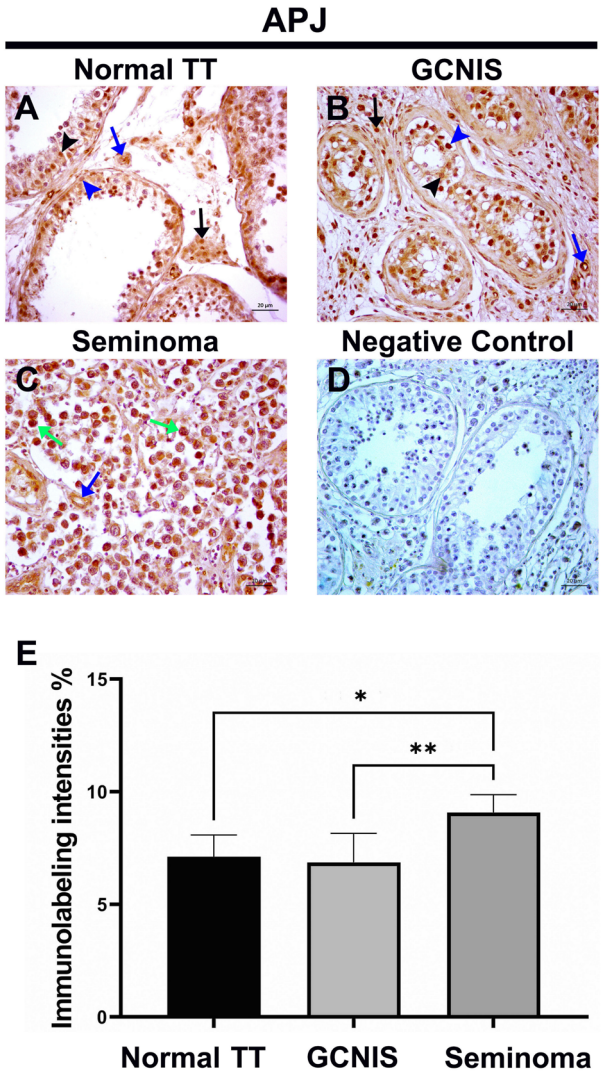

Figure 2. APJ immunostaining. It was observed that APJ immunohistochemical reactivity was significantly increased in seminoma compared to normal TT and GCNIS. A, Normal TT; B, GCNIS (germ cell neoplasia in situ); C, Seminoma; D, Negative control. Germ cells (blue arrowhead), Sertoli cells (black arrowhead), Leydig cells (black arrow), endothelial cells (blue arrow), and germ cell tumour cells (green arrow). E, Semi-quantitative analysis of APJ immunolabeling intensity. The symbol "*" denotes the significance of the difference between normal TT and seminoma ($P<0.05$).'**' indicates the significance between GCNIS and seminoma ($P<0.05$)

Table 1. Semi-quantitative distribution of Apelin and APJ labeling in normal testicular tissue, germ cell neoplasia in-situ(GCNIS) and seminoma of human testis

Cells	Normal TT		GCNIS		Seminoma	
	Apelin	APJ	Apelin	APJ	Apelin	APJ
Germ cells	0	(+)	++	+++	-	-
Sertoli cells	0	+++	(+)	++	-	-
Tumor cells	-	-	-	-	+++	+++
Leydig cells	+++	+++	+++	+++	-	-
Endothelial cells	0	+++	(+)	+++	(+)	+

0: negative; (+): weak positive; +: positive; ++: dense positive; +++: very dense positive

DISCUSSION

Although testicular cancer deaths are low compared to other cancers, patient mortality rates rise yearly. While 360 patients died from testicular cancer in 2012, it is estimated that 460 people will die in 2022 in the United States (4, 35). The expected death rate in the last 10 years has increased by approximately 28% according to these data. TGCT constitutes the majority of testicular cancers. More than half of TGCT patients have seminoma (2). The cure rates of seminoma patients are quite high. However, resistance to treatment and relapse that occurs in patients in the following process reduce the success of the treatment of the disease and even cause death (8). The Apelin/APJ axis is a pathway expressed in many tissues in the body and plays a role in different physiological processes (13,15). This axis has important roles in angiogenesis and lymphangiogenesis (16), and it is also expressed in cancer tissues. This pathway supports angiogenesis (17), proliferation (18), invasion, metastasis (20,21), and lymphangiogenesis (10), which are observed especially in aggressive cancers. In this study, we investigated seminoma, which constitutes most of TGCT and its precursor GCNIS, and the expression changes of apelin and APJ proteins in normal testes.

Previous studies determined that apelin and APJ are important for angiogenesis in cancer tissue (17), and their expression increased microvascular proliferation in brain tumours (18). In this study, we determined that apelin expression was increased in GCNIS and seminoma compared to normal TT. Hypoxia, commonly observed in cancer tissues, increases apelin expression, which supports angiogenesis, invasion, and metastasis in tumour tissue (20,21). In addition, the increased apelin expression has been shown to support lymphangiogenesis and lymph node metastasis (10). High apelin expression in cancer patients reduces their total survival (16,17,22-32). It can be concluded that a significant increase in apelin in seminoma carcinogenesis may cause angiogenesis, lymphangiogenesis, invasion, migration, and lymph node metastasis, which causes a poor prognosis in patients.

APJ contributes to the aggressiveness of cancer cells which was demonstrated by the previous studies. In this study, we found that APJ protein expression in seminoma was increased compared to normal TT and GCNIS. Apelin and APJ expressions were higher in colorectal cancer tissues compared to healthy tissues (30). APJ promotes angiogenesis in muscle-invasive bladder cancer (17). High APJ expression in patients with renal cell carcinoma has been directly associated with the aggressiveness of the disease (33). In mouse models of hepatocellular (37), glioblastoma (38), cholangiocarcinoma (22) and breast cancer (39) cancers, APJ inhibitors reduced tumour growth, angiogenic factors and vascular density (36). It is clear that APJ is a good target for inhibiting angiogenesis, growth, migration, and metastasis in cancer when all these studies are evaluated together. If agents targeting the APJ are used in treating seminoma, it can be predicted that angiogenesis, tumour growth, migration, and metastasis may be reduced. As a result, mortality may decrease, and relapse-free survival times may be prolonged.

CONCLUSION

In conclusion, the apelin/APJ axis is a pathway whose expression is increased in cancer patients. This increased expression promotes angiogenesis, lymphangiogenesis, migration, tumour growth, and metastasis. In different cancer mouse models that directly target the apelin and APJ proteins for therapeutic purposes, it has been shown that tumour growth was slowed, microvascular density and the expression of angiogenic factors were decreased (36). The use of treatments targeting apelin and APJ proteins in seminoma may be a new hope to prevent the increasing incidence and mortality of the disease in recent years. Further studies are needed before apelin/APJ inhibitors can be used clinically.

Financial disclosures: *The authors received no support from any financial institution or organization for this study.*

Conflict of Interest: *The authors declare that they have no competing interest.*

Ethical approval: *Ethical approval was obtained from Akdeniz University Clinical Research Ethics Committee (KAEK-511).*

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