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Research Article

DNASE I IMPACT ON BREAST CELLS CO-CULTURED WITH OVARIAN CANCER CELLS: A STUDY OF CELLULAR INTERACTIONS AND VIABILITY

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ABSTRACT

Objective: Cell-free DNAs (cf-DNAs) are released into the bloodstream through cell apoptosis, necrosis, or active secretion, often originating from cancer cells. These cf-DNAs have been associated with cancer development and metastasis, although their precise role remains under debate. DNase I, an enzyme that degrades extracellular DNA, has shown potential to impact cf-DNAs and influence cancer progression. This study investigates the effects of ovarian cancer cells on the proliferation and viability of non-tumorigenic breast cells, with a focus on DNase I's role.

Materials and Methods: Human ovarian cancer cells (OVCAR-3) and normal human breast cells (MCF-10A) were cultured under standard conditions (37°C, 5% CO_2). Co-culture experiments were conducted by incubating cells separately in plates and inserts, with or without DNase I, for 72 hours. Cell viability was assessed using the trypan blue exclusion test, while proliferation and adhesion were measured with an XTT assay.

Results: DNase I significantly reduced OVCAR-3 proliferation ($p<0.001$) and adhesion ($p<0.01$) without affecting MCF-10A cells. DNase I also decreased OVCAR-3 cell viability but did not significantly impact MCF-10A viability. Genetic analysis identified p53 exon 7 mutations and methylation of APC1A, APC1B, and RASSF1A genes in OVCAR-3 cells, which were unaffected by DNase I. No mutations or methylation were detected in MCF-10A cells.

Conclusion: The results suggest that the impact of DNase I on the proliferation and viability of cancer cells is significant and warrants further investigation. The potential effects of prolonged exposure between different cell types could yield even more compelling findings.

Keywords: cf-DNA, OVCAR-3, MCF-10A, Cancer, DNase I

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INTRODUCTION

DNA fragments, known as cell-free DNAs (cf-DNAs), can be released into the bloodstream through processes such as apoptosis, cell death, or active secretion by living cells, including those from cancerous tumors. Research has suggested that cf-DNAs shed by tumor cells into the bloodstream may contribute to cancer spread(1). It has long been recognized that cancer cells can disseminate throughout the body via metastatic processes. Free DNAs, discovered in 1948, have been shown in various in vivo studies to integrate into the genome of certain cells through horizontal gene transfer (2-4). Data from 1982 suggested that dominant oncogenes found in tumor cells could be transferred to normal cells (5). This led to the hypothesis that "cancer metastasis might occur through the transfection of cell-free DNAs containing dominant genes from the primary tumor into susceptible cells in the target organ"(3). The finding that cell-free DNAs can reflect the invasive and prognostic characteristics of the original cancer spurred laboratory research into their clinical potential. Studies have shown that DNA fragment levels, which are usually low, increase as malignancy progresses(5). These observations underscore the role of cell-free DNA in cancer development and metastasis. Research focusing on the relationship between tumor-specific DNA changes in primary tumors and blood samples has emphasized alterations in methylation, microsatellites, oncogenes, and tumor suppressor genes(6-8) .

Point mutations or changes in chromosomal integrity within various genomic regions of tumor cells can influence oncogenes, tumor suppressor genes, and genes related to metastasis, such as HIF-1 (Hypoxia-Inducible Factor-1) (9). It is reported that over 50% of human cancers carry gene mutations (10). Mutations in exons 5-8 of the p53 gene are commonly found in primary tumors and are frequently detected in plasma as well (2). Mutant p53 plays a crucial role in the development of metastasis by either blocking or activating downstream target genes, leading to DNA damage and the activation of multiple oncogenes. For example, OVCAR-3 cells exhibit a specific mutation (743G>A) in exon 7 of the p53 gene (11, 12).

Mutations in cancer-related genes are typically not due to regulatory abnormalities alone. Metastasis is also facilitated by epigenetic modifications, such as chromatin remodeling in CpG islands and gene silencing errors related to hyper-methylation (13). DNA methylation-induced gene silencing leads to gene inactivation

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at the transcriptional level (14, 15). While CpG islands in the promoter regions of approximately 50% of genes remain unmethylated in normal cells, CpG dinucleotides in repetitive sequences are methylated. In cancer cells, widespread hypo-methylation across the genome is often observed, along with hyper-methylation of CpG islands in promoter regions, resulting in gene silencing crucial for the inactivation of tumor suppressor genes (16). The inactivation of these genes, such as the RASSF1A gene, is vital for cancer progression. One significant epigenetic mechanism implicated in the development of ovarian cancer is the methylation of tumor suppressor genes. Research has shown that patients with primary or metastatic breast cancer exhibit RASSF1A methylation in their plasma and serum (16).

In normal cells, CpG islands in the promoter regions of about half of all genes remain unmethylated, while CpG dinucleotides in repetitive sequences are methylated. Cancer cells frequently display widespread genome-wide hypo-methylation along with hyper-methylation of CpG islands in promoter regions, leading to gene silencing and the inactivation of tumor suppressor genes, which is crucial for cancer development (16). The inactivation of tumor suppressor genes like RASSF1A is essential for cancer progression. Epigenetic mechanisms, such as RASSF1A methylation, have been identified in the plasma and serum of patients with primary or metastatic breast cancer, underscoring their role in cancer development.

Studies have indicated that promoter hyper-methylation and the reduced expression of the tumor suppressor gene adenomatous polyposis coli (APC) play significant roles in cancer development. The association between ovarian cancer and APC promoter hyper-methylation has been well-established. Research has shown that APC promoter hyper-methylation is more prevalent in ovarian cancer patients than in healthy and benign controls, suggesting that this could increase the risk of developing ovarian cancer (17). It has been proposed that RASSF1A and APC genes could serve as epidemiological biomarkers in individuals at risk for breast cancer due to the high incidence of promoter hyper-methylation in cancers (18).

DNase I, a member of the endonuclease enzyme class, breaks down phosphodiester bonds between sxrch has shown that metastases can form due to the leakage of free DNA from tumor cells into the circulation. Numerous studies have investigated the role of DNase I in metastasis formation. This study aimed to determine whether certain genes and structural features of OVCAR-3 tumor cells can be transferred to healthy

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MCF-10A cells via free DNA. Additionally, the differences between the presence and absence of DNase I in these two cell types (cancerous and non-cancerous) were explored for the first time in the literature.

MATERIALS AND METHODS

Cell Culture

The human ovarian cancer cell line OVCAR-3 (ATCC, HTB-16, Washington D.C., USA) was cultured in 25 cm² and 75 cm² flasks (Thermo Fisher Scientific, USA) using RPMI-1640 medium (ATCC, Washington, USA) supplemented with 20% FBS (Gibco, UK) and 0.01 mg/ml bovine insulin (Sigma, USA), and maintained in a humidified incubator at 37°C with 5% CO2 (Sanyo, Japan). The human normal breast cell line MCF-10A (ATCC, CRL-10317, Washington D.C., USA) was cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, UK), which was supplemented with 10% Fetal Bovine Serum (Gibco, UK) and 2 mM L-Glutamine, in 25 cm² and 75 cm² flasks (Thermo Fisher Scientific, USA). These cells were incubated under the same conditions at 37°C with 5% CO_2 (Sanyo, Japan).

Table 1. Experimental Groups

Group no	Groups
1	OVCAR-3
2	OVCAR-3 DNase I
3	MCF10A
4	MCF10A DNase I
5	Co-culture OVCAR-3
6	Co-culture MCF10A
7	Co-culture OVCAR-3 DNse I
8	Co-culture MCF10A DNase I

DNase I Treatment

A DNase I stock solution (10 mg/ml) was prepared by dissolving 10 mg of powdered DNase I (Sigma-Aldrich, Germany) in 1 ml of the appropriate medium. Cells were then treated with DNase I at a final

concentration of 0.1 mg/ml for 72 hours. For co-culture experiments, sterile inserts (Thermo Scientific, USA) with a 6.5 mm diameter and a pore size of 0.4 μm were placed in a 24-well plate.

Proliferation Assay

Cell proliferation was assessed using the XTT assay (Biological Industries, Israel). A 5 ml reaction solution, containing 100 μl of activation solution, was prepared, and 50 μl of this mixture was added to each well containing the cell samples. After a 2-hour incubation at 37°C, absorbance was measured at 450 nm using a Multiskan GO spectrophotometer (Thermo Scientific, USA). The proliferation rates were determined using raw data and standard curves. Each experiment was conducted at least three times for consistency.

Adhesion Assay

The cells were detached from the flask surface using trypsin, counted, and $7x10^3$ cells were transferred to 7 separate wells of 96-well plates for each group. The cells were incubated for 24 hours to allow adhesion to the flask surface. After performing the appropriate procedures for each group in the wells designated for adhesion, the cells were washed three times with PBS, and 100 μl of fresh medium was added. Then, the XTT assay was applied. The adhesion of the cells was determined by substituting the results into the formula.[1-(absorbance of unwashed well- absorbance of washed well)]/absorbance of unwashed well)]

Cell Viability Assay

Cell viability was assessed using the trypan blue exclusion method. After trypsinization, a portion of the cell suspension was mixed with 10 μl of trypan blue dye (Biological Industries, Israel), placed on slides, and analyzed using a cell counter (JuLI™ Br, Nanoentek Inc., Seoul, Korea), with results expressed as the percentage of viable cells.

DNA Extraction

DNA extraction was performed using the Direct PCR kit (Thermo Fisher Scientific, USA), following the manufacturer's instructions. Dilution buffer and DNA release solution were added to the cells and media,

followed by vortexing. The mixture was incubated at room temperature for 5 minutes, then heated to 98°C. A PCR mixture was prepared using the supernatant, and DNA concentrations were measured with a Multiskan GO spectrophotometer (Thermo Scientific, USA). Samples were stored at -20°C until further analysis.

Primer Design and PCR Protocols

APC1A and APC1B Methylation Analysis: Methylated and unmethylated primers were designed for the APC1A and APC1B gene regions. The forward primer sequences for APC1A were 5′-TATTGCGGAGTGCGGGTC-3′ (methylated) and 5′-GTGTTTTATTGTGGAGTGTGGGTT-3′ (unmethylated), while the reverse primer sequences were 5′-TCGACGAACTCCCGACGA-3′ (methylated) and 5′-CCAATCAACAAACTCCCAACAA-3′ (unmethylated). For APC1B, the forward primer sequences were 5′-TAGAATAGCGAACGAGTGTTC-3′ (methylated) and 5′-GATAGAATAGTGAATGAGTGTTT-3′ (unmethylated), and the reverse primer sequences were 5′-TCCGACGACCACACCCCG-3′ (methylated) and 5′-CTTCCAACAACCACACCCCA-3′ (unmethylated). A 25 µl reaction mixture was prepared using 12.5 µl of One Taq Quick-Load 2x Master Mix (BioLabs, UK), 2 µl of forward primer, 2 µl of reverse primer, 2 µl of bisulfite-converted DNA, and dH2O to reach the final volume. The PCR conditions included an initial denaturation at 95°C for 15 minutes, followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 30 seconds. A final extension step was performed at 72°C for 10 minutes.

RASSF1A Methylation Analysis: For the RASSF1A gene, methylated and unmethylated primers were designed. The forward primer sequences were 5′-CGAGAGCGCGTTTAGTTTCGTT-3′ (methylated) and 5′-GGGGGTTTTGTGAGAGTGTGTTT-3′ (unmethylated), while the reverse primer sequences were 5′-CGATTAAACCCGTACTTCGCTAA-3′ (methylated) and 5′-CCCAATTAAACCCATACTTCACTAA-3′ (unmethylated). The PCR reaction setup and cycling conditions were identical to those used for APC1A and APC1B analysis. PCR products were separated on a 2% agarose gel at 120V for 30 minutes and visualized using a SynGene UV imaging system.

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p53 Exon 7 Mutation Analysis: Primers targeting p53 exon 7 were used, with the forward primer sequence 5′-TCCTAGGTTGGCTCTGACTGT-3′ and the reverse primer sequence 5′-AGTGGCCCTGACCTGGAGTCT-3′. The reaction mixture consisted of 12.5 µl of One Taq Quick-Load 2x Master Mix, 2 µl of forward primer, 2 µl of reverse primer, 2 µl of the DNA sample, and dH_2O to complete a 25 µl volume. The PCR protocol included an initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 62°C for 1 minute, and extension at 72°C for 1 minute. A final extension step was performed at 72°C for 10 minutes. After PCR amplification, samples were mixed with 95% formamide, heated at 80°C for 10 minutes, and chilled on ice. A 30% polyacrylamide gel electrophoresis was performed at 120V for 4 hours at 4°C, and the bands were visualized using a silver staining method.

Statistical Analysis

The normality of the quantitative data was evaluated using the Shapiro-Wilk test. For groups that exhibited a normal distribution, one-way analysis of variance (ANOVA) was used, followed by Tukey HSD test for multiple comparisons. Statistical significance was determined for p-values less than 0.05. The results were reported as mean ± standard deviation in tables and graphs. All statistical analyses were performed using IBM SPSS Statistics 21.0 software.

RESULTS

Proliferation Analysis

A statistical difference was observed between the groups ($p<0.001$). DNase I significantly reduces the proliferation of OVCAR-3. The untreated OVCAR-3 cells had a proliferation rate of (32,581 ± 4,784), while DNase I-treated OVCAR-3 cells (OVCAR-3 DNase I) showed a reduced proliferation rate of (22,714 ± 4,059) ($p<0.05$). This demonstrates that DNase I effectively decreases cancer cell proliferation. In contrast, the MCF-10A cells (non-cancerous breast cells) showed minimal impact from DNase I, with MCF10A having a proliferation rate of (31,445 ± 3,469) and MCF10A DNase I exhibiting (32,909 ± 3,969) ($p>0.05$). (Table 2).

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Table 2. Determination of Proliferation in Cells

Groups	Mean ± standard deviation	p value	Multiple Comparison
1	32581 ± 4784	p<0.001	1-2: p<0.05 6-8: p<0.05
2	22714 ± 4059		
3	31445 ± 3469		
4	32909 ± 3969		
5	27090 ± 5199		
6	26571 ± 6618		
7	26895 ± 5736		
8	35152 ± 4499		

Adhesion Analysis

A statistically significant difference was observed between the groups (p<0.01). DNase I had a strong effect on the adhesion of OVCAR-3 cells. Untreated OVCAR-3 cells had an adhesion value of (69,126 ± 8,500), while DNase I-treated OVCAR-3 cells (OVCAR-3 DNase I) showed a significantly lower adhesion value of (47,925 ± 10,028) (p<0.01) (Table 3).

Table 3. Determination of Adhesion in Cells

Groups	Mean ± standard deviation	p value	Multiple Comparison
1	69126±8500	p<0.01	1-2: p<0.01
2	47925±10028		
3	63291±3584		
4	57132±6195		
5	60443±6556		
6	65229±6443		
7	56471±10549		
8	63639±4683		

Cell Viability

Cell viability analysis shows that DNase I selectively impacts cancer cells. Untreated OVCAR-3 cells had a viability rate of 87.2%, which significantly dropped to 68.1% after DNase I treatment. In contrast, MCF-

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10A cells (non-cancerous breast cells) remained largely unaffected by DNase I, with untreated cells showing 81.3% viability and DNase I-treated cells at 77.5%. This indicates that DNase I effectively targets cancer cells while preserving the viability of non-cancerous cells (Table 4).

Table 4. Determination of Viability in Cells

Groups	Viability %
1	% 87,2
2	% 68,1
3	% 81,3
4	% 77,5
5	% 80,4
6	%83,3
7	%71,6
8	%82,2

Gene Mutation and Methylation Analysis

Gene mutation analysis showed that p53 exon 7 mutations were present in OVCAR-3 cells, confirming their cancerous nature, while no mutations were found in MCF-10A cells. Additionally, APC1A, APC1B, and RASSF1A gene methylation was detected in both untreated and DNase I-treated OVCAR-3 cells, indicating DNase I may degrade hyper-methylated cfDNA in cancer cells. In contrast, no methylation was observed in MCF-10A cells, showing that DNase I selectively affects cancer cells without impacting non-cancerous cells (**Table 5**).

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Table 5. Gene Mutation and Methylation Analysis

Condition	p53 Exon 7	APC1A Methylated	APC1A Unmethylated	APC1B Methylated	APC1B Unmethylated	RASSF1A Methylated	RASSF1A Unmethylated
OVCAR-3 Untreated	+	+	-	+	-	+	-
OVCAR-3 DNase I Treated	+	+	-	+	-	+	-
MCF-10A Untreated	-	-	-	-	-	-	-
MCF-10A DNase I Treated	-	-	-	-	-	-	-
Co-culture OVCAR-3 Untreated	+	+	-	+	-	+	-
Co-culture OVCAR-3 DNase I Treated	+	+	-	+	-	+	-
Co-culture MCF-10A Untreated	-	-	-	-	-	-	-
Co-culture MCF-10A DNase I Treated	-	-	-	-	-	-	-

DISCUSSION

Molecular diagnostic markers have become indispensable tools for detecting cancer metastasis and tumor formation, significantly contributing to personalized cancer treatments. These markers provide detailed insights into the tumor's genetic profile, essential for selecting appropriate therapies such as chemotherapy or surgery. Circulating free DNA (cfDNA) is a promising non-invasive biomarker that reflects cancer's invasive and prognostic characteristics. Studies have emphasized its importance in understanding cancer progression and metastasis, highlighting its potential in clinical research and tailored treatment approaches (20-23).

cfDNA is released from tumor cells primarily through apoptosis and necrosis, carrying mutations and epigenetic modifications that may drive metastasis. A pivotal study using a rat model of colon cancer demonstrated that oncogenic material from tumor cells could be transferred to normal cells, contributing to metastatic processes. The transfer of the CAT marker gene to lung tissues after injecting plasma from cancer-bearing rats was one of the first experimental validations of cfDNA-mediated metastasis, often referred to as

"genometastasis" (2, 4). These findings have been supported by more recent studies, confirming that cfDNA can promote the dissemination of oncogenic DNA, leading to cancer spread in distant organs (20, 22).

DNase I, an enzyme traditionally associated with nucleic acid metabolism, has gained attention for its potential therapeutic role in cancer treatment due to its ability to degrade cfDNA. Early studies presented conflicting evidence regarding its effectiveness, but recent research confirms DNase I's capacity to reduce cancer progression by breaking down hyper-methylated cfDNA, a feature of cancer cells. This enzymatic degradation prevents the transfer of oncogenic material to healthy cells, limiting metastasis. Our study focused on the selective targeting of hyper-methylated cfDNA fragments by DNase I, revealing its potential as a cancer treatment tool (24). The enzyme's ability to degrade cfDNA makes it a promising candidate for future cancer therapies aimed at reducing tumor metastasis (25).

DNA methylation is a key epigenetic process involved in cancer development, as it often silences tumor suppressor genes. CpG dinucleotides, which constitute over 70% of vertebrate DNA, are frequently methylated in cancer cells, leading to the inactivation of critical genes such as APC and RASSF1A (26). These genes play essential roles in controlling cell proliferation and apoptosis, and their silencing contributes significantly to cancer progression (27). DNase I's ability to degrade hyper-methylated cfDNA fragments suggests it may reverse gene silencing, reactivating tumor suppressor genes and inhibiting tumor growth (25). This therapeutic potential offers a promising avenue for epigenetic cancer therapies, particularly when combined with other targeted treatments.

One key finding in DNase I research is its selective action on cancer cells. Previous studies demonstrated that DNase I effectively reduced the viability of MDA-MB-231 breast cancer cells without harming healthy bone cells. This specificity is linked to the hyper-methylation of APC1A, APC1B, and RASSF1A genes in cancer cells, while healthy cells remain unaffected (28). Our study expanded on this, showing that DNase I's effectiveness may vary depending on the cellular environment. For example, no significant differences in gene methylation were observed between MCF-10A and OVCAR-3, suggesting that the enzyme's activity may be cell-type specific. These findings indicate DNase I could be developed as a targeted therapy for specific cancer types.

Despite DNase I's promising results in degrading cfDNA and selectively targeting cancer cells, further research is needed to fully understand its potential as a cancer therapy. In particular, studies should explore its long-term effects, especially when combined with other epigenetic therapies, to determine its efficacy across different cancer types and genetic profiles (24). Additionally, interactions between cancer and healthy cells, particularly how cancer cells influence normal cell proliferation and viability, remain critical areas for future research (26). DNase I's ability to reverse gene silencing by degrading hyper-methylated cfDNA fragments could pave the way for novel cancer treatments, but more comprehensive studies are required to confirm these findings.

CONCLUSION

In conclusion, molecular diagnostic markers like cfDNA have proven essential in understanding cancer metastasis and guiding personalized treatment plans. DNase I has emerged as a promising therapeutic tool for targeting hyper-methylated cfDNA, potentially reactivating silenced tumor suppressor genes and inhibiting cancer progression. While further research is required to validate DNase I’s long-term efficacy, its selective action on cancer cells offers hope for developing more effective cancer therapies in the future.

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Authorship contributions

All authors have contributed equally to the work

Data availibity statement

The authors state that the data supporting the study's results can be found in the article. Additionally, the raw data can be obtained from the corresponding author upon a reasonable request.

Declaration of competing interest

The Authors declare no conflict of interest pertaining to the current work

Ethics

Since the study was conducted on commercially purchased cell cultures, an ethics committee approval document is not required.

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