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Evaluation of DNA Damage in Patients with a Neuroendocrine Tumor

Nöroendokrin Tümörlü Hastalarda DNA Hasarının Değerlendirilmesi

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Abstract

Objective: Neuroendocrine tumors (NETs) develop from the neuroendocrine cells of the endocrine system. As these tumors are extremely slow growing compared with other cancers, they often take years to reach a measurable dimension, thus leading to the late diagnosis, which has adverse effects on the survival and quality of life of patients. There is a link between many types of cancer and genomic instability, thus the markers associated with genomic instability can be used for early diagnosis of the disease or cancer-related changes. Comet assay is the most commonly used method to test genomic instability or DNA damage. To the best of our knowledge, no data are available on DNA damage in patients with NET. This study aimed to investigate the possible risk of DNA damage in a patient with NETs using the comet assay.

Material and Methods: The study included 23 patients with NET and 20 age- and sex-matched healthy participants. The DNA damage was determined using the comet assay for leukocytes obtained from the peripheral blood samples of patients and control participants.

Results: We found that the DNA damage was markedly higher in the patients with NETs than control participants.

Conclusion: Our data suggest that genomic instability contributes to the development of NETs. However, further investigations are needed to support our results, as it is a preliminary report on DNA damage risk in patients with NETs.

Keywords: Neuroendocrine tumors; comet assay; genomic instability; DNA damage

Özet

Amaç: Nöroendokrin tümörler (NET) endokrin sistemin nöroendokrin hücrelerinden oluşan tümör grubunu temsil etmektedir. Bunların çoğu diğer kanserlere kıyasla çok yavaş büyür, belirtilere neden olmaları yıllar sürer ve geç evrede tanı konur. Genetik değişiklikler ile kanser arasında bir ilişkinin varlığı birçok çalışmayla desteklenmiş olup, genomik kararsızlık ile ilişkilendirilmiş belirteçlerin, birçok hastalığın erken tanısında kullanılmasının yararlı olabileceği ileri sürülmektedir. Komet tekniği genomik karasızlık/DNA hasarını test etmek için en sık kullanılan yöntemdir. Bildiğimiz kadarıyla, NET'lü hastalarda genomik DNA hasarı ilgili herhangi bir veri bulunmamaktadır. Çalışmamızda komet yöntemi ile tedavi almayan NET'lü hastalarda olası DNA hasarlarının araştırılması amaçlandı.

Gereç ve Yöntemler: Araştırmamız, NET teşhisi konmuş ve herhangi bir tedavi almamış 23 hastada ve bu hastalara benzer yaş ve cinsiyette olan 20 sağlıklı kişide yapıldı. Hasta ve sağlıklı kontrollerden alınan periferal kan örneklerinden elde edilen lökositlerinde DNA hasarı komet assay yöntemiyle belirlendi.

Bulgular: NET'lü hastalarda DNA hasar oranının sağlıklı kişilere göre önemli seviyede yüksek olduğu bulundu ($p<0.05$).

Sonuç: Verilerimiz genomik kararsızlığın NET'lerin oluşuma katkıda bulunabileceğini göstermektedir. Fakat, sunulan çalışma literatürde bir ilk olduğundan dolayı çalışmamızı desteklemek için daha fazla hasta sayısı ile yapılacak çalışmalara gerek olduğu düşünülmektedir.

Anahtar kelimeler: Nöroendokrin tümörler; komet analizi; genomik kararsızlık; DNA hasarı

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Introduction

Neuroendocrine tumors (NETs) originate from a group of neuroendocrine cells of the endocrine system, which develop into tumors. NETs grow extremely slowly compared with most other cancers, thus take a long time to reach measurable dimensions and for symptoms to appear, leading to the late diagnosis that may negatively affect the quality of life of the patients (1).

Cancer is a multistep process. A relationship between genetic changes and cancer has been supported by several studies, and it is now well established that there is a link between several types of cancer and genomic instability (2-5). It has been suggested that the markers associated with genomic instability can be explored for early diagnosis of the disease or cancer-related changes (6-8). Therefore, micronucleus, sister chromatid exchange, chromosomal aberrations, or comet assay parameters can be used as cancer risk biomarkers (9). Comet assay is one of the most commonly used methods for measuring DNA strand breaks in eukaryotic cells (5,10), as it is an extremely simple and sensitive technique compared with other techniques (10, 11).

Patients with NETs are characterized by certain mutations and chromosomal aberrations (12-14). Unrepaired DNA damage has been the most crucial characteristic of several cancers; however, its presence in patients with NETs is unknown. We believe that DNA damage may contribute to the NET development and may be used as a prognostic biomarker for the diagnosis or treatment of NET. Thus, in the present study, we aimed to investigate if there is an unrepaired DNA damage in basal proportion in patients with NET by using comet assay (10, 11).

Material and Methods

Patient group: The study included 23 patients with NETs from the Departments of Endocrinology and Metabolism, Oncology, Surgery, Pathology, Radiology, Nuclear Medicine, and Gastroenterology at Medical Faculty of Erciyes University. Out of 23 patients, 12 were women and 11 were men aged 18-58 years. All patients were newly diagnosed and had not received medical or surgical treatment for NETs.

Control group: The control group included healthy participants matched with age and sex. None of them had received any drug for medical or other reasons. All participants were asked to complete a standardized questionnaire to obtain relevant current health status and lifestyle and information on medical history.

This study was approved by the Erciyes University Ethics Committee (Protocol No. 2013/364, 21.05.2013). All patients and healthy participants provided written informed consent. This study was conducted in accordance with the Declaration of Helsinki and local laws.

Blood sample collection and lymphocyte isolation

After completing the questionnaire, blood samples (1 mL) were collected in heparinized tubes from each patient and processed on the same day. The trypan blue exclusion test for estimating cell viability was performed, and a viability rate of ≥99% was detected. In addition, 100 µL of the blood sample was suspended in 1 mL of phosphate buffered saline (PBS). Subsequently, 100 µL of Histopaque was added and the cells were centrifuged at 1800 rpm for 30 min at room temperature. The lymphocyte band between the sample layer and the Histopaque solution was then removed. The lymphocytes were washed twice with PBS and then centrifuged for 5 min at 1800 rpm and 4 °C temperature (15).

Comet assay

All chemicals were purchased from Sigma-Aldrich (St. Louis, USA).

Preparation of slides: The comet assay was performed according to the method of Singh et al. (16). Microscope slides were coated with 0.65% high hot melting agarose (300°C) prepared in PBS. The slides were dried overnight at room temperature and stored at 4°C. Leukocytes isolated from the heparinized blood samples (100 µL) were mixed with 0.65% low melting agarose and then placed on microscope slides. The slides were stored at 4°C for 30 min to solidify. Subsequently, the slides were placed in cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris HCl [pH 10], 1% Triton X-100, and 10% dimethyl sulfoxide) for 45 min at 4°C in

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dark (to allow DNA unfolding). After incubation in the lysis solution, slides were exposed to alkaline buffer (electrophoresis buffer: 0.1 M EDTA, 10 M NaOH buffer, pH 7.5) for 30 min at room temperature in the dark (to completely degrade RNA) (15).

Electrophoresis: The slides placed in electrophoresis buffer were then subjected to electrophoresis for 30 min at 15 V and 300 mA. To prevent additional DNA damage, all processes were performed in the dark at 4 °C. The slides were then washed three times (5 min each) with 0.4 M Tris HCl buffer (neutralization buffer, pH 7.5) to neutralize the excess alkali and remove detergents and stained with ethidium bromide (EtBr; 2 µg/mL) (15).

Staining and scoring for DNA damage: 50 µL of EtBr (2 µg/mL) was added to each slide. The slides were covered with a coverslip and immediately analyzed using a fluorescence microscope (Nikon) (15, 17). The DNA damage was calculated by taking images of 100 randomly selected cells at 400× magnification using a fluorescent microscope and analyzed visually according to comet appearance (15,17).

Each image was classified according to the intensity of the fluorescence in the comet tail and was provided a value of 0, 1, 2, 3, or 4 (from undamaged class 0 to maximally damaged class 4) (15, 17). The total comet score was calculated using the formula: (total comet score=0 (n)+1 (n)+2 (n)+3 (n)+4 (n), where n represents the number of cells in each class (17-19). The slides were analyzed under blind conditions by at least two different individuals.

Statistical analysis

The differences between the control participants and patients were measured using the Mann-Whitney U test for two independent samples. Spearman's ρ correlation test was used to determine the relationship between age and basal DNA damage. The p-value of less than 0.05 was considered statistically significant. Data were analyzed using GraphPad program (Prism 5).

Results

The clinical findings of patients are shown in Table 1. The comet score in 100 peripheral blood mononuclear cells of patients and con-

Table 1. Profile of patients at the time enrolment to the study.

Case No	Age at blood collection	Gender	Primary tumor site
1	65	M	Stomach
2	62	F	Unknown
3	61	F	Duodenum
4	55	M	Stomach
5	32	F	Duodenum
6	29	F	Lungs
7	52	M	Lungs
8	62	M	Pancreas
9	51	M	Ileum
10	69	M	Bladder
11	52	M	Pancreas
12	54	F	Stomach
13	37	F	Stomach
14	44	M	Pancreas
15	57	F	Liver
16	55	F	Unknown
17	49	F	Unknown
18	46	M	Small intestine
19	74	F	Unknown
20	50	F	Unknown
21	51	M	Unknown
22	50	F	Unknown
23	28	M	Pancreas

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trol participants is shown in Table 2. The cells with damaged DNA appeared like a comet, whereas undamaged cells showed an intact nucleus without a tail (Figure 1). The basal DNA damage frequency (%) between the study and control groups is shown in Table 3. The study group showed significantly higher basal DNA damage than the control groups ($p<0.05$; Table 3). Furthermore, a significant positive correlation was observed between the basal DNA damage and age of control participants ($r=0.45$, $p=0.044$; Figure 2a). However, no significant relationship was noted between the basal DNA damage and age of patients ($r=0.097$, $p=0.66$; Figure 2b).

Discussion

NETs are a heterogeneous group of malignancies (20, 21). They are typically less aggressive compared with other solid tumors; therefore, NETs are considered rare tumors (22). The majority of NETs are known as sporadic. However, it is reported that NETs can also occur because of inherited familial syndromes (13, 20, 23). Although the genetic basis of NETs is unclear, several genes responsible for NETs have been identified. These genes are the members of crucial pathways involved in the maintenance of telomere length, chromatin modification, cell proliferation, response to DNA damage, and cell metabolism. It was shown that the genes from familial predisposition syndromes are similarly mutated in sporadic NETs (24). Moreover, some of the mutated genes identified in sporadic tumors were associated with familial cancer syndromes causing NETs (22). These genes associated with neuroendocrine tumorigenesis have functions in DNA repair and mTOR pathways (22). However, no data exist on the basal

Table 2. Mean value of each comet class per 100 cells (±standard deviation) in patients and controls.

Comet Class	0 (Mean±SD)	1 (Mean±SD)	2 (Mean±SD)	3 (Mean±SD)	4 (Mean±SD)
Patients	25.13±5.50	25.83±3.75	25.91±3.76	14.35±2.64	10.00±1.89**
Controls	42.40±7.35	33.75±4.54	24.00±5.28	11.35±2.11	4.2±0.70

SD: Standard Deviation, ** $p<0.0001$.

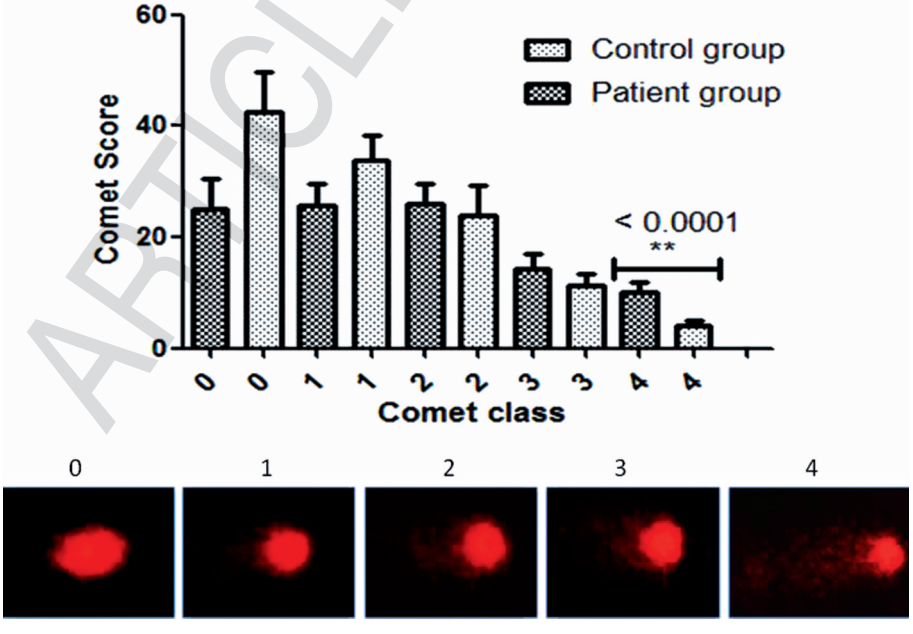


Figure 1: DNA damage assessed by using the comet assay. Mean comet score and comet classes in the control and patient groups. The cells were assessed visually and received class 0 (undamaged) to 4 (maximally damaged), according to the size and shape of the tail.

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Table 3. Basal DNA damage frequency (TCS% according to the formula) in the patients with NET and controls.

Groups	Age (years) (Mean±SD)	DNA damage (%) (Mean±SD)
Patients with NET (n=23)	51.96±2.37	1.78±0.124*
Controls (n=20)	50.40±1.50	1.42±0.94
p-value	0.59	0.031

SD: Standard Deviation, TCS: Total Comet Score* p<0.05.

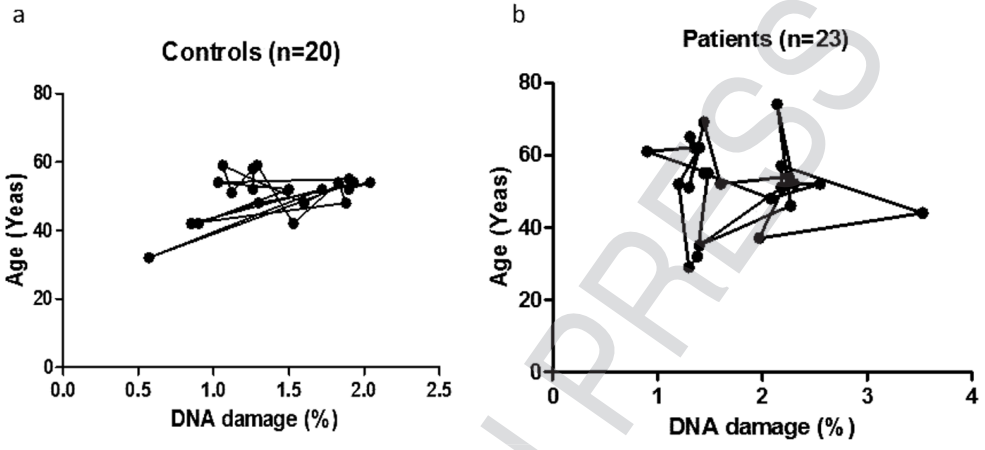


Figure 2: Correlation between the age and DNA damage frequencies in the controls **(a)** and patients **(b)**.

DNA damage level in patients with NET. Comet test is a powerful technique for quantifying and analyzing DNA damage and has the advantage of showing DNA damage in individual cells (25-27). Our comet assay findings showed increased DNA damage in patients with NET.

Chromosomal instability, one of the causes of NET tumorigenesis, is related to DNA methylation rather than genomic changes (12, 28-31). However, in this study, the comet frequency was higher in the peripheral blood mononuclear cells of patients with NETs. Increased comet frequency reflects genomic alterations in patients with NETs. Alterations in multiple pathways such as DNA replication and repair of DNA damage mutations can lead to genomic instability (32). Further, the structurally altered DNA or DNA strand breaks also promote genome instability. The comet assay is used to measure structural DNA damage (33). In contrast to previous studies (12, 28-31), our findings also indicated the presence of genomic instability induced by structural DNA damage in patients with NETs. As genomic instability is considered a driving force for numerous cancers (32), we believe that structural DNA damage may be responsible for NET formation.

Recent studies indicated that epigenetic modifications are likely to be a major factor for neuroendocrine tumorigenesis, whereas mutated oncogenes only play a minor role in pathogenesis (13). However, different mechanisms such as defective DNA repair, replication, and chromosome maintenance may contribute to NET development (34). In addition, our findings showed that genomic instability is caused by structural DNA damage in patients with NET. Therefore, we believe that genomic instability may contribute to the initiation or progression of NET.

In patients with Nijmegen syndrome or Xeroderma Pigmentosum, who have DNA breakage, it may be possible to use the comet assay as an aid to diagnosis (18). Furthermore, it is suggested that comet assay can be used as a reliable clinically potential tool to identify a disease (5, 33). Therefore, the DNA damage determined using the comet assay may be a biomarker for NET.

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Conclusion

Our findings indicated that the increased structural DNA alterations may promote tumorigenesis in NETs, and the DNA damage determined using the comet assay may be a biomarker for the diagnosis of NETs. However, further studies with large sample sizes are needed to support our preliminary findings.

Financial Resources

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Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: Zuhal Hamurcu, Fahri Bayram, Hamiyet Dönmez Altuntaş; Design: Zuhal Hamurcu, Fahri Bayram, Nesrin Delibaşı; Control/Supervision: Zuhal Hamurcu; Data Collection and/or Processing: Ümmühan Abdülrezzak, Figen Öztürk, Ender Doğan, Erdoğan Sözüer, Alper Yurci, Gülten Sezgin Can, Şebnem Gürsoy, Mustafa Kula; Analysis and/or Interpretation: Fahri Bayram; Literature Review: Zuhal Hamurcu, Fahri Bayram, Ümmühan Abdülrezzak, Hamiyet Dönmez Altuntaş; Writing the Article: Zuhal Hamurcu, Fahri Bayram, Hamiyet Dönmez Altuntaş; Critical Review: Zuhal Hamurcu, Fahri Bayram; Materials: Fahri Bayram, Ümmühan Abdülrezzak, Ender Doğan, Erdoğan Sözüer, Alper Yurci, Gülten Sezgin Can, Şebnem Gürsoy, Mustafa Kula.

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