www.biodicon.com Biological Diversity and Conservation

ISSN 1308-8084 Online ISSN 1308-5301 Print

Research article/Araştırma makalesi
DOI: 10.46309/biodicon.2020.746487
13/2 (2020) 153-159

The effect of DNase I on free DNAs and its relationship with metastasis: A preliminary results

Aylin DAL ULUTAŞ [1], Didem TURGUT COŞAN [*1,2]
ORCID: 0000-0002-3382-9451**;** 0000-0002-8488-6405

[1] Eskişehir Osmangazi University, Graduate School of Natural and Applied Sciences, Department of Biotechnology and Biosafety, Eskişehir, Turkey
[2] Eskişehir Osmangazi University, Faculty of Medicine, Department of Medical Biology, Eskişehir, Turkey

Abstract

In breast cancer, p53 is generally mutant and plays an important role in the development of metastasis, including bone metastasis. However, mutations are not always responsible for regulation disorders in cancer-related genes. Epigenetic changes such as hyper-methylation-related gene silencing errors and chromatin remodeling in CpG islands contribute to the development of cancer and metastasis. Free DNAs play an important role in cancer and metastasis formation. Studies show that DNA fragment levels are low in normal conditions and an increase in malignancy. In our study, invasive breast cancer cells MDA-MB-231 and hFOB 1.19 cells representing bone tissue, which is one of the most metastasized tissues of breast cancer, were cultured in the same environment (co-culture). In both cells, the presence of hypermethylation in the mutant p53 exon 8 region and APC1A, APC1B, and RASSF1 genes in MDA-MB-231 cells was observed. Appropriate primer sequences were selected for p53 exon8 amplifications and APC1A, APC1B, and RASSF1A methylation analysis. After PCR treatment applied for mutant p53 exon8 determinations, the products were subjected to electrophoresis in a 30% homogeneous polyacrylamide gel. Methylation Specific PCR (MSP) was performed by the APC1A, APC1B, and RASSF1A gene regions. PCR products were analyzed by conducting 2% agarose gel electrophoresis. The results were also evaluated in environments where DNase I was added. The results of our study provide researchers with a model that shows that some gene and structural features of tumor cells can be transported to normal cells via free DNA. Also, it is aimed to reveal the effect of DNase I in providing or preventing this transport.

Key words: cell-free DNA, breast cancer cell, metastasis, bone cell, DNase I k

---------- * ----------

DNase I 'in serbest DNAlar üzerine etkisi ve metastaz ile ilişkisi: İlk sonuçlar

Özet

Meme kanserinde p53 genellikle mutanttır ve kemik metastazı da dahil olmak üzere metastaz gelişiminde önemli bir rol oynar. Bununla birlikte, mutasyonlar kansere bağlı genlerdeki regülasyon bozukluklarından her zaman sorumlu değildir. CpG adalarında hipermetilasyonla ilişkili gen susturma hataları ve kromatin yeniden modellenmesi gibi epigenetik değişiklikler, kanser ve metastaz gelişimine katkıda bulunur. Serbest DNA'lar kanser ve metastaz oluşumunda önemli bir rol oynar. Çalışmalar, DNA fragment seviyelerinin normal koşullarda düşük olduğunu ve malignitede arttığını göstermektedir. Çalışmamızda, meme kanserinin metastaz yapma eğiminde olduğu dokularından biri olan kemik dokusunu temsil eden hFOB 1.19 hücreleri ile invaziv meme kanser hücreleri MDA-MB-231 aynı ortamda kültüre edilmiştir (ko-kültür). Her iki hücrede de, mutant p53 ekson 8 bölgesinde hipermetilasyon ve MDA-MB-231 hücrelerinde APC1A, APC1B ve RASSF1 genlerinin varlığı gözlendi. P53 ekson8 amplifikasyonları ve APC1A, APC1B ve RASSF1A metilasyon analizi için uygun primer sekansları seçildi. Mutant p53 ekson8 saptamaları için PCR işleminden sonra, ürünler %30 homojen bir poliakrilamid jel içinde elektroforeze tabi tutuldu. APC1A, APC1B ve RASSF1A gen bölgelerinde, Metilasyon Spesifik PCR (MSP) işlemi gerçekleştirildi. PCR ürünleri %2

[*] *Corresponding author* / Haberleşmeden sorumlu yazar: Tel.: +905333662680; Fax.: +902222393772; E-mail: dcosan@gmail.com
 Received: 01.06.2020; Published: 15.08.2020 BioDiCon. 909-0601

agaroz jel elektroforezi yapılarak analiz edildi. Sonuçlar DNase I'in eklendiği ortamlarda da değerlendirildi. Çalışmamız araştırmacılara, tümör hücrelerinin bazı genlerinin ve yapısal özelliklerinin, serbest DNA yoluyla normal hücrelere taşınabileceğini gösteren bir model sunmaktadır. Ayrıca, DNase I'in bu taşınmayı sağlama veya önlemedeki etkisini ortaya koymaktadır.

Anahtar kelimeler: cell-free DNA, breast cancer cell, metastasis, bone cell, DNase I

1. Introduction

It has been known for a long time that tumor cells move through the body and form metastases. Subsequent studies have led to the suggestion that this metastasis could be via free DNA released from tumor cells into the blood. Free DNAs, which are DNA fragments released into the bloodstream as a result of cell apoptosis, necrosis, or active release of living cells, can be released from cancerous cells into plasma and serum. These DNAs, which were first determined in 1948, have been shown in various studies in which horizontal gene transfer takes place by integrating some cells into the genome [1]. These data were obtained in 1982 by Pulciani et al. it proves the view that "dominant oncogenes present in tumor cells can be transferred to normal cells" [2]. These studies led to the view that “metastasis can be realized by transfection of sensitive cells localized in the target organ with free DNA containing the dominant gene belonging to the primary tumor”. With the discovery that free DNA can reflect invasive and prognostic features in cancer, laboratory studies on its applicability in the clinic have begun. Studies show that DNA fragment levels are low in normal conditions and an increase in malignancy [1]. These studies reveal the importance of free DNA in cancer and metastasis formation.

Studies determining tumor-specific DNA exchanges between primary tumor and blood in breast cancer patients have focused on oncogene and tumor suppressor gene mutations, methylation states, and microsatellite changes [1,3]. Point mutations or chromosome integrity changes occur in various regions of the tumor cell's genome. These changes may affect proto-oncogenes, tumor suppressor genes, and metastasis-related genes such as HIF-1 (Hypoxia-Inducible Factor-Hypoxia-Inducible Factor-1) [1,2].

In primary tumor, mostly p53 gene exon 5-8 mutations occur and in many cases, the same mutations have been identified in cell-free DNA in plasma parallel to tumor tissue [4,5]. In breast cancer development, the p53 gene is usually mutant and plays an important role in many stages of metastasis development [6].Mutant p53 oncoprotein activates or blocks downstream target genes, causing DNA damage and various oncogene activations in cells. According to the International Agency for Research on Cancer (IARC) data, 70% of p53 missense mutations cause breast cancer development [7].p53 plays a very important role in the development of most metastases, including bone metastasis [8,9].

Regulation disorders in cancer-related genes do not always result in mutation. Epigenetic changes such as hyper-methylation-related gene silencing errors and chromatin remodeling in CpG islands also contribute to the development of metastasis [10,11]. DNA methylation causes gene silencing, causing the gene to be inactivated at the transcriptional level [11,12]. In normal cells, CpG dinucleotides in repeat sequences are methylated, while CpG islets in the promoter regions of 50% of genes are un-methylated. In cancer cells, with diffuse hypo-methylation in the genome, hyper-methylation is observed in CpG islets in the promoter regions. Promotor region hypermethylation causes silencing of the subsequent gene, which is especially important in the inactivation of tumor suppressor genes [11,12].Studies have shown that RASSF1A and APC are methylated in the plasma and serum of primary or metastatic breast cancer patients [11].Because of the high rate of promoter hyper-methylation in RASSF1A and APC genes in breast cancer, it has been suggested that these genes can be used as an epidemiological biomarker in individuals at risk for breast cancer. The most common hypermethylated gene region in human cancers is RASSF1A, and the CpG islands are highly hyper-methylated in breast cancer patients [12].

The effects of free DNAs on metastasis in vitro conditions and the role of DNase I have been investigated in various studies. The effect of DNase I, which plays an important role in the nucleic acid metabolism in the blood with the determination of oncogenic free DNA, has been a matter of curiosity. Studies on the anti-tumoral effect of DNase I started from the 1960s and continue until today [13]. DNase I’s are a large family of endonuclease enzymes that break down the phosphodiester bonds of double chain DNA between pyrimidine bases [14]. Generally, it is thought that the free DNAs in the plasma are cut by DNases [3,15]. It is interpreted that the reason why cancer patients have higher levels of free DNA in the plasma of healthy individuals may be circular DNase inhibitors in some sources and low DNase levels in some sources [15,16]. It is reported that DNase I activity is seen more frequently in regions where methylation is repeated [17]. Previous studies suggest that free DNA released from tumor cells into the blood may form metastases. There are several studies about the role of DNase I in the development of metastasis due to its role in free DNA breakdown [12]. MDA-MB-231 cells have been shown in previous studies to be sensitive to DNase I [15].

In this study, it is aimed to determine the transfer of free DNA fragments released from MDA-MB-231 cells to hFOB cells in the co-culture medium and to determine the effect of DNase I on this transfer. In our study, MDA-MB-231, which represents invasive breast cancer cells, and hFOB 1.19 cells, which represent bone tissue, one of the tissues where breast cancer metastasizes at the highest rate, were kept in the same environment (co-culture). Thus, we predicted

that p53 exon8 and methylated/un-methylated gene regions specific for MDA-MB-231 cells can be detected in hFOB 1.19 cells. Previous studies have shown that DNase I affects oncogenic free DNAs released from MDA-MB-231 cells. For this reason, we added DNase I to the culture medium and thus evaluated the results from a different perspective. We did not know which of the two possible results would be in the presence of DNase I. The first possibility is to increase DNA transfer to hFOB 1.19 cells by causing more oncogenic free DNA released to the environment due to the sensitivity of MDA-MB-231 cells to DNase I. The second possibility is that the free DNA released from MDA-MB-231 cells was not transferred to hFOB 1.19 cells since it was cleaved with DNase I in the medium. You can find the results and comments in the following lines of the article.

2. Materials and methods

Human breast cancer cells MDA-MB-231 and human osteoblast cells hFOB 1.19 were obtained from ATCC (Washington D.C., USA). The cells were seeded into T-25 and T-75 cm2 flask (Greiner, Cellstar, Germany) using Dulbecco's Modified Eagle's Medium-F12, supplemented with %10 Fetal Bovin Serum (FBS; Gibco, UK) in 5% CO_2 at 34,5°C. The cells were co-cultured with MDA-MB-231 on the plate base and hFOB 1.19 cells in the inserts. The cells in the co-culture medium were administered DNase I at a concentration of 0.1 mg/ml for 72 hours. Cells were removed from the flask base with trypsin after 72 hours of incubation, and DNAs were isolated from these cells using the Thermo Scientific Phusion Human Specimen Direct PCR kit (Thermo Fisher Scientific, USA). Following the kit procedure, 20 µl Dilution Buffer and 0.5 µl DNARelease Additive were added to the tubes. After vortexing, it was incubated for 2-5 minutes at room temperature. Samples were incubated at room temperature for 2-5 minutes after the vortex step. Then, incubation was continued at 98°C for 2 minutes. 20 µl of the PCR mixture was created by taking 0.5 µl of the supernatant. Following the kit protocol, 10 µl 2x Phusion Human Specimen PCR Buffer, 0.4 µl Universal Control Primer, 0.4 µl Phusion Human Specimen DNA Polymerase, 0.5 µl sample and enough distilled water was added to complete the total volume to 20 µl. The PCR parameters were 98°C for 1 second, 72°C for 1 minute, 72°C for 15 seconds for 40 cycles followed by a final elongation at 72°C for 5 min. The concentrations of the DNA samples obtained were determined on the Multiskan ™ GO Microplate Spectrophotometer device and stored at -20°C until use. To perform methylation analysis, bisulfite conversion was performed in the samples. EZ DNA Methylation-GoldTM Kit was used for this application. Primer sequences were selected for the p53 exon8 (E-8) and the methylation analysis of APC1A, APC1B, and RASSF1A gene regions (methylated and un-methylated) (Table 1, Table 2, Table 3).

Table 1. Primer sequences for p53 exon 8 amplification

p53	**Forward Primer Dizisi**	**Reverse Primer Dizisi**
E-8	5'-GGGACAGGTAGGACCTGATTTCCTT-3'	5'-ATCTGAAGGCATAACTGCACCCTTGG-3'

Table 2. Methylated and un-methylated primer sequences for the APC1A and APC1B genes

APC	**Forward Primer Dizisi**	**Reverse Primer Dizisi**
1A-M	5'-TATTGCGGAGTGCGGGTC-3'	5'-TCGACGAACTCCCGACGA-3'
1A-UM	5'-GTGTTTTATTGTGGAGTGTGGGTT-3'	5'-CCAATCAACAAACTCCCAACAA-3'
1B-M	5'- TAGAATAGCGAACGAGTGTTC-3'	5'-TCCGACGACCACACCCCG-3'
1B-UM	5'-GATAGAATAGTGAATGAGTGTTT-3'	5'-CTTCCAACAACCACACCCCA-3'

1A-M: APC1A Methylated gene region; **1A-UM:** APC1A Un-methylated gene region; **1B-M**: APC1B Methylated gene region; **1B-UM**: APC1B Un-methylated gene region.

Table 3. Methylated and un-methylated primer sequences for the *RASSF1A* gene

RASSF1	**Forward Primer Dizisi**	**Reverse Primer Dizisi**
M	5'-CGAGAGCGCGTTTAGTTTCGTT-3'	5'-CGATTAAACCCGTACTTCGCTAA-3'
UM	5'-GGGGGTTTTGTGAGAGTGTGTTT-3'	5'-CCCAATTAAACCCATACTTCACTAA-3'

M: RASSF1A Methylated gene region; **UM:** RASSF1A Un-methylated gene region

For determination of p53 exon 8, 25 µl PCR mix was prepared using 12.5 µl One Taq ® Quick-Load 2x Master Mix (BioLabs, UK), 2 µl forward primer, 2 µl reverse primer, 2 µl DNA sample and sufficient amount of distilled water. PCR was performed at 94°C for 1 minute, 68°C for 1 minute, 70°C for 1 minute, 40 cycles. After PCR, 30% homogeneous polyacrylamide gel was prepared and electrophoresis was performed at +4°C.

Methylation Specific PCR (MSP) was performed following the APC1A, APC1B, and RASSF1A gene regions. Using appropriate primers, 12.5 µl One Taq ® Quick-Load 2x Master Mix, 2 µl forward primer, 2 µl reverse primer, 2 µl bi-sulfide converted DNA sample and 25 µl MSP mixture were prepared using a sufficient amount of water. Then,

the MSP procedure was performed according to each primer. For APC1A and APC1B; 95 cycles of 15 seconds, 60°C 45 seconds, 72°C 30 seconds, 35 cycles, RASSF1A 95°C 1 minute, 56°C 1 minute, 72°C 1 minute 40 cycles of PCR were applied. The PCR products obtained were analyzed by 2% agarose gel electrophoresis method..

3. Results

The presence of mutant p53 exon 8 region, which is absent in normal cells, was detected in hFOB 1.19 normal osteoblast cells co-cultured with breast cancer cells. It was observed that APC1A, APC1B, and RASSF1 gene regions were hyper-methylated in MDA-MB-231 cells, and APC1A, APC1B, and RASSF1 gene regions were un-methylated in hFOB 1.19 osteoblast cells. In our study, it was found that these normally un-methylated regions in hFOB 1.19 osteoblast cells were methylated in co-culture. In our study, all these results were also investigated after DNase I added to the co-culture medium. It was observed that the mutant p53 exon 8 region was not found in hFOB 1.19 osteoblast cells in the environment of DNase I. It was also determined that hyper-methylated regions seen in co-culture medium in hFOB cells were not seen after DNase I administration (**Figure 1**).

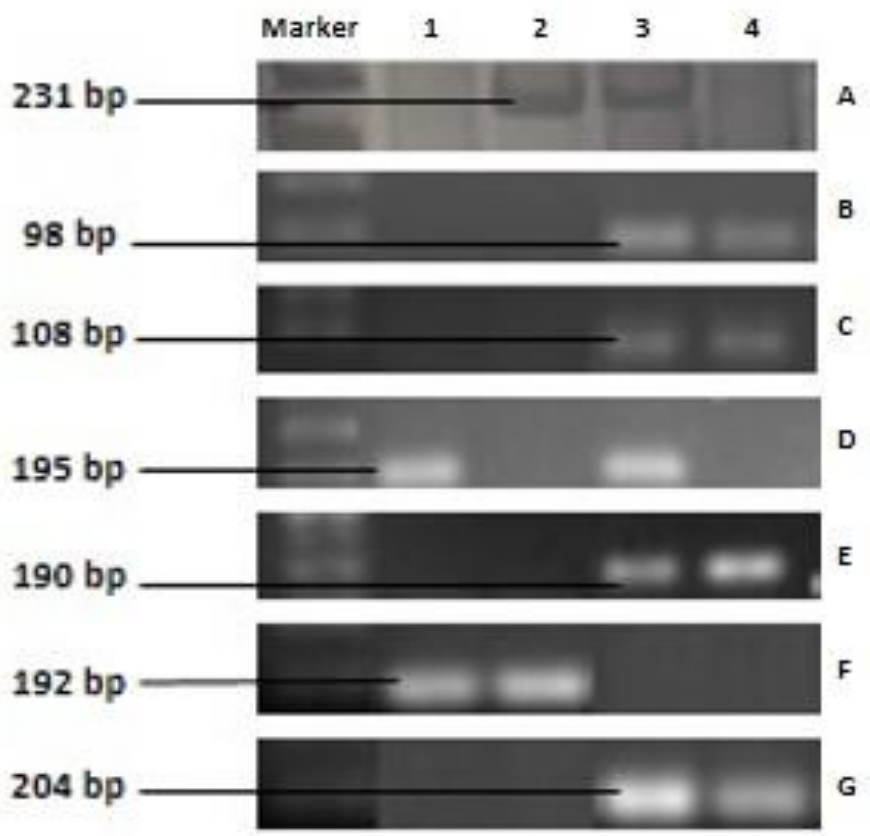

Figure 1. For P53-exon 8; hFOB1.19 cells (well 1), MDA-MB-231 cells (well 2), hFOB1.19 cells in co-culture (well 3), hFOB1.19 cells treated with DNase I in co-culture (well 4) (**A**), for APC1A-methylated region; hFOB1.19 cells (well 1), hFOB1.19 cells administered DNase in co-culture (well 2), MDA-MB-231 cells (well 3), hFOB1.19 cells in co-culture (well 4) (**B**), for APC1A- un-methylated region; MDA-MB-231 cells (well 1), MDA-MB-231 cells applied DNase I in co-culture (well 2), hFOB1.19 cells in co-culture (well 3), hFOB1.19 cells administered DNase I in co-culture (well 4) (**C**), for APC1B-methylated region; MDA-MB-231 cells (well 1), hFOB1.19 cells (well 2), hFOB1.19 cells in co-culture (well 3), hFOB1.19 cells applied DNase I in co-culture (well 4) (**D**), for APC1B- un-methylated; MDA-MB-231 cells (1), MDA-MB-231 cells treated with DNase I in co-culture (well 2), hFOB1.19 cells (well 3), hFOB1.19 cells treated with DNase I in co-culture (well 4) (E), for RASSF1 methylated region; MDA-MB-231 cells (well 1), hFOB1.19 cells in co-culture (well 2), hFOB1.19 cells (well 3), hFOB1.19 cells treated with DNase I in co-culture (well 4) (F), for RASSF1 un-methylated region; MDA-MB-231 cells (1), MDA-MB-231 cells (well 2) treated with DNase in co-culture, hFOB1.19 cells (well 3) in co-culture, hFOB1.19 cells treated with DNase in co-culture (well 4) (G).

4. Conclusions and discussion

Mutant p53 is a very important oncogene in the development of breast cancer and in the metastasis of many cancers, including bone metastasis, and this mutation can develop as a genetic or spontaneous [4,5,7-9]. Osteoblast differentiation is also regulated by the p53 signal pathway [16]. In our study, MDA-MB-231 cells, which are mutant and invasive breast cancer cells in terms of the p53 gene, and hFOB 1.19, normal osteoblast cells, were co-cultured. MDA-MB-231 cells used in our study have p53 exon 8 AGA-> AAA (Arg (R) -Lys (K)) missense mutation [11]. In our study, it was found that the p53 exon 8 (mutant) gene not found in hFOB 1.19 cells was also found in hFOB 1.19 cells co-cultured with MDA-MB-231 cells. This suggests that this can be achieved through oncogenic free DNA fragments of tumor cells. Free DNAs play an important role in cancer formation [18]. The effects of free DNAs on metastasis in vitro conditions and the role of DNase I have been investigated in various studies [1,17]. Although there are studies in the current literature showing its effect on methylation with DNase I activity, there is no research yet showing whether it has effects in terms of gene mutations.

In our study, the cancer cell-specific p53 exon 8 (mutant) gene was found in hFOB cells co-cultured with MDA-MB-231 cells in the environment without DNase I. It suggests that these tumor specific regions may have been transported to hFOB cells via oncogenic free DNA fragments. It was observed that there was no p53 exon 8 (mutant) gene in hFOB 1.19 cells co-cultured with MDA-MB-231 cells in the environment with DNase I. Therefore, it was concluded that DNase I has the potential to prevent this transition. In mammalian cells, the epigenetic system forms DNA methylation, histone modification, and non-coding RNAs. Studies show that gene silencing through methylation, which is one of the most important mechanisms of epigenetic control, can be a biological biomarker in cancer [19]. Jeronimo et al. conducted a methylation analysis of 23 gene regions in primary tumor tissue. It is suggested that these genes can be separated from invasive carcinoma and fibroadenoma due to the absence of hyper-methylation in normal tissue [20]. In many studies, it has been determined that APC and RASSF1A gene methylation play an important role in the development of breast cancer. Methylation gene pattern studies show that methylation can be used as an effective biomarker in cancer cells because normal cells contain non-methylated gene regions, cancer cells contain methylated gene regions. Swellam et al. in their study, they determined that methylated APC was higher in metastatic breast cancer patients than in benign breast cancer patients. It was also stated in the study that there was no hyper-methylated APC in healthy controls. Therefore, it has been stated that among the serum-based molecular markers, APC is very important in the early diagnosis of breast cancer [21]. Many studies show that the RASSF1A gene has low methylation levels in healthy individuals, and is hyper-methylated in breast cancer patients [8,11, 22-26]. Fackler et al. reported that tumor tissue has higher RASSF1A promoter methylation patterns than healthy tissue, as well as methylated genes in normal tissue around the tumor [27]. Also, it has been suggested that circular hyper-methylated RASSF1A in serum detected in metastatic breast cancer patients is associated with poor prognosis and maybe a very important prognostic biomarker [22, 27-28]. Another study in breast cancer patients found that RASSF1A and APC methylations were significantly higher than controls. It has been proposed that hyper-methylation seen in the promoter of these genes can also be used as an epidemiological biomarker in individuals at risk for breast cancer [12]. Another study in breast cancer patients found that RASSF1A and APC methylations were significantly higher than controls. It has been proposed that hyper-methylation seen in the promoter of these genes can also be used as an epidemiological biomarker in individuals at risk for breast cancer [12].

It has been previously determined that MDA-MB-231 cells are sensitive to DNase I [19]. Following this reported literature information, it was observed in our study that the viability of DNase I sensitive MDA-MB-231 cells decreased in the environment containing DNase I. In our study, in addition to evaluations for the p53 mutant gene, it was planned to demonstrate the methylation status of APC1A, APC1B, and RASSF1A genes in hFOB 1.19 cells cultured with MDA-MB-231 cells.

Accordingly, it was found that these genes, which are methylated in MDA-MB-231 cells, are not seen in the hFOB 1.19 cells cultured alone, but in the methylated state in the co-cultured hFOB 1.19 cells. There are studies in the literature to reveal the effect of DNase I in cancer [29, 30]. In a study evaluating the DNase I activity in the methylated and non-methylated gene regions, it was determined that the DNase I cut-out was more common in the regions where repeated methylation occurred [31].

When the data obtained from our study were evaluated, it was determined that the APC1A, APC1B and RASSF1A genes in the hFOB 1.19 cells kept in co-culture medium were hyper-methylated, whereas the APC1A, APC1B, and RASSF1A genes remained un-methylated in the environment containing DNase I. In the literature, DNase I has been reported to inhibit tumor cell proliferation as a potential antitumor agent and also reduce metastatic capacity [32,33].

Acknowledgements

This study was supported by Eskişehir Osmangazi University Scientific Research Project Committee with Project Support Number 2014575.

References

[1] García-Olmo, D. C., Gutiérrez-González, L., Samos, J., Picazo, M. G., Atiénzar, M., García-Olmo, D. (2006). Surgery and hematogenous dissemination: comparison between the detection of circulating tumor cells and of tumor DNA in plasma before and after tumor resection in rats. *Annals of Surgical Oncology*, 13(8), 1136-1144.

[2] Pulciani, S., Santos, E., Lauver, A. V., Long, L. K., Aaronson, S. A., Barbacid, M. J. N. (1982). Oncogenes in solid human tumours. *Nature*, 300(5892):539.

[3] Silva, J. M., Garcia, J. M., Dominguez, G., Silva, J., Miralles, C., Cantos, B., et al (2002). Persistence of tumor DNA in plasma of breast cancer patients after mastectomy. *Annals of Surgical Oncology*, 9(1):71-76 .

[4] Hartmann, A., Blaszyk, H., McGovern, R., Schroeder, J., Cunningham, J., De, E. V. et al (1995). p53 gene mutations inside and outside of exons 5-8: the patterns differ in breast and other cancers. *Oncogene*, 10(4):681-688.

[5] Runnebaum, I. B., Nagarajan, M., Bowman, M., Soto, D., Sukumar, S. (1991). Mutations in p53 as potential molecular markers for human breast cancer. *Proc Natl Acad Sci U S A*, 88(23):10657-10661.

[6] Gasco, M., Shami, S., Crook, T. J. (2002). The p53 pathway in breast cancer. *Breast Cancer Research*, 4(2):70.

[7] Petitjean, A., Mathe, E., Kato, S., Ishioka, C., Tavtigian, S. V., Hainaut, P., et al (2007). Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database. *Human Mutation*, 28(6):622-629.

[8] Powell, E., Piwnica-Worms, D., Piwnica-Worms, H. (2014). Contribution of p53 to metastasis. *Cancer Discov*, 4(4):405-414

[9] Yang, P., Du, C., Kwan, M., Liang, S., Zhang, G. J. (2013). The impact of p53 in predicting clinical outcome of breast cancer patients with visceral metastasis. *Scientific Reports*, 3:2246.

[10] Kulis, M., Esteller, M. (2010). Methylation and cancer. *Adv. Genet*, 70:27-56.

[11] Müller, H. M., Widschwendter, A., Fiegl, H., Ivarsson, L., Goebel, G., Perkmann, E., et al. (2003). DNA methylation in serum of breast cancer patients: an independent prognostic marker. *Cancer Research*, 63(22):7641-7645.

[12] Lewis, C. M., Cler, L. R., Bu, D-W., Zöchbauer-Müller, S., Milchgrub, S., Naftalis, E. Z, et al. (2005). Promoter hypermethylation in benign breast epithelium in relation to predicted breast cancer risk. *Clinical Cancer Research*, 11(1):166-172.

[13] de Lamirande, G. (1961). Action of deoxyribonuclease and ribonuclease on the growth of Ehrlich ascites carcinoma in mice. *Nature*, 192(4797):52.

[14] Lahm, A., Suck, D. (1991). DNase I-induced DNA conformation: 2 Å structure of a DNase I-octamer complex. *Journal of Molecular Biology*, 222(3):645-667.

[15] Leon, S. A., Shapiro, B., Servi, P., Parsons, R. G. (1981). A comparison of DNA and DNA-binding protein levels in malignant disease. *European Journal of Cancer*, 17(5):533-538.

[16] Gahan, P., Stroun, M. (2010). The biology of circulating nucleic acids in plasma and serum (CNAPS). Extracellular Nucleic Acids, Springer.

[17] Lazarovici, A., Zhou, T., Shafer, A., Machado, A. C. D., Riley, T. R., Sandstrom, R., et al (2013). Probing DNA shape and methylation state on a genomic scale with DNase I. PNAS, 110(16):6376-6381.

[18] Papadopoulou, E., Davilas, E., Sotiriou, V., Georgakopoulos, E., Georgakopoulou, S., Koliopanos, A., et al. (2006). Cell-free DNA and RNA in plasma as a new molecular marker for prostate and breast cancer. *Ann N Y Acad Sci*, 1075(1):235-243.

[19] Armenante, F., Merola, M., Furia, A., Tovey, M., Palmieri, M. (1999). Interleukin-6 repression is associated with a distinctive chromatin structure of the gene. *Nucleic Acids Research*, 27(22):4483-4490.

[20] Jeronimo, C., Monteiro, P., Henrique, R., Dinis-Ribeiro, M., Costa, I., Costa, V. L., et al. (2008). Quantitative hypermethylation of a small panel of genes augments the diagnostic accuracy in fine-needle aspirate washings of breast lesions. *Breast Cancer Research and Treatment*, 109(1):27-34.

[21] Swellam, M., Abdelmaksoud, M. D., Sayed Mahmoud, M., Ramadan, A., Abdel-Moneem, W., Hefny, M. M. (2015). Aberrant methylation of APC and RARβ2 genes in breast cancer patients. *IUBMB Life*, 67(1):61-68.

[22] Dulaimi, E., Hillinck, J., de Caceres, I. I., Al-Saleem, T., Cairns, P. (2004). Tumor suppressor gene promoter hypermethylation in serum of breast cancer patients. *Clin Cancer Res*, 10(18):6189-6193.

[23] Hoque, M. O., Feng, Q., Toure, P., Dem, A., Critchlow, C. W., Hawes, S. E., et al. (2006). Detection of aberrant methylation of four genes in plasma DNA for the detection of breast cancer. Journal of Clinical Oncology: *Official Journal of the American Society of Clinical Oncology*, 24(26):4262-4269.

[24] Hu. X-C., Wong, I. H., Chow, L. W. C. (2003). Tumor-derived aberrant methylation in plasma of invasive ductal breast cancer patients: clinical implications. *Oncology Reports*, 10(6):1811-5181.

[25] Skvortsova, T., Rykova, E., Tamkovich, S., Bryzgunova, O., Starikov, A., Kuznetsova, N., et al. (2006). Cell-free and cell-bound circulating DNA in breast tumours: DNA quantification and analysis of tumour-related gene methylation. *Br J Cancer*, 94(10):1492.

[26] Yazici, H., Terry, M. B., Cho, Y. H., Senie, R. T., Liao, Y., Andrulis, I., et al. (2009). Aberrant methylation of RASSF1A in plasma DNA before breast cancer diagnosis in the Breast Cancer Family Registry. *Cancer Epidemiol Biomarkers Prev*, 18(10):2723-2725.

[27] Fackler, M. J., Bujanda, Z. L., Umbricht, C., Teo, W.W., Cho, S., Zhang, Z., et al. (2014). Novel methylated biomarkers and a robust assay to detect circulating tumor DNA in metastatic breast cancer. *Cancer Res*, 74(8):2160-2170.

[28] Yazici, H., Terry, M. B., Cho, Y. H., Senie, R. T., Liao, Y., Andrulis, I., et al (2009). Aberrant methylation of RASSF1A in plasma DNA before breast cancer diagnosis in the Breast Cancer Family Registry. *Cancer Epidemiol Biomarkers Prev*, 18(10):2723-2725.

[29] Golonka, R. M., Yeoh, B. S., Petrick, J. L., Weinstein, S. J., Albanes, D., Gewirtz, A. T., et al. (2019). Deoxyribonuclease I Activity, Cell-Free DNA, and Risk of Liver Cancer in a Prospective Cohort. *JNCI Cancer Spectrum*, 2(4):pky083.

[30] Kochanek, S., Renz, D., Doerfler, W. (1993). Differences in the accessibility of methylated and unmethylated DNA to DNase I. *Nucleic Acids Res*, 21(25):5843-5845.

[31] Lu, R., Wang, Y., Xu, X., Xie, S., Wang, Y., Zhong, A., et al. (2018). Establishment of a detection assay for DNA endonuclease activity and its application in the screening and prognosis of malignant lymphoma. *BMC Biochem*, 19(1):6.

[32] Barták, B., Nagy, Z., Spisák, S., Tulassay, Z., Dank, M., Igaz, P., et al. (2018). In vivo analysis of circulating cell-free DNA release and degradation. *Orv Heti*l, 159(6):223-233.

[33] Trejo-Becerril, C., Pérez-Cardenas, E., Gutiérrez-Díaz, B., De La Cruz-Sigüenza, D., Taja-Chayeb, L., González-Ballesteros, M., et al. (2016), Antitumor effects of systemic DNAse I and proteases in an in vivo model, *Integrative Cancer Therapies*, 15(4):NP35-NP43.