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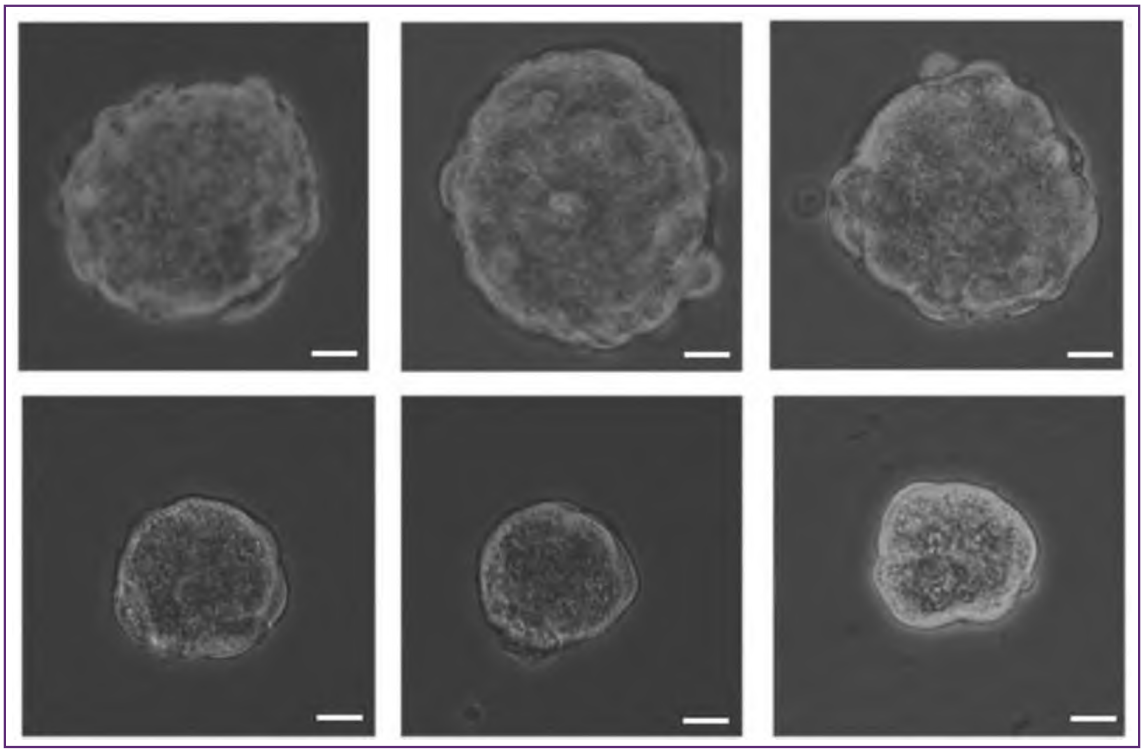

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Research Article

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Determining the Expression Levels of Lncrna PVT1 and Mir-128 in the Human Lung Carcinoma Cell Line

İnsan Akciğer Karsinomu Hücre Hattında Lncrna PVT1 ve Mir-128'in İfade Düzeylerinin Belirlenmesi

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Abstract

Objective: In the presented research, we aimed to examine the expression of microRNA-128 (miR-128) and long non-coding RNA (lncRNA) the plasmacytoma variant translocation 1 (PVT1) in non-cancerous cells BEAS-2B and human lung carcinoma A549 to identify any potential roles these molecules might play in the cancer development in lung tissue.

Material and Methods: The cell lines used in this research were A549 and BEAS-2B. Total RNA was isolated from these cell lines, and then complementary DNA (cDNA) was synthesised. Using the quantitative real-time PCR (RT-qPCR) method, the expression levels of lncRNA PVT1 and miR-128 were evaluated. The $2^{-\Delta\Delta Ct}$ method was employed to analyse fold changes by normalising to U6 snRNA and GAPDH expressions.

Results: Elevated PVT1 expression was determined in the lung cancer cells ($p<0.01$). Nevertheless, miR-128 expression did not change statistically significantly ($p>0.05$). Even though miR-128 is a potential binding target of lncRNA PVT1 according to in silico analysis, the expression profile does not correlate.

Conclusion: Our evaluation of the relationship between lncRNA PVT1 and miR-128 in cancerous cells A549 compared with non-cancerous cells BEAS-2B reveals that PVT1 expression is significantly

Öz

Amaç: Araştırmamızın amacı, normal akciğer epitel hücre hatlarında (BEAS-2B) ve insan akciğer karsinomunda (A549), mikroRNA-128 (miR-128) ve uzun kodlamayan RNA (lncRNA) PVT1 ekspresyonunu inceleyerek, bu moleküllerin akciğer kanserinin gelişimi ve ilerlemesinde oynayabileceği potansiyel rolleri belirlemektir.

Gereç ve Yöntemler: Bu araştırmada kullanılan hücre hatları A549 ve BEAS-2B'dir. Gerçek zamanlı kantitatif PCR (RT-qPCR) yöntemi kullanılarak PVT1 ve miR-128'in ifade seviyeleri değerlendirildi. $2^{-\Delta\Delta Ct}$ yöntemi, U6 snRNA ve GAPDH ifadelerine göre normalize ederek kat değişimlerini incelemek için kullanıldı.

Bulgular: Akciğer kanseri hücrelerinde PVT1 ekspresyonunun yükseldiği belirlendi ($p<0,01$). Bununla birlikte, miR-128 ekspresyonu istatistiksel olarak anlamlı bir şekilde değişmedi ($p>0,05$). Mir-128, in-silico analizde lncRNA PVT1'in potansiyel bir bağlanma hedefi olmasına rağmen, ekspresyon profili sonuçlarına göre korelasyon görülmedi.

Sonuç: Normal akciğer epitel hücrelerine kıyasla akciğer kanseri hücrelerinde lncRNA PVT1 ve miR-128 arasındaki ilişkinin incelendiği çalışmamız, PVT1 ekspresyonunun akciğer kanseri hücrelerinde önemli ölçüde yüksek olduğunu, buna karşın

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elevated in lung cancer cells, whereas miR-128 expression remains unchanged. Further investigation is needed to explore whether the interaction between lncRNA PVT1 and miR-128 is indirect.

Keywords Lung cancer · A549 cell line · PVT1 · miR-128 · RT-qPCR

miR-128 ekspresyonunun değişmeden kaldığını ortaya koymaktadır. LncRNA PVT1 ve miR-128 arasındaki etkileşimin dolaylı olup olmadığını keşfetmek için daha fazla çalışmaya ihtiyaç vardır.

Anahtar Kelimeler Akciğer kanseri · A549 hücre hattı · PVT1 · miR-128 · RT-qPCR

INTRODUCTION

Lung cancer (LC) is a prominent cause of cancer-associated mortality on a global scale. Each year, it affects 1.8 million individuals worldwide, resulting in 1.6 million deaths. Lung cancer is divided into two main subtypes. The first is small cell lung cancer (SCLC), which is present in 15% of new cases. Second is non-small-cell lung cancer (NSCLC), which represents about 85% of all newly diagnosed lung cancers (1). Challenges associated with detecting LC at an early stage persist because of the inadequacy of practical diagnostic tools and methods (2). Therefore, we need to explore new molecular markers and methodologies to enhance our elucidation of the molecular mechanisms behind this disease.

MicroRNAs (miRNAs) represent a distinct class of endogenous, small non-coding RNAs ranging from 18 to 27 nucleotides in length. They are essential for post-transcriptional gene regulation via attachment to the 3′ untranslated region (UTR) of specific mRNAs. They also participate in a broad spectrum of cellular activities, such as cell proliferation, metabolic processes, and programmed cell death. miRNAs have been recognised as key regulators in various cancer types, particularly through alterations in their expression profiles that influence cancer pathogenesis. Emerging evidence underscores the significant dysregulation of these non-coding RNA types in various cancers. In tumour tissues, specific miRNAs (OncomiRs) are overexpressed, whereas others (tumour suppressor miRNAs) are underexpressed. Reduced levels of tumour suppressor miRNAs and increased expression of oncomiRs contribute to key cancer hallmarks such as sustained proliferation, evasion of cell death mechanisms, evasion of immune surveillance, invasion, and metastasis. Differential expression of miRNAs has been observed in tumour tissues of patients with LC (3-5).

lncRNAs do not possess open reading frames (ORFs) and thus cannot encode proteins. The intricate functions of lncRNAs in cellular processes involve complex interactions with biological molecules such as DNA, RNA, proteins, and other cellular components. lncRNAs interact with RNA-binding proteins and can modulate transcription factors, activating or suppressing target genes at the post-transcriptional or post-translational levels. Recent investigations have underscored their pivotal involvement in tumorigenesis, functioning as oncogenes or tumour suppressors. They are intricately involved in critical biological processes such as cellular proliferation, apoptosis evasion, migration, and invasion. Consequently, research on lncRNAs holds substantial promise in elucidating the mechanisms underlying tumour initiation and progression (6-8).

An important aspect is that miRNAs and lncRNAs are capable of interacting with each other, creating intricate regulatory networks within cells. For instance, miRNAs can target and regulate the stability and abundance of lncRNAs. Conversely, lncRNAs can function as molecular sponges or competitive decoys for miRNAs, modulating their activity by sequestering them away from their mRNA targets. Moreover, miRNAs and lncRNAs can cooperatively regulate common target genes, enhancing the complexity and precision of gene expression control (9).

PVT1 is a key oncogenic factor in the progression of various malignancies. It operates as a competing endogenous RNA (ceRNA), modulating miRNA activity by serving as a molecular sponge. It binds to miRNAs, hindering their interaction with target mRNAs. However, this sponging effect of PVT1 is disrupted under cancerous conditions (10, 11). miR-128 is recognised as a prominent tumour suppressor that inhibits tumour growth, migration, and metastasis by downregulating oncogenic processes. miR-128 is involved in a range of diseases and cellular mechanisms, including cell proliferation, epithelial-mesenchymal transition (EMT), tumorigenesis, and angiogenesis. Recent research indicates that lncRNA PVT1 promotes the progression of different cancer types by functioning as a sponge for miR-128 (12-14). Accordingly, this research aims to shed light on the potential roles of lncRNA PVT1 and miR-128 in lung cancer (LC) development and progression by examining their expression levels in cancerous cells compared with non-cancerous BEAS-2 B cells.

MATERIALS AND METHODS

Cell line and culture conditions

Incubation of the A549 cell line (ATCC CRM-CCL-185 ™) and the BEAS-2B cell line (ATCC CRL-3588 ™) were conducted at 37 °C, the ratio of CO_2 was 5% and humidity 95% in CO_2 incubator (Panasonic, MCO-170M-PE). The maintenance of both cell lines was conducted in a culture medium composed of RPMI-1640 (Gibco), with the incorporation of Foetal Bovine Serum (Gibco)

and penicillin–streptomycin (Gibco), at concentrations of 10% and 1%, respectively. Once the cells reached confluence, they were subcultured by adding a Trypsin-EDTA solution (Gibco), followed by centrifugation and re-suspension in fresh media.

Total RNA extraction and complementary DNA Synthesis

Total RNA extraction, including miRNA, was performed from all cells using miRNeasy Tissue/Cells Advanced Mini Kit (Qiagen) following the instructions. The RNA purity and quality were evaluated with a spectrometric measurement (NanoDrop, Thermo) by measuring the A260/A280 and A260/A230 ratios. The synthesis of complementary DNA was performed by reverse transcription using an RT² First Strand Kit (Qiagen). Additionally, the miRCURY LNA RT Kit (Qiagen) was used to reverse transcribe for the miRNA experiments. The total RNA yield of all samples was equalised to 500 ng for cDNA synthesis. After reverse transcription, cDNA concentrations were spectrophotometrically evaluated to check the synthesis step. All cDNA concentrations were between 1400 ng/µL and 1450 ng/µL, and these were then used in the RT-qPCR experiment.

Real-time quantitative PCR

Commercially available and wet-lab validated target primer sets, RT² lncRNA qPCR Assays (Qiagen) and miRCURY LNA miRNA PCR Assay (Qiagen) were used for determining lncRNA and miRNA expression levels, respectively. Detailed information on the primer assays is shown in Table 1. Real-time PCR application based on SYBR Green fluorescence was conducted to detect the lncRNA and miRNA levels by using RT² SYBR Green ROX FAST Mastermix (Qiagen). We performed the PCR in two steps: 1) 95°C for 10 min and 2) 40 cycles consisting of 95°C for 15 s and 60°C for 60 s, respectively. The fluorescence intensity was measured to obtain the Ct (threshold cycle) value for all samples at the end of each cycle. Single peaks are observed in the melting curves for all assays, confirming specific amplification. We determined the hsa-miR-128-3p and PVT1 relative expression levels between the cell line A549 and the non-cancer BEAS-2B cell line by $2^{-\Delta\Delta Ct}$ method by normalising to U6 snRNA and GAPDH expressions (15), and all samples were carried out in triplicate, using biological and technical replicates.

In silico Analysis of lncRNA-miRNA Interactions

It is well established that lncRNAs frequently act as ceRNAs or molecular sponges, thereby binding miRNAs and modulating their biological activities (16). To further investigate the mechanism of PVT1 in lung cancer cell lines, we examined whether it affects the expression profile of miR-128. For this purpose, we used the online bioinformatics tool Starbase-ENCORI (https://rnasysu.com/encori/, access date: 18 February 2025) to show the binding probability of PVT1 with miR-128 directly or not.

Table 1. miRNA and lncRNA assay information

lncRNA Name	Gene ID or miRbase Accession Number	GeneGlobe ID
PVT1	5820	LPH17013A
GAPDH	2597	LPH31725A
U6 snRNA	26827	YP02119464
hsa-miR-128-3p	MIMAT0000424	YP00205995

ID: Identification, lncRNA: long non-coding RNA

Statistical analysis

GraphPad Prism (v8.0) was used to perform the statistical evaluation. Fold change values less than one were converted to fold regulation using the following formula: Fold Regulation = (−1 / fold change value). Fold changes values ≥ 2 and ≤ −2 were interpreted as upregulation and downregulation, respectively. Student's t-test was performed to compare the differences among groups, $p<0.05$ regarded as statistically significant.

RESULTS

lncRNA PVT1 may interact with miR-128 based on in silico analysis

We used Starbase-ENCORI as a bioinformatics tool to forecast the binding possibility of PVT1 with miR-128 (Figure 1). Data from Starbase-ENCORI indicate that PVT1 may bind to miR-128 and downregulate its expression. (CLIP Data: in silico prediction, TMDM score: 1.4343). According to the results of the in silico prediction based on the TMDM score, PVT1 has a relatively high binding capacity with hsa-miR-128-3p.

Real-time quantitative PCR (RT-qPCR) experiment for miR-128 and PVT1

We analysed the expression levels of PVT1 and miR-128 to test whether there was an interaction between their expressions in the lung cell lines. We observed the elevated expression of PVT1 in lung cancerous cell A549, while miR-128 expression was not different compared with the non-cancerous cells BEAS-2B. PVT1 expression increased 4.03-fold; this alteration was statistically significant ($p<0.01$). miR-128 expression was downregulated 1.05-fold, but no significant statistically ($p>0.05$). The expression results of miR-128 and PVT1 are indicated in Figures 2 and 3, respectively. Table 2 also shows the fold regulation and p-value.

miRNA	GeneName	GeneType	TargetSite	Alignment	Type	TDMDScore
hsa-miR-128-3p	PVT1	lncRNA	chr8:127855212-127855234[+]	Target: 5' UAGGAGUCCUGCUGUCACUGUGG 3' miRNA : 3' UUUCUCUGG-CCA-AGUGACACU 5'	7mer-m8	1.4343
hsa-miR-128-3p	PVT1	lncRNA	chr8:128099409-128099429[+]	Target: 5' CACCACCCAGCAUCACUGUGG 3' miRNA : 3' UUUCUCUGGCCAAGUGACACU 5'	7mer-m8	0.7701

Figure 1. lncRNA PVT1 can bind directly to hsa-mir-128-3p. Possible binding regions between PVT1 and miR-128 are shown in the Alignment section.

Table 2. Statistical results and fold regulation values

	Average ΔCt A549	Average ΔCt Control (BEAS-2B)	$2^{-\Delta\Delta Ct}$ A549	p value	Standard Deviation	Fold Regulation
hsa-mir-128-3p	2.55	2.48	0.95	>0.05	0.033	−1.05
lncRNA PVT1	6.17	8.18	−2.01	<0.01*	0.012	4.03
U6 snRNA	0.00	0.00	1.00	>0.05	-	-
GAPDH	0.00	0.00	1.00	>0.05	-	-

Student's t-test: p<0.05*, Ct: Cycle threshold, snRNA: small nucleolar RNA, GAPDH, and u6 snRNA: Reference gene

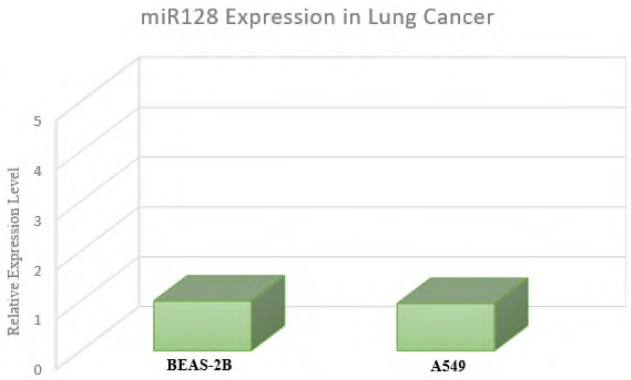

Figure 2. Relative expression level of hsa-miR-128-3p in the lung carcinoma cell line A549. There was no statistically significant difference in the miR-128 expression level between BEAS-2B and A549 cells (p>0.05). Lung cells were cultivated for 48 h before the qRT–PCR experiment, which was performed to determine the fold change.

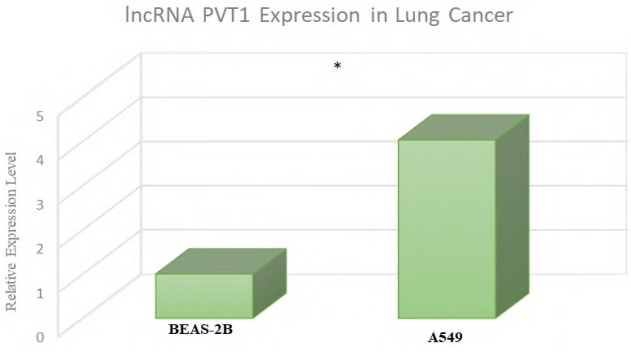

Figure 3. Relative expression level of PVT1 in the lung carcinoma cell line A549. The elevated PVT1 expression level was statistically significant (p<0.01) according to the fold change results obtained from qRT–PCR experiment after 48 h of lung cell culture

DISCUSSION

miRNAs and lncRNAs contribute significantly to the modulation of various biological processes and are often linked to human disorders, including cancer. Understanding the interactions between these two classes of non-coding RNAs is crucial for unravelling the full complexity of gene regulatory networks and enlightening their potential effect for diagnosis as a marker in terms of disease pathophysiology.

The dysfunction of miRNAs has been demonstrated in malignant disorders like cancer via several mechanisms, including the overexpression or downregulation of miRNA genes, improper regulation of miRNAs transcriptionally, and disruptions in the biosynthesis mechanism of miRNA. miR-128 is known as a significant tumour suppressor that inhibits cell migration, tumour growth, and metastasis through its upregulation in cancer cells (17). Numerous studies have demonstrated that miR-128 regulates the progression of cancer by upregulating or downregulating. A recent study showed that the levels of miR-128 are higher in the benign type than in the invasive one for prostate cells (18). A further study has demonstrated a tumour-suppressive function for miR-128-3p in hepatocellular carcinoma (HCC) development and progression (19). miR-128 has been demonstrated to inhibit cell growth and trigger apoptosis in gastric cancer cells (20). miR-128-3p, in a cellular model, has been studied for its ability to decrease cell viability and suppress the ability to form colonies by inducing apoptosis (21). It has been demonstrated that decreased miR-128-3p expression in tissue samples from patients with lung cancer is linked to TNM stage and tumour size (22). Nonetheless, in this study, we showed that the difference in the level of mir-128 expression between the lung carcinoma cells A549 and the non-cancerous cells

BEAS-2B was not statistically significant. Despite the proposal of miR-128 as a broadly applicable cancer-related target, our findings indicate that it does not function as a central mediator in the development of LC, thereby invalidating its use as a therapeutic strategy in LC.

Several cancers have been correlated with aberrant PVT1 expression. Recent studies showed upregulated PVT1 expression in multiple types of cancers in cancer tissues and cell lines, including NSCLC, oral squamous cell carcinoma, gallbladder cancer, pancreatic carcinoma, glioma, and oesophageal cancer. Furthermore, its elevated expression was related to the undesired clinical features of the tumours, such as poor prognosis, invasion, metastasis, higher grade, increased migration and proliferation of cells, and tumour progression (23-29). Studies have shown that lncRNA PVT1 affects tumour progression by inhibiting miRNA expression. PVT1 elimination improved the radiosensitivity through its sponging effect on miR-195 in non-small cell LC (28). PVT1 may facilitate the cell migration and growth in pancreatic carcinoma by sponging miR-488. (29). PVT1 also promotes tumour progression by acting as a sponge endogenously for miR-128 in glioma (12). miR-128 is an essential target of PVT1 in cancer. It was shown that PVT1 contributes to the cell proliferation in breast cancer and epithelial-mesenchymal transition through its binding to miR-128 and FOXQ1 (13). EMT progression is a key factor for the migration of NSCLC cells. Because many human malignancies stem from epithelial tissues, a deeper understanding of the EMT processes could contribute significantly to our knowledge of solid tumour development. Recent studies have identified PVT1 as a key regulator of EMT progression, unveiling a previously unrecognised mechanism underlying this process (29). Downregulation of PVT1 increased the miR-128 level and reduced proliferation in oesophageal squamous cell carcinoma. However, it has been demonstrated that reducing the miR-128 expression level while simultaneously knocking down PVT1 reverses the reduced proliferation, invasion, and migration ability (30). These results emphasise the connection between lncRNA PVT1 and miR-128, operating through various cellular mechanisms linked to EMT and proliferation. Based on the findings, it can be concluded that lncRNA PVT1 and miR-128 bind to their respective target mRNAs competitively; thus, malignancy may develop in cases where PVT1 predominates in the cell due to hampering miR-128 tumour suppressive activity. In our study, we found elevated PVT1 expression in A549 compared with BEAS-2B. However, this increased expression was not accompanied by a decrease in miR-128 expression. This discordance may show that PVT1 does not tend to bind with miR-128 directly, especially in the A549 cell line. As a limitation of our study, we did not use alternative lung cancer cell lines and did not perform induction or inhibition experiments. Further investigations with different cell lines and induction/inhibition experiments should be performed to confirm our findings.

We employed the bioinformatics tool Starbase to support our hypothesis that the expression of PVT1 may inhibit miR-128 expression in LC cells. It has been established that PVT1 has two target sites to bind to miR-128. However, only one site had a high TMDM score, which indicated a higher possibility of interaction between the miRNA and lncRNA. Unlike in silico prediction, elevated PVT1 expression did not directly affect miR-128 expression. We have concluded that PVT1 may act as an endogenous RNA that binds to miR-128 targets competitively. We need to investigate further how lncRNA PVT1 may function as a sponge for miR-128 by modulating its activity. For instance, whole transcriptome sequencing can be used to analyse the alterations in the mRNA profile following the knockdown of miR-128 or lncRNA PVT1.

In conclusion, we examined whether there is any interplay among lncRNA PVT1 and miR-128 in lung cancer cells compared to non-cancerous cells. Our findings reveal that PVT1 expression is elevated in lung cancer cells, while the expression level of miR-128 remains unchanged. This research adds valuable insights to the literature by elucidating the etiopathogenesis of lung cancer through the interaction of PVT1 and miR-128. However, additional analyses are needed to assess the mechanistic interaction between PVT1 and miR-128.

Ethics Committee Approval: For this type of study, there is no need for written informed consent and formal ethics approval.

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Research Article

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Impact of HLA-B Allele Variability on HIV-1 Viral Load and CD4⁺ T Cell Counts

HLA-B Alel Değişkenliğinin HIV-1 VİRAL Yük ve CD4⁺ T Hücre Sayilari Üzerindeki Etkisi

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Abstract

Objective: Human Immunodeficiency Virus-1 (HIV-1) has high morbidity and mortality and specifically targets CD4⁺ T cells, a unique reservoir for HIV-1. CD4⁺ T cells, which have a role in regulating the immune response and activating other immune cells, recognise HIV-1 antigens presented by Human Leukocyte Antigen (HLA) Class I molecules. HLA-B is most strongly linked to the potential disease outcomes when compared with other HLA classes. It is important to detect HLA-B alleles to determine their effects on HIV-1 infection. By demonstrating the difference in HLA B allele frequency between HIV-1 uninfected and people living with HIV-1 (PLWH), we aimed to demonstrate the correlation between the presence of these alleles in PLWH and the CD4⁺ T cell count and viral replication levels.

Material and Methods: We evaluated the HLA-B allele frequency and its association with HIV-1 infection outcomes in 412 PLWH and 406 healthy individuals. After purification of the genomic DNAs, we identified the HLA-B alleles using PCR-SSP and Luminex technology PCR-SSO methods.

Results: We found that the HLA-B*07, *18, *35, *44, and *51 alleles occurred at a frequency greater than 5% in the patient group. In PLWH, the frequency of the HLA-B*57 allele was observed to be lower than that in the control group. The HLA-B*57:01 allele positivity was determined as 1.6%. All patients with HLA-B*57:01 allele positivity had VL <100,000 copies/ml. Patients with the HLA-B*07 and HLA-B*35 alleles exhibited lower CD4⁺ T cell counts (cells/mm³) and higher HIV RNA levels (copies/mL).

Öz

Amaç: İnsan immün yetmezlik virüsü-1 (HIV-1) yüksek morbidite ve mortaliteye sahiptir ve özellikle HIV-1 için benzersiz bir rezervuar olan CD4⁺ T hücrelerini hedef alır. İmmün yanıtı düzenlemede ve diğer immün hücreleri aktive etmede rol oynayan CD4⁺ T hücreleri, İnsan Lökosit Antijeni (HLA) Sınıf I molekülleri tarafından sunulan HIV-1 antijenlerini tanır. HLA-B, diğer HLA sınıflarıyla karşılaştırıldığında hastalık sonuçlarıyla en güçlü şekilde ilişkilidir. HIV-1 enfeksiyonu üzerindeki etkilerini belirlemek için HLA-B allellerinin varlığını tespit etmek önemlidir. HIV-1 ile enfekte olmayan ve HIV-1 ile yaşayan kişiler (PLWH) arasındaki HLA-B alel sıklığındaki farkı göstererek, bu allellerin PLWH'deki varlığı ve CD4⁺ T hücre sayısı ile viral replikasyon seviyeleri arasındaki ilişkiyi göstermeyi amaçladık.

Gereç ve Yöntemler: 412 PLWH ve 406 sağlıklı bireyde HLA-B alel sıklığını ve HIV-1 enfeksiyon sonuçlarıyla ilişkisini değerlendirdik. Genomik DNA'lar saflaştırıldıktan sonra PCR-SSP ve Luminex teknolojisi PCR-SSO yöntemleri ile HLA-B allellerini tanımladık.

Bulgular: HLA-B*07, *18, *35, *44 ve *51 allellerinin hasta grubunda %5'ten daha yüksek bir sıklıkta olduğunu gördük. PLWH'de HLA-B*57 alelinin sıklığının kontrol grubuna göre daha düşüktü ve HLA-B*57:01 alel pozitifliği %1,6 olarak görüldü. HLA-B*57:01 alel pozitifliği olan tüm hastalarda viral load (VL) <100.000 kopya/ml idi. HLA-B*07 ve HLA-B*35 allellerine sahip hastalar daha düşük CD4⁺ T hücre sayıları (hücre/mm³) ve daha yüksek HIV RNA seviyeleri (kopya/mL) sergilediler.

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Conclusion: Our findings strongly imply the involvement of other molecular mechanisms, extending beyond the traditional role of class I HLA molecules in antigen presentation. Research into how HLA-B alleles influence HIV-1 infection and disease progression will help us understand who is more susceptible to HIV-1 and how the disease will evolve in different individuals. Further research is essential on this factor, as it holds significant implications for the ongoing, years-long pursuit of an effective HIV vaccine.

Keywords HIV-1, HLA-B, CD4⁺T cell, HLA-B*57:01

Sonuç: Bulgularımız antijen sunumunda sınıf I HLA moleküllerinin geleneksel rolünün ötesine uzanan diğer moleküler mekanizmaların dahil olduğunu ima etmektedir. HLA B alellerinin HIV-1 enfeksiyonunu ve hastalık ilerlemesini nasıl etkilediğine dair araştırmalar, kimin HIV-1'e daha duyarlı olduğunu ve hastalığın farklı bireylerde nasıl evrimleşeceğini anlamamıza yardımcı olacaktır. Bu faktör üzerinde daha fazla araştırma yapılması önemlidir çünkü etkili bir HIV aşısının yıllarca süren devam eden arayışı için önemli çıkarımlar taşımaktadır.

Anahtar Kelimeler HIV-1, HLA-B, CD4⁺ T hücre, HLA-B*57:01

INTRODUCTION

Human Immunodeficiency Virus-1 (HIV-1), which allows life-threatening opportunistic infections and cancers to develop, has infected an estimated 84 million people worldwide and caused 40 million deaths (1, 2). This retrovirus has high morbidity and mortality and specifically targets CD4⁺ T cells, a unique reservoir for HIV-1 (1-3). CD4⁺ T cells, which have a role in regulating the immune response and activating other immune cells, recognise HIV-1 antigens presented by Human Leukocyte Antigen (HLA) Class I molecules. The spread of HIV-1 within the body of an infected individual reduces the functional capacity of CD4⁺ T cells, driving the cells towards immune exhaustion and as a result, causing the Acquired Immunodeficiency Syndrome (AIDS), which is defined as the final stage of the disease. AIDS is characterised by significant immune deficiency and a reduction in CD4⁺ T cell levels, often falling below 200 cells per cubic millimetre, contributing to increased vulnerability to opportunistic infections and certain cancers (4). The time to AIDS has been extended with the use of efficient combination antiretroviral therapy (cART) (5). However, despite the increased use of cART, it continues to exist latently in many anatomical regions, and this latent reservoir is one of the key reasons why a cure for HIV remains elusive (6, 7). This clinical latency is the biggest obstacle to treatment success (8).

The kinetics of the cellular immune response and progression to AIDS vary depending on the host's immunogenetic profiles. HLA, which has a highly polymorphic structure, is the most striking among these immunogenetic profiles. It is a predominant determinant of the host adaptive immune response that influences HIV disease progression and shows a strong association with advancement to AIDS. HLA class I molecules (HLA-A, HLA-B, and HLA-C), encoded by HLA class I genes, trigger the CD8⁺ T cell response against HIV-1 (3, 4, 6). Studies have shown that HIV-infected patients with specific HLA class I alleles control viral replication more effectively and are less exposed to CD4⁺ T cell loss. HLA-B is most strongly linked to the potential disease outcomes when compared with other HLA classes. The immune response that HLA B-bound CD8⁺ T cells mount against the virus is critical in shaping the course of the infection and influencing how quickly the disease progresses (4).

It has been reported that certain HLA alleles have a protective role because they better control HIV-1 replication, prolong the transition to AIDS, and reduce CD4⁺ T cell loss. In terms of their positive contributions to disease prognosis, the HLA-B*27 and *57 alleles are the most frequently encountered alleles, and HIV-1 needs more time to adapt to the host with these alleles (9-12). Some HLA-B alleles such as HLA-B*57:01 have features that include the Bw4 epitope. The presence of the Bw4 epitope allows important antigenic structures, such as the HIV-1 gag protein, to be recognised effectively by CD8⁺T and NK cells. This situation leads to a long-term non-progressive (LTNP) clinical course in HIV-1 patients (13, 14).

On the other hand, certain HLA alleles worsen the disease prognosis, shorten the transition to AIDS and trigger CD4⁺ T cell loss much more. Additionally, the HLA-B*35 allele has been linked to poor prognosis in many studies due to its negative effect on disease progression (12, 15-19). The ability of HLA-B alleles to provide partial control over viremia and to lead to significantly different disease outcomes highlights the importance of these genetic variants in shaping the course of HIV-1 infection. It is important to detect these alleles to determine their effects on HIV-1 infection (19). By demonstrating the difference in the HLA-B allele frequency between HIV-1 uninfected and people living with HIV-1 (PLWH), we aimed to demonstrate the correlation between the presence of these alleles in PLWH and the CD4⁺ T cell count and viral replication levels.

MATERIAL AND METHODS

Subjects

HIV-1 infected diagnosed patients over ≥ 18 years of age who applied to Istanbul University-Cerrahpasa Hospital Infectious Diseases Clinic were included in the study. This PLWH-control comparative study included 412 HIV-1-positive patients with confirmed HIV infection based on clinical and laboratory data. The study included 406 bone marrow and kidney donors who

were not diagnosed with HIV-1 infection as a healthy control group. Patients' age, gender, HLA-B*57:01, CD4⁺ T lymphocyte and HIV RNA results were evaluated retrospectively through the patient files and hospital automation system. In our study, the data on CD4⁺ T cell counts and HIV RNA levels measured at the time of the initial clinic visit were considered.

Ethical approval for this study was obtained by the Clinical Research Ethics Committee of İstanbul University-Cerrahpaşa, Cerrahpasa Medical Faculty (1154166/12.11.2024). The study was conducted at the HLA Tissue Typing Laboratory of Istanbul University-Cerrahpasa Hospital, which holds accreditation from the Ministry of Health. Our laboratory routinely undergoes external quality control assessments every year through the Balkan External Qualification Testing Program.

HLA B genotyping

Peripheral whole blood samples from both the patient and control group donors were collected in EDTA-coated tubes. Genomic DNA was purified using QIAGEN DNA isolation kits on the EZ1 advanced XL magnetic bead-based workstation (Qiagen, Hilden, Germany). The LIFECODES HLA-B eRES SSO Typing Kit (IMMUCOR, Stanford, CT, USA) targeting the antigen-binding region located in exons 2 and 3 of the HLA B gene sequence was used to determine specific genetic variations in genomic DNA. The HLA-B*57:01 Typing Kit (Olerup SSP AB, Sweden) was used to determine the HLA-B*57:01 alleles in patients. We identified specific HLA B alleles using PCR-SSO (Polymerase Chain Reaction-Sequence Specific Oligonucleotide Probe) and performed the analysis using Luminex technology (Luminex Corporation, Austin, USA).

Statistical analysis

Data belonging to the patients and control group donors were obtained from the hospital automation system. Data were analysed using IBM SPSS Statistics version 25.0 for Windows (SPSS, Chicago, USA). To compare the data, Pearson's chi-square test was employed, and the association between patients and control group donors was assessed through 95% confidence intervals (CI) and odds ratios (OR). Variables detected as statistically significant as $p<0.05$ were considered significant.

RESULTS

The demographic data and HLA-B*57:01 allele frequencies of 412 PLWH and 407 healthy control donors are presented in Table 1. Because the HLA alleles show a codominant inheritance pattern, the HLA-B allele frequencies of HIV-1 infected patients and healthy control donors are evaluated as "2n" level in Tables 2 and 3. The HLA-B alleles were characterised at a two-digit resolution, while the HLA-B*57 alleles were characterised at a four-digit resolution. We found that the HLA-B*07, *18, *35, *44, and *51 alleles occurred at a frequency greater than 5% in the patient group, (5.3%, 6.6%, 20.1%, 6.6% and 14.6%, respectively). In the group of healthy control donors, the HLA-B alleles that occurred at frequencies higher than 5% included *18, *35, *44, *49, and *51 (5.9%, 18.4%, 6.9%, 5.5%, and 16.16%, respectively). In PLWH, the frequency of the HLA-B*57 allele was observed to be lower than that in the control group (OR: 3.099 [95% CI: 1.310–7.330], p = 0.007), as indicated in Table 2. All seven patients with the HLA-B*57 allele were identified as positive for the HLA-B*57:01 variant, which was tested to assess their sensitivity to abacavir, a drug known to inhibit reverse transcriptase and block HIV replication. HLA-B*57:01 allele positivity was determined as 1.6%. The association between the patients' HLA-B alleles and their VL or CD4 count at the initial assessment was also investigated. As shown in Table 4, all patients with HLA-B*57:01 allele positivity had VL <100,000 copies/ml [p=0.063]. However, as indicated in Table 3, the CD4⁺ T cell counts were variable. The 51 AIDS patients had CD4⁺ T cell counts <200 cells/mm³ and their VL were >10⁶. HLA-B*35 was observed at the highest frequency in these patients.

Table 1. Gender and age distribution of the study group and HLA-B*57:01 positivity

Study group	n(%)	Mean age±SD	HLA-B*57:01 positivity
HIV⁺ male	376(91.3)	36.21±11.71	7(100%)
HIV⁺ female	36(8.7)	41.89±11.32	0(0)
Total HIV+	412(100)	36.71±11.77	7(100%)
HC male	211(52)	50.06±14.27	-
HC female	195(48)	47.35±13.96	-
Total HC	406(100)	48.76±14.17	-

HIV: Human Immunodeficiency Virus, HC: Healthy Control

Table 2. Distribution of the HLA-B alleles

Genotype	HIV (2n=824) (2n=824)	Control (2n=812)	Statistical analysis	
HLA B*	n-AF (%)	n-AF (%)	OR-(95% CI)	p
07	44(5.3)	37(4.6)	0.84(0.54-1.32)	0.465
08	23(2.8)	21(2.6)	0.92(0.50-1.68)	0.798
13	18(2.2)	26(3.2)	1.481(0.80-2.72)	0.203
14	21(25)	21(2.6)	1.01(0.55-1.87)	0.962
15	33(4.0)	27(3.3)	0.82(0.49-1.38)	0.465
18	54(6.6)	48(5.9)	0.89(0.60-1.33)	0.591
27	23(2.8)	25(3.1)	1.10(0.62-1.96)	0.730
35	166(20.1)	135(18.4)	0.79(0.61-1.01)	0.066
37	9(1.1)	10(1.2)	1.12(0.45-2.79)	0.793

Genotype	HIV (2n=824) (2n=824)	Control (2n=812)	Statistical analysis	
38	32(3.9)	26(3.2)	0.81(0.48-1.38)	0.456
39	13(1.6)	11(1.4)	0.85(0.38-1.92)	0.708
40	38(4.6)	34(4.2)	0.90(0.56-1.45)	0.676
41	16(1.9)	19(2.3)	1.21(0.61-2.37)	0.578
42	1(0.1)	2(0.2)	2.03(0.18-22.45)	0.555
44	54(6.6)	56(6.9)	1.05(0.71-1.55)	0.782
45	1(0.1)	2(0.2)	2.03(0.18-22.45)	0.555
46	1(0.1)	2(0.2)	2.03(0.18-22.45)	0.555
47	1(0.1)	0(0)	-	-
48	4(0.5)	4(0.5)	1.01(0.25-4.07)	0.983
49	30(3.6)	45(5.5)	1.55(0.96-2.49)	0.066
50	30(3.6)	30(3.7)	1.01(0.60-1.70)	0.954
51	120(14.6)	135(16.6)	1.17(0.89-1.52)	0.250
52	25(3.0)	25(3.1)	1.01(0.57-1.78)	0.958
53	6(0.7)	7(0.9)	1.18(0.39-3.54)	0.760
54	2(0.2)	1(0.1)	0.50(0.04-5.60)	0.572
55	31(3.8)	22(2.7)	0.71(0.40-1.24)	0.229
56	1(0.1)	1(0.1)	1.01(0.06-16.25)	0.992
57	**7(0.8)**	**21(2.6)**	**3.09(1.31-7.33)**	**0.007**
58	17(2.1)	19(2.3)	1.13(0.58-2.20)	0.703
78	2(0.2)	0(0)	-	-
81	1(0.1)	0(0)	-	-

HLA: Human leukocyte antigen, HIV: Human immunodeficiency virus, HC: Healthy control, AF: Allele frequency, OR: Odds ratio, CI: Confidence interval, 2n: represents the presence of 2 HLA alleles

Patients with the HLA-B*07 and HLA-B*35 alleles exhibited lower CD4+ T cell counts (cells/mm³) and higher HIV RNA levels (copies/mL). This relationship was found to be statistically significant (for CD4+T cell count (cell/mm³) [p=0.026], [p=0.044] and for HIV-1 RNA levels (copies/mL) [p=0.041], [p=0.054] respectively). Although statistically significant, the HLA-B*45 and *54 alleles were excluded from the evaluation due to insufficient sample size.

DISCUSSION

The dynamic of HLA molecules that present antigens to the immune system determines how the immune system responds to viruses. The effect of this dynamic is seen in both cell-mediated and antibody-mediated responses, which aim to eliminate viral infection most effectively. On account of this, HLA molecules play a crucial role in shaping the host's adaptive immune responses and have a significant impact on the advancement of HIV-1 infection (20).

Variations in HLA alleles result from differences in how the immune system displays the HIV-1 virus, which also causes differences in disease outcomes (20). The presence of specific HLA alleles that generate a stronger immune response against HIV-1 results in a more gradual disease advancement. The first large population study on this subject demonstrated that the HLA-B*27 and B*57 alleles have the strongest impact on slowing the disease progression and are associated with a slower transition to AIDS (11).

Table 3. CD4+T cell distribution of HLA-B alleles in patients

Genotype		CD4+T cell (cell/mm³)				Genotype		CD4+T cell (cell/mm³)			
HLA B*		<350 n(%)	350-500 n(%)	>500 n(%)	p	HLA B*		<350 n(%)	350-500 n(%)	>500 n(%)	p
07	**0**	**227(29.1)**	**131(16.8)**	**422(54.1)**	**0.026**	46	0	248(30.1)	138(16.8)	437(53.1)	0.531
	1	**21(47.7)**	**7(15.9)**	**16(36.4)**			1	0(0)	0(0)	1(100.0)	
08	0	243(30.)3	135(16.9)	423(52.8)	0.499	47	0	248(30.1)	138(16.8)	437(53.1)	0.643
	1	5(21.7)	3(13.0)	15(65.2)			1	0(0)	0(0)	1(100.0)	
13	0	242(30.0)	136(16.9)	428(53.1)	0.805	48	0	246(30.0)	137(16.7)	437(53.3)	0.524
	1	6(33.3)	2(11.1)	10(55.6)			1	2(50.0)	1(25.0)	1(25.0)	
14	0	244(30.4)	134(16.7)	425(52.9)	0.535	49	0	235(29.6))	133(16.8)	426(53.7)	0.243
	1	4(19.0)	4(19.0)	13(61.9)			1	13(43.3)	5(16.7)	12(40.0)	
15	0	238(30.0)	134(16.9)	422(53.1)	0.850	50	0	238(30.0)	133(16.8)	423(53.3)	0.920
	1	10(33.3)	4(13.3)	16(53.3)			1	10(33.3)	5(16.7)	15(50.0)	
18	0	234(30.4)	132(17.1)	404(52.5)	0.290	51	0	214(30.4)	121(17.2)	369(52.4)	0.548
	1	14(25.9)	6(11.1)	34(63.0)			1	34(28.3)	17(14.2)	69(57.5)	
27	0	245(30.6)	134(16.7)	422(52.7)	0.175	52	0	243(30.4)	132(16.5)	424(53.1)	0.423
	1	3(13.0)	4(17.4)	16(69.6)			1	5(20.0)	6(24.0)	14(56.0)	
35	**0**	**190(28.9)**	**104(15.8)**	**364(55.3)**	**0.044**	53	0	247(30.2)	138(16.9)	433(52.9)	0.300

Genotype		CD4⁺T cell (cell/mm³)				Genotype		CD4⁺T cell (cell/mm³)			
HLA B*		<350 n(%)	350-500 n(%)	>500 n(%)	p	HLA B*		<350 n(%)	350-500 n(%)	>500 n(%)	p
	1	**58(34.9)**	**34(20.5)**	**74(44.6)**			1	1(16.7)	0(0)	5(83.3)	
37	0	244(29.9)	136(16.7)	435(53.4)	0.482	54	0	247(30.0)	137(16.7)	438(53.3)	0.265
	1	4(44.4)	2(22.2)	3(33.3)			1	1(50.0)	1(50.0)	0(0)	
38	0	241(30.4)	131(16.5)	420(53.0)	0.514	55	0	238(30.0)	134(16.9)	421(53.1)	0.839
	1	7(21.9)	7(21.9)	18(56.3)			1	10(32.3))	4(12.9)	17(54.8)	
39	0	243(30.0)	136(16.8)	432(53.3)	0.801	56	0	248(30.1)	138(16.8)	437(53.1)	0.643
	1	5(38.5)	2(15.4)	6(46.2)			1	0(0)	0(0)	1(100.0)	
40	0	239(30.4)	133(16.9)	414(52.7)	0.449	57	0	246(30.1)	137(16.8)	434(53.1)	0.974
	1	9(23.7)	5(13.2)	24(63.2)			1	2(28.6)	1(14.3)	4(57.1)	
41	0	242((30.0)	134(16.6)	432(53.5)	0.425	58	0	241(29.9)	135(16.7)	431(53.4)	0.553
	1	6(37.5)	4(25.0)	6(37.5)			1	7(41.2)	3(17.6)	7(41.2)	
42	0	248(30.1)	138(16.8)	437(53.1)	0.643	78	0	248((30.2)	138(16.8)	436(53.0)	0.413
	1	0(0)	0(0)	1(100.0))			1	0(0)	0(0)	2(100.0)	
44	0	238(30.9)	128(16.6)	404(52.5)	0.155	81	0	248(30.1)	137(16.6)	438(53.2)	0.083
	1	10(18.5)	10(18.5)	34(63.0)			1	0(0)	1(100)	0(0)	
45	0	248(30.1)	138(16.8)	437(53.1)	0.643						
	1	0(0)	0(0)	1(100.0)							

Table 4. Viral load distribution of HLA-B alleles in patients

Genotype		HIV RNA (copies/mm³)				Genotype		HIV RNA (copies/mm³)			
HLA B*		$<10^5$ n(%)	10^5–10^6 n(%)	$>10^6$ n(%)	p	HLA B*		$<10^5$ n(%)	10^5–10^6 n(%)	$>10^6$ n(%)	p
07	**0**	**443(56.8)**	**240(30.8)**	**97(12.4)**	**0.041**	46	0	461(56.0)	254(30.9)	108(13.1)	0.676
	1	**19(43.2)**	**14(31.8)**	**11(25.0)**			1	1(100)	0(0)	0(0)	
08	0	448(55.9)	248(31.0)	105(13.1)	0.874	47	0	461(56.0)	254(30.9)	108(13.1)	0.676
	1	14(60.9)	6(26.1)	3(13.0)			1	1(100.0)	0(0)	0(0)	
13	0	454(56.3)	246(30.5)	106(13.2)	0.448	48	0	460(56.1)	252(30.7)	108(13.2)	0.597
	1	8(44.4)	8(44.4)	2(11.1)			1	2(50.0)	2(50.0)	0(0)	
14	0	446	251	106(13.2)	0.159	49	0	449(56.5)	241(30.4)	104(13.1)	0.289
	1	16(11.8)	3(6.5)	2(2.8)			1	13(43.3)	13(43.3)	4(13.3)	
15	0	446(56.2)	247(31.1)	101(12.7)	0.213	50	0	447(56.3)	243(30.6)	104(13.1)	0.759
	1	16(53.3)	7(23.3)	7(23.3)			1	15(50.0)	11(36.7)	4(13.3)	
18	0	425(55.2)	244(31.7)	101(13.1)	0.109	51	0	396(56.3)	216(30.7)	92(13.1)	0.967
	1	37(68.5)	10(18.5)	7(13.0)			1	66(56.3)	38(31.7)	16(13.3)	
27	0	446(55.7)	249(31.1)	106(13.2)	0.416	52	0	450(56.3)	244(30.5)	105(13.1)	0.598
	1	16(69.6)	5(21.7)	2(8.7)			1	12(48.0)	10(40.0)	3(12.0)	
35	**0**	**377(57.3)**	**204(31.0)**	**77(11.7)**	**0.054**	53	0	458(56.0)	253(30.9)	107(13.1)	0.751
	1	**85(51.2)**	**50(30.1)**	**31(18.7)**			1	4(66.7)	1(16.7)	1(16.7)	
37	0	456(56.0)	253(31.0)	106(13.0)	0.386	**54**	**0**	**462(56.2)**	**254(30.9)**	**106(12.9)**	**0.001**
	1	6(66.7)	1(11.1)	2(22.2)			**1**	**0(0)**	**0(0)**	**2(100.0)**	
38	0	444(56.1)	241(30.4)	107(13.5)	0.168	55	0	444(56.0)	243(30.6)	106(13.4)	0.512
	1	18(56.3)	13(40.6)	1(4.2)			1	18(58.1)	11(35.5)	2(6.5)	
39	0	456(56.2)	247(30.5)	108(13.2)	0.120	56	0	461(56.0)	254(30.9)	108(13.1)	0.676

Genotype		HIV RNA (copies/mm³)				Genotype		HIV RNA (copies/mm³)			
HLA B*		<10⁵ n(%)	10⁵–10⁶ n(%)	>10⁶ n(%)	p	HLA B*		<10⁵ n(%)	10⁵–10⁶ n(%)	>10⁶ n(%)	p
	1	6(46.2)	7(53.8)	0(0)			1	1(100.0)	0(0)	0(0)	
40	0	440(56.0)	240(30.5)	106(13.5)	0.307	**57**	**0**	**455(55.7)**	**254(31.1)**	**108(13.2)**	**0.063**
	1	22(57.9)	14(36.8)	2(5.3)			**1**	**7(100.0)**	**0(0)**	**0(0)**	
41	0	453(56.1)	247(30.6)	108(13.4)	0.221	58	0	453(56.1)	247(30.6)	107(13.3)	0.516
	1	9(56.3)	7(43.8)	0(0)			1	9(52.9)	7(41.2)	1(5.9)	
42	0	461(56.0)	254(30.9)	108(13.1)	0.676	78	0	462(56.2)	252(30.7)	108(13.1)	0.393
	1	1(100.0)	0(0)	0(0)			1	0(0)	2(100.0)	0(0)	
44	0	425(55.2)	241(31.3)	104(13.5)	0.143	81	0	462(56.1)	253(30.7)	108(13.1)	0.325
	1	37(68.5)	13(24.1)	4(7.4)			1	0(0)	1(100.0)	0(0)	
45	**0**	**462(56.1)**	**254(30.9)**	**107(13.0)**	**0.036**						
	1	**0(0)**	**0(0)**	**1(100.0)**							

What distinguishes these two alleles from others is their carrying of the Bw4 epitope, the ligand for the NK cell receptor KIR3DL1, and their mechanism that delays the adaptation of HIV-1 to the host HLA, which is associated with a slower progression to AIDS. Patients expressing these alleles tended to have lower viral loads and remained healthy for longer periods of time before progressing to AIDS.

Subsequent research has likewise confirmed a connection between these HLA-B alleles and the progression of HIV-1 disease (10, 18, 21-23). A recent study uncovered multiple protein layers that play a key role in the HIV-1 elite controller phenotype of HLA-B*57:01/B*57:03 alleles (24). Kawashima et al. showed that the HLA-B*51:01 allele has a differential effect on disease outcome depending on geographic and evolutionary factors (25). The initial protective effect of this allele in HIV B clade infection has diminished over time with the emergence of mutant HIV-1 strains, but it has been found to be associated with disease susceptibility in HIV C clade infection (16). Similarly, the HLA-B*35:01 allele exhibits variability in its association with disease susceptibility or protection depending on the population or clade being studied (18, 19, 26). Ngumbella et al. demonstrated that those expressing the HLA-B*58:01 allele provided more effective viremia control, whereas those expressing the HLA-B*58:02 allele had a poorer prognosis. These two variations of the HLA gene differ by only three amino acids in the part of the protein responsible for binding peptides important for immune system function. They also observed that patients with the HLA-B*58:02 allele exhibited lower CD4⁺ T cell counts and higher VL (27). Fellay et al. also identified significant associations between HLA-B and HIV-1 VL and progression to AIDS (28). Similarly, a study conducted in 2015 on 6,300 HIV-1 infected patients reported that the HLA molecule had a significant effect on viral load and disease advancement (29). Pelak et al. showed that the HLA-B*57:03 allele influenced the viral load change. Their study confirmed that the HLAB* 57:03 allele was the most important common variant affecting viral load change in African Americans, consistent with the HLA-B*57:01 allele being the most important common variant observed in individuals of European descent (30). These individuals tended to have lower viral loads and remained healthier for a longer period before progressing to AIDS. A study of 1.318 individuals in Thailand showed that the HLA-B*46:01 allele carries the epitope for the NK cell inhibitory receptor and is linked to faster disease advancement and a decrease in the absolute CD4+ T cell count (20). Zhang et al. demonstrated that the rare protective allele, HLA-B*67:01, is particularly effective in controlling infection among Japanese patients, resulting in a lower viral load and higher CD4⁺ T cell count (31).

While the HLA-B*57:01 allele offers a protective effect, it also confers an increased risk of hypersensitivity to abacavir. Kolou et al. found that the frequency of HLA-B*57:01 was 0.1% in West and Central Africa (32). Small et al. found the frequency of HLA-B*57:01 to be higher (3.4%) in Europeans than in Africans and Americans (33). In a study conducted in Northern Poland, the frequency of the HLA-B*57:01 allele was reported to be 5.8%, whereas a separate study in Brazil observed a significantly higher frequence of 19.9% (34, 35).

In the first study conducted in Turkey, the frequency of HLA-B*57:01 in HIV-1 infected patients was determined as 3.0% (36). Toplu et al. reported that they could not detect HLA-B*57.01 in any of the 47 HIV-positive patients (37). Darbas et al. reported the HLA-B*57:01 allele frequency as 1.2%, and Büyüktuna et al. reported it as 3.6% (38, 39). In our recent multicenter study involving 867 PLWH, the frequency of the HLA-B*57:01 allele was reported to be 1.6% (40). The prevalence of this allele in

our current study was consistent with that in our other study. Moreover, the HLA-B*57 allele was higher in the healthy control donors, indicating that it may be significant when compared with HIV-1-infected patients. The HLA-B*27 allele was similar in both groups and was not statistically significant.

As we have previously noted, GWAS (Genome-wide association studies) have consistently revealed that variation in the HLA-B alleles is the most crucial host-dependent determinant of HIV-1 VL and disease progression (28, 30, 41-43). In our current study, which is the first study conducted in our country, we found that patients with HLA-B*07 and *35 alleles had higher VL and lower CD4+T cell counts. As stated in previous studies, it confirms the idea that the HLA-B*07 and *35 alleles are associated with faster progression of the disease. In addition, all patients with the HLA-B*57 allele had low viral loads. All of these patients had the HLA-B*57:01 allele, which also confirms the idea that the HLA-B*57:01 allele is linked to the slow progression of the disease. Although the HLA-B*45 and *54 alleles were found to be statistically significant, they were excluded from the evaluation because of the limited sample size.

In summary, our study correlates with the literature. The limitation of our study is that the HLA B alleles were studied as two digits. In addition, patients were not evaluated for the presence of co-infections or malignancies that could affect the results of CD4+T cell counts.

CONCLUSION

These findings strongly imply the involvement of other molecular mechanisms, extending beyond the traditional role of class I HLA molecules in antigen presentation. Research into how HLA B alleles influence HIV-1 infection and disease progression will help us understand who is more susceptible to HIV-1 and how the disease will evolve in different individuals. This information is crucial for developing effective treatments and vaccines and for understanding how human genetic factors affect the spread of HIV in populations. Further research is essential on this factor, as it holds significant implications for the ongoing, years-long pursuit of an effective HIV vaccine.

Ethics Committee Approval	This study was approved by İstanbul University-Cerrahpaşa Ethics Committee (Date: 12.11.2024, No: 1154166).
Informed Consent	Written informed consent was obtained from all the participants of the study.
Peer Review	Externally peer-reviewed.
Author Contributions	Conception/Design of Study- M.D., E.Y., F.S.O.; Data Acquisition- M.D., S.Y.K., B.M.; Data Analysis/Interpretation- M.D., E.Y., H.Ş.Ç., F.S.O.; Drafting Manuscript- M.D., E.Y., H.Ş.Ç., F.S.O.; Critical Revision of Manuscript- M.D., E.Y., H.Ş.Ç., F.S.O.; Final Approval and Accountability- M.D., E.Y., H.Ş.Ç., F.S.O.
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Research Article

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The Role of DHRS2 in the Regulation of BRCA1 Expression in Non-small Cell Lung Cancer

DHRS2'nin Küçük Hücre Dışı Akciğer Kanserinde BRCA1 Ekspresyonunun Düzenlenmesindeki Rolü

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Abstract

Objective: Non-small cell lung cancer (NSCLC) is a leading cause of cancer mortality and is often diagnosed late. *DHRS2* regulates lipid metabolism, hormones, and oxidative stress, acting as a tumour suppressor by stabilising p53 and inhibiting *MDM2*. *Tp53* and *BRCA1* are crucial for DNA repair and tumour suppression, with *BRCA1* as a prognostic marker in NSCLC. However, the interaction between *DHRS2* and *BRCA1* in NSCLC remains unclear. This study aimed to examine how *DHRS2* expression influences *BRCA1* levels in NSCLC cells, providing insight into potential therapeutic targets.

Material and Methods: *DHRS2* overexpression was induced in NSCLC cells (A549, H1299) and normal bronchial epithelial cells (BEAS-2B) using an expression vector and confirmed by quantitative PCR (qPCR). The effect of *DHRS2* overexpression on *BRCA1* expression levels was examined. In addition, *BRCA1* expression levels in NSCLC subtypes were analysed using GEPIA2, while genomic alterations in *BRCA1* and *DHRS2* were investigated via cBioPortal-TCGA.

Results: *DHRS2* overexpression led to a decrease *in BRCA1* expression in A549 cells (p53 wild-type) but had no effect in H1299 cells (p53-null). In BEAS-2B cells, *DHRS2* overexpression also resulted in *BRCA1* suppression. GEPIA2 analysis showed significantly higher *BRCA1* expression in tumour tissues. Genomic analysis revealed frequent *BRCA1* alterations, indicating instability, whereas *DHRS2* had fewer mutations, implying a regulatory role.

Conclusion: This study demonstrates that *DHRS2* modulates *BRCA1* expression through a p53-dependent mechanism. Genetic alterations in *BRCA1* and *DHRS2* indicate their potential involvement in NSCLC tumorigenesis. Future studies should further investigate the mechanisms mediated by *DHRS2* and p53 to elucidate their roles in cancer progression.

Keywords *DHRS2* · *BRCA1* · non-small cell lung cancer · DNA damage response

Öz

Amaç: Küçük hücre dışı akciğer kanseri (KHDAK), kansere bağlı ölümlerin önde gelen nedenlerinden biridir ve genellikle ileri evrede teşhis edilir. *DHRS2*, lipid metabolizması, hormon regülasyonu ve oksidatif stresle ilişkili olup, p53 stabilizasyonu ve *MDM2* inhibisyonu yoluyla kanser progresyonunu etkileyen önemli bir tümör baskılayıcıdır. *TP53* ve *BRCA1*, DNA hasar yanıtı ve tümör baskılama süreçlerinde kritik roller oynarken, *BRCA1* aynı zamanda KHDAK'de prognostik bir belirteç olarak öne çıkmaktadır. Bu çalışma, KHDAK hücrelerinde *DHRS2* ekspresyon düzeyleri ile *BRCA1* arasındaki etkileşimleri inceleyerek, moleküler mekanizmalarını daha iyi anlamayı ve yeni terapötik hedefler ile tedavi stratejileri geliştirmeyi amaçlamaktadır.

Gereç ve Yöntemler: Bu çalışmada ekspresyon vektörü kullanılarak akciğer kanser hücre hatları (A549, H1299) ve normal bronş epitel hücre hattında (BEAS-2B), *DHRS2* aşırı ekspresyonu sağlanmış ve quantitative PCR (qPCR) ile doğrulanmıştır. Ardından, *DHRS2* aşırı ekspresyonunun *BRCA1* ekspresyon seviyeleri üzerindeki etkisi incelenmiştir. Ayrıca KHDAK'nin alt tiplerinde *BRCA1* ekspresyon düzeyleri GEPIA2, *BRCA1* ve *DHRS2* genlerindeki genomik değişiklikler ise cBioPortal-TCGA ile incelenmiştir.

Bulgular: *DHRS2*'nin aşırı ekspresyonu, A549 hücrelerinde (p53 wild type) *BRCA1* ekspresyonunu azaltırken, H1299 hücrelerinde (p53 null) herhangi bir değişikliğe neden olmamaktadır. BEAS-2B hücre hattında ise *DHRS2*'nin aşırı ekspresyonu *BRCA1* ekspresyonunu baskılamaktadır. GEPIA2 verileri, *BRCA1*'in tümör dokularında normal dokulara kıyasla belirgin şekilde arttığını göstermektedir. Genomik değişiklik analizi, *BRCA1*'deki amplifikasyonlar ve delesyonlar, genomik instabiliteyi işaret ederken, *DHRS2*'nin daha az mutasyon sergilemesi, düzenleyici rolünü ortaya koymaktadır.

Sonuç: Elde ettiğimiz bulgular *DHRS2*'nin *BRCA1* ekspresyonunu modüle edebileceğini ve p53'ün bu süreçte kritik rol oynayabileceğini göstermektedir. *BRCA1* ve *DHRS2*'deki genetik değişiklikler, KHDAK tümörigenezinde etkili olduğunu düşündürmektedir, gelecekteki çalışmalarla *DHRS2* ve p53 aracılı mekanizmaları daha ayrıntılı incelemelidir.

Anahtar Kelimeler *DHRS2* · *BRCA1* · Küçük hücreli dışı akciğer kanseri · DNA hasar yanıtı

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INTRODUCTION

Lung cancer is one of the most common types of cancer, accounting for approximately 26% of cancer-related deaths worldwide (1). Non-small cell lung cancer (NSCLC) accounts for 80% of lung cancer, and NSCLC is divided into lung squamous cell carcinoma (LUSC) and lung adenocarcinoma (LUAD). NSCLC is a group of diseases that exhibit genetic and cellular heterogeneity, but early diagnosis is difficult as there are usually no obvious symptoms in the early stages. NSCLC follows an aggressive course, often leading patients to be diagnosed with locally advanced or metastatic disease. While surgical intervention is preferred in early-stage patients, multimodal treatment modalities such as radiotherapy and chemotherapy combination are used in advanced-stage patients (2).

The Short-Chain Dehydrogenase/Reductase (SDR) superfamily plays important roles in the metabolism of lipids, amino acids, carbohydrates, steroid hormones, and xenobiotics and participates in redox sensing mechanisms. *DHRS2*, a member of the SDR family, has a structural domain that interacts with the coenzyme NADP. The organelle localisation of *DHRS2* protein is mainly in the cytoplasm, nucleus, and mitochondria. When it is in the nucleus, it is found mainly in the karyoteca (3). The highest levels of *DHRS2* are found in the parotid gland and ovary, and relatively low expressions are found in the liver, placenta, mammary glands, and testis (4). As an NADPH-dependent carbonyl reductase, *DHRS2* reduces dicarbonyl compounds and protects cells against carbonyl cytotoxicity. *DHRS2*, which plays an important role in lipid metabolism and hormone regulation, is also associated with mitochondrial oxidative stress and protects cells against oxidative damage. It contributes to cancer progression, particularly through p53 stabilisation and *MDM2* inhibition. The high expression of *DHRS2* may increase sensitivity to treatment in some types of cancer (e.g. Hodgkin's lymphoma, gastric cancer and colorectal cancer) and shows a protective function against oxidative stress. Furthermore, the inhibition of *DHRS2* offers an approach to combat resistance in cancer therapy. It is important to study the regulatory mechanisms of *DHRS2* to better understand its potential in cancer therapy. Although *DHRS2* has become prominent primarily for its tumour suppressor functions, its regulatory role in DNA repair processes and these mechanisms are not yet fully understood, and the function of *DHRS2* in cancer biology stands out as a research area that needs to be examined in more depth (5).

The p53 protein is a crucial tumour suppressor that responds to DNA damage by regulating genes involved in the DNA damage response (DDR) (6). While *BRCA1* was previously associated with breast and ovarian cancer, it has recently gained interest as a prognostic and predictive marker in other tumours like NSCLC. *BRCA1* is recognised as a multifunctional tumour suppressor protein that plays an essential role in a range of critical cellular processes, including cell cycle regulation, DNA replication, mitotic spindle assembly, transcriptional control, and the higher-order organisation of chromatin (7). Additionally, *BRCA1* is integral to the DDR and apoptosis. Beyond its tumour-suppressive functions, *BRCA1* also modulates cellular responses to cytotoxic chemotherapy. Therefore, *BRCA1* is being investigated as a potential predictive marker in NSCLC treatment (8).

This study aimed to investigate possible interactions between *DHRS2* expression levels and *BRCA1*. To determine the effects of the *DHRS2* gene on NSCLC and the associated molecular mechanisms, *BRCA1* expression levels were examined in one non-cancerous human lung epithelial cell line (BEAS-2B) and two NSCLC cell lines [A549 (p53 wild type) and H1299 (p53 deficiency)] which were induced with the *DHRS2* expression vector. Investigating the interactions between *BRCA1* and *DHRS2* in the context of NSCLC may provide valuable insights into novel therapeutic targets and treatment pathways against this deadly disease.

MATERIAL AND METHODS

Expression of the *BRCA1* gene in NSCLC

Gene expression levels were analysed using the GEPIA2 database. Tumour tissues from LUAD and LUSC patients were compared with corresponding normal tissues. Data were expressed in Transcripts Per Million (TPM) units, and statistically significant differences were assessed using appropriate statistical analyses.

Genomic Alteration Analysis of *BRCA1* and *DHRS2* in the LUAD and LUSC samples

Genomic alterations in *the BRCA1* and *DHRS2* genes in LUAD and LUSC were analysed using the cBioPortal for Cancer Genomics (https://www.cbioportal.org/). Data from the TCGA (The Cancer Genome Atlas) Firehose Legacy and TCGA Nature 2014 datasets were used for this analysis. The genomic alterations, including missense mutations, splice mutations, truncating mutations, amplifications, and deep deletions, were identified and visualised by the OncoPrint tool.

Cell culture

The human non-small cell lung cancer cell lines (A549 and H1299) and the regular bronchial epithelial cell line (BEAS-2B), which were stored in a liquid nitrogen tank (-196°C), were obtained from the Istanbul University Aziz Sancar Institute of

Experimental Medicine. Both the H1299 and BEAS-2B cell lines were cultured in RPMI-1640 medium (Thermo Fisher Gibco, ABD) supplemented with 10% heat-inactivated fetal bovine serum (sourced from South America; Capricorn, Germany) and 1% penicillin-streptomycin (P/S) (Capricorn, Germany). The cells were incubated at 37°C with 5% CO_2. Similarly, A549 cells were grown in DMEM/F12 medium (Thermo Fisher Gibco, ABD) formulated by ATCC, supplemented with 10% FBS and 1% P/S, and incubated at 37°C in a 5% CO_2 ambiance.

Plasmids and Transfection

A commercially purchased expression vector containing the *DHRS2* gene (OriGene, USA) was used. We established three groups: 1. *DHRS2-overexpressing* group, 2. non-target (NT) control group (in which cells were transfected with the same vector but with the gene region removed), and 3. no template control (NTC) group (a group without transfection treatment). Commercially available E. coli strains (DH5α) were used as competent cells to replicate the expression vector. The vectors were transformed into cells according to the protocol and then selected according to antibiotic resistance. After the selected colonies had been grown in LB medium, we isolated the vectors using the Miniprep isolation kit (Invitrogen, ABD). We transfected *DHRS2* containing vector and empty vector into cells separately according to the manufacturer's protocol of Lipofectamine™ 3000 (Invitrogen, ABD) transfection agent. The cells were harvested 24 and 48 h after transfection. RNA was then isolated from these cells for analysis, and the overexpression efficiency was evaluated using qPCR.

Quantitative PCR (qPCR)

Total RNA was extracted using the PureLink® RNA Mini Kit (Invitrogen-Thermo Fisher, ABD). Subsequently, complementary DNA (cDNA) was synthesised from RNA using the High Capacity cDNA Reverse Transcription Kit (Thermo Scientific, Massachusetts, USA), following the manufacturer's protocol. Quantitative PCR was performed in quintuplicate on the LightCycler 480 system using the Bioline SensiFast SYBR® No-Rox kit ((Meridian, Bioline, ABD). QPCR was performed under the following conditions: Fluorescence signals were acquired during pre-denaturation at 95°C for 5 min, followed by amplification at 95°C for 10 s, 64°C for 20 s, and 72°C for 10 s for 45 cycles. The melting curve analysis was then performed at 95°C for 5 s and 65°C for 1 min, with a final hold at 4°C for 30 s. Target gene expression levels were normalised to the internal control gene TATA-binding protein and then calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are provided in Table 1.

Table 1. Primer sequences of the target genes

Gene	Primer Sequence	
	Forward 5'– 3'	Reverse 5'– 3'
DHRS 2	GCT GTC ATC CTG GTC TCT TCC	CTG GAA CCA CGC AGT TTA CC
BRCA1	CCG AAG AGG GGC CAA GAA AT	ACA GAC ACT CGG TAG CAA CG
TBP	ACT TGA CCT AAA GAC CAT TGC AC	CTT GAA GTC CAA GAA CTT AGC TGG

Statistical analysis

Statistical analyses were performed using SPSS version 25.0 (IBM SPSS Corp., Armonk, NY, USA) and GraphPad Prism (version 8.0.2, Boston, USA). The expression levels of the *DHRS2* and *BRCA1* genes were compared using Student's t-test, Mann–Whitney U test, and one-way analysis of variance (ANOVA). A p-value of less than 0.05 was considered statistically significant.

RESULTS

Expression Levels of the *BRCA1* Gene in the LUAD and LUSC Samples

Gene expression analysis using the TCGA dataset in GEPIA revealed that *BRCA1* expression levels were significantly elevated in LUAD and LUSC tumour tissues compared with normal tissues. In both the LUAD and LUSC groups, the gene expression levels were higher in tumour tissues, whereas the normal tissues exhibited lower expression levels (Figure 1).

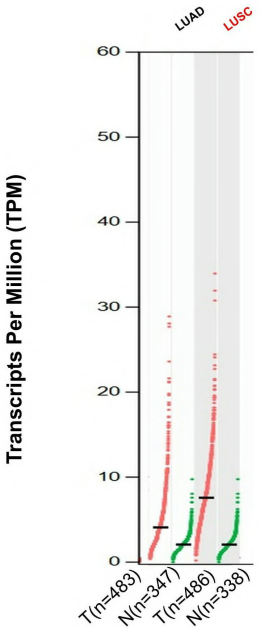

Figure 1. The expression levels of the *BRCA1* gene in the LUAD and LUSC samples and normal tissues in the GEPIA database.

Genomic alteration analysis of the *BRCA1* and *DHRS2* genes in LUAD and LUSC

The genomic alteration analysis revealed distinct mutation patterns in *BRCA1* and *DHRS2* across the LUAD and LUSC samples. *BRCA1* exhibited frequent amplifications (red) and deep deletions (blue), indicating potential genomic instability and its role in tumour progression. Additionally, various mutations, including missense (green), splice (orange), and truncating mutations (yellow), were observed, demonstrating possible functional disruptions. In contrast, *DHRS2* primarily showed amplification events (red), with fewer detected mutations, including some missense mutations (green). Overall, the alteration frequency for both genes was approximately 3% among the analysed samples, highlighting their potential involvement in NSCLC tumorigenesis (Figure 2a). Additionally, Figure 2b demonstrates the differences in the types and frequencies of genetic alterations in the *BRCA1*

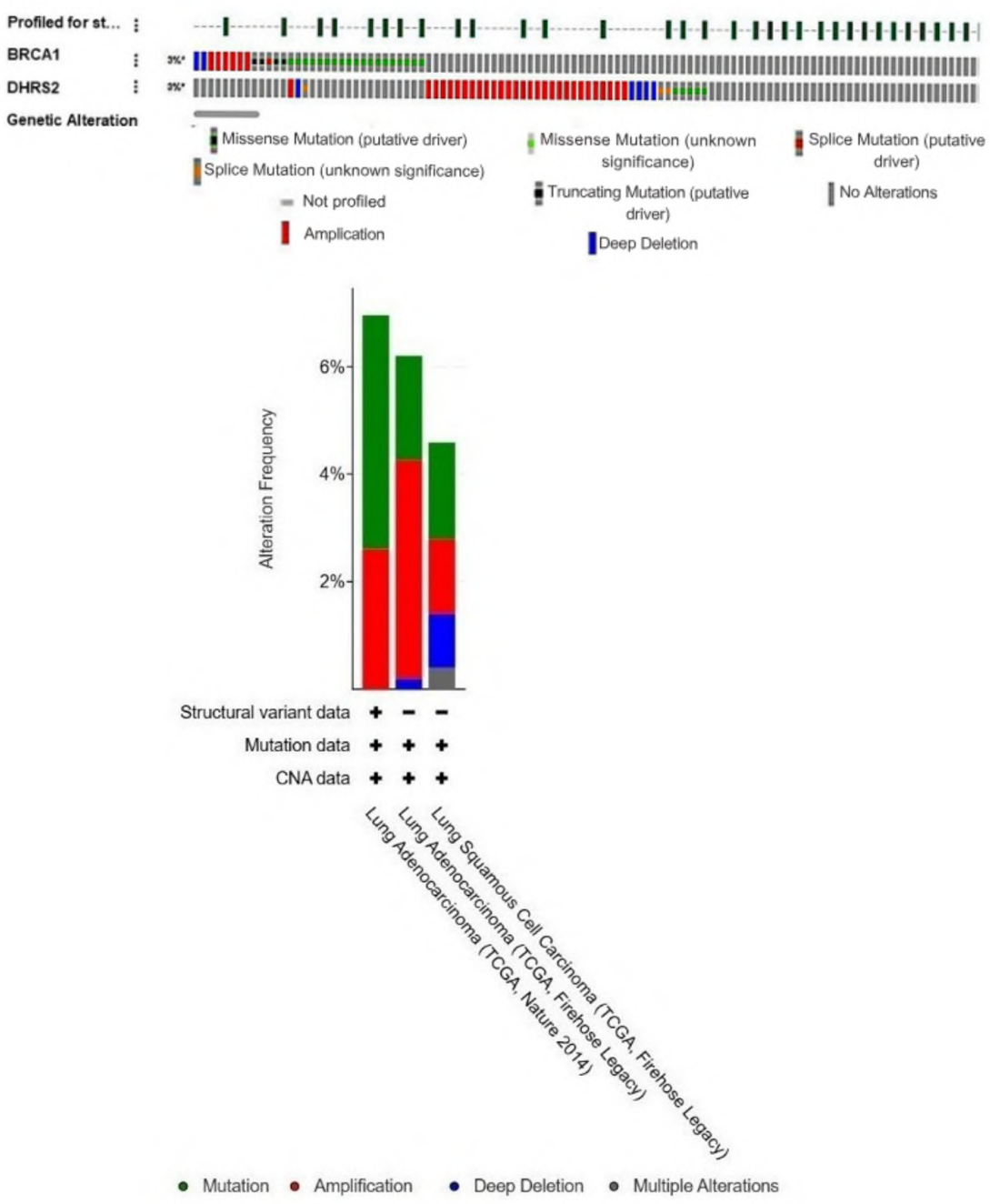

Figure 2. Genomic alterations in the *BRCA1* and *DHRS2* genes in NSCLC analysed by the cBioPortal database. (a) OncoPrint of the gene alterations in these two genes in the LUAD and LUSC cohorts (the different colours indicate different types of genetic alterations, of which amplification accounts for the largest proportion). (b) Details of the gene alteration types in the *BRCA1* and *DHRS2* in the LUAD and LUSC cohorts. CNA, copy number alteration; TCGA, The Cancer Genome Atlas; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma.

and *DHRS2* genes across various LUAD and LUSC databases. According to the results, alterations in these genes were observed in approximately 5% of the total patient population. The most common type of alteration was mutation (green), which was detected in both cancer types. An increase in the copy number (red) was observed in certain patient groups, while in some patients, the *BRCA1* and *DHRS2* genes were completely deleted (blue, deep deletion). The proportion of samples with multiple alterations was low (grey). Furthermore, differences in the rates of genetic alterations were observed between LUAD and LUSC, with *BRCA1* amplifications being more frequent in LUSC (Figure 2b). Together, these results suggest that the genomic alterations of these genes might play an essential role in cancer onset and progression.

DHRS2 Modulates *BRCA1* Expression in Lung Cancer and Normal Bronchial Epithelial Cells

DHRS2 overexpression was performed in A549, H1299 and BEAS-2B cells and confirmed the successful construction of A549 *DHRS2* OE, H1299 *DHRS2* OE and BEAS-2B *DHRS2* OE cell lines by qPCR analysis.

In this study, we evaluated the changes in *BRCA1* expression levels in A549, H1299, and BEAS-2B cell lines overexpressing the *DHRS2* gene (Figure 3). The findings suggest that the effect of *DHRS2* on *BRCA1* regulation is related to the cell type, p53 levels, and transfection method used:

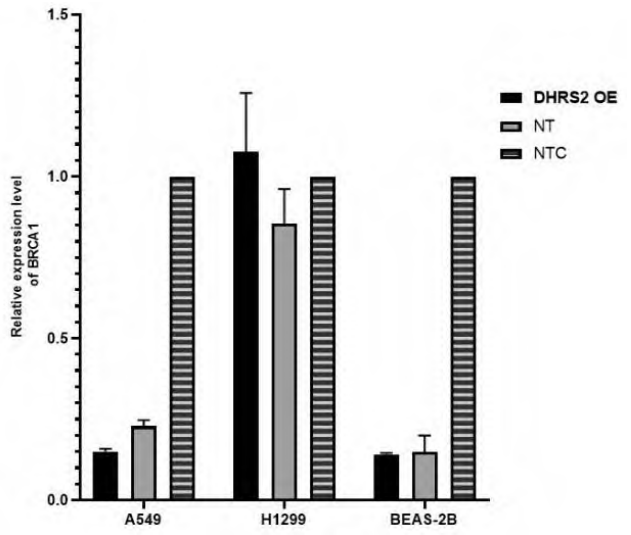

Figure 3. Relative expression level of the *BRCA1* gene in the A549 *DHRS2* OE, H1299 *DHRS2* OE and BEAS-2B *DHRS2* OE, groups ($p<0.001$).

A549 cell line (p53 wild type): *BRCA1* expression was significantly reduced in the *DHRS2* overexpressed group, but the expected difference between the NT and the overexpressed groups was not observed. This suggests that lipofectamine affects *BRCA1* expression.

H1299 cell line (p53 null): *BRCA1* expression was unchanged between the control and DHRS2-overexpressing groups. This result supports that p53 deficiency may abrogate the effect of *DHRS2* on *BRCA1*.

In the BEAS-2B cell line (normal human bronchial epithelial cell): *BRCA1* expression was found at lower levels in the *DHRS2* overexpressing group compared with the control groups. This indicates that *DHRS2* can suppress *BRCA1* expression in healthy cells.

DISCUSSION

Non-small cell lung cancer is one of the most common cancers worldwide and has a high risk of developing metastases at later stages. The prognosis of these patients is generally poor and their response to treatment is limited (2). Therefore, early diagnosis and treatment is of great importance in NSCLC patients.

In recent years, there has been a growing interest in the role of the SDR (dehydrogenase/reductase) enzyme family in the carcinogenesis and progression of cancers. In particular, the *DHRS2* gene is located on chromosome 14q11.2 and the loss of this region is a common chromosomal alteration in various cancers, suggesting that *DHRS2* may be a tumour suppressor gene. However, whether *DHRS2* functions as an oncogene or a tumour suppressor gene varies depending on the specific cancer type (9). Overexpression of *DHRS2* in some cancer types, particularly breast cancer, is associated with poor prognosis. This may increase the invasion of cancer cells and lead to accelerated metastatic processes. The catalytic and non-catalytic effects of *DHRS2* in metabolic processes also play an important role in cancer progression and the development of resistance to treatment. Increased expression of *DHRS2* may enhance the resistance of cancer cells to oxidative stress and inhibit cellular invasion. However, decreased expression of *DHRS2* has also been observed in some cancers. Decreased *DHRS2* expression in oesophageal, ovarian, gastric cancer and leukaemia cells leads to increased oxidative stress, disruption of the cell cycle and inhibition of cancer cell proliferation. This may weaken the tumour suppressor function of *DHRS2* and contribute to developing resistance to therapy (10). Furthermore, *DHRS2* has been reported to be associated with tumour genomic instability, and this mechanism plays a critical role in cancer progression (11). *DHRS2*'s regulation of p53 and inhibition of *MDM2*'s E3 ubiquitinate activity is one of the crucial mechanisms affecting the biological behaviour of cancer cells (9). *DHRS2* increases the stability of p53 by binding with *MDM2*, which contributes to the maintenance of cellular homeostasis. This interaction may lead to attenuation of tumour suppressive

effects, especially in cancer types where the function of p53 is impaired (12). *DHRS2* has NADPH-dependent dicarbonyl reductase activity, which plays a role in neutralising ROS. It has been shown that the NADP/NADPH ratio decreases in cells overexpressing *DHRS2* and increases in cells in which *DHRS2* is inactivated. In parallel, a decrease in mitochondrial ROS levels was observed in cells lacking *DHRS2* expression. Increased ROS levels can promote DNA damage and lead to oxidative stress in the cell. High levels of oxidative stress can cause cell death, whereas low levels of stress activate DNA repair mechanisms, promoting cell growth, migration, and invasion. ROS-induced DNA damage is frequently observed in cancer cells (9).

Interactions between *BRCA1* and p53 also play an important role in cancer development. *BRCA1* plays a critical role in maintaining genetic stability, whereas p53 regulates the cell cycle and apoptosis and exerts tumour suppressive effects. However, p53 dysfunction can lead to impaired *BRCA1* function and increased cellular resistance to DNA damage. This may lead to increased resistance to therapy, especially in sporadic breast cancer (13). The interaction of *DHRS2* with *BRCA1* and p53 may play an important role in tumour suppression. The fact that *DHRS2* regulates the homeostasis of cancer cells by increasing p53 stability may allow further potentiation of its tumour suppressive effects. However, the interactions of *DHRS2* with *BRCA1* and p53 need to be examined in more detail.

The findings indicate that *BRCA1* gene expression is elevated in LUAD and LUSC tumours, indicating its potential role in the tumorigenesis of these lung cancer subtypes. Given *BRCA1*'s established function in DNA repair mechanisms, its involvement in lung cancer pathogenesis should be further explored.

Integrating the GEPIA2 database analysis with our cell culture experiments supports the notion that *BRCA1* plays a crucial role in LUAD and LUSC, while *DHRS2*'s regulatory influence on this mechanism is context-dependent. Our data demonstrate that *BRCA1* and *DHRS2* interactions may serve as potential biomarkers or therapeutic targets in lung cancer, warranting further investigation into their clinical relevance.

Genomic Alterations in *BRCA1* and *DHRS2*

The observed genetic alterations in *BRCA1* and *DHRS2* reveal their potential implications in NSCLC tumorigenesis. Frequent copy number variations, including amplifications and deletions in *BRCA1*, indicate its role in genomic instability and tumour progression. This aligns with previous studies highlighting *BRCA1*'s involvement in DNA damage response and repair pathways. Interestingly, *DHRS2* exhibited fewer mutations, implying that its function in NSCLC may be modulated through regulatory mechanisms rather than direct genetic alterations. Further studies are required to elucidate whether *DHRS2*'s tumour-suppressive role directly influences *BRCA1* expression and how these alterations correlate with patient prognosis and therapeutic responses.

Functional Role of *DHRS2* in *BRCA1* Regulation

Our results show that *DHRS2*'s effect on *BRCA1* regulation may be associated with a p53-dependent mechanism and is highly cell type-specific. In A549 cells (p53 wild-type), *DHRS2* overexpression significantly downregulated *BRCA1* expression, supporting a potential p53-mediated regulatory mechanism. The absence of this effect in H1299 cells (p53-null) reinforces the hypothesis that p53 may be required for *DHRS2*-induced *BRCA1* suppression.

Additionally, the lack of expected differences between the NT and *DHRS2*-overexpressing groups in A549 cells implies that Lipofectamine, the transfection reagent, may independently influence *BRCA1* expression. This highlights the necessity of evaluating transfection methods when studying gene regulation. Future studies should validate *DHRS2*'s precise effect on *BRCA1* using alternative transfection strategies to minimise confounding variables.

Finally, *BRCA1* downregulation in BEAS-2B cells upon *DHRS2* overexpression indicates that *DHRS2* may regulate *BRCA1* even in normal bronchial epithelial cells. This indicates that *DHRS2* exerts regulatory functions in normal and cancerous cells through distinct mechanisms, which may have important implications for tumour biology.

This study highlights the differential genetic alterations in *BRCA1* and *DHRS2* across the LUAD and LUSC subtypes. The observed mutation and copy number variations demonstrate that these genes play key roles in lung cancer pathogenesis. The higher frequency of *BRCA1* amplifications in specific patient cohorts further supports its association with DNA repair processes and tumour-suppressive mechanisms. Meanwhile, the role of *DHRS2* in cancer progression remains to be elucidated, particularly regarding its potential tumour-suppressive or oncogenic functions in different cancer types.

Our findings indicate that *BRCA1* and *DHRS2* alterations may serve as potential biomarkers and therapeutic targets in lung cancer. However, validation in patient cohorts and functional studies is necessary to confirm their clinical utility. Future research should focus on elucidating the interactions between *DHRS2*, *BRCA1*, and p53, as well as optimising experimental designs to eliminate potential technical biases.

Ethics Committee Approval: This study was conducted using a cell line and does not require ethics committee approval.
Peer Review: Externally peer-reviewed.
Author Contributions: Conception/Design of Study- N.A., B.S.Y.; Data Acquisition- V.Z.; Data Analysis/Interpretation- V.Z., N.A., B.S.Y., S.S.E.; Drafting Manuscript- V.Z.; Critical Revision of Manuscript- N.A., B.S.Y., S.S.E.; Final Approval and Accountability- V.Z., N.A., B.S.Y., S.S.E.
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Research Article

Open Access

CDK2 Depletion Impairs Tumour Growth in the 3D Luminal Breast Cancer Model via CRISPR-CAS9 Gene Editing

CDK2 Geninin CRISPR-CAS9 ile Hedeflenmesi 3B Luminal Meme Kanseri Modellerinde Tümöral Büyümeyi Baskılar

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Abstract

Objective: Cyclin-Dependent Kinase 2 (CDK2) is a key regulator of the G1/S transition and genomic stability. While recent studies have linked CDK2 activity to therapy resistance in hormone receptor-positive breast cancer, its functional role under tumour-relevant conditions remains unclear. This study aims to elucidate the context-specific requirement of CDK2 in luminal breast cancer models.

Material and Methods: CDK2 was knocked out in T47D cells using CRISPR-Cas9. Phenotypic effects were evaluated in both 2D monolayer and 3D spheroid cultures. Quantitative PCR validated gene knockout. Colony formation and GFP-based competition assays assessed 2D proliferation, while 3D spheroid size was quantified using ImageJ.

Results: CDK2 depletion resulted in a 40% reduction in 2D colony formation and a 50% decrease in spheroid size. The fitness disadvantage was more pronounced in 3D cultures, suggesting increased dependency on CDK2 in complex microenvironments.

Conclusion: CDK2 plays a critical role in sustaining tumour growth and structural organization, particularly in 3D environments that mimic the tumour microenvironment. These findings highlight CDK2 as a potential therapeutic target in luminal breast cancer, especially in the context of anti-oestrogen resistance.

Keywords CDK2 · Luminal breast cancer · 3D culture · CRISPR-Cas9

Öz

Amaç: Siklin-Bağımlı Kinaz 2 (CDK2), G1/S geçişi ve genomik stabilitenin önemli bir düzenleyicisidir. Son çalışmalar, hormon reseptörü pozitif meme kanserinde CDK2 aktivitesini tedavi direnciyle ilişkilendirse de, tümörle ilişkili koşullarda fonksiyonel rolü net değildir. Bu çalışmanın amacı, luminal meme kanseri modellerinde CDK2'ye özgü bağlama-bağımlı gerekliliği ortaya koymaktır.

Gereç ve Yöntemler: CDK2, CRISPR-Cas9 sistemi kullanılarak T47D hücrelerinde devre dışı bırakıldı. Fenotipik etkiler hem 2D monolayer hem de 3D sferoid kültür modellerinde değerlendirildi. Gen silinmesi kantitatif PCR ile doğrulandı. 2D proliferasyon kolon oluşumu ve GFP-temelli rekabet analizleriyle incelendi. 3D sferoid boyutları ImageJ ile ölçüldü.

Bulgular: CDK2 silinmesi, 2D kolon oluşumunda yaklaşık %40, 3D sferoid boyutunda ise yaklaşık %50 oranında azalmaya neden oldu. Özellikle 3D ortamda CDK2'ye olan bağımlılık daha belirgin hale geldi.

Sonuç: CDK2, özellikle tümör mikroçevresini taklit eden 3D yapılarda tümöral büyüme ve yapısal bütünlüğün korunmasında kritik rol oynamaktadır. Bulgular, CDK2'nin luminal meme kanserinde, özellikle anti-östrojen direnci bağlamında potansiyel bir terapötik hedef olabileceğini göstermektedir.

Anahtar Kelimeler CDK2 · Luminal meme kanseri · 3B hücre kültürü · CRISPR-Cas9

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INTRODUCTION

Cyclin-dependent kinase 2 (CDK2) is a serine/threonine protein kinase that plays a central role in cell cycle progression, particularly at the G1/S transition, where it facilitates DNA replication (1). CDK2 becomes catalytically active through its association with cyclin E and cyclin A, enabling the phosphorylation of key substrates that regulate replication and genome stability, such as Retinoblastoma protein (Rb), members of the minichromosome maintenance (MCM) complex, and the cyclin-dependent kinase inhibitor $p27^{Kip1}$ (2-7).

The activity of CDK2 is tightly regulated by cyclins and CDK inhibitors, including $p21^{Cip1}$ and $p27^{Kip1}$, which bind and suppress CDK2 function to maintain controlled cell proliferation (8, 9). Interestingly, CDK2 can phosphorylate $p27^{Kip1}$, promoting its degradation and contributing to a positive feedback loop that favours cell cycle progression (6).

Although CDK2 is not considered a primary oncogenic driver like CDK4/6, its overexpression has been reported in several malignancies, including bladder, cervical, and breast cancers (4, 10-13). In acute myeloid leukaemia (AML), the HDAC3-AKT-P21-CDK2 signaling pathway's activation is associated with poorer event-free and overall survival (10). Bladder cancer shows increased CDK2 levels alongside reduced expression of the CDK2-targeting microRNA, miR-3619, correlated with advanced tumour stages and grades (11). In breast cancer, higher MTHFD2 levels, which interact with CDK2, are linked to worse overall survival and higher tumour grades (12-14).

In breast cancer, CDK2 has emerged as a potential therapeutic target because of its elevated expression in aggressive tumour subtypes and its interaction with oncogenic pathways. Notably, CDK2-mediated phosphorylation of EZH2 at threonine 416 enhances histone methyltransferase activity, leading to transcriptional repression via H3K27me3—an epigenetic mechanism implicated in cancer progression (15, 16). In luminal breast cancer, CDK2 activity may play a subtype-specific role in sustaining proliferation and contributing to endocrine therapy resistance (17-20). Previous studies have shown that CDK2 is essential for the tumorigenic activity of the low-molecular-weight (LMW) isoform of cyclin E and that its inhibition can reduce the growth of anti-oestrogen-resistant breast cancer cells (21-23).

Understanding cancer development and improving therapeutic strategies increasingly depend on the use of advanced experimental models that better reflect in vivo conditions (24-26). While 2D cultures have long served as standard models for assessing gene function, three-dimensional (3D) systems provide a more physiologically relevant environment that mimics in vivo tumour architecture. These models are critical for uncovering context-specific dependencies that may not be evident in traditional monolayer cultures (27, 28).

In this study, we used CRISPR-Cas9 gene editing system to knock out CDK2 in the luminal breast cancer cell line T47D. By comparing the phenotypes in 2D monolayers and 3D spheroid cultures, we aimed to uncover the context-dependent functional role of CDK2 in breast cancer cell proliferation and structural integrity. Our findings demonstrate that CDK2 is a key regulator of tumour-like growth in complex microenvironments and a potential therapeutic target in hormone-responsive breast cancer.

MATERIAL AND METHODS

Cell Culture

T47D (HTB-133™, ATCC) cell line was cultured with RPMI-1640 (Capricorn Scientific, Germany) supplemented with heat-inactivated 10% FBS (Biowest, France) and 100U/mL penicillin/streptomycin (Biowest, France). The HEK293T (CRL-3216™, ATCC) cell line was cultured with DMEM (Sigma, USA) supplemented with heat-inactivated 10% FBS (Biowest, France) and 100U/mL penicillin/streptomycin (Biowest, France). All cells were cultured at 37°C and 5% CO_2 concentration. To ensure their integrity and prevent contamination, all the lines were subjected to weekly mycoplasma testing.

Cloning of sgRNAs

CDK2 and non-targeting (NT) oligonucleotides were designed (Table 1) and cloned following the protocol recommended by the Zhang laboratory (29, 30). The pLenti Crispr-v2 plasmid (a gift from Feng Zhang (Addgene # 52961, USA) was digested with the enzyme BsmBI-v2 (NEB, USA) at 37°C for 30 minutes, and the resulting linearised backbone products were purified using the gel clean-up kit (MN NucleoSpin, Germany). Phosphorylation and annealing of complementary sgRNA oligos were carried out using 100 µM oligos, 1x T4 PNK Buffer (NEB), 1 mM ATP, and 5U T4 PNK (NEB, USA) under the following thermal conditions: incubation at 37°C for 30 minutes, denaturation at 95°C for 5 minutes, followed by gradual cooling at 5°C/min until reaching 25°C. Cloning was performed by T4 Ligase and confirmed through Sanger sequencing.

Packaging Using Lentiviral Vectors

HEK293T cells (4×10^6 cells/10 cm^2 dish) were transfected with 2.5 µg pRSV-Rev (Addgene #12253, USA), 3.5 µg pCMV-VSVG (Addgene #8454, USA), 5 µg pMDLg/pRRE (Addgene #12251, USA), and 10 µg target plasmids using polyethylenimine (PEI,

sigma) at a 1:3 DNA: PEI ratio and incubated for 18 hours. After adding fresh medium, the virus-containing supernatant was collected at 48- and 72-h post-transfection, filtered through a 0.45 µm PES filter, and stored at −80°C. pMDLg/pRRE, pRSV-Rev, and pCMV-VSVG plasmids are third-generation packaging plasmids containing the DNA elements required for lentiviral vector production.

Table 1. Sequences of DNA oligonucleotides used for the synthesis and cloning of single-guide RNA (sgRNA) constructs

	OLIGO 1 (FORWARD)	OLIGO 2 (REVERSE)
sgNT-1	CACCGGTCGTGAAGTGCATTCGATC	AAACGATCGAATGCACTTCACGACC
sgNT-2	CACCGGTAGCGAACGTGTCCGGCGT	AAACACGCCGGACACGTTCGCTACC
sgNT-3	CACCGGACCGGAACGATCTCGCGTA	AAACTACGCGAGATCGTTCCGGTCC
CDK2-1	CACCGAAGCAGAGAGATCTCTCGGA	AAACTCCGAGAGATCTCTCTGCTTC
CDK2-2	CACCGCATGGGTGTAAGTACGAACA	AAACTGTTCGTACTTACACCCATGC
CDK2-3	CACCGTCTGAGGTTTAAGGTCTCGG	AAACCCGAGACCTTAAACCTCAGAC

Lentiviral Transduction of T47D Cells

T47D cells were seeded into 10 cm² cell culture plates at a density of 4 million. The cells were then incubated for 24 hours at 37°C and 5% CO_2. On the following day, NT and CDK2 viruses were applied to the cells at a concentration of 8 µg/mL Polybrene (Sigma-Aldrich, Japan). The virus-treated cells were further incubated for 24 hours at 37°C and 5% CO_2. Subsequently, fresh medium was added to the cells. For antibiotic selection, the cells were incubated with a medium containing 4,5 µg/mL. puromycin for 48 hours, allowing for the selection of cells infected with the virus by the end of the 48 hours. At the end of this selection period, two separate cell lines were established: one infected with the CDK2-targeting virus and the other with the non-targeting (NT) control virus. The NT-infected cells were used as controls in all subsequent analyses.

RNA Isolation, cDNA Synthesis, and qPCR

RNA isolation was performed using Trizol (Invitrogen™, USA) following the manufacturer's protocol. cDNA synthesis from the isolated RNA was carried out using the iScript cDNA Synthesis Kit (Bio-Rad, USA). Quantitative PCR was performed using the LightCycler (Roche, Switzerland) qPCR instrument to measure changes in gene expression. All qPCR results were calculated using the ΔΔCt method relative to control cell populations and were normalised to the geometric mean of ß-actin levels, with 3 technical replicates per sample. The primers used for quantitative PCR (qPCR) are detailed in Table 2.

Table 2. Sequences of DNA oligonucleotides used for the synthesis and cloning of single-guide RNA (sgRNA) constructs

GENE NAME	PRIMER TYPE	SEQUENCE
ACTB	Forward	CATGTACGTTGCTATCCAGGC
	Reverse	CTCCTTAATGTCACGCACGAT
CDK-2	Forward	ATGGATGCCTCTGCTCTCACTG
	Reverse	CCCGATGAGAATGGCAGAAAGC

GFP-based Competition Assay

To assess the relative proliferative capacity of the knockout (KO) cells compared with the wild-type (WT) control cells, a GFP-based competition assay was performed. Non-targeting (NT) control T47D cells were used as the WT population and labelled with GFP using the pLenti CMV GFP Puro plasmid (Addgene #17448, a gift from Eric Campeau & Paul Kaufman). These GFP-labelled NT control cells were then co-cultured with unlabelled CDK2 knockout (KO) T47D cells at a 1:3 ratio. The GFP fluorescence signal served as a marker to distinguish NT (WT) control cells from CDK2 KO cells within the mixed population. The cultures were analysed by flow cytometry every 3 days to determine the proportion of GFP-positive (NT control) and GFP-negative (KO) cells. Temporal changes in the GFP signal were used to evaluate the competitive growth dynamics between the two populations, thereby providing functional insights into the impact of CDK2 loss on cell proliferation.

Clonogenic Assays

Cells were initially seeded into 6-well plates at a density of 4×10^3 cells per well. Following seeding, the cells were allowed to proliferate for 15 days. After this incubation period, the experimental groups were fixed with methanol and stained with crystal violet. This staining process enables the visualisation and quantification of the colonies, as crystal violet binds to the cellular proteins and nucleic acids, resulting in a visible purple colour. The number and size of the colonies were then assessed to evaluate cell proliferation and clonogenic potential.

3D Spheroid Assay

For spheroid generation, 7×10^5 breast cancer cells were resuspended in the culture medium supplemented with 10% methylcellulose (MC). MC stock solution was prepared by autoclaving 6 g of MC powder (Sigma-Aldrich, Japan) in a 500 mL flask. 20% MC RPMI containing 20% FBS (Biowest, France) and 200 U/mL penicillin/streptomycin (Biowest, France) was used for all assays. The cells were incubated at 37°C with 5% CO_2 for 6 days, and spheroid formation was monitored daily. Spheroid areas were quantified using ImageJ

(NIH, USA); at least 50 spheroids per replicate were manually traced and measured.

RESULTS

CDK2 Classified as a Strongly Selective Gene

The CDK2 gene, on chromosome 12q13, encodes a key enzyme that regulates the cell cycle and kinase-mediated processes (31) (Figure 1). The ATP-binding domain, characterised by a hydrophobic pocket, facilitates the ATP binding necessary for kinase function (32). Additionally, the peptide-binding (catalytic) domain enables the phosphorylation of substrate proteins when CDK2 complexes with cyclins A or E (32). Cyclin binding induces conformational changes, activating the ATP-binding site for efficient substrate phosphorylation (33). CDK2 is classified as a strongly selective gene in the Cancer Dependency Map (DepMap, https://depmap.org/portal/gene/CDK2?tab=overview), a distinction based on divergent findings between CRISPR and RNA interference (RNAi) approaches.

The RNA sequencing data from various breast cancer cell lines revealed that CDK2 expression was significantly elevated (Figure 2A). This overexpression is linked to aggressive tumour characteristics (34), demonstrating that CDK2 may play a pivotal role in tumour progression.

Given its association with cell cycle regulation (35), particularly during the G1/S phase transition, CDK2's heightened activity could lead to increased proliferation rates in breast cancer cells, contributing to more aggressive and invasive phenotypes.

CDK2 plays a pivotal role in the progression of breast cancer, with its expression often elevated in tumour tissues compared to normal tissues (34), correlating with more advanced tumour stages and poorer survival outcomes (34). CDK2 is a critical regulator in the progression of breast cancer, exhibiting significantly higher expression levels in tumour tissues compared to adjacent normal tissues (Figure 2B). This upregulation of CDK2 is frequently associated with more advanced stages of the disease, demonstrating its involvement in the transition to aggressive tumour phenotypes. Furthermore, clinical studies have demonstrated that elevated CDK2 expression correlates with poorer survival outcomes, indicating that it may serve as a prognostic biomarker for breast cancer progression (Figure 2C). The dysregulation of CDK2 is believed to enhance cell proliferation, disrupt cell cycle control, and contribute to tumour growth, thereby underscoring its potential as a therapeutic target in the management of breast cancer (34).

Impact of CDK2 Knockout on 2D Colony Formation Dynamics in Luminal Breast Cancer

To investigate the effect of the *CDK2* gene in T47D cells, it was initially targeted using the CRISPR-Cas9 system (29). The knockout (KO) was confirmed by quantitative PCR (qPCR), which showed a significant reduction (Difference between means ± SEM: −0.7135±0.01315; 95% CI: −0.7500 to −0.6770; $p<0.0001$) in *CDK2* gene level compared with the control cells (Figure 3A). Following this, functional assays were performed to further evaluate the consequences of CDK2 KO. In the competition assay, CDK2 KO cells exhibited a mild fitness disadvantage under competition, showing an approximately 20% decrease (Difference between means ± SEM: −21.56±5.670; 95% CI: −34.19 to −8.926; $p=0.0035$) in relative cell growth compared to control cells (Figure 3B). Moreover, CRISPR-Cas9-mediated knockout of CDK2 resulted in a 40% reduction (Difference between means ± SEM: −42.06±6.316; 95% CI: −59.59 to −24.52; $p=0.0026$) in the colony-forming ability in the 2D monolayer culture relative to the control cells (Figure 3C, Figure 3D). This finding suggests that while CDK2 plays a contributory role in promoting proliferation within the 2D monolayer culture, its absence does not completely abrogate growth. The observed reduction in colony-forming ability may be attributed to partial compensation by other cell cycle regulators, indicating a degree of redundancy in the regulatory networks governing cell proliferation.

CDK2's Crucial Role in 3D Spheroid Growth Mimics the Tumour Microenvironment

In contrast to the observations in 2D cultures, the knockout of CDK2 in T47D breast cancer cells exhibited a significant effect when these cells were cultured in three-dimensional (3D) spheroid models. The 3D culture system provides a more complex and biologically relevant microenvironment, closely resembling the in vivo tumour architecture, which allows cells to interact with each other and the extracellular matrix (ECM) in a way that is not possible in 2D (27, 28). These interactions in 3D cultures enable the formation of more structured, multicellular formations that better mimic tumour-like growth patterns (25). In this context, CDK2 knockout led to a marked reduction in the spheroid size. Quantitative analysis revealed that spheroids formed by CDK2 KO cells were markedly smaller in size, measuring approximately 50% less (Difference between means ± SEM: −0.4667±0.03480; 95% CI: −0.5633 to −0.3700; $p=0.0002$) (Figure 4A and Figure 4B). This substantial reduction in the spheroid area indicates that CDK2 plays a critical role in supporting tumour-like growth within 3D environments.

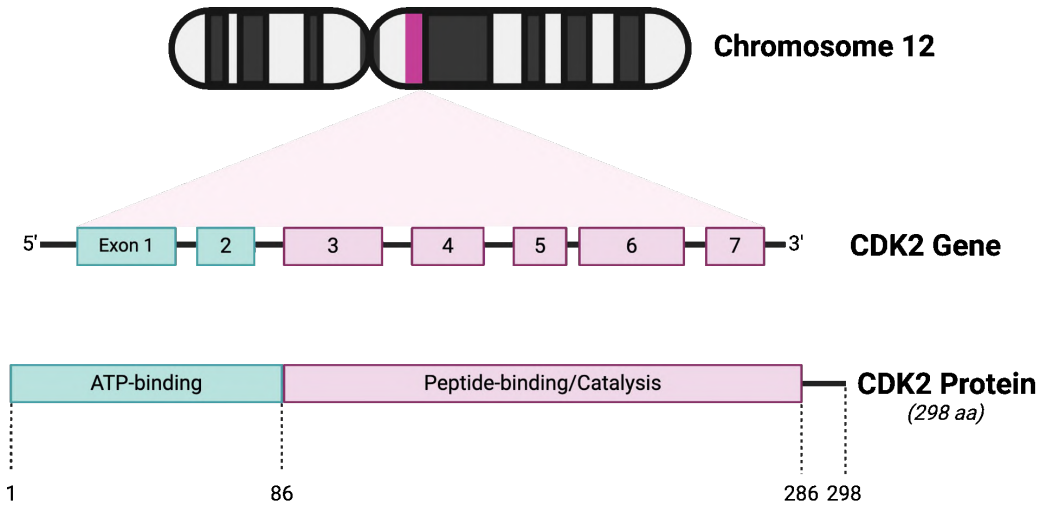

Figure 1. CDK2 Gene Structure and Functional Domains. The CDK2 gene located on chromosome 12q13 encodes a key cell cycle regulator. It has 7 exons encoding functional regions, including an ATP-binding domain (green) crucial for kinase activity and a catalytic domain (pink) responsible for substrate phosphorylation.

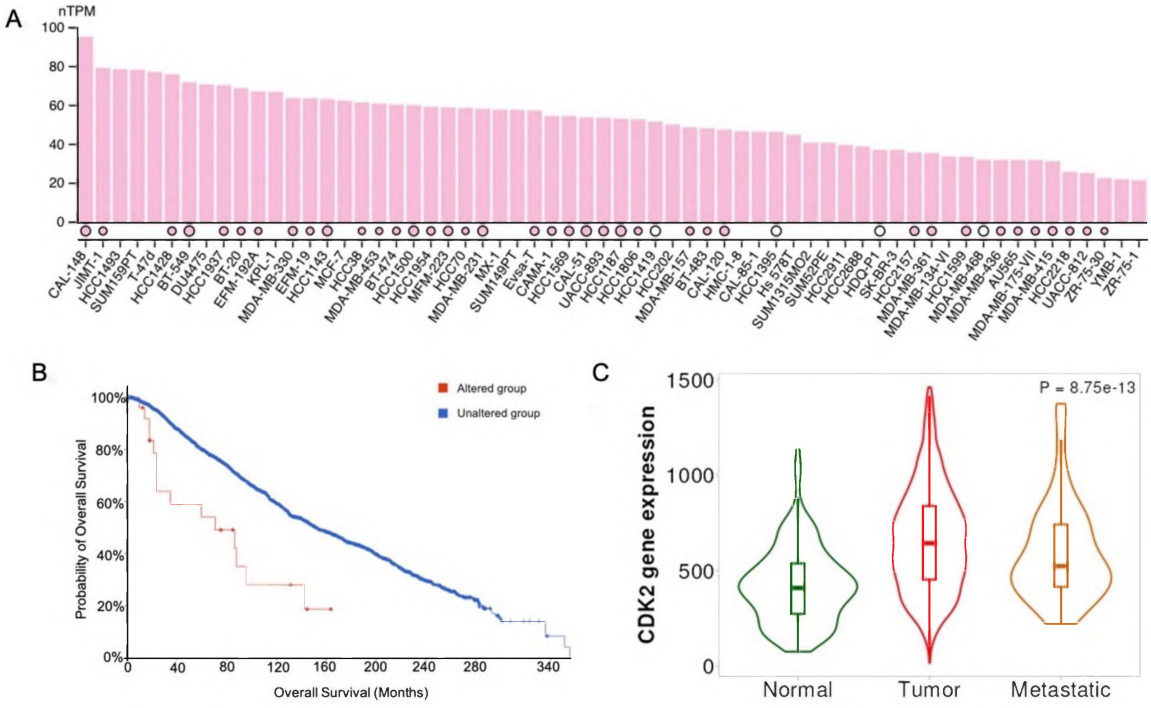

Figure 2. Elevated CDK2 Expression in Breast Cancer and Its Clinical Implications. (A) Bar plot showing CDK2 expression levels (measured in TPM, transcripts per million) across various breast cancer cells. The x-axis represents the cells, while the y-axis indicates CDK2 expression (The Human Protein Atlas). **(B)** Kaplan-Meier survival curve illustrating overall survival for patients with altered (mutated, red) and unaltered (wild-type, blue) CDK2 expression (cBioportal). **(C)** Violin plot comparing CDK2 expression levels in normal breast tissue, primary tumours, and metastatic samples (TNMPlot website).

The observed impairment in spheroid development indicates that the loss of CDK2 compromises the capacity of breast

cancer cells to effectively adapt to conditions that closely resemble the in vivo tumour microenvironment. In 3D cultures, cells experience distinct cell-cell and cell-matrix interactions, as well as variations in nutrient and oxygen gradients, which are pivotal for promoting cellular proliferation and survival (36, 37). The diminished spheroid size in CDK2 KO cells points to a disruption in these essential interactions, likely impacting not only cell proliferation but also the structural integrity and cohesiveness of the spheroids.

Furthermore, the findings imply that CDK2 is involved in key signaling pathways that facilitate the maintenance of cellular homeostasis within a 3D microenvironment. This role may involve the regulation of essential processes such as apoptosis, nutrient uptake, and the establishment of effective intercellular communication. The pronounced effect of CDK2 depletion in this model highlights its importance in enabling breast cancer cells to thrive in complex microenvironments, ultimately contributing to tumour growth and progression (25, 38)

Overall, the results underscore the necessity of CDK2 for sustaining tumour architecture and functionality in 3D cultures, demonstrating that targeting CDK2 could be a viable strategy for disrupting tumour growth in clinical settings. Further investigations are warranted to elucidate the specific molecular mechanisms through which CDK2 regulates spheroid formation and maintenance, as well as its broader implications for breast cancer therapy.

DISCUSSION

This study reveals a context-dependent requirement for CDK2 in luminal breast cancer, particularly within 3D culture systems that better recapitulate the in vivo tumour microenvironment. While CDK2 KO in 2D monolayer cultures resulted in only a moderate reduction in colony-forming ability, its depletion in 3D spheroid models caused a pronounced impairment in spheroid size and architecture. These findings underscore the significance of CDK2 not only in proliferation but also in maintaining structural integrity under physiologically relevant conditions.

Interestingly, our results are consistent with previous reports suggesting that CDK2 is not a universal driver of tumorigenesis but may be indispensable in specific cellular contexts, such as in hormone receptor-positive breast cancer and under therapeutic pressure. Previous studies have indicated that CDK2/cyclin E complexes are integral to the proliferation of oestrogen receptor-positive breast cancer cells, particularly those resistant to endocrine therapies (20). The reduction in spheroid size following CDK2 KO may reflect a decreased proliferative capacity among anti-oestrogen-resistant cells in the tumour microenvironment. This raises the intriguing possibility that targeting CDK2 could enhance the effectiveness of existing endocrine therapies by contributing to the survival of resistant subpopulations within the tumour mass.

CDK2 facilitates the G1/S transition and promote efficient DNA replication. Its absence can disrupt these processes, particularly under stress conditions such as DNA damage (39). Research indicates that while other cyclin-dependent kinases (CDKs) may compensate for some functions of CDK2, they do not fully replace its role in orchestrating cell cycle responses during replication stress or DNA damage (40, 41) Specifically, CDK2 is essential for activating the ATR/Chk1 pathway, which is crucial for a robust DNA damage response (39) Without CDK2, cells may experience delayed progression through the S/G2 phases, leading to increased sensitivity to stressors and potentially resulting in premature cell cycle exit (13, 38).

Beyond its canonical role in the G1/S transition, CDK2 has been shown to phosphorylate epigenetic regulators such as EZH2 (15, 16), enhancing H3K27 trimethylation and repressing tumour suppressor gene expression. Although this study did not directly assess epigenetic changes, the profound effect of CDK2 loss in 3D cultures-where the chromatin state and microenvironmental signaling may differ substantially from 2D-suggests a possible epigenetic component to the observed phenotype. Future work should explore whether CDK2 inhibition alters histone modifications or transcriptional programs in 3D settings.

The observed impairment in spheroid development demonstrates that the loss of CDK2 compromises the capacity of breast cancer cells to effectively adapt to conditions that closely resemble the in vivo tumour microenvironment. This phenotype may also reflect a disruption in a stem- or progenitor-like subpopulation that plays a key role in maintaining tumour architecture and regenerative capacity. CDK2 has previously been implicated in supporting self-renewal and survival in specific cellular contexts, indicating that its loss may impair the function or maintenance of cancer stem-like cells in 3D cultures. Future studies should investigate whether CDK2 inhibition affects stemness-associated gene expression programs or lineage plasticity within tumour spheroids.

In 3D cultures, cells experience distinct cell-cell and cell-matrix interactions, as well as variations in nutrient and oxygen gradients, which are pivotal for promoting cellular proliferation and survival (36, 37).

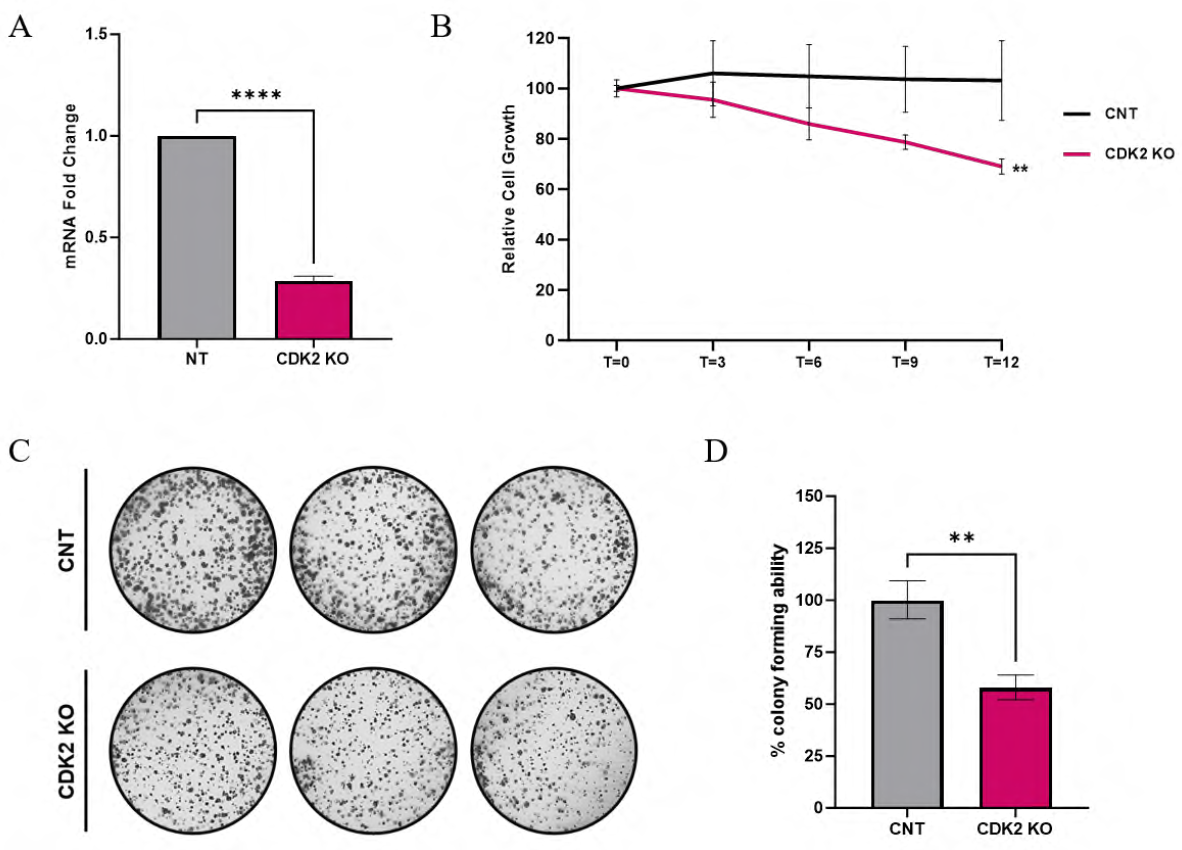

Figure 3. Validation of CDK2 Knockout and Its Impact on Proliferation in T47D Cells. (A) Quantitative PCR confirmed the effective knockout of CDK2 in T47D cells following CRISPR-Cas9-mediated gene editing (Difference between means ± SEM: −0.7135 ± 0.01315; 95% CI: −0.7500 to −0.6770; P<0.0001). **(B)** Relative cell growth was assessed using a competition assay, showing a significant reduction in proliferative capacity upon CDK2 depletion (Difference between means ± SEM: −21.56 ± 5.670; 95% CI: −34.19 to −8.926; p= 0.0035). **(C, D)** Colony formation assays revealed a marked decrease in clonogenic potential following CDK2 knockout, with the reduction in colony numbers compared to control (Difference between means ± SEM: −42.06 ± 6.316; 95% CI: −59.59 to −24.52; P=0,0026). All data are presented as mean ± standard deviation (SD) from at least three independent biological replicates. Statistical significance was determined using an unpaired two-tailed Student's t-test. p < 0.05 was considered statistically significant ($p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***).

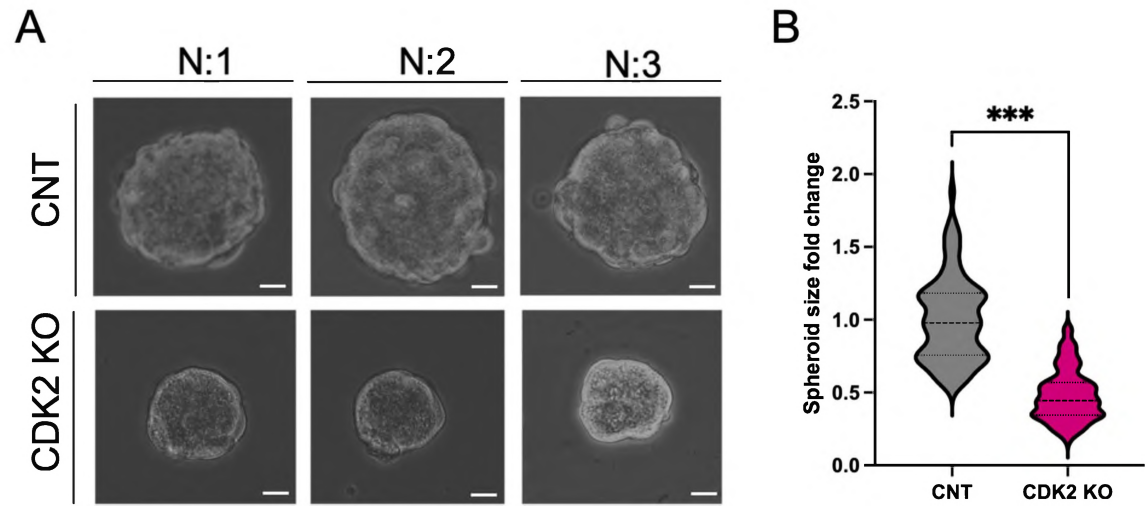

Figure 4. Impact of CDK2 Knockout on Spheroid Size in 3D Cultures of T47D Breast Cancer Cells. (A) Representative brightfield images of spheroids derived from control and CDK2 knockout T47D cells grown in a methylcellulose-based 3D culture for 6 days. **(B)** Quantification of spheroid size (projected 2D area) revealed a significant reduction in spheroid dimensions following CDK2 loss, indicating impaired 3D growth capacity (Difference between means ± SEM: −0.4667 ± 0.03480; 95% CI: −0.5633 to −0.3700; P=0,0002). Data represent the mean ± SD of at least three independent experiments. Statistical significance was assessed using an unpaired two-tailed Student's t-test. p < 0.05 was considered statistically significant ($p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***).

The diminished spheroid size in CDK2 KO cells points to a disruption in these essential interactions, likely impacting not only cell proliferation but also the structural integrity and cohesiveness of the spheroids.

Furthermore, the findings imply that CDK2 is involved in key signaling pathways that facilitate the maintenance of cellular homeostasis within a 3D microenvironment. This role may involve the regulation of essential processes such as apoptosis, nutrient uptake, and the establishment of effective intercellular communication. The pronounced effect of CDK2 depletion in this model highlights its importance in enabling breast cancer cells to thrive in complex microenvironments, ultimately contributing to tumour growth and progression (25, 38).

Overall, the results underscore the necessity of CDK2 for sustaining tumour architecture and functionality in 3D cultures, demonstrating that targeting CDK2 could be a viable strategy for disrupting tumour growth in clinical settings. Further investigations are warranted to elucidate the specific molecular mechanisms through which CDK2 regulates spheroid formation and maintenance, as well as its broader implications for breast cancer therapy.

Importantly, our findings highlight the need to integrate 3D models into functional genomics workflows, especially when evaluating candidate therapeutic targets (24, 42). Genes like CDK2, which may not appear essential in standard 2D cultures, could emerge as context-specific vulnerabilities in more complex systems.

From a translational standpoint, targeting CDK2 in luminal breast cancer-particularly along with endocrine therapies-may offer a strategy to overcome therapy resistance. Previous studies have shown that CDK2 inhibition sensitises resistant breast cancer cells to anti-oestrogens (20, 43-45), and our results support the notion that CDK2 function is critical for sustaining tumour-like growth in 3D.

Despite these insights, our study has limitations that warrant further exploration. Future research should aim to elucidate the specific molecular pathways affected by CDK2 loss, particularly those related to cell adhesion, migration, and invasion-the mechanisms underlying the 3D-specific dependency on CDK2.

Additionally, investigating the therapeutic potential of CDK2 inhibitors in combination with other targeted therapies may yield promising results for patients with luminal breast cancer exhibiting high CDK2 activity. Finally, it is important to evaluate whether CDK2-dependent epigenetic regulation contributes to survival in tumour spheroids.

In conclusion, our study highlights the critical role of CDK2 in luminal breast cancer cell proliferation and survival, especially within complex 3D tumour models (Figure 5). The significant impact of CDK2 KO on spheroid size underscores its importance in maintaining tumour architecture and growth in conditions that closely mimic the tumour microenvironment. These findings demonstrate that CDK2 represent a potential therapeutic vulnerability, particularly in the context of anti-oestrogen resistance. Further studies are warranted to elucidate its precise role in breast cancer progression and treatment response. Understanding these dynamics is crucial for developing effective therapeutic strategies aimed at enhancing patient outcomes in breast cancer treatment.

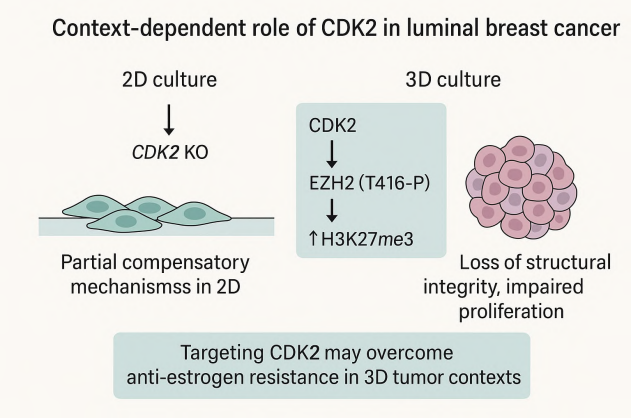

Figure 5. Context-dependent Role of CDK2 in Luminal Breast Cancer. CRISPR-Cas9-mediated knockout of CDK2 in T47D luminal breast cancer cells has a modest effect on colony formation in 2D cultures but leads to a significant reduction in spheroid growth and structural integrity in 3D cultures. This schematic summarises our findings and incorporates hypotheses based on previous studies indicating that CDK2 may regulate epigenetic factors such as EZH2 and H3K27me3. Although not directly tested here, these mechanisms may contribute to the observed 3D-specific phenotypes. Targeting CDK2 could represent a potential strategy to sensitise hormone receptor-positive breast cancers to therapy, particularly in resistant or tumour-like contexts.

Ethics Committee Approval: Since the study was not conducted on animals or humans, ethics committee approval is not required.

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- G.K., E.G.; Data Acquisition- G.K., E.G.; Data Analysis/Interpretation- G.K., E.G.; Drafting Manuscript- G.K.; Critical Revision of Manuscript- G.K., E.G.; Final Approval and Accountability- G.K.

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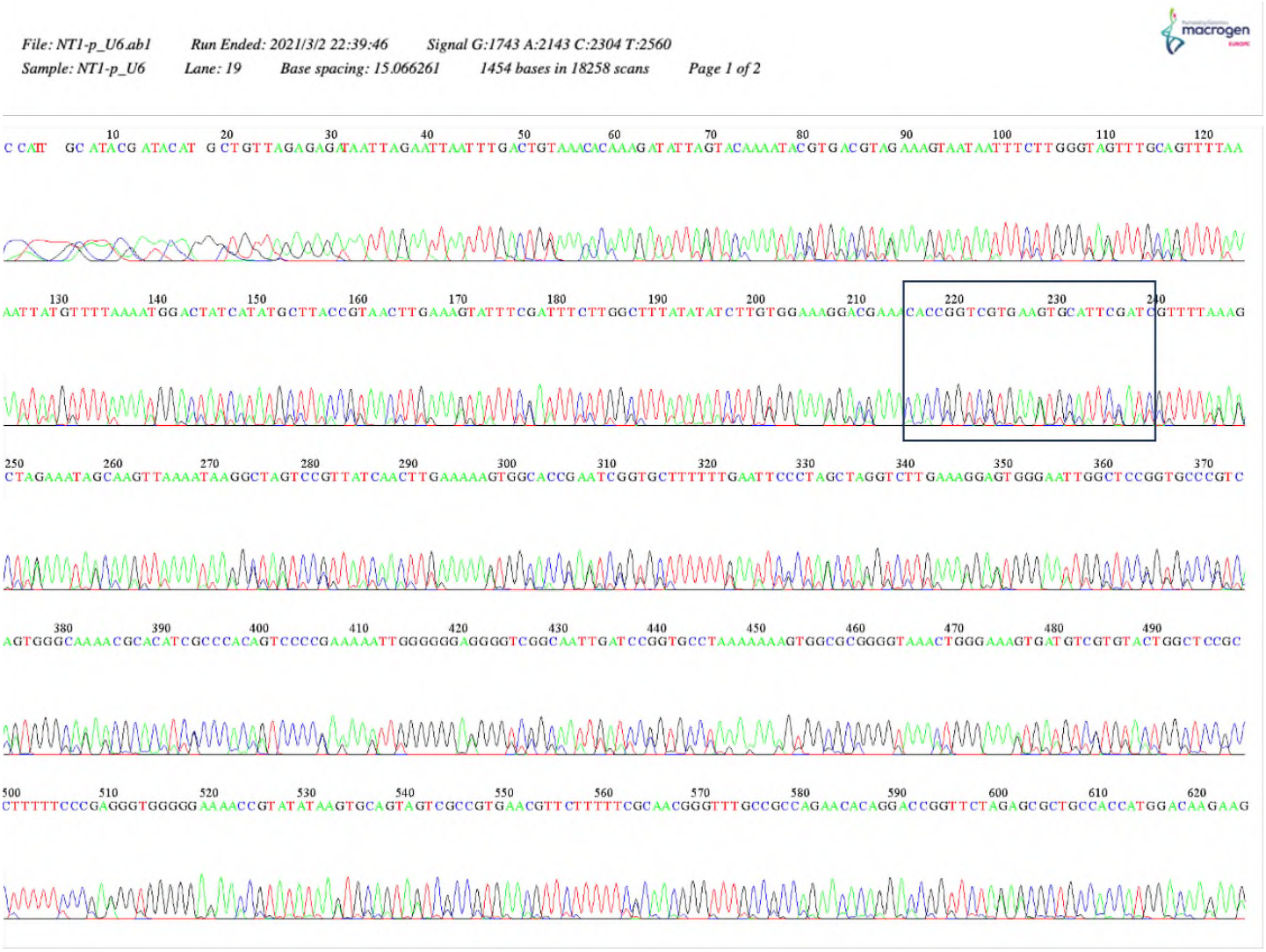
Appendix

Supplementary Figure 1. Validation of sgRNA cloning into the pLentiCRISPRv2 vector by Sanger sequencing. Chromatogram confirms the correct insertion of the target sgRNA sequence into the pLentiCRISPRv2 backbone.



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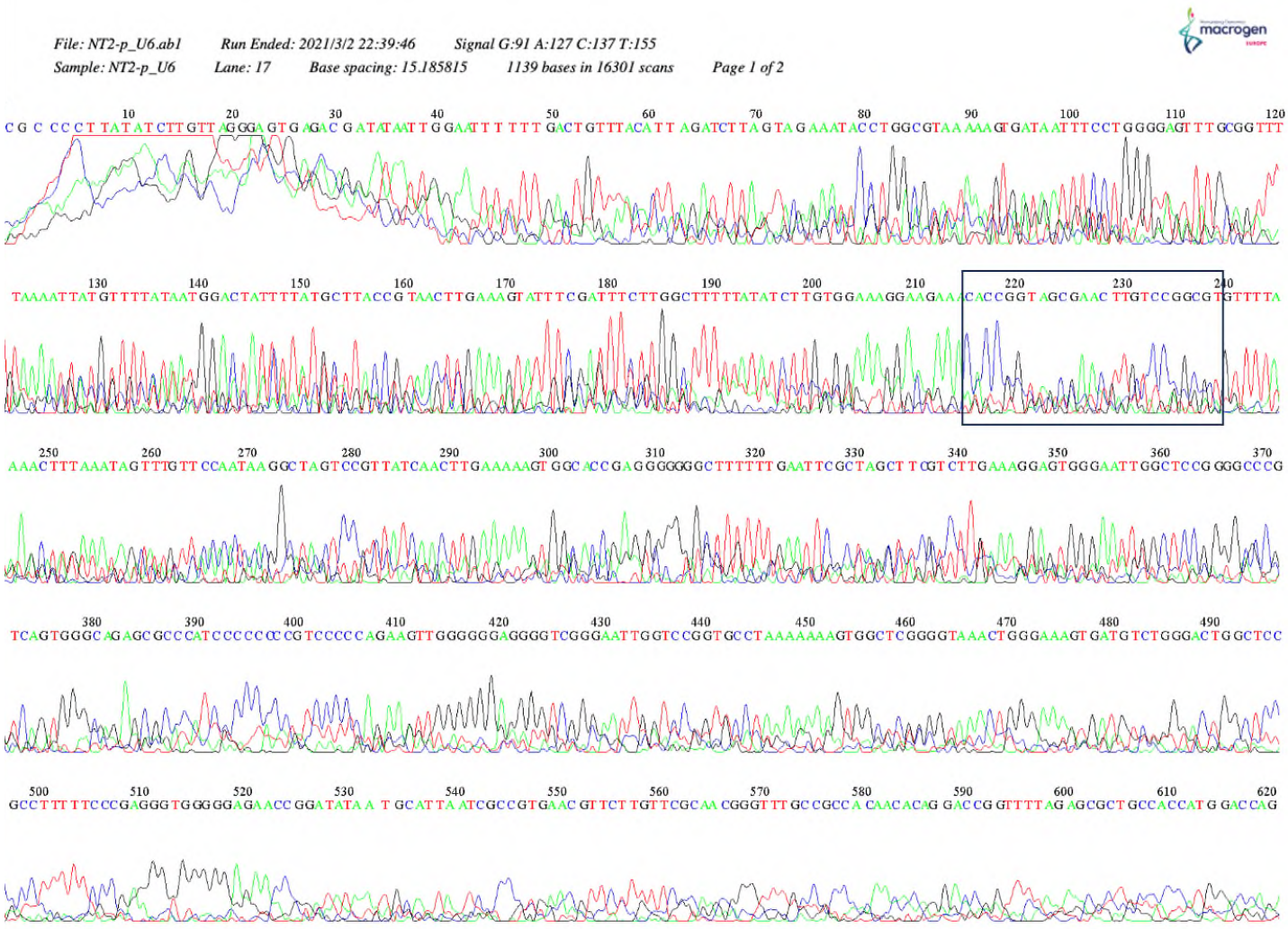
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macrogen

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macrogen

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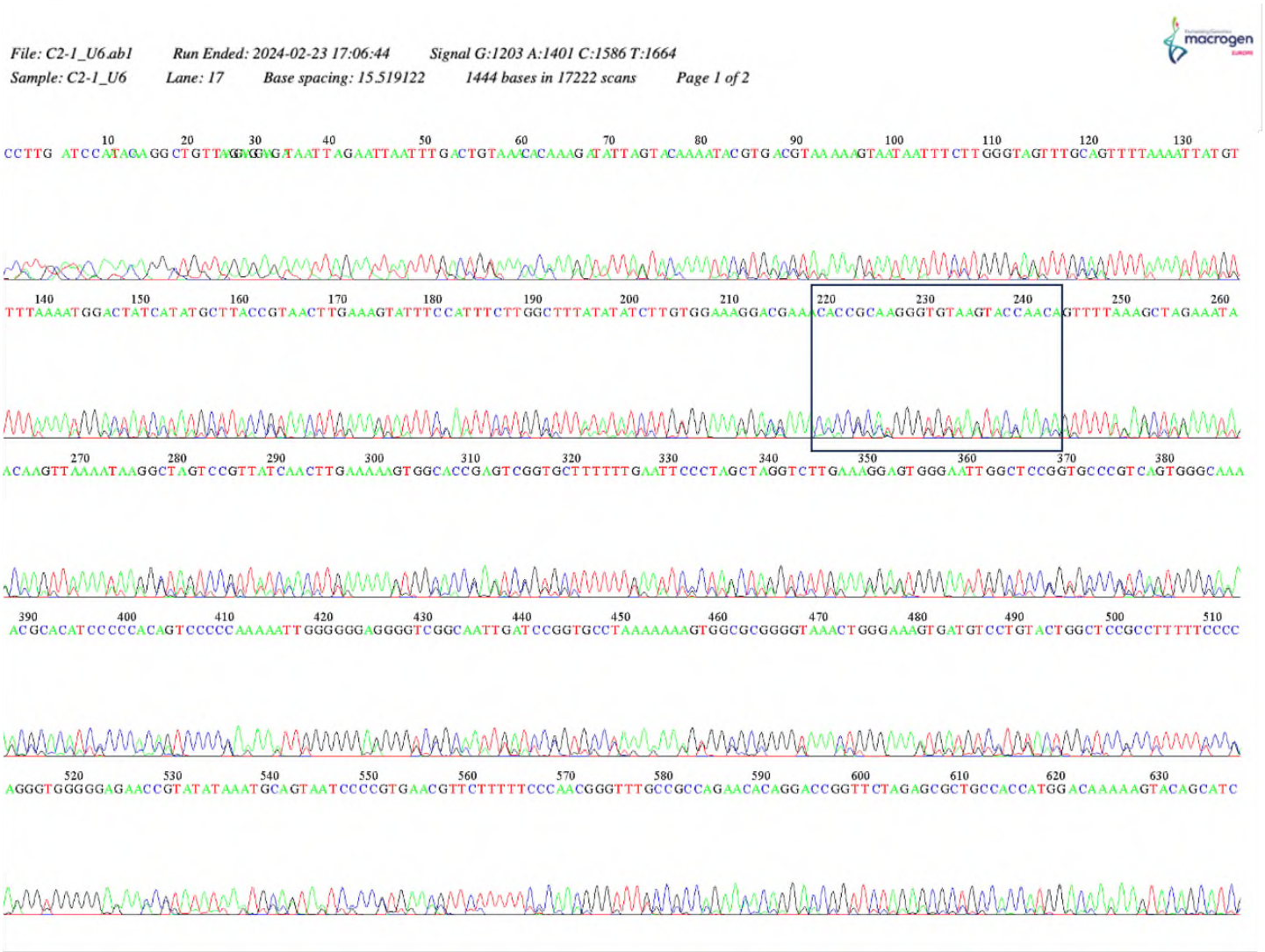
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macrogen

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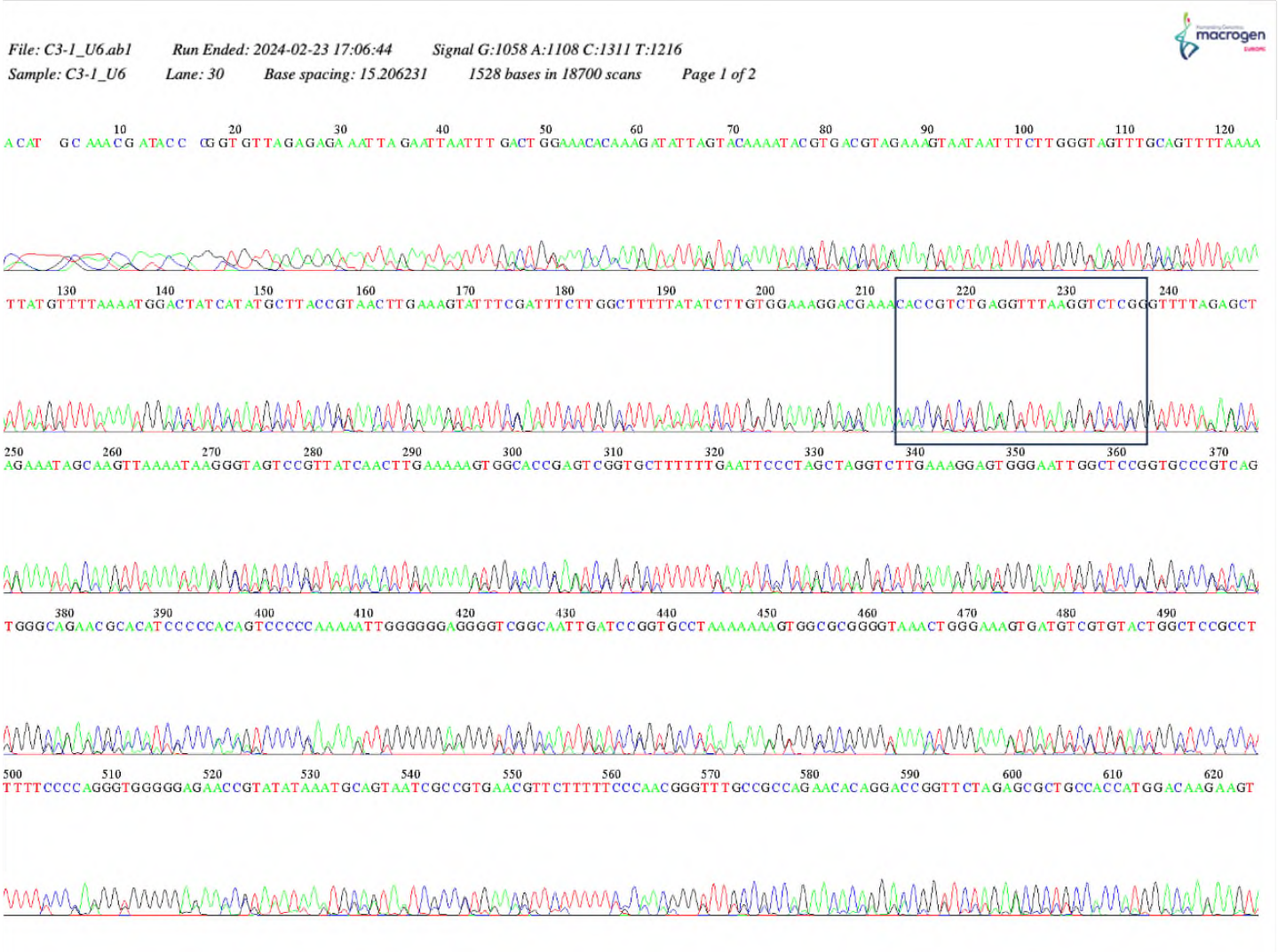
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macrogen

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Research Article **Open Access**

The Role of Liquid Biopsy in Patients Treated with Gamma Knife Radiosurgery for Brain Metastasis

Beyin Metastazı için Gamma Knife Radyocerrahisi ile Tedavi Edilen Hastalarda Likit Biyopsinin Rolü

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Abstract

Objective: The treatment modality for brain metastases (BM) is surgical resection or radiotherapy. Gamma Knife radiosurgery (GKR) treatment prevents toxicity to healthy brain tissue; therefore, this modality is preferred for treating selected patients with BM. Our aim in this study was to investigate the role of cfDNA levels, exosome levels, and miR-208a expressions as biomarkers for diagnosis and predicting the outcome in patients with BM treated with GKR.

Material and Methods: We included nine patients with brain metastasis who received GKR in this study. Samples were taken from patients at different time intervals (before GKR, 1 month after GKR, and at recurrence). Eight age- and gender-matched eight controls were used as healthy individuals. cfDNA, exosome, serum miRNA, and serum exosomal miRNA were isolated from the patient and control groups.

Results: The levels of cfDNA and exosomes were higher in BM patients than in the healthy group. Exosome and cfDNA levels decreased after GKR and increased at recurrence. The expression of serum miR-208a and serum exosomal miR-208a was increased after GKR. The expression of serum miR-208a was significantly higher in patients with lung cancer than in healthy controls and non-lung cancer patients. The expression of serum exosomal miR-208a was found to be significantly higher in patients with lung cancer than in healthy controls.

Öz

Amaç: Beyin metastazlarının (BM) tedavi yöntemi cerrahi rezeksiyon ve/veya radyoterapidir. Gamma Knife radyocerrahisi (GKR) tedavisi, sağlıklı beyin dokusuna toksisiteyi önler. Bu nedenle, BM'li seçilmiş hastaların tedavisinde tercih edilen bir modalitedir. Bu çalışmada, GKR ile tedavi edilen BM hastalarında tanı koyma ve tedavi sonucunu öngörmede biyobelirteç olarak cfDNA seviyeleri, eksozom seviyeleri ve miR-208a ekspresyonunun rolünün araştırılması amaçlandı.

Gereç ve Yöntemler: Bu çalışmaya GKR uygulanan beyin metastazlı dokuz hasta dahil edildi. Hastalardan farklı zaman aralıklarında (GKR öncesi, GKR sonrası 1. ay ve nüks döneminde) örnekler alındı. Yaş ve cinsiyet açısından eşleştirilmiş sekiz sağlıklı birey kontrol grubu olarak kullanıldı. Hasta ve kontrol grubundan cfDNA, eksozom, serum miRNA ve serum ekzozomal miRNA izole edildi.

Bulgular: BM hastalarında cfDNA ve eksozom düzeyleri sağlıklı gruba kıyasla daha yüksek bulundu. GKR sonrası cfDNA ve eksozom düzeyleri azaldığı, ancak nüks döneminde tekrar arttığı tespit edildi. Serum miR-208a ve serum ekzozomal miR-208a ekspresyonu GKR sonrası artmış bulundu. Serum miR-208a ekspresyonu, akciğer kanseri hastalarında sağlıklı kontrollere ve akciğer kanseri olmayan hastalara kıyasla anlamlı derecede daha yüksek olduğu saptandı. Serum ekzozomal miR-208a ekspresyonu ise akciğer kanseri hastalarında sağlıklı kontrollere kıyasla anlamlı derecede yüksek bulundu.

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Conclusion: Our results showed that cfDNA and exosome levels might be used as diagnostic markers for BM patients.

Keywords Brain metastasis · cfDNA · exosome · miRNA

Sonuç: Bulgularımız, cfDNA ve eksozom düzeylerinin BM hastaları için tanısal belirteç olarak kullanılabileceğini göstermektedir.

Anahtar Kelimeler Beyin metastazı · cfDNA · eksozom · miRNA

INTRODUCTION

The process of metastasis consists of sequential and interrelated steps during the migration of malignant tumour cells to distant microenvironments (1). The occurrence of metastasis requires malignant cells to spread in the bloodstream and new discontiguous niches, use angiogenesis for nutrient requirements and evade immune cells in the receiving tissue (1, 2). The metastasis of systemic cancers-frequently lung, breast, melanoma, and gastrointestinal tumors-to the brain is more common than primary brain tumours. The preferred treatment for brain metastases (BM) is surgical resection or radiotherapy (3, 4). After surgery alone, the average survival ranges from 4 to 6 months; with surgery and radiation, the average survival time can reach 12 months. An important cause for the poor outcome is the local and distant recurrences of the tumours (5).

Researchers have shown that concomitant therapy with tumour resection and whole-brain radiation therapy improved the survival time of patients with BM. However, the Gamma Knife radiosurgery (GKR) treatment modality prevents toxicity to normal brain tissue. Therefore, GKR limits the development of radiation-induced neurocognitive diseases and decreases the performance status (4, 6, 7).

In some cases, there are difficulties in distinguishing BMs from other brain tumours, such as glioblastoma and lymphoma. Appropriate diagnosis is of the utmost importance in effectively treating patients with BM. The challenges in diagnosing and monitoring BM patients directed researchers to find new non-invasive methods such as liquid biopsy (LB). LB provides information about the molecular structure of the tumour through the analysis of samples taken from the body fluids (blood, saliva, urine, and cerebrospinal fluid) of patients. LB is more applicable to monitor the response to treatment following the initial diagnosis and treatment regimens. Furthermore, analysis of circulating tumour cells (CTCs), extracellular vesicles (EVs) such as exosomes, and circulating cell-free DNA (cfDNA) can potentially reflect the tumour genome and transcriptome (8). In addition, the molecular examination of cfDNAs obtained by LB and the exosomal content including DNA, RNA, proteins, and micro-RNAs (miRNAs) can be performed (9, 10).

However, in the literature, molecular studies conducted on patients' peripheral blood for diagnostic purposes are very limited in patients with BM (10). There is no study showing the role of exosome and cfDNA levels in diagnosis and their changes after GKR treatment. Therefore, we aimed to investigate the role of exosome levels, cfDNA levels, and miR-208a expression as biomarkers for diagnosis and predicting the outcome in patients with BM treated with GKR.

MATERIAL AND METHODS

Patient selection

We included nine patients who had brain metastasis (primary tumour type; five lung cancer, two breast cancer, one colon cancer, and one ovary cancer) who received hypofractionated GKR in this study. The radiation treatment was applied in three fractions in all patients. The patients' characteristics, including the type of primary cancer, sex, age, and survival time, were reviewed retrospectively. Gender- and age-matched controls were used as healthy individuals. The samples were collected at three different time periods: 1) Before GKR; 2) 1 month after GKR; and 3) At recurrence. This study was conducted in line with the principles of the Declaration of Helsinki. The Ethical Committee of Bezmialem Vakif University approved this study (Date: 13.01.2020, No: 2020-538). All patients signed the informed consent form to participate in the study.

cfDNA Isolation and Measurement

One ml of the serum sample of BM patients and healthy individuals was used to extract cfDNA using the ChargeSwitch gDNA Serum Kit (Invitrogen, Life Technologies) according to the manufacturer's guidelines using the EasySep Magnet (StemCell Technologies, Canada). Isolated DNA samples were stored at –20°C. Qubit® 2.0 fluorometer device was used with a Qubit dsDNA High Sensitivity Assay (Invitrogen, Life Technologies) according to the manufacturer's guidelines to measure cfDNA levels.

Exosome Isolation and Measurement

Five hundred µL of serum sample was used to isolate exosomes with Total Exosome Isolation Reagent (Invitrogen™, Massachusetts, USA) according to the manufacturer's protocols. The pellet was dissolved in PBS and stored at –20°C. EXOCET Exosome Quantitation Kit (System Bioscience, CA, USA) was used according to the manufacturer's guidelines to measure the exosome numbers.

miRNA Isolation and Quantification

The exosome samples were pelleted at 110.000 x g for 70 minutes with a Beckman Coulter Allegra 25R centrifuge (CA, USA). The miRNeasy serum/plasma kit (Qiagen, Hilden, Germany) was used to isolate miRNAs from 200 µL of serum and 30 µL of pelleted exosome samples according to the manufacturer's protocol. The extracted RNA samples were stored at -80ºC.

miRNA cDNA libraries were prepared using the miRNA All-In-One cDNA Synthesis Kit (ABM, Richmond, Canada) according to the manufacturer's guidelines. The qPCR method was used to detect the expression level of miR-208a by using specific primers, and normalisation was done with U6 snRNA. The SensiFAST SYBR No-Rox Kit (Meridian Bioscience, Ohio, USA) was used for qPCR in the conditions 95°C 3 min, 95°C 30 sec, 55°C 30 sec, 72°C 30 sec.

Statistical analysis

IBM SPSS 22 (IBM SPSS Corp., Armonk, NY, USA) was used for the statistical analysis. The Wilcoxon Signed Rank test was used to compare exosome and cfDNA levels and the expression of miRNA in the patient group for different time intervals. The Mann-Whitney-U test was used to compare exosome and cfDNA levels and the expression of miRNA in the patient group versus the control group. The Pearson Correlation test was used to analyse the correlation between the clinical data and the experimental results. Statistical significance was set at $p<0.05$.

RESULTS

Patient characteristics

Nine patients who underwent GKR for brain metastasis and eight healthy controls were included in the study. The median age of the patients was 58 years (range: 43-72 years). The median age of the healthy controls was 57 years (range: 41-70 years). The median tumour volume was 11.26 cm^3 (range: 3.18-28.54 cm^3). The median overall survival was 9.5 months (range: 2.8-36.8 months). The median total radiation dose was 25.5 Gy (range: 22.5 and 27 Gy). GKR was applied in 3 fractions for all patients. The characteristics of the patients are presented in Table 1. There was no correlation between the clinical data and the experimental results.

cfDNA levels

The serum cfDNA levels of BM patients and healthy controls were compared to investigate the role of serum cfDNA in diagnosing BM patients. The mean serum cfDNA levels of BM patients (n=9) (518.7 ng/mL) were higher than those of healthy controls (n=8) (325.9 ng/mL), although the difference was not significant. When the patients were grouped as lung cancer and non-lung cancer patients, lung cancer patients (n=5) (620.5 ng/mL) had a higher level of cfDNA compared to non-lung cancer patients (n=4) (391.6 ng/mL) and controls (n=8) (325.9 ng/mL) (Figure 1).

Table 1. Characteristics of the patients who underwent GKR treatment

Characteristic	Value
Cases (F/M), n	5/4
Median age (range), years	58 (43-72)
Median tumour volume, cm3 (range)	11.26 (3.18-28.54)
Median total radiation dose, Gy (range)	25.5 (22.5-27)
Primary Tumour Type, n (%)	
Lung	5 (56)
Breast	2 (22)
Colon	1 (11)
Ovary	1 (11)
Chemotherapy, n (%)	
Yes	6 (67)
No	3 (33)
Radiation therapy, n (%)	
Yes	9 (100)
No	0 (0)
Median overall survival time, months (range)	9.5 (2.8-36.8)

In addition, serum cfDNA levels were measured at different time intervals (before GKR, one month after GKR, and at recurrence) to elucidate the effect of GKR on the cfDNA level of BM patients. The serum cfDNA level was reduced 1 month after GKR (373.8 ng/mL) compared with the cfDNA level before GKR (518.7 ng/mL) in BM patients. Additionally, the serum cfDNA level was the highest at recurrence (647.9 ng/mL) among all time intervals. There was a decreasing trend for cfDNA in non-lung cancer patients after GKR (before GKR 391.6 ng/mL, one month after GKR 343.8 ng/mL, at recurrence 257.8 ng/mL). However, the cfDNA level was increased at recurrence for lung cancer patients (before GKR 620.5 ng/mL, one month after GKR 397.9 ng/mL, at recurrence 882 ng/mL) (Figure 1).

Exosome levels

The serum exosome levels of BM patients and healthy controls were compared to investigate the role of exosomes in diagnosing BM. The mean serum exosome levels of BM patients (n=9) (6507.9 U/mL) were higher than those of healthy controls (n=8) (5744.4 U/mL), although the difference was not significant. Patients with lung cancer (n=5) (8680.2 U/mL) had the highest level of exosomes compared to non-lung cancer patients (n=4) (3792.4 U/mL) and healthy controls (n=8) (5744.4 U/mL) (Figure 2).

The serum exosome levels were measured at different time intervals (before GKR, one month after GKR, and at recurrence) to elucidate the effect of GKR on the exosome level of BM patients. The serum exosome levels decreased one month after GKR (5655.5 U/mL) compared with those before GKR (6507.9 U/mL) in BM patients. Besides, the serum exosome level was highest at recurrence (8269.4 U/mL) among all time intervals. A similar trend was observed in lung cancer patients in different time intervals (before GKR 8680.2 U/mL, one month after GKR 6031.8 U/mL, and at recurrence 11203.9 U/mL). The serum exosome level of non-lung cancer patients fluctuated during the following time (before GKR 3792.4 U/mL, one month after GKR 5185.1 U/mL, at recurrence 3378.7 U/mL) (Figure 2).

The expression of miR-208a in serum and serum exosomes

The expression of serum miR-208a was higher in BM patients compared with the healthy control group. The expression of serum miR-208a was significantly higher in patients with lung cancer (n=5) than in healthy controls (p=0.008) and non-lung cancer patients (n=4) (p=0.05) (Figure 3).

Serum miR-208a expression increased 1 month after GKR and decreased at recurrence. Serum miR-208a expression increased one month after GKR and decreased at recurrence in both lung and non-lung primary cancer patients (Figure 3).

The expression of serum exosomal miR-208a was higher in the BM patient group than in the healthy ones. The expression of serum exosomal miR-208a was found to be significantly higher in lung cancer patients (n=5) than in healthy controls (n=8) (p=0.04). The expression of serum exosomal miR-208a was higher in lung cancer patients than in non-lung cancer patients, although the difference was not significant (Figure 4).

The expression of serum exosomal miR-208a was increased one month after GKR compared with that before GKR. The expression of serum miR-208a and serum exosomal miR-208a was decreased at recurrence compared with that at one month after GKR. The expression of serum exosomal miR-208a was increased one month after GKR and decreased at recurrence in patients with primary lung cancer. The expression of serum exosomal miR-208a fluctuated during the follow-up time in non-lung primary cancer patients (Figure 4).

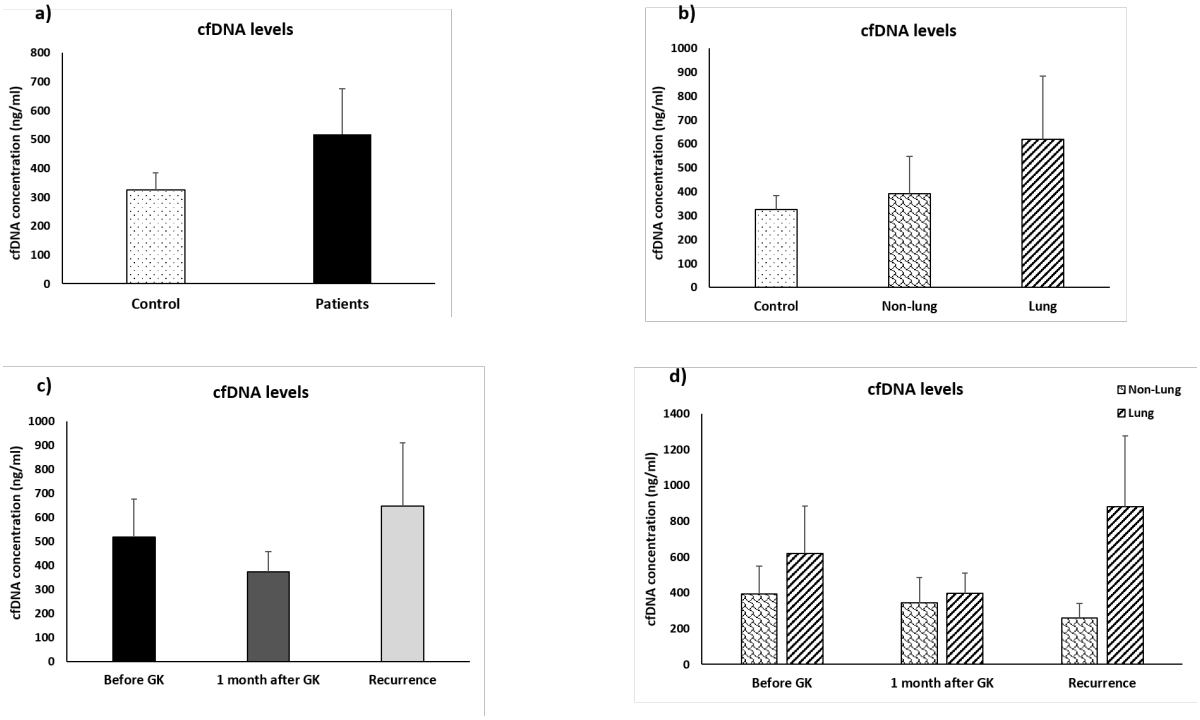

Figure 1. cfDNA levels in BM patients and controls. a) cfDNA levels are higher in BM patients (n=9) compared to the control group (n=8). b) Lung cancer patients had higher levels of cfDNA than non-lung cancer patients and controls. c) cfDNA levels decreased 1 month after GKR compared to before GKR and increased at recurrence in BM patients. d) The level of cfDNA decreased over time in non-lung cancer patients. The cfDNA levels decreased after GKR and increased at recurrence in patients with lung cancer

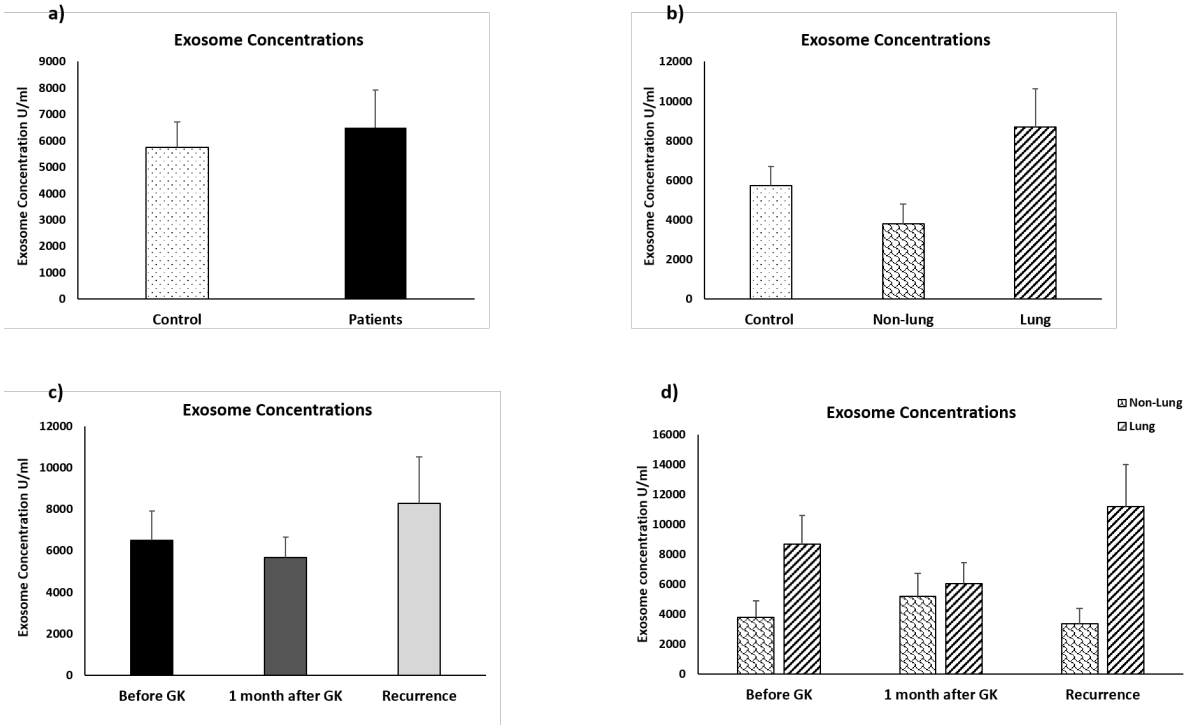

Figure 2. The levels of exosomes in BM patients. a) Exosome levels are higher in BM patients (n=9) compared to healthy individuals (n=8), and controls. b) Primary lung cancer patients had the highest level of exosomes compared to non-lung primary patients and controls. c) Exosome levels decreased 1 month after GKR compared to before GKR and increased at recurrence in BM patients. d) The exosome levels fluctuated during the follow-up time in non-lung cancer patients. The exosome levels decreased after GKR and increased at recurrence in primary lung cancer patients

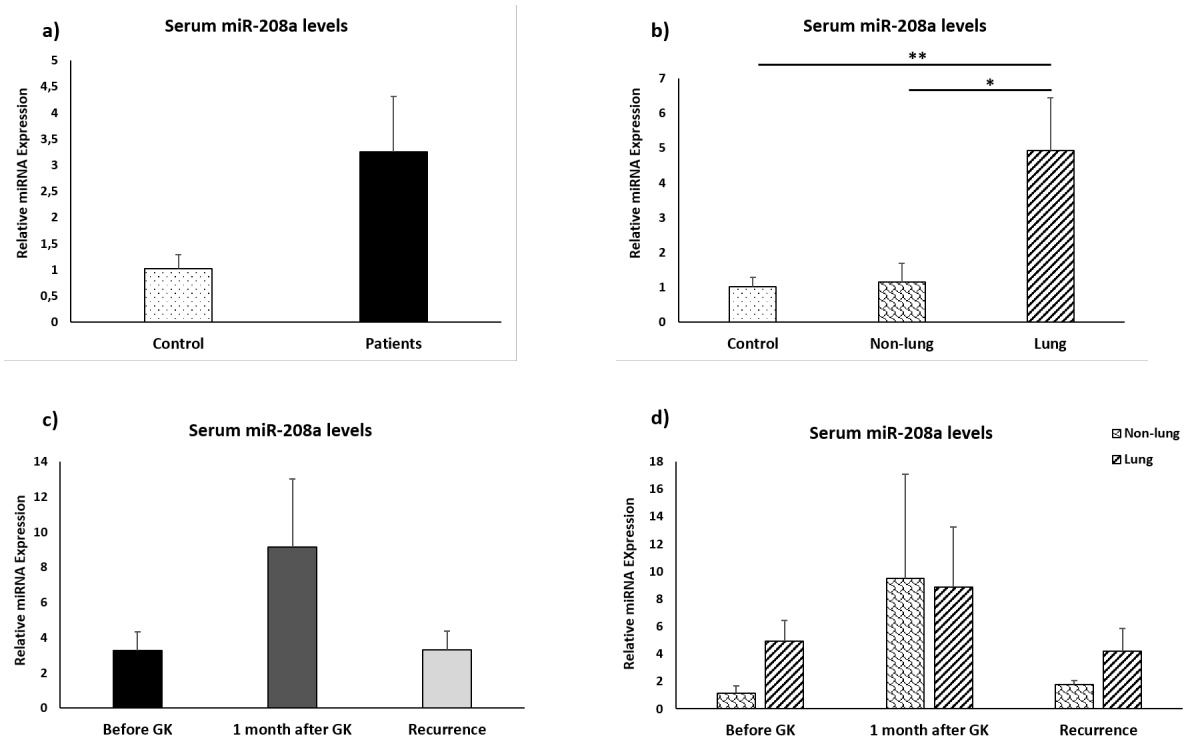

Figure 3. The expression of serum miR-208a in BM patients and controls. a) The expression of serum miR-208a was higher in BM patients than in healthy controls. b) Serum miR-208a levels were significantly higher in primary lung cancer patients than in non-lung primary patients (p=0.05) and controls (p=0.008). c) Serum miR-208a expression was increased 1 month after GKR and decreased at recurrence. d) Serum miR-208a expression was increased one month after GKR and decreased at recurrence in both lung and non-lung primary cancer patients (*p<0.05, **p<0.01)

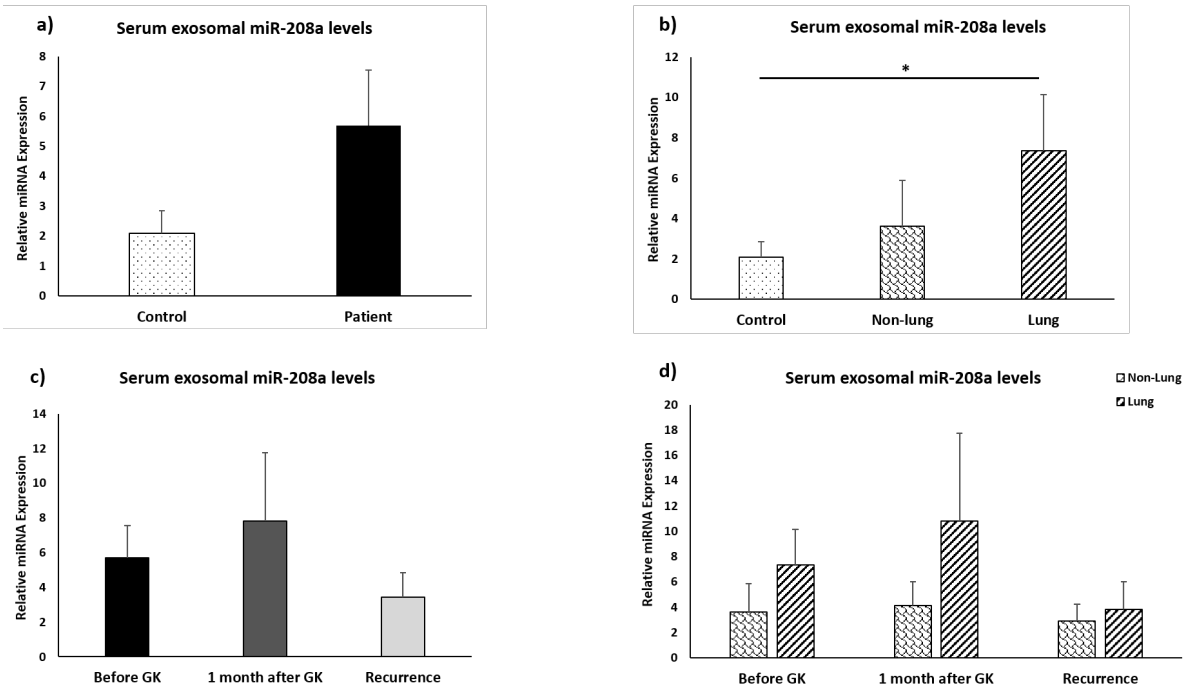

Figure 4. The expression of serum exosomal miR-208a in BM patients and controls. a) The expression of serum exosomal miR-208a was higher in BM patients than in healthy controls. b) The expression of serum exosomal miR-208a was significantly higher in primary lung cancer patients than in controls (p=0.04). The expression of serum exosomal miR-208a was higher in primary lung cancer patients compared with non-lung primary cancer patients, although the difference was not significantly significant. d) The expression of serum exosomal miR-208a was increased one month after GKR and decreased at recurrence in primary lung cancer patients. The expression of serum exosomal miR-208a fluctuated during the follow-up time in non-lung primary cancer patients (*p<0.05, **p<0.01)

DISCUSSION

We tested the hypothesis of whether cfDNA, exosome, and miR-208a could be diagnostic biomarkers in patients with BM. The results showed that the expression of serum miR-208a was significantly higher in patients with lung cancer compared to those with non-lung cancer and healthy controls. Additionally, exosome and cfDNA levels were higher in BM patients compared with the control group. The cfDNA and exosome levels decreased after GKR and increased at recurrence, although there was no statistical significance.

Although the LB is a highly common technique in the monitoring of several types of cancers, the LB research for BM patients is highly limited and challenging due to the short survival time of these patients. Cheok et al. investigated the mutations present in the CSF, blood, and brain metastasis tissue samples of BM patients. Mutations of selected patients matched 80% in CSF and tissue samples and 20% in blood and tissue samples. They suggested that LB from CSF can be an alternative method to tissue biopsy (11). Similarly, Wu et al. compared the genomic status of DNA from primary tumour and BM tissue, CSF, and plasma of BM patients. As a consequence, they showed that CSF cfDNA was more representative of the mutational burden of BM tissue than plasma. Therefore, CSF is a highly reliable source for LB research in patients with BM (12). However, there are drawbacks to the collection of CSF with lumbar puncture that can cause neurological complications and side effects. There are few reports regarding the usage of blood and especially serum samples in the usage of LB in BM patients. However, serum cfDNA is a reliable material in the diagnosis and monitoring of different types of cancers (13-15). We aimed to use more applicable material to obtain from cancer patients and during the follow-up time. Additionally, the decrease after GKR may represent the shrinkage of tumour tissue in the brain, and its increase at recurrence may represent the recurrence of tissue intracranially.

Exosomes are extracellular vesicles that play a role in intercellular communication by carrying DNA, mRNA, miRNA, protein, and lipid particles. Researchers proposed that tumour-derived exosomes play various roles in the metastasis of primary tumours by contributing to angiogenesis, tumour growth, and invasion (16). The feature of exosomes contributing to intercellular communication between primary tumour-derived exosomes and receiving brain cells plays a key role in the formation of BM (17). Therefore, exosomes can be used as prognostic and diagnostic markers for BM because of their existence in different body fluids, contribution to metastasis, and acting as cargo (18). In our study, we found that the number of exosomes was higher in BM patients compared to the healthy control group in

their serum samples. Although exosome levels have not been studied in BM patients, there are several papers regarding the number of exosomes in other malignancies such as breast cancer, ovarian cancer, and hepatocellular carcinoma (19-21). Exosome levels decreased after GKR, which may indicate the effectiveness of the GKR, and increased at recurrence, which may be related to the regrowth of tumour bulk.

Although there are a limited number of studies regarding exosomes in BM, Li et al. collected plasma samples of five patients with BM before and after radiotherapy and performed miRNA-sequencing from exosomes for the first time. They found 35 differentially expressed miRNAs between before and after radiotherapy specimens, which can be defined as non-invasive biomarkers (22). Similarly, Catelan et al. investigated the expression of serum exosomal miR-21, miR-124-3p, and miR-222 in BM and high-grade glioma patients before and after surgical/radiological operations. The expression of miR-21 was increased and miR-124-3p was decreased in patients with BM compared with the control group. Additionally, the expression of miR-21 significantly declined after surgical/radiosurgical operations (23).

Tang et al. demonstrated the up-regulated expression of miR-208a after radiotherapy in patients with lung cancer using an miRNA microarray. They concluded that radiation may induce the increase of miR-208a expression and play a role in the radiosensitivity of lung cancer (24). As Tang et al. showed the effect of radiation on the expression of miR-208a, we also found that serum miR-208a expression was increased after GKR in lung cancer patients and non-lung cancer patients and decreased at recurrence. Additionally, the serum miR-208a expression was significantly higher in patients with lung cancer than in controls and non-lung patients. The serum exosomal miR-208a expression was found to be significantly higher in lung cancer patients compared with the healthy controls. Therefore, we showed that miR-208a could be a biomarker related to the treatment response after radiation in patients with BM. Besides, the small size of the patient group is the limitation of the study.

CONCLUSION

Our results revealed that LB using patients' serum cfDNA, exosome, and miRNAs might be a non-invasive method for diagnosis in patients with BM. However, further investigations are warranted.

Ethics Committee Approval	This study was approved by Bezmialem Vakif University (Date: 13.01.2020, No: 2020-538).
Informed Consent	Written informed consent was obtained from all the participants of the study.
Peer Review	Externally peer-reviewed.
Author Contributions	Conception/Design of Study- I.K., M.A.H.; Data Acquisition- B.K., E.B.E.; Data Analysis/ Interpretation- K.A., S.M., B.K.; Drafting Manuscript- B.K., M.A.H.; Critical Revision of Manuscript- M.A.H.; Final Approval and Accountability- M.A.H., I.K.
Conflict of Interest	The authors declare that there is no conflict of interest.
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Research Article

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After Covid-19 and the February 6 Earthquakes: The Impact of Global Health Crises on Adolescent Mental Health and Social Relationships

Covid-19 ve 6 Şubat Depremleri Sonrası: Küresel Sağlık Krizlerinin Adölesan Ruh Sağlığı ve Sosyal İlişkiler Üzerindeki Etkileri

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Abstract

Objective: The aim of this study was to determine the relationship between global health crises and adolescent mental health and to examine changes in their social relationships.

Methods: This cross-sectional study was conducted with late adolescents between November 1 and December 15, 2024, in an online environment. Data were collected in 10-15 minutes using a Demographic Information Form, Social Relationships Scale (SRS), and Positive Mental Health Scale (PMHS), and were distributed to social media groups based on voluntary participation. Descriptive statistics, Pearson correlation analysis, independent t-tests, and ANOVA tests were used for data analysis.

Results: Global health crises, particularly the COVID-19 pandemic and the February 6 earthquakes, were associated with changes in adolescents' mental health and social relationships ($p<0.05$). These crises have been found to be associated with increased levels of anxiety, fear, and other mental health issues ($p<0.05$). Additionally, demographic factors (such as gender, family structure, family communication, and family income) have been associated with more pronounced changes in adolescents' psychological and social development over the long term ($p<0.05$).

Conclusion: Nurses should monitor adolescents' mental health and provide early support, educate families to strengthen stress management and parenting skills, establish connections with social support networks, and raise public awareness about the long-term impacts of crises.

Keywords Adolescence · Mental Health · Global Health · Adolescent Psychology · Earthquakes · COVID-19

Öz

Amaç: Küresel sağlık krizlerinin adölesanların ruh sağlığı ile olan ilişkilerini belirlemek ve sosyal ilişkilerindeki değişimlerini incelemeyi amaçlamaktayız.

Gereç ve Yöntemler: Kesitsel türde yürütülen bu araştırma, geç dönem adölesanlarla 01 Kasım-15 Aralık 2024 tarihleri arasında elektronik ortamda gerçekleştirilmiştir. Veriler, Tanıtıcı Bilgi Formu, Sosyal İlişkiler Ölçeği (SİÖ) ve Pozitif Mental Sağlığı Ölçeği (PMSÖ) kullanılarak 10-15 dakikada toplanmış ve gönüllülük esasına dayanarak sosyal medya gruplarına gönderilen anketlerle toplanmıştır. Veri analizinde tanımlayıcı istatistikler, Pearson korelasyon analizi, bağımsız t-testi ve ANOVA testi kullanılmıştır.

Bulgular: Küresel sağlık krizleri, özellikle COVID-19 pandemisi ve 6 Şubat depremleri, ergenlerin ruh sağlığı ve sosyal ilişkilerinde değişimlerle ilişkilendirilmiştir ($p<0.05$). Bu krizler, kaygı, korku ve diğer ruh sağlığı sorunlarıyla ilişkili bulunmuştur ($p<0.05$). Ayrıca, demografik faktörler (cinsiyet, aile yapısı, aile içi iletişim ve aile geliri gibi) ergenlerin psikolojik ve sosyal gelişiminde uzun vadede daha belirgin değişimlerle ilişkilendirilmiştir ($p<0.05$).

Sonuç: Hemşireler, ergenlerin ruhsal sağlıklarını izleyip erken destek sunmalı, ailelere eğitim vererek stres yönetimi ve ebeveynlik becerilerini güçlendirmeli, sosyal destek ağlarıyla bağlantı kurarak destek sağlamalı ve kriz sonrası toplumsal farkındalık oluşturmalıdır.

Anahtar Kelimeler Adölesan · Ruh Sağlığı · Küresel Sağlık · Ergen Psikolojisi · Depremler · COVID-19

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INTRODUCTION

Global health crises are events that deeply affect the social structure of societies, individuals' psychological states, and overall healthcare services (1). The COVID-19 pandemic and the earthquakes in February 6, 2023, in particular, are large-scale health crises that have had significant impacts not only on physical health but also on mental health and social relationships (2-4). The late adolescence period (ages 18-21) is a critical process during which individuals develop their identities, form social relationships, and mature emotionally (5-7). Stress and uncertainty experienced during this period can negatively affect adolescents' mental health and limit their social interactions.

During global health crises, adolescents have faced emotional challenges such as social isolation, uncertainty, and anxiety (1, 4). School closures, social distancing rules, and changes in family dynamics have had a determining impact on the social relationships and mental well-being of young people during this period (1, 2). In this context, there is a need for applicable and scalable mental health interventions for individuals affected by numerous adversities (8). Adolescents' mental health and social relationships can be influenced not only by large external factors such as health crises but also by individual characteristics and environmental factors. In this regard, demographic factors (such as age, gender, and socioeconomic status) can lead to significant differences in adolescents' psychological well-being and social interactions. For example, individuals from lower socioeconomic backgrounds may experience more stress and trauma during health crises, while gender differences may also play a decisive role in access to social support systems or coping mechanisms (9, 10).

The mental health of children and adolescents, as well as changes in anxiety and depression symptoms, can vary over time and by region (11). However, the earthquake that occurred on February 6th has caused trauma for many adolescents, and stress factors related to this event have been reported to lead to the development of acute stress disorder (ASD) or other psychiatric disorders (9, 12). This study aims to understand how adolescents' mental health and social relationships were affected in the long term after global health crises such as the COVID-19 pandemic and the February 6th earthquakes in Turkey. In this context, the study aims to analyse the effects of health crises on adolescents' psychological and social development using current data, thereby filling gaps in the existing literature and contributing to a deeper understanding of the issue.

The main objective of the study was to identify the relationship between such global health crises and adolescents' mental health, understand the changes in their social relationships, and examine the relationship between demographic factors and these changes. The findings will guide healthcare professionals and policymakers in better understanding the needs of adolescents and providing them with appropriate support. In addition, this research aims to contribute to the development of intervention strategies for adolescents' mental health.

In conclusion, global health crises such as the COVID-19 pandemic and the February 6th earthquakes have led to significant changes in adolescents' mental health and social relationships. However, the nature and extent of these changes have not been adequately studied, and there is a significant gap in the existing literature. This study aims to highlight the mental well-being of young people and contribute to improving support programs, designing school- and community-based support, and developing effective intervention strategies.

Within this context, the present study seeks to address several key questions: What changes have been observed in adolescents' mental health following major global crises such as the COVID-19 pandemic and the February 6 earthquakes? How have adolescents' social relationships been influenced during these periods? What is the relationship between demographic factors and adolescents' mental health and social relationships? Finally, what changes have occurred in adolescents' mental health and social relationships over the long term as a result of these crises?

MATERIAL AND METHODS

Research purpose and design

This study, conducted with a cross-sectional design, aims to understand the relationship between global health crises and adolescents' psychosocial health, as well as to examine the relationship between demographic factors and these changes. Specifically, it investigates how adolescents' mental well-being and social interactions changed during and after health crises. The research was conducted with late adolescents between November 1st and December 15th, 2024, in an electronic environment. The questionnaire forms were presented to the participants electronically and took approximately 10-15 minutes to complete.

The criteria for participant selection were as follows: Adolescents who can speak and understand Turkish, do

not have communication problems, have no psychiatric diagnoses, are not using medications, are willing to participate in the study, have experienced the February 6th earthquake in Kahramanmaraş, and are active members of a social media group. Participants were limited to adolescents aged 21 and under.

Data collection tools

Data in this study were collected using three different tools: the Demographic Information Form, the Social Relationships Scale (SRS), and the Positive Mental Health Scale (PMHS). These tools were designed to assess the participants' sociodemographic characteristics, social relationships, and mental health.

Demographic Information Form: The researcher prepared this form based on the relevant literature (5, 10, 13-15). The form includes questions about adolescents' sociodemographic characteristics and their emotional responses to the COVID-19 pandemic and the February 6th earthquakes — such as loss of a loved one, mood changes, whether the impact of the crises persists, and the effects of these events on mental health and social communication.

Social Relationships Scale (SRS): Developed by Köse and colleagues in 2022, the SRS is a 5-point Likert-type scale ranging from "Very Distant (1)" to "Very Close (5)". The scale consists of three subdimensions and 10 items, with no items requiring reverse scoring. The subdimensions are as follows: Family Relationships (Items 1-3), Relational/Neighbour Relationships (Items 4-6), and Friendship Relationships (Items 7-10). Since the number of items is not equal across the subdimensions, scoring can be done by dividing the sum of the items by the number of items in the subdimension or the total scale. Higher scores on the scale indicate closer social relationships. The total scale scores range from 10 to 50. The internal consistency coefficients for the scale are .79 for the total scale and .73, .74, and .67 for the subdimensions (16). In this study, the internal consistency coefficients were found to be .83 for the total scale and .83, .81, and .67 for the subdimensions.

Positive Mental Health Scale (PMHS): Developed by Lukat et al. (2016), the PMHS consists of 9 items. This scale primarily assesses emotional well-being, without correlating with theories related to overall well-being in mental health. It was designed to evaluate a single holistic positive emotional concept related to mental health. Yılmaz Akbaba and Eldeleklioğlu adapted the scale into Turkish (14). The items are measured using a 4-point Likert scale, where (1) is "not true" and (4) is "true". All items are positively worded. A higher score on the scale indicates better positive mental health. The internal consistency reliability coefficient was found to be between .84 and .93 (14). In this study, the internal consistency coefficient for the total scale was determined to be .91.

Data collection

Before the main data collection, a pilot test was conducted with 10 students who were excluded from the final sample to assess the clarity of the questionnaire forms. Following the pilot test, it was determined that there were no unclear questions. The researchers conducted the research through electronic means by sending a consent form, survey questions, and scales to adolescent social media groups. Participants were asked to complete the survey forms on a voluntary basis. To prevent potential biases, the participants' information was kept completely confidential, which eliminated social pressure. Standardised scales were used to ensure that the results were unbiased, and the validity and reliability of the tools were tested.

Sample size

In this study, a snowball sampling method was used, and sample size calculations were not made in advance. Data saturation was determined on the basis of post hoc power analysis. The analysis revealed that with a sample size of 522, a 5% margin of error, and a small effect size (0.2), the power was found to be 99.8%. This indicates that the study has high reliability and validity.

Ethical considerations

Before starting the research, necessary permissions were obtained for the scales used, and ethical approval was granted by the Kahramanmaraş Sütçü İmam University Social and Humanities Ethics Committee (Date: 18.10.2024, No. 2024-21). Additionally, written consent was obtained from both the adolescents' parents and the adolescents themselves.

Statistical methods

Descriptive statistics such as frequency, percentage, mean, and standard deviation were used in the data evaluation. Before analysing the data, normality checks were performed. As the skewness and kurtosis statistics of the scale averages were within acceptable limits (±1.5), the data were considered to follow a normal distribution. Therefore, the parametric methods were preferred for the analysis. The internal consistency coefficient was calculated using the Cronbach's alpha test. For data analysis, Pearson correlation analysis was used, and independent t-tests and ANOVA were applied for comparing scale scores across groups. Statistical significance was set at $p \leq 0.05$.

RESULTS

In this section, the changes in the mental health and social relationships of the adolescents participating in the study in relation to global health crises are examined. Additionally, it analyzes how adolescents' mental well-being and social interactions have changed during and after the crises, as well as the underlying reasons for these changes. The findings from the data provide a clearer understanding of these effects.

The average age of the adolescents who participated in the study was 18.78±1.00, with 74.6% being female, 87.3% having a nuclear family, and 60.5% reporting that their income was equal to their expenditures. It was found that 13.0% of the adolescents had lost a close one during the pandemic, and 33.7% had lost a close one during the February 6th earthquakes. Furthermore, 72.5% stated that they still felt the effects of these two crises. Regarding mental health and social communication, 75.7% of adolescents reported experiencing negative changes in their mental health during the pandemic, while 55.8% noted negative changes in their social communication. In the context of the earthquake, 52.5% of adolescents reported experiencing negative changes in their mental health, and 62.3% noted negative changes in their social communication. Adolescents reported experiencing intense feelings of stress (46.0%), anxiety (41.5%), and uncertainty (27.0%) during the pandemic, while the earthquake led to intense feelings of fear (56.0%), anxiety (30.3%), and stress (27.7%) (Table 1).

Table 1. Descriptive Statistics for Participants (n=552).

Descriptive Characteristics	Mean±SD	
Age	**18.78±1.00**	
	n	**%**
Age		
17 years old	71	12.9
18 years old	139	25.2
19 years old	179	32.4
20 years old	163	29.5
Gender		
Female	412	74.6
Male	140	25.4
Family type		
Nuclear family	482	87.3
Extended family	42	7.6
Broken family	28	5.1
Family Income Status		
Our income is less than our expenses	170	30.8
Our income is equal to our expenses	334	60.5
Our income is more than our expenses	48	8.7
Loss of a Loved One During the Pandemic		
Yes	72	13.0
No	480	87.0
Loss of a Loved One During the Earthquake		
Yes	186	33.7
No	366	66.3
Perceived Impact of Crises (COVID-19 and Earthquake)		
Yes	400	72.5
No	152	27.5
Did the Pandemic Negatively Affect Mental Health?		
Yes	418	75.7
No	134	24.3
Did the Pandemic Negatively Affect Social Communication?		
Yes	308	55.8
No	244	44.2
Did the Earthquake Negatively Affect Mental Health?		
Yes	290	52.5
No	262	47.5
Did the Earthquake Negatively Affect Social Communication?		
Yes	344	62.3
No	208	37.7
Feelings Experienced During the Pandemic *		
Stress	254	46.0
Anxietyı	229	41.5
Fear	64	11.6
Uncertainty	149	27.0
Other (Panic, Depression, Worry, Unhappiness, Sadness, etc.)	132	23.9
Feelings Experienced During the Earthquake *		
Stres	153	27.7
Anxiety	167	30.3
Fear	309	56.0
Uncertainty	60	10.9
Other (Insomnia, Restlessness, Panic, Hopelessness, Worry, etc.)	224	40.6

*: Percentages have been calculated within each variable. Mean (M): Average, sd: Standard deviation, %: Percentage within the total,

In Table 2, the correlation analysis conducted to determine the strength and direction of the relationship between the research variables shows a strong positive relationship between them ($p<0.01$). These findings indicate that increases in social relationships and their dimensions are positively

related to improvements in adolescents' positive mental health (Table 2).

Table 2. Results of Correlation Analysis for the Variables of the Study (n=552)

Variables	1	2	3	4	5
Family Relationships (1)	1				
Relatives/Neighbour Relations (2)	0.401**	1			
Friendship Relations (3)	0.443**	0.566**	1		
SR (4)	0.743**	0.808**	0.824**	1	
PMHS (5)	0.310**	0.210**	0.224**	0.311**	1

**p<0.01

When the adolescents' mean scores for Family Relationships (FR), Relatives/Neighbour Relationships (RNR), Friendship Relationships (FRR), Social Relationships (SR), and Positive Mental Health Scale (PMHS) were analysed based on gender, it was found that male adolescents had significantly higher levels of social communication and positive mental health ($p<0.05$). However, no significant difference was found in the Family Relationships (FR) dimension ($p>0.05$).

When the adolescents' FR, RNR, FRR, SR, and PMHS scores were examined based on whether they experienced family conflict, it was found that adolescents without family problems had significantly better levels of social communication and positive mental health ($p<0.001$).

When the adolescents' FR, RNR, FRR, SR, and PMHS scores were analysed based on whether they had lost a close one during the earthquake, it was found that those who had lost a close one had significantly weaker social communication and positive mental health levels ($p<0.05$).

When the adolescents' FR, RNR, FRR, SR, and PMHS scores were examined based on whether they felt the effects of the crises, it was found that those who felt the effects of the crises had significantly weaker social communication and positive mental health levels ($p<0.05$). However, no significant difference was observed in the Family Relationships (FR) dimension ($p>0.05$).

An analysis of adolescents' FR, RNR, FRR, SR, and PMHS scores based on self-reported changes in social communication during the pandemic showed that those who reported changes tended to have lower levels of social communication and positive mental health ($p<0.05$). However, no significant difference was observed in the Family Relationships (FR) dimension ($p>0.05$).

An analysis of adolescents' FR, RNR, FRR, SR, and PMHS scores based on self-reported changes in social communication following the earthquake indicated that those who reported such changes tended to have lower levels of social communication and positive mental health ($p<0.05$).

When the adolescents' FR, RNR, FRR, SR, and PMHS scores were analysed based on whether they experienced anxiety after the pandemic, it was found that those who experienced anxiety had significantly weaker social communication and positive mental health levels ($p<0.05$). However, no significant difference was observed in the FRR and PMHS mean scores ($p>0.05$).

When the adolescents' FR, RNR, FRR, SR, and PMHS scores were analysed based on whether they experienced fear after the earthquake, it was found that those who experienced fear had significantly weaker social communication and positive mental health levels ($p<0.05$). However, no significant difference was found in the FRR mean scores ($p>0.05$).

There was a significant difference in the FR, RNR, and SR mean scores based on family structure ($p<0.05$), but no significant difference was observed in the RNR and PMHS scores ($p>0.05$). Further analysis revealed that the significant difference was primarily due to individuals from broken families.

It was found that there was a significant difference in the FR, RNR, FRR, and SR mean scores based on family income level ($p<0.05$), but no significant difference was observed in the PMHS mean scores ($p>0.05$). Further analysis revealed that the significant difference was primarily due to individuals from families whose income was lower than their expenditures (Table 3).

DISCUSSION

In this section, the findings of this research examining the relationship between global health crises and the psychosocial health of adolescents will be discussed comprehensively. The analysis will focus on how the mental well-being and social interactions of adolescents have changed during and after health crises, addressing the underlying reasons for these changes. The alignment or divergence of these findings with previous literature will also be evaluated, and the contributions of this study to both the literature and practical applications will be discussed. Additionally, the potential impacts of these findings on adolescents and the guidance they offer for future research will be explored.

The results of the study indicate that the gender variable is significantly related to social communication and positive mental health levels, with this relationship being more pronounced among males.

Table 3. Examination of Adolescents' Family Relationships (FR), Relatives/Neighbour Relations (RNR), Friendship Relations (FRR), Social Interaction (SR), and Positive Mental Health Scale (PMHS) Averages According to Socio-Demographic Variables (n=552)

Variables	FR	RNR	FRR	SR	PMHS
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Gender					
Female	3.79±0.87	2.71±0.85	2.89±0.77	3.09±0.64	23.09±6.56
Male	3.85±0.89	3.01±0.72	3.05±0.78	3.25±0.62	26.10±6.22
Test	t=-0.675 p=0.500	**t=-4.154 p<0.001**	**t=-2.127 p=0.034**	**t=-2.636 p=0.009**	**t=-4.738 p<0.001**
Having Issues with Family					
Yes	3.25±0.86	2.46±0.81	2.71±0.89	2.79±0.68	21.06±5.92
No	3.91±0.83	2.85±0.82	2.97±0.75	3.19±0.61	24.40±6.59
Test	**t=-6.764 p<0.001**	**t=-4.075 p<0.001**	**t=-2.662 p=0.003**	**t=-5.521 p<0.001**	**t=-4.459 p<0.001**
Losing a Loved One in an Earthquake					
Yes	3.68±0.84	2.60±0.76	2.75±0.73	2.98±0.56	22.37±6.01
No	3.87±0.88	2.87±0.85	3.02±0.79	3.20±0.66	24.61±6.76
Test	**t=-2.391 p=0.017**	**t=-3.655 p<0.001**	**t=-3.815 p<0.001**	**t=-4.172 p<0.001**	**t=-3.805 p<0.001**
Feeling the Impact of Crises					
Yes	3.81±0.88	2.70±0.80	2.86±0.75	3.08±0.61	23.11±6.30
No	3.80±0.85	3.00±0.86	3.11±0.82	3.26±0.69	25.81±6.98
Test	t=0.036 p=0.972	**t=-3.885 p<0.001**	**t=-3.408 p<0.001**	**t=-3.082 p=0.002**	**t=-4.362 p<0.001**
Did the Pandemic Affect My Social Communication?					
Yes	3.77±0.93	2.67±0.83	2.81±0.80	3.05±0.64	23.01±6.33
No	3.85±0.80	2.92±0.80	3.09±0.72	3.22±0.62	24.92±6.79
Test	t=-1.051 p=0.294	**t=-3.569 p<0.001**	**t=-4.237 p<0.001**	**t=-3.098 p=0.002**	**t=-3.413 p<0.001**
Did the Earthquake Affect My Social Communication?					
Yes	3.70±0.93	2.72±0.83	2.82±0.80	3.04±0.66	22.88±6.48
No	3.98±0.75	2.90±0.82	3.11±0.70	3.27±0.57	25.47±6.49
Test	**t=-3.808 p<0.001**	**t=-2.474 p=0.014**	**t=-4.174 p<0.001**	**t=-4.045 p<0.001**	**t=-4.539 p<0.001**
Anxiety Experienced After the Pandemic					
Yes	3.88±0.81	2.88±0.81	2.98±0.79	3.20±0.60	23.78±6.74
No	3.70±0.94	2.65±0.83	2.87±0.76	3.03±0.68	23.95±6.41
Test	**t=2.218 p=0.027**	**t=3.172 p=0.002**	t=1.622 p=0.105	**t=3.076 p=0.002**	t=-0.292 p=0.770
Fear Experienced After the Earthquake					
Yes	3.66±0.87	2.70±0.78	2.89±0.73	3.05±0.60	23.11±6.73
No	3.92±0.86	2.85±0.86	2.96±0.81	3.19±0.66	24.44±6.44
Test	**t=-3.521 p<0.001**	**t=-2.137 p=0.033**	t=-1.145 p=0.253	**t=-2.611 p=0.009**	**t=-2.342 p=0.020**
Family Structure					
Nuclear family	3.85±0.85	2.78±0.81	2.93±0.77	3.14±0.62	23.72±6.60
Extended family	3.90±0.79	2.92±0.89	3.11±0.71	3.24±0.62	24.71±6.80
Broken family	2.92±0.89[a]	2.71±0.95	2.61±1.00[a]	2.74±0.84[a]	24.92±6.25
Test	**f=15.752 p<0.001**	f=0.659 p=0.518	**f=3.380 p=0.035**	**f=5.974 p=0.003**	f=0.823 p=0.440
Famiyl Income Status					
Our income is less than our expenses	3.58±0.96[a]	2.65±0.97[a]	2.76±0.96[a]	2.97±0.76[a]	22.92±6.72
Our income is equal to our expenses	3.91±0.80	2.86±0.74	3.00±0.69	3.19±0.57	24.28±6.64
Our income is more than our expenses	3.88±0.86	2.76±0.76	3.11±0.56	3.22±0.52	24.16±5.63

Variables	FR	RNR	FRR	SR	PMHS
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Test	f=8.615 p<0.001	f=3.701 p=0.025	f=6.715 p<0.001	f=7.596 p<0.001	f=2.452 p=0.087

Mean (M): Average, sd: Standard deviation, %: Percentage within the total, f: One-Way ANOVA, t: Independent Samples t-test. Within each group, comparisons were made using the group-specific statistical tests, and the superscripts a, b, and c indicate differences within the groups. Values with the same superscript are statistically similar.

Previous studies conducted during the COVID-19 pandemic and the February 6th earthquakes have identified gender as a risk factor for the development of mental health issues (3, 4, 10, 17-20). Globally, children and adolescents have experienced more mental health problems due to the pandemic, with gender being one of the most influential factors in this context (21, 22). This research, based on self-reporting and data collection from a specific region, highlights that the female gender is a strong predictor of psychosocial distress after major crises. The relationship between gender and social communication as well as positive mental health is crucial for paediatric nursing practices. This highlights the need for nurses to consider gender differences when assessing the mental health needs of adolescents. In particular, the more pronounced relationship among males (4, 10, 18, 19), calls for a gender-sensitive approach in care. Nurses should provide appropriate, effective, and individualised support by considering gender-based differences alongside the trauma adolescents have experienced. By analysing the gender factor, nurses can make targeted interventions to address the psychosocial needs of their patients, contributing to the healthy development of individuals.

The study also revealed that adolescents experiencing social communication problems with their families and the environment during the crises (pandemic and earthquake) had significantly lower levels of social communication and positive mental health (23, 24). Both the pandemic and the earthquake have caused considerable mental health stress for over 14 million individuals and families worldwide (9, 17, 18, 25, 26). Low-quality family relationships, exposure to pandemic-related discrimination, and other psychosocial factors reported by both parents and adolescents have exacerbated mental distress in youth. However, social and family support, in conjunction with positive coping strategies, has been associated with better outcomes (3, 18, 21, 25). During crises such as the pandemic and earthquakes, difficulties in family relationships have been associated with changes in children's psychological health. Paediatric nurses should observe family dynamics in such situations and provide guidance and support to the family. Offering family-oriented education and support programs to address communication issues within the family could be a critical approach. By fostering social and family support, nurses can help children develop better coping skills, which may contribute to healthier psychosocial development.

Crises like the COVID-19 pandemic have significantly impacted adolescent mental health, leading to increased levels of fear, anxiety, and other negative emotions, which have weakened their social communication and positive mental health. These crises have created an atmosphere of fear, anxiety, loneliness, depression, stress, and burnout (1-4, 8, 10, 12). Studies conducted among children and adolescents have shown that during these periods, symptoms such as anxiety, depression, loneliness, and stress are among the most prevalent mental health problems (3, 12). In particular, anxiety has been found to have a significant negative relationship with health needs during the crisis, with these needs increasing substantially (10, 27, 28). Crises have increased anxiety and stress among students, adversely affecting adolescents' self-efficacy (29). National paediatric data show a slight increase in anxiety levels compared to the period before the pandemic (19) and before the earthquake (4). Anxiety weakens adolescents' functionality, leading to negative approaches to their health. Therefore, it is crucial to develop effective strategies and policies to alleviate the fear and anxiety adolescents experience and to meet their health needs appropriately. Nurses should consider not only the physical health of adolescents but also their mental health, providing support to help them cope with emotional challenges.

To alleviate adolescents' anxiety levels, it is important for nurses to collaborate with families to strengthen strategies implemented at home and assist adolescents in developing their social skills in social environments such as school. Furthermore, providing psychological support to adolescents, offering coping skills training, and monitoring anxiety levels for intervention are necessary.

Crises such as the pandemic can lead to an increase in anxiety levels, weakening adolescents' functionality and leading to negative health outcomes. Nurses should develop effective strategies to help adolescents manage their anxiety and provide interventions to improve both their mental and physical health.

Family structure and socioeconomic status been significantly related to social communication and positive mental health levels. After major crises such as the pandemic and the

earthquake, disruptions in daily routines and financial losses in families have increased feelings of anxiety, stress, and uncertainty in children and adolescents (3, 4, 26). It has been observed that the behaviours of elementary school-aged children were significantly affected by the COVID-19 pandemic, with the negative outcomes varying according to family circumstances (22). Crises such as the COVID-19 pandemic and the earthquake have led to more mental health problems among children and adolescents, with socioeconomic status, parenting quality, family functioning, and social support being some of the most influential factors (21, 26). Family structure, socioeconomic status, parenting quality, family functioning, and social support directly impact adolescents' mental health. During crises, financial losses and disruptions in daily routines in families increase feelings of anxiety, stress, and uncertainty, leading to mental health problems in children and adolescents. Nurses should collaborate with families during this process to understand the family dynamics and provide guidance. Strengthening social support systems for families facing socioeconomic difficulties is a key role for nurses. Additionally, providing training and support for parents can strengthen healthy parenting skills, improve family functioning, and reduce the negative impact on children's mental health. In conclusion, paediatric nurses should develop various interventions to protect and improve the mental health of children and adolescents by considering family factors during crises. Strengthening social support and family functioning is an effective way to support the mental health of children and adolescents.

CONCLUSION

After global health crises, such as the COVID-19 pandemic and the February 6th earthquakes, both crises have negatively impacted adolescents' mental health, leading to an increase in anxiety, depression, stress, and other mental health problems.

The COVID-19 pandemic and the February 6th earthquakes have been associated with changes in adolescents' social relationships.

During these crises, adolescents' social relationships weakened due to losses and environmental factors, which, in turn, negatively impacted their mental health.

Demographic factors such as gender, family structure, family communication, and family income status have been shown to have a negative relationship with adolescents' mental health and social relationships.

The long-term effects of these crises, particularly the COVID-19 pandemic and the February 6th earthquakes, have resulted in significant changes in adolescents' mental health and social relationships. These crises have worsened adolescents' mental health, increasing anxiety, fear, stress, and uncertainty.

Nurses should monitor adolescents' mental health and provide psychological support by detecting symptoms of anxiety and depression early.

Nurses can offer educational and guidance services to families. Stress management, healthy communication, and parenting skills within the family should be strengthened. Nurses should support families in developing positive attitudes towards their adolescents.

Nurses should assess adolescents' social support resources and, when necessary, connect them with networks such as schools, community centres, and social services for additional support.

Nurses can provide training on stress management, emotion regulation, and problem-solving skills to adolescents.

Nurses should monitor adolescents' mental health in the post-crisis period and intervene early if symptoms of anxiety or depression are observed. Early diagnosis and treatment can prevent long-term mental health problems.

Nurses should raise awareness in the community about the long-term effects of crises and increase social sensitivity to mitigate the negative effects on adolescents' mental health.

Ethics Committee Approval: This study was approved by Kahramanmaraş Sütçü İmam University Social and Humanities Ethics Committee (Date: 18.10.2024, No. 2024-21)

Informed Consent: Written consent was obtained from both the adolescents' parents and the adolescents themselves.

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A Pedodontic Approach to Second Premolar Agenesis in Children

İkinci Küçük Azı Dişi Eksikliğinde Pedodontik Yaklaşım

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Abstract

Tooth agenesis is a very common craniofacial malformation in humans. Second molar agenesis is the most common condition after third molar agenesis. Second premolars play an important role in maintaining occlusion, and their absence may cause various problems. Various treatment alternatives are applied in the presence of retained deciduous second molars and congenital second premolar agenesis: Preservation and restorative treatment of the deciduous second molar, spontaneous space closure by extraction of the deciduous second molar before the eruption of the first molar, space closure with controlled slicing and hemisection of the deciduous second molar, orthodontic space closure following extraction of the deciduous second molar, autotransplantation, implant replacement, conventional fixed bridge and resin-bonded bridge. It is critical to choose the treatment plan that will give the best results in the long term. During treatment planning, various factors such as the condition of the deciduous second molar, the age of the patient, the space requirement for correction of the malocclusion, the facial profile, the patient's treatment preference and the duration of treatment should be taken into consideration. In this review, treatment planning for second premolar agenesis will be analysed, focusing on various strategies to ensure optimal oral health and function.

Keywords Hypodontia · congenital missing teeth · second premolar

Öz

Diş eksikliği, insanlarda çok yaygın görülen kraniyofasial malformasyondur. İkinci küçük azı eksikliği, üçüncü büyük azı dişi eksikliğinden sonra en sık karşılaşılan durumdur. İkinci küçük azı dişleri oklüzyonu sağlamada önemli bir rol oynamakta ve bu dişlerin eksikliği çeşitli sorunlara neden olabilmektedir. Retine süt ikinci azı dişi varlığında ve konjenital ikinci küçük azı dişi eksikliğinde çeşitli tedavi alternatifleri uygulanmaktadır: Süt ikinci azı dişin korunması ve restoratif tedavisi, birinci azı dişin sürmesinden önce süt ikinci azı dişinin çekilmesi ile spontan boşluk kapatılması süt ikinci azı dişinin kontrollü kesimi ve hemiseksiyon ile boşluk kapatılması, süt ikinci azı dişinin çekimini takiben ortodontik olarak boşluğun kapatılması, ototransplantasyon, implant uygulaması, geleneksel sabit köprü uygulaması, rezin bağlı köprü uygulaması. Uzun vadede en iyi sonuçları verecek tedavi planının seçimi oldukça önemlidir. Tedavi planlaması sırasında süt ikinci azı dişinin durumu, hastanın yaşı, maloklüzyonun düzeltilmesi için yer gereksinimi, yüz profili, hastanın tedavi tercihi ve tedavi süresi gibi çeşitli faktörler göz önünde bulundurulmalıdır. Bu derlemede ikinci küçük azı dişi eksikliği ile ilgili tedavi planlamaları incelenerek optimal ağız sağlığı ve fonksiyonu sağlamak için uygulanan çeşitli stratejilere odaklanılmaktadır.

Anahtar Kelimeler Hipodonti · ikinci küçük azı · konjenital diş eksikliği

INTRODUCTION

Tooth agenesis is the most prevalent craniofacial anomaly in humans (1, 2). Various classifications are used to describe the number of missing permanent teeth. When 1 to 5 teeth are absent (excluding third molars), it is referred to as "hypodontia," whereas the absence of six or more teeth is termed "severe hypodontia" or "oligodontia." The complete absence of all permanent teeth is defined as "anodontia" (3).

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Congenital tooth agenesis can arise from a range of internal and external factors. Among the permanent teeth, second molar agenesis is the most frequently observed condition after the third molars. Second premolars play a critical role in maintaining proper occlusion, and their absence may result in various clinical challenges. This review explores treatment strategies for second premolars, focusing on approaches to optimise oral health and function.

Prevalence

The prevalence of hypodontia varies between 2.3% and 15.7% among different ethnic groups, depending on the population studied, and recent epidemiological studies consistently report that mandibular second premolars are among the most commonly missing teeth in non-syndromic hypodontia (4, 5). Specifically, the left mandibular second premolar was noted as the most commonly missing individual tooth (5).

Excluding the third molars, the mandibular second premolars are the most frequently missing teeth, with a prevalence of 2.9%-3.2%, followed by the maxillary lateral incisors (1.6-1.8%), maxillary second premolars (1.4-1.6%), and mandibular incisors (0.2-0.4%), while other teeth are rarely absent (6).

Unilateral agenesis is more common in the second premolars. For bilateral agenesis, the occurrence in mandibular second premolars ranges from 43.5% to 47.7%, whereas in maxillary second premolars, it is 46.3% to 52.2% (6).

Diagnosis of Congenital Second Molar Agenesis

The timing of diagnosis is a key consideration in cases of tooth agenesis. Delayed mineralisation of tooth germs, such as those of the mandibular second premolars, may lead to a false-positive diagnosis of agenesis in radiographic examinations. Mineralisation of mandibular second premolars generally begins between 3 and 3.5 years of age but may occasionally start several years later. Studies have shown that mandibular second premolars diagnosed as agenesis at age 7 can subsequently develop after the age of 10. Thus, no definitive diagnosis of agenesis should be made before the age of 7 (7).

A thorough evaluation is required when a permanent tooth has not erupted more than 1 year beyond its expected timeframe or if it has not emerged within 6 months of its contralateral counterpart. Additional indicators include asymmetric eruption patterns, the presence of retained deciduous teeth, or ankylosis of the mandibular deciduous molars (8).

Aetiology

Various hypotheses regarding the aetiology of hypodontia have been proposed in the literature. The diversity of these theories highlights a multifactorial origin involving both genetic regulation and environmental influences. Tooth morphogenesis involves the expression of over 300 genes, with *PAX9*, *MSX1*, *AXIN2*, and *EDA* being the most commonly implicated in nonsyndromic hypodontia (9).

Tooth agenesis resulting from MSX1 gene variants demonstrates a characteristic pattern of tooth absence. Nieminen et al. reported that the second premolars and maxillary first premolars were the most frequently missing teeth associated with MSX1 mutations (10). In line with these findings, Zheng et al. identified the maxillary and mandibular second premolars, along with the mandibular first premolars, as the most commonly affected teeth in individuals with MSX1-related agenesis (11). Similarly, Zhao et al. confirmed that among patients with non-syndromic congenital tooth agenesis attributed to MSX1 variants, the second premolars and maxillary first premolars were most frequently absent (12).

Patients with tooth agenesis have been reported to exhibit a higher prevalence of associated dental anomalies such as taurodontism, microdontia, enamel hypoplasia, and distoangulation of the second premolars (13).

Treatment Planning for Congenital Second Premolar Agenesis

Mandibular second premolar agenesis ranks as the most frequent form of tooth agenesis after third molars. Determining the treatment approach for missing mandibular second premolars requires consultation with an orthodontist. Several treatment options are available for managing retained deciduous second molars and congenital agenesis of second premolars, including the following:

Maintenance and restorative treatment of the deciduous second molar

- Spontaneous space closure by extraction of the deciduous second molar before the eruption of the first permanent molar

Space closure with controlled slicing and hemisection of the deciduous second molar

- Orthodontic space closure following the extraction of the deciduous second molar
- Autotransplantation
- Implant replacement
- Conventional fixed bridge

- Resin-bonded bridge

Selecting the treatment plan that ensures optimal long-term results is crucial. Factors such as the condition of the deciduous second molar, the patient's age, space requirements for malocclusion correction, facial profile, patient preferences, and treatment duration should be carefully evaluated during planning. In cases involving multiple missing premolars, deciduous molar extraction may be an option in mild Class III cases. For patients with crowding, the missing premolar site can serve as an extraction site. Extraction of the mandibular second deciduous molar may help alleviate crowding, allow for anterior teeth retraction in patients with incisor proclination, or both. Conversely, early extraction of the second deciduous molar can result in decreased arch length, tipping of adjacent teeth, alveolar bone resorption, and extrusion of opposing teeth. In cases of mandibular retrusion, Class II molar relationships, or polydiastema, preserving the deciduous second molar is recommended because it may help prevent future malocclusion (14). Recent studies have highlighted an increasing clinical focus on treatment planning for second premolar agenesis, particularly regarding long-term space management, preservation of primary molars, and interdisciplinary approaches involving paediatric dentists and orthodontists (15, 16).

The current treatment options for second premolar agenesis have been summarised to support the clinical decision-making process (Table1).

Table 1. Clinical Decision-Making Guide

Clinical Condition	Recommended Treatment Option	Notes
The retained deciduous molar is intact, no infraocclusion, no root resorption	Preservation or restoration	Maintain until complications arise or skeletal maturity is reached
Maintain until complications arise or skeletal maturity is reached	Extraction + Orthodontic space closure	Particularly effective in Class I/II profiles
Advanced infraocclusion or ankylosis	Early extraction	Consider timing for implant planning
Orthodontic closure not feasible, patient still growing	Autotransplantation	Best when the donor tooth has an open apex
Skeletally mature patient with adequate bone	Implant placement	Ensure the completion of facial growth with cephalometric confirmation

Maintenance and Restorative Treatment of the Deciduous Second Molar

When evaluating patients with retained primary teeth, overall health, motivation, expectations, and oral condition should be assessed first. Locally, tooth shape, colour, structural integrity, gingival alignment, and occlusion must be examined. Radiographic analysis should consider the root length, morphology, apical condition, and periodontal support. For implant planning, the vertical bone height and inter-root space are crucial.

Research indicates that maxillary and mandibular deciduous canines and second molars generally exhibit a better prognosis than other primary teeth (17). Additionally, studies suggest that root resorption in deciduous teeth slows with age, and clinically significant resorption is rarely observed after the age of 20 (14). If both the crown and root are in good condition and the tooth remains functionally and aesthetically acceptable, it may be preserved unless orthodontic extraction is necessary. This approach allows the tooth to maintain bone and soft tissue integrity, remaining in function with minimal intervention (18). In a systematic review of the literature evaluating the survival rate of primary molars in cases of premolar agenesis, it was reported that 82% to 89% of the retained primary molars remained in good condition over a follow-up period of 5 to 13 years, suggesting that this approach represents a clinically viable treatment option (19).

It has also been reported that if infraocclusion is mild and non-progressive, retaining the tooth may be advantageous, as the alveolar crest can decrease by 25% within 4 years after extraction. Thus, an early extraction of primary teeth could compromise future implant placement (20).

For cases where infraocclusion persists despite a sound crown and root structure, or when aesthetic concerns arise, the retained deciduous tooth may be restored using direct composite applications or indirect restorations, such as composite, porcelain, or gold onlays. Some studies proposed that onlays are effective in restoring infraoccluded deciduous molars, preventing both the tipping of adjacent teeth and the extrusion of opposing teeth (21).

Since the longevity of deciduous teeth is comparable to that of implants or other fixed restorations, they can be preserved if no pathology is present and their crown-root structure is adequate (18).

The application of direct or indirect restorations in deciduous teeth can postpone or even eliminate the necessity for more invasive procedures while being a more cost-effective option. Managing retained primary teeth in this manner aligns with the principles of minimally invasive dentistry and should be

considered when appropriate. Further research is required to support clinical decision-making regarding the prognosis of retained primary teeth and their restorations (8).

Pulp Treatment of the Primary Second Molars

The primary objective of restorative treatments is to preserve pulp vitality. When pulp exposure occurs in deciduous molars, pulpotomy may be indicated. The commonly used pulpotomy agents include formocresol, ferric sulphate, calcium hydroxide, and mineral trioxide aggregate (MTA) (22). Research has demonstrated that MTA minimises microleakage, supports tissue regeneration upon contact with pulp or periradicular tissues, maintains pulp vitality, promotes the formation of a tubular hard dentine bridge, and exhibits low toxicity. Compared with formocresol, MTA has shown greater long-term clinical and radiographic success in deciduous teeth and is the most biocompatible pulpotomy material. Unlike formocresol, MTA does not trigger adverse reactions, making it a preferred choice for pulpotomy. Although its cost is relatively high, MTA is considered a viable alternative for extending the longevity of deciduous teeth (23).

Root Canal Treatment of Primary Second Molars

Because of their thin enamel, prominent pulp horns, and prolonged retention, primary second molars are highly susceptible to caries and pulpal involvement. Pulpectomy aims to preserve these teeth until their natural exfoliation. However, complex root canal morphology and accessory canals pose significant challenges in effective cleaning and obturation (24). The quality of root canal sealing is essential for success. Inadequate sealing allows bacterial survival, while effective obturation cuts off nutrient supply, leading to bacterial elimination. MTA, used in both pulpotomy and pulpectomy, has demonstrated antibacterial effects against Enterococcus faecalis, enhancing its effectiveness (25). Moreover, treatment outcomes are better in cases of irreversible pulpitis than in necrotic pulp conditions (26). Radiographic studies confirm that MTA provides superior results compared to gutta-percha, particularly in cases with permanent tooth agenesis, due to its excellent sealing and antimicrobial properties (24).

Controlled Slicing and Hemisection in Deciduous Second Molars

Hemisection of the mandibular primary molars has been suggested as a preventive approach against alveolar bone atrophy in cases where the second premolars are congenitally absent (27). This approach serves as an effective method to prevent long-term complications and minimise the need for future restorations, facilitating the natural medialization of permanent molars. The technique involves removing 1.5-2 mm from the distal crown of the second deciduous molar, followed by hemisection of the distal crown and root once the first molar has shifted mesially. The second deciduous molar is then extracted. This controlled cutting technique helps preserve the buccolingual ridge while preventing both vertical and horizontal bone loss. Performing this procedure at an early age (8-9 years) has been associated with higher success rates, ensuring controlled medialization of the first permanent molar. However, studies indicate that the likelihood of success diminishes as the child grows older. A notable drawback of this method is the necessity for two separate dental visits—one for hemisection and another for extracting the deciduous molar. For the initial cutting of the distal crown, topical anaesthesia is generally sufficient. Special attention must be given to protecting the first permanent molar during the procedure (28).

Space Closure

Various factors should be considered when determining the appropriate approach for space closure in cases of second premolar agenesis.

Age

The timing of diagnosis significantly influences treatment planning. If the condition is identified before the second molar develops, all treatment options remain viable. However, if diagnosed after the completion of permanent dentition, space closure may have aesthetic implications.

Facial Shape

The patient's facial structure plays a crucial role in deciding whether to maintain or close spaces. In hypodivergent individuals, tooth extraction should be avoided to prevent an increase in overbite, and the space should be preserved. Conversely, in hyperdivergent individuals, extraction of the second deciduous molar and subsequent space closure is recommended.

Skeletal and Dental Abnormalities

Orthodontic space closure is often a preferred approach for young patients due to its long-term stability. Since no additional space maintenance or restorations are needed after the growth is complete, the decision should be based on a comprehensive orthodontic diagnosis. Factors such as arch length, facial profile, and occlusion must be carefully evaluated. Orthodontic space closure is particularly indicated when there is inadequate space, incisor proclination, or lip protrusion. Additionally, retaining deciduous molars may impact occlusion due to the discrepancy between the mesiodistal crown widths of the deciduous second molar and the permanent second premolar.

Number of Missing Teeth

The extent of missing teeth also influences the choice of treatment. In cases of severe oligodontia, closing the spaces may result in a significantly shortened dental arch, which could compromise masticatory function.

Integrity of the Deciduous Molar

The long-term retention of healthy deciduous second molars is associated with favourable clinical outcomes in some patients. However, several factors must be considered, including the risk of root resorption, Bolton tooth size discrepancies, pulpal pathology, crowding, ankylosis, and infraocclusion. In many cases, these concerns favour the extraction of the deciduous second molar (29). Infraocclusion, in particular, is linked to permanent tooth loss and root resorption, making it a critical factor in extraction decisions for patients with remaining growth potential. Ankylosed deciduous second molars can lead to infraocclusion and vertical bone defects. Therefore, extraction is recommended to allow the alveolar crest to shift occlusally as adjacent teeth continue to erupt. If space maintenance is required for future implant placement, the alveolar ridge width may decrease over time, increasing the likelihood of requiring bone grafting (30).

A study reported that between 11 and 20 years of age, 60% of the mesial roots and 46% of the distal roots of deciduous second molars exhibited resorption. Findings also indicate that root resorption rates decline with age, and these teeth remain occlusally stable beyond the age of 20. However, their potential loss in later years would necessitate closing a significant space (14).

Extraction of Ankylosed Deciduous Teeth

Ankylosis is frequently observed in deciduous molars exhibiting infraocclusion. When these teeth are extracted, significant alveolar bone loss may occur, which can compromise future implant placement and disrupt the overall orthodontic treatment plan (31).

Patient and Parent Motivation

Orthodontic space closure is a long-term treatment that demands a high level of cooperation and motivation from both the patient and their parents (32).

A wider range of treatment options is available for younger patients. However, after the age of nine, these options become more restricted, and spontaneous space closure is no longer a feasible approach. Early extraction of the second deciduous molar has been proposed to prevent the tipping of adjacent teeth and to facilitate spontaneous space closure through the mesial movement of the first molar as it erupts. Nonetheless, some studies indicate that congenital second premolar agenesis cannot be reliably diagnosed before the age of nine. Another study reported that spontaneous space closure can successfully occur when the second deciduous molar is extracted around 8-9 years of age, before the root development of the mandibular first premolar is complete and before the eruption of the permanent second molar (33).

Autotransplantation

Autotransplantation has gained renewed interest in recent years as a viable treatment option for children with second premolar agenesis, especially when donor teeth such as developing third molars are available. Studies have demonstrated favourable long-term outcomes when performed during the optimal stage of root development (34, 35). When orthodontic space closure is not feasible following the extraction of a second deciduous molar, maintaining the space and alveolar bone for future implant placement becomes challenging. In such cases, autotransplantation serves as a viable alternative, especially in growing patients. This technique, which requires technical precision and careful case selection, should be planned within a multidisciplinary framework, considering factors such as age, extraction timing, adjacent tooth condition, and the patient's general health and motivation. Unlike implants, autotransplantation enables biological integration and can promote alveolar bone development during skeletal growth. The success of the procedure largely depends on the preservation of the periodontal ligament, while complications such as root resorption, apical periodontitis, and ankylosis often related to ligament damage remain major risks. Immediate transplantation after extraction is recommended to minimise resorption and improve prognosis (36).

Even in cases of replacement resorption, the surrounding bone typically remains intact, allowing the transplanted tooth to remain functional until implant placement becomes appropriate. While resorption tends to progress more rapidly in younger patients due to higher bone turnover, transplanted teeth can remain clinically functional for extended periods. In adolescents or adults with limited vertical bone growth, ankylosed teeth may remain asymptomatic with slow resorption. When necessary, occlusal and proximal function may be restored using composite restorations or fixed prosthetics (37).

Optimal timing of the procedure is linked to the donor tooth's stage of development. The highest success rates were observed when the root reached approximately three-quarters of its total length and the apex remained open,

supporting continued pulpal circulation and apexogenesis. If the apex is closed, endodontic treatment is recommended to prevent pulp necrosis and subsequent inflammatory resorption (38).

There is variation in the literature regarding the splinting duration and the timing of orthodontic intervention. Some authors recommend delaying tooth movement for several months, whereas others support initiating orthodontic forces as early as 6-8 weeks post-transplantation. In cases with compromised periodontal support or a high risk of ankylosis, early orthodontic movement promotes periodontal ligament regeneration and may help prevent ankylosis (39).

Root morphology is another determinant of success. Teeth with curved or multiple roots are less suitable because of the increased risk of damage to the periodontal ligament and cementum during extraction (40). Overall, autotransplantation has emerged as a biologically and economically favourable alternative to implants in the management of congenital tooth agenesis (41).

Implant Replacement

Osseointegrated single-tooth implants are the preferred treatment when space closure is not feasible. They are considered the most biologically conservative option for replacing congenitally missing teeth. However, implants are contraindicated in growing patients as they interfere with skeletal development. Orthodontists must ensure adequate bone height and width while managing vertical and mesiodistal positioning with proper root parallelism. For implant placement, retaining the deciduous second molar until the vertical facial growth is complete is recommended. Serial cephalometric radiographs should be used to assess facial growth, which typically continues until approximately age 17 in females and 21 in males (42).

Studies indicate that 4 years after mandibular second deciduous molar extraction, the alveolar crest undergoes a 25% reduction, reaching 30% after 7 years. Resorption occurs more on the buccal side, leading to lingual implant shifting. Adjusting occlusal forces can help prevent abutment and crown fractures. Because deciduous second molars are wider than premolars, reducing their width can prevent occlusal issues (20).

Congenital second premolar agenesis is often associated with ankylosis of the deciduous molar. If ankylosis occurs early, the alveolar bone fails to develop vertically, leading to severe defects when extraction is delayed. This complicates the implant placement and may require bone grafting. Timely extraction considering the patient's growth potential is critical. If vertical defects develop, repositioning the mandibular first premolar into the second premolar space can be an alternative to grafting. Another preventive measure is extracting the deciduous first molar once half of the first premolar root has developed to stimulate alveolar ridge formation (43).

In cases of molar extraction due to caries, infraocclusion, or severe root resorption, a space maintainer is needed until implant placement. Options include a band/crown loop, nance palatal arch, or lingual arch (32).

Fixed Prosthesis

When a deciduous molar has a poor prognosis due to caries, root resorption, or periodontal problems, extraction followed by prosthetic rehabilitation is necessary. For young patients with healthy adjacent teeth, resin-bonded bridges (RBBs) are preferred over conventional bridges due to their minimally invasive nature and ability to preserve tooth structure (44). RBBs, first introduced in the 1970s, have improved with resin cements that bond to both enamel and metal. Despite slightly lower survival rates (~88%) compared to implants, they offer advantages such as shorter treatment time, reduced cost, and less need for anaesthesia, making them suitable for children and anxious patients (41).

Clinical success depends on sufficient enamel for bonding, proper design (e.g., 180° wrap), and minimising forces in cases of bruxism (45). Aesthetic concerns with metal retainers can be addressed using opaque cement or adjusted design approaches. With appropriate case selection, RBBs represent a functional and cost-effective interim solution for young patients (46).

Literature Review

A study investigating the survival of mandibular second primary molars in cases of missing mandibular second premolars analysed radiographs from 99 patients (37 males and 62 females) ranging in age from 12 years to adulthood. In cases of bilateral tooth loss, only one deciduous molar was randomly selected for inclusion to ensure statistical independence among all evaluated molars. The radiographic assessment focused on the distance between the missing premolar and the adjacent premolar, the degree of infraocclusion, and the extent of root resorption. The average age at the final assessment was 24 years and 7 months. Throughout the follow-up period, only 7 out of the 99 retained deciduous molars were lost due to significant root resorption, severe infraocclusion, or caries. Based on these findings, it was concluded that over 90% of patients with retained deciduous molars in cases of mandibular second premolar agenesis could expect long-term tooth survival (45).

A study involving 34 patients (20 males and 14 females) with 52 missing second premolars compared two treatment approaches: controlled slicing and extraction of the deciduous second molar. The aim was to facilitate the physiological mesial shift of the permanent molars, thereby preventing long-term complications and minimising the need for restorative treatment. Of the 52 missing premolars, 42 (81%) were located in the mandible, while 10 (19%) were in the maxilla. Among these cases, 28 (54%) were managed using controlled slicing, 14 (27%) underwent extraction of the mandibular deciduous second molars, and 10 (19%) had the maxillary deciduous second molars extracted. All 10 maxillary extraction cases resulted in successful outcomes. The bodily movement of the maxillary first molar was more effective than that of the mandibular first molar. Due to the root anatomy of the maxillary deciduous second molar, controlled slicing is not recommended in these cases, as extraction of the deciduous molar provides a more favourable condition for the permanent molar to move truncally. Among the 20 missing teeth in the 8-9 year-old group treated with controlled slicing, 18 (90%) demonstrated the most favourable clinical outcomes, while the remaining 2 (10%) exhibited moderate responses. In contrast, in the 10-11 year-old group, controlled slicing was associated with a higher tendency for moderate or poor results, with only 2 cases (25%) achieving good clinical outcomes. In the extraction group within the 8-9 year-old category, only 2 cases (28.5%) showed favourable outcomes, 3 cases (42.8%) had moderate responses, and 2 cases (28.5%) had poor results. Similar trends were observed in the older age group. When analysing the clinical responses across both treatment approaches regardless of age, controlled slicing yielded the best outcomes, with 71.4% achieving favourable results and 21.4% showing moderate responses. Conversely, the extraction group exhibited a greater tendency towards moderate to poor outcomes, with 71.5% falling into these categories. The overall success rate of controlled slicing exceeded 90%, significantly outperforming the extraction method, which was associated with poor outcomes in more than 75% of cases (28).

A study comparing 2 groups of 30 patients with congenital second molar agenesis examined the effects of space closure treatment using hemisection. Among these patients, 23 underwent hemisection with space closure, involving 4 molar extractions—one group had their first molars extracted, while the other group had their second molars extracted. The degree of anchorage loss was analysed and compared between these 2 groups. Additionally, to establish an untreated control group, the changes observed in a Bolton sample of 9-to 12-year-olds were also evaluated. To enhance the correction process in the hemisection-treated group, patients were further divided into two subgroups: those who underwent upper tooth extractions and those who did not. The hemisection groups demonstrated a statistically significant reduction in the distal movement of the upper anterior teeth, as well as a decrease in the retraction of the upper and lower lips. Additionally, there was a significant increase in the mesial movement of the lower molar and an improvement in the molar relationships. The corrections in molar positioning were statistically significant, with a greater degree of molar relationship correction observed in the hemisection group compared with the premolar extraction group. However, there was no statistically significant difference in the overjet changes between the groups. This treatment approach enhances facial fullness by preserving the upper premolars. Hemisection plays a role in minimising the distal movement of the anterior teeth and reducing the flattening effect on the facial profile that can occur with space closure. The benefits provided by hemisection treatment make it possible to avoid extractions in the upper arch, significantly improving the overall treatment outcome (47).

This study aimed to evaluate space closure and occlusal changes following the extraction of the lower second premolar and the upper second premolar on the side of agenesis in 11 patients (mean age: 11.8 years), consisting of 7 girls and 4 boys, all with normal occlusion and lower second premolar agenesis. Treatment started once the first premolar had occluded, and patients were monitored over a 4-year period. Dental cast models were obtained at the start of treatment and at 1, 2, and 4 years post-extraction. Additionally, lateral cephalograms were taken at the beginning of treatment and after 2 and 4 years. Measurements of space closure, sagittal movements, rotational movements, tilting of the first molar and first premolar, and dental midline shifts were assessed using photographs of the dental casts. The sagittal movement of the incisors was evaluated through lateral cephalograms. Findings indicated that most extraction space closed within the first year, with 55% closure in the upper jaw and 46% in the lower jaw. By the end of the four-year follow-up, 89% of the extraction space in the upper jaw and 80% in the lower jaw had closed, leaving an average remaining extraction space of 0.9 mm in the upper jaw and 2 mm in the lower jaw. In the upper jaw, 70% of the extraction space was closed through the mesial and rotational movements of the first molars. The upper premolar exhibited distal movement only during the first year of observation. In the lower jaw, extraction space closure was primarily achieved through mesial rotational movement and tilting of the first molars, as well as distal movement and tilting of the first premolars. Unilateral

extraction did not impact the upper midline; however, a statistically significant shift towards the extraction side was observed in the lower midline. Based on these results, the extraction of the lower second deciduous molar, combined with the extraction of the upper second premolar, is proposed as a viable treatment approach. However, in certain cases, active space closure may be necessary (48).

A study evaluating the treatment of second molar agenesis through autotransplantation and preoperative orthodontic preparation examined 80 patients (39 girls and 41 boys), in whom a total of 110 teeth were transplanted to replace the missing lower second molars. The age range for patients receiving transplanted premolars was 10.2-22 years, while those undergoing molar transplantation were between 17.1 and 21 years old. At the 4-year follow-up, failure was observed in 8 of 99 teeth with incomplete root formation and in 2 of 11 teeth with complete root formation. Overall, 100 transplanted teeth (91%) were classified as successful. Among the transplanted teeth, 58 were placed in the left mandible and 52 in the right mandible. In 101 cases, the deciduous molar was still present at the time of transplantation. Orthodontic treatment was performed in 52 cases, with 11 patients requiring preoperative orthodontic intervention specifically to enlarge the space for autotransplantation. The remaining patients underwent orthodontic treatment for reasons unrelated to tooth loss. On average, the duration of preoperative orthodontic treatment was 12 months.

Four years post-transplantation, all transplanted teeth exhibited either partial or complete pulp chamber degradation. A total of 44 molars and 66 premolars were autotransplant to replace missing premolars, with the premolars extracted primarily due to crowding. The success rate was 92% for teeth with incomplete root formation and 82% for those with fully developed roots. These findings indicate that when carefully planned and appropriately timed, autotransplantation offers a favourable prognosis for patients with lower second molar deficiencies (49).

CONCLUSION

The management of second premolar agenesis requires an interdisciplinary approach, considering patient age, skeletal growth, occlusion, and the condition of the retained deciduous molar. Preserving the deciduous molar is an option if it remains functional, but risks like root resorption and infraocclusion must be monitored.

If extraction is necessary, spontaneous space closure, orthodontic treatment, or autotransplantation may be considered. Orthodontic space closure is ideal when feasible, while autotransplantation is a good alternative for growing patients. Implants and prostheses are best suited for skeletally mature patients to avoid complications.

A personalised treatment plan is essential to balance function, aesthetics, and long-term stability. Further research is needed to refine the treatment protocols for optimal outcomes.

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