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Qualification and quantification of plasma cell-free DNA after long-term storage conditions in patients with benign prostatic hyperplasia (BPH): a pilot study

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Abstract

Objectives: Free DNA is used as a cancer biomarker due to its low cost, high applicability, and fast, reliable results compared to invasive methods. This study aimed to evaluate the quantification of plasma-free DNA after long-term storage conditions and perform qualification through Single Nucleotide Polymorphism (SNP) screening based on this DNA.

Methods: Plasma-free DNA samples were quickly isolated from the peripheral blood of both the Benign Prostatic Hyperplasia (BPH) and control group participants and then maintained at −80 °C for four years. Upon thawing, first, free DNA was purified and fluorometric measurements were taken to determine the amount of DNA. Subsequently, the rs6983267, rs12628, and rs1799939 SNPs were screened in the *CCAT2*, *HRAS*, and *RET* genes, respectively.

Results: Significant results were obtained from the fluorometric measurements in terms of single-stranded DNA (ssDNA) ($p<0.001$). However, there was no significant difference in SNPs rs6983267, rs12628, and rs1799939 in the BPH group compared to the healthy individuals.

Conclusions: The data show that fluorometric ssDNA measurements are suitable for quantifying free DNA. The fact that SNP screening can be done successfully in both healthy people and BPH patients suggests that plasma-free DNA can be stored in the laboratory under appropriate conditions.

Keywords: *CCAT2*; free DNA; *HRAS*; *RET*; single nucleotide polymorphism (SNP).

Introduction

Benign Prostatic Hyperplasia (BPH) is a common disease that occurs in men over 50 years, and it is caused by increased proliferation of stromal (smooth muscle and connective tissue) and epithelial cells of the prostate gland [1, 2]. This disease's prevalence increases with age to approximately 100%. Clinically speaking, BPH causes bladder outlet obstruction formation by compressing the urethra, which leads to further problems, including lower urinary tract symptoms, infections, and urine retention. Although the etiology of BPH has not yet been definitively revealed, both non-modifiable risk factors, such as age, genetics, and geography, and modifiable risk factors, including diabetes, diet, inflammation, metabolic syndrome, cardiovascular disease, obesity, physical activity, and sex steroid hormones, are involved in BPH formation [2].

An Single Nucleotide Polymorphism (SNP), which is seen on an average of 1.9 kilobases in the human genome [3], alters the function and structure of encoded proteins based on the presence of genes' coding regions, and their presence in genes' regulatory regions increases the risk of common diseases. It is sometimes possible to encounter SNPs in noncoding regions of the genome [4]. As in many genetically based diseases, SNP screening can provide important information in understanding the occurrence and progression of BPH [5]. Although a genetic marker with high penetrance has not yet been identified for BPH, it is thought that SNPs in some genes, especially those related to inflammation, may be associated with BPH development [6–8].

Cell-free DNA or free DNA in the bloodstream are residual DNA fragments of cells that have been destroyed by mechanisms such as cell senescence, apoptosis, and

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necrosis. These DNA fragments, which have a half-life of approximately 2 h and are degraded by macrophages, are generally assumed to increase in disease states, including various types of cancer [9, 10]. Parallel to next-generation sequencing development, there is ongoing research investigating the use of free DNA isolated from serum or plasma as a diagnostic tool in cancer and many other clinical cases [11, 12].

In this study, to investigate free DNA's usability value in BPH diagnoses and the suitability of commercial kits used in the isolation, quantification, and qualification processes, free DNA was purified. Next, the amounts of single-stranded and double-stranded free DNA in patients diagnosed with BPH before, immediately after surgery, and 30 days after surgery were compared with a healthy control group. Subsequently, SNPs located in the *CCAT2* (rs6983267), *HRAS* (rs12628), and *RET* (rs1799939) genes were investigated to determine the quality and suitability of free DNA. Before these comparisons, plasma-free DNA samples obtained by centrifugation were stored for four years at −80 °C to reveal whether these samples were suitable for use after long-term storage.

Materials and methods

Collection of blood samples

The present study protocol was reviewed by the Ethics Committee of the Rectorate of Erzincan Binali Yıldırım University (approval No. 44495147/52–7690). Informed consent was submitted by all subjects when they were enrolled. Peripheral blood samples were obtained from 45 patients (mean age 58, minimum 52, and maximum 80) who presented to the urology clinic of Erzincan Mengücek Gazi Training and Research Hospital and were diagnosed with BPH at three different times: preoperatively (BPH_1), immediately after surgery (BPH_2), and 30 days after surgery (BPH_3). A control group was formed with 40 healthy individuals (mean age 57, minimum 50, and maximum 76), and their blood samples were also collected. The prostate-specific antigen (PSA) values of the patients with BPH were below 4. Blood was collected from both the BPH and control groups over the same period of time (i.e., within two months).

Approximately 20 mL of blood samples taken from patients with BPH and healthy individuals were immediately centrifuged at 2,500×*g* for 10 min in a gel-free tube with EDTA. The supernatant was placed in a sterile tube, which was then centrifuged at 16,000×*g* for 10 min. The new supernatant was separated from the pellet after the last centrifugation, transferred to a new sterile tube, and kept at −80 °C until analysis.

DNA isolation and quantification

After the samples were stored for 4 years, the Plasma/Serum Cell-Free Circulating DNA Purification Micro Kit purchased from Norgen (Cat. 55500) was used to purify free DNA from the plasma. Free DNA was purified from the samples following the manufacturer's instructions. Fluorometric measurements were taken using a Qubit 4 fluorometer (Thermo Fisher, Cat. Q33226) for the quantification of free DNA. The Qubit™ ssDNA Assay Kit (Thermo Fisher, Cat. Q10212) was used for single-stranded DNA (ssDNA) measurements, and the Qubit™ dsDNA HS Assay Kit (Thermo Fisher, Cat. Q32851) for double-stranded DNA (dsDNA) measurements.

SNP analysis

Free DNA samples obtained from 40 controls and 45 patients with BPH_1 were used to perform the SNP analysis. Genotyping was performed with the QuantStudio 3 device (Thermo Fisher, Cat. A28566). The primers and probes of rs6983267, rs12628, and rs1799939 SNPs belonging to the *CCAT2*, *HRAS*, and *RET* genes were obtained from Applied Biosystems (TaqMan SNP Genotyping assays).

Statistical analysis

Categorical variables were presented as numbers (%), while descriptive statistics for continuous variables were summarized as mean ± standard deviation and median (minimum–maximum) values. The chi-squared test was used in the analysis of categorical variables, and the odds ratio was calculated for disease risk. The assumption of normal distribution in continuous variables was verified with the Shapiro–Wilk normality test. In cases where the amount of DNA was not normally distributed, the Kruskal–Wallis ANOVA was used in analysis and Dunn's test was applied for multiple comparisons. Corrected p values were given in multiple comparisons. In the analysis of the data, $p<0.05$ was considered statistically significant. Statistical analyses were performed using IBM SPSS ver. 22 (Armonk, NY, IBM Corp.).

Results

According to the data obtained, the fluorometric measurements of ssDNA showed statistically significant differences in the comparisons between the groups ($p<0.001$). In terms of dsDNA, there was no difference between the groups ($p=0.48$). The pairwise comparisons of ssDNA revealed significant differences between the control group and BPH_1 and between the control group and BPH_2 ($p<0.001$ for both) (Table 1, Figures 1 and 2).

There was no significant difference between the control and BPH groups in relation to the allele frequencies and genotype ratios of SNPs in the *CCAT2*, *HRAS*, and *RET* genes. In addition, GG vs. GT + TT and GG + GT vs. TT comparisons for the *CCAT2* gene, TT vs. TC + CC and TT + TC vs. CC comparisons for the *HRAS* gene, and GG vs. GA + AA and GG + GA vs. AA comparisons for the *RET* gene did not reveal any statistically significant differences (Tables 2–4).

Table 1: Descriptive statistics of the DNA amounts in the control, preoperative (BPH_1) and postoperative (BPH_2 and BPH_3) samples of the patients. DNA concentrations were calculated in ng/μL. Data are presented as mean ± standard deviation and median (minimum–maximum) values.

	Control	BPH_1	BPH_2	BPH_3	p-Value
ssDNA	0.139 ± 0.074 0.127 (0.030–0.310)	0.408 ± 0.723 0.240 (0.060–4.270)	0.444 ± 0.690 0.245 (0.030–3.530)	0.224 ± 0.154 0.160 (0.040–0.620)	**0.000**
dsDNA	0.088 ± 0.081 0.055 (0.050–0.470)	0.085 ± 0.052 0.052 (0.050–0.260)	0.130 ± 0.143 0.065 (0.050–0.610)	0.095 ± 0.086 0.050 (0.050–0.380)	**0.479**

BPH, benign prostatic hyperplasia; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA. The p-Values were obtained from the comparisons between the groups.

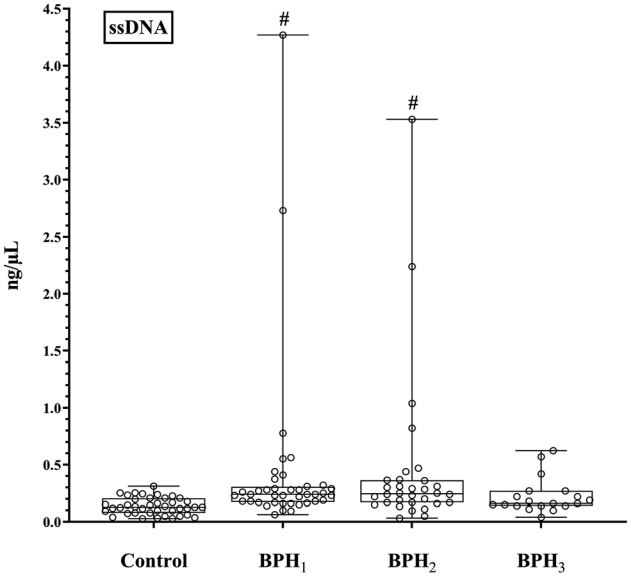


Figure 1: Graphical comparison of the ssDNA amounts in the control, preoperative (BPH_1) and postoperative (BPH_2 and BPH_3) samples of the patients. #: statistically significant increase compared with the control group ($p<0.001$).

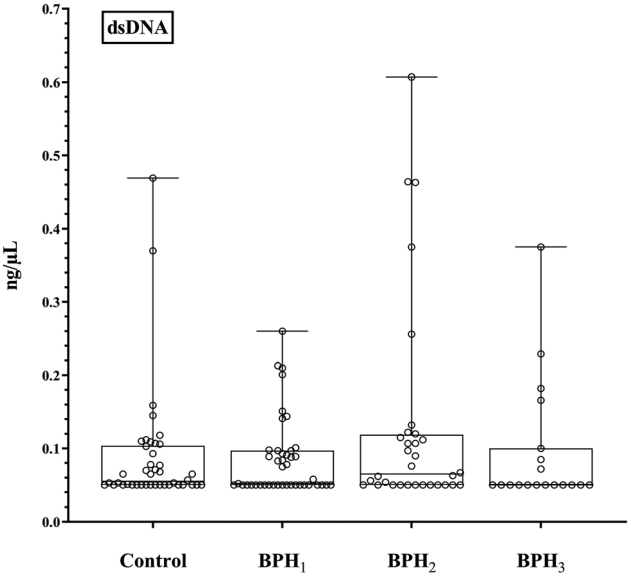


Figure 2: Graphical comparison of the dsDNA amounts in the control, preoperative (BPH_1) and postoperative (BPH_2 and BPH_3) samples of the patients.

Discussion

After obtaining plasma-free DNA samples by centrifugation, these samples were stored at −80 °C for four years. At the end of this period, the free DNA samples were purified, their levels were measured, and SNP studies were carried out using these samples (control group and BPH_1). The central aim of this study was to determine whether the plasma-free DNA that was isolated and kept under appropriate conditions would preserve its integrity (i.e., whether plasma-free DNA was suitable for long-term storage). Although it is known that the amount of free DNA isolated from serum is generally higher than that of plasma, in this study, we preferred the latter as the source of free DNA.

From past to present, studies have been conducted to investigate whether high quantities of free DNA can be indicators of many diseases, especially cancer when compared to free DNA quantities in healthy people, and whether the amount of free DNA can be used as a marker in the diagnosis of diseases. In light of the data available, it can be assumed that the amount of free DNA is generally high in any disease state. Many studies have reported that free DNA levels are higher in patients with BPH than in the control group [13–15]. In our study, although the results obtained from the dsDNA kit were nonsignificant for the comparisons between multiple measurements and pairwise comparisons, there were significant differences in ssDNA between the control group and BPH_1 and between the control group and BPH_2. The lack of a significant difference between the control group measurement and BPH_3 could indicate that the course of ssDNA is parallel to the healing process of the disease. Although the Qubit® ssDNA Kit allows for the quantification of ssDNA and

Table 2: Single nucleotide polymorphism recorded in the *CCAT2* gene (rs6983267).

CCAT2 gene			Group		p-Value	OR (95% CI)
			Control	BPH		
Allele	G	n	41	48	0.677	Ref.
		%	52.6	55.8		
	T	n	37	38		0.88 (0.47–1.62)
		%	47.4	44.2		
Codominant	GG	n	12	14	0.880	Ref.
		%	30.8	32.6		
	GT	n	17	20		1.01 (0.37–2.76)
		%	43.6	46.5		
	TT	n	10	9		0.77 (0.24–2.52)
		%	25.6	20.9		
Dominant	GG	n	12	14	0.862	Ref.
		%	30.8	32.6		
	GT + TT	n	27	29		0.92 (0.36–2.34)
		%	69.2	67.4		
Recessive	GG + GT	n	29	34	0.614	Ref.
		%	74.4	79.1		
	TT	n	10	9		0.77 (0.28–2.15)
		%	25.6	20.9		

BPH, benign prostatic hyperplasia; OR, odds ratio; CI, confidence interval; Ref., reference.

Table 3: Single nucleotide polymorphism recorded in the *HRAS* gene (rs12628).

HRAS gene			Group		p-Value	OR (95% CI)
			Control	BPH		
Allele	T	n	44	54	0.517	Ref.
		%	56.4	61.4		
	C	n	34	34		0.81 (0.44–1.52)
		%	43.6	38.6		
Codominant	TT	n	12	18	0.584	Ref.
		%	30.8	40.9		
	TC	n	20	18		0.60 (0.23–1.58)
		%	51.3	40.9		
	CC	n	7	8		0.76 (0.22–2.66)
		%	17.9	18.2		
Dominant	TT	n	12	18	0.337	Ref.
		%	30.8	40.9		
	TC + CC	n	27	26		0.64 (0.26–1.59)
		%	69.2	59.1		
Recessive	TT + TC	n	32	36	0.978	Ref.
		%	82.1	81.8		
	CC	n	7	8		1.02 (0.33–3.12)
		%	17.9	18.2		

BPH, benign prostatic hyperplasia; OR, odds ratio; CI, confidence interval; Ref., reference.

oligonucleotides, it is known to be nonspecific to ssDNA. This kit also detects the amount of dsDNA and RNA by eliminating contaminating proteins or nucleotides. The Qubit dsDNA HS Assay Kit was specifically designed to quantify dsDNA. Free DNA, which is the target of macrophages after their extracellular release, cannot be expected to be present only in the form of dsDNA. Oligonucleotides and ssDNA are expected to be found in blood tissue with natural breakages in free DNA. In addition, dsDNA will be partially converted to oligonucleotides and ssDNA by breakages that occur during the isolation and storage of the samples. The higher amount of ssDNA than that of dsDNA, despite the same parameters being used, indicates the presence of not only oligonucleotides and ssDNA but also dsDNA in the samples. In this respect, fluorometric ssDNA measurement is very sensitive and accurate in the quantification of free DNA. Similar results are available in the literature [10, 16, 17].

In the second part of the current study, SNPs associated with different cancer types, which are located in the *CCAT2*, *HRAS*, and *RET* genes, were studied using free DNA as an indicator of the quality of the stored DNA samples. The rs6983267 SNP is located within the *CCAT2* gene. *CCAT2* is a gene localized in the 8q24 genomic region and generates a long non-coding CCAT2 transcript (colon cancer–associated transcript 2). Although the exact functionality of the CCAT2 transcript remains unknown, it has been reported that the genomic region containing rs6983267 physically interacts with the *MYC* proto-oncogene as a transcriptional enhancer, and the alleles of this SNP bind differently to transcription factor 7-like 2 [18]. The *CCAT2* gene is considered to have high upregulation in many cancer types, especially colorectal cancer, and thus, this gene plays a role in the tumorogenesis process through overexpression. It is also interesting that the G risk allele in the rs6983267 location in colon cancer leads to the formation of more CCAT2 transcripts [19]. Although rs6983267 is associated with prostate cancer, it has been observed that this SNP is not a risk factor for the development of BPH in many different populations [20–22], which is in agreement with the data obtained in this study.

In mammals, the *RAS* gene family has three known members with different localizations in the genome: *HRAS* in the 11p15.5 position with a 3 kb length, *KRAS* in the 1p13 position with a 35 kb length, and *NRAS* in the 12p12.1 position with a 7 kb length. The *RAS* genes provide the expression of HRAS, KRAS, and NRAS proteins, which are closely related to each other and play a role in the cell-signal communication mechanism as small GTP-binding proteins. In *RAS* genes, mutations usually occur in codons

Table 4: Single nucleotide polymorphism recorded in the *RET* gene (rs1799939).

RET gene			Group		p-Value	OR (95% CI)
			Control	BPH		
Allele	G	n	44	61	0.085	Ref.
		%	56.4	69.3		
	A	n	34	27		0.57 (0.30–1.09)
		%	43.6	30.7		
Codominant	GG	n	17	25	0.354	Ref.
		%	43.6	56.8		
	GA	n	10	11		0.75 (0.26–2.15)
		%	25.6	25.0		
	AA	n	12	8		0.45 (0.15–1.34)
		%	30.8	18.2		
Dominant	GG	n	17	25	0.229	Ref.
		%	43.6	56.8		
	GA + AA	n	22	19		0.59 (0.25–1.40)
		%	56.4	43.2		
Recessive	GG + GA	n	27	36	0.181	Ref.
		%	69.2	81.8		
	AA	n	12	8		0.50 (0.18–1.39)
		%	30.8	18.2		

BPH, benign prostatic hyperplasia; OR, odds ratio; CI, confidence interval; Ref., reference.

12, 13, and 61 [23–25]. Codon 12 and 13 mutations increase the affinity of the RAS molecule to GTP, while the mutation of codon 61 inactivates the autocatalytic GTPase function of the RAS molecule. In both cases, permanent RAS activation is provided, and the cell is introduced into the oncogenesis process with the chronic stimulation of downstream targets along the MAPK- and PI3K/AKT-signaling pathways [26]. In addition to these known polymorphisms, the rs12628 SNP, localized in codon 27 (81 T→C) of exon 1 in the *HRAS* gene, is important because it has been associated with many types of cancer [27]. An interesting finding of a previous study was that the 81 T→C polymorphism in follicular tumors of the thyroid increased total HRAS mRNA expression [28]. It is difficult to establish a link between rs12628 and urogenital carcinoma risk [29]. While some studies reported that the C allele decreased the risk of bladder cancer [16, 30], others showed that the C allele frequency was increased in individuals with bladder cancer [31]. The current study is the first to examine the rs12628 SNP in patients with BPH, and there was no significant difference in this polymorphism between the BPH and healthy control groups.

The *RET* protooncogene localized at 10q11.2 encodes the RET protein, a transmembrane tyrosine kinase receptor [32]. This receptor plays a role, especially in the development of the enteric nervous, endocrine, and renal systems; thus, many cancer types, such as multiple endocrine neoplasia type 2, thyroid, lung, colorectal, breast, and salivary gland, occur with different rearrangements in the *RET* proto-oncogene [33, 34]. SNP rs1799939 (c.2071G→A) is located in exon 11 of the *RET* protooncogene. In the presence of the A allele, the p.Gly691Ser variant of the RET protein is formed. The G691S variant allele is considered a risk factor for the formation of many different cancers, including lung cancer [35]. SNP rs1799939, which is relatively new compared to the other RAS polymorphisms, has not been the subject of studies on prostate cancer or BPH. The data obtained from our study suggest that SNP rs1799939 is not a risk factor for BPH in the Turkish population.

In this study, the SNP genotype could not be determined for the *CCAT2*, *HRAS*, and *RET* genes in one individual in the control group. In the BPH group, primary amplification could not be performed in two individuals for the *CCAT2* gene and in one individual for the *HRAS* and *RET* genes.

Conclusions

Our results showed that plasma-free DNA samples obtained from healthy individuals and patients with BPH yielded successful results in quantification and qualification tests after being isolated under laboratory conditions for a long time, which is in accordance with the established hypothesis and suggests that free DNA can be used in retrospective research and applications. Considering the current popularity of free DNA, these data are important in the literature.

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Informed consent: Informed consent was obtained from all individuals included in this study.

Ethical approval: Research involving human subjects complied with all relevant national regulations, institutional policies and is in accordance with the tenets of the Helsinki Declaration (as revised in 2013), and has been approved by the authors' Institutional Review Board (Ethics Committee of the Rectorate of Erzincan Binali Yildirim University) (approval No. 44495147/52–7690).

References

1. McVary KT. BPH: epidemiology and comorbidities. Am J Manag Care 2006;12:122–8.
2. Patel ND, Parsons JK. Epidemiology and etiology of benign prostatic hyperplasia and bladder outlet obstruction. Indian J Urol 2014;30:170–6.
3. Sachidanandam R, Weissman D, Schmidt SC, Kakol JM, Stein LD, Marth G, et al. International SNP Map Working Group. A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. Nature 2001;409: 928–33.
4. Syvänen AC. Accessing genetic variation: genotyping single nucleotide polymorphisms. Nat Rev Genet 2001;2:930–42.
5. Konwar R, Chattopadhyay N, Bid HK. Genetic polymorphism and pathogenesis of benign prostatic hyperplasia (BPH). BJU Int 2008;102:536–44.
6. Yoo KH, Kim SK, Chung JH, Chang SG. Association of IL10, IL10RA, and IL10RB polymorphisms with benign prostate hyperplasia in Korean population. J Kor Med Sci 2011;26:659–64.
7. Jiao Y, Wang L, Gu X, Tao S, Tian L, Na R, et al. LILRA3 is associated with benign prostatic hyperplasia risk in a Chinese population. Int J Mol Sci 2013;14:8832–40.
8. El Ezzi AA, Zaidan WR, El-Saidi MA, Al-Ahmadieh N, Mortenson JB, Kuddus RH. Association of benign prostate hyperplasia with polymorphisms in VDR, CYP17, and SRD5A2 genes among Lebanese men. Asian Pac J Cancer Prev 2014;15: 1255–62.
9. Sorenson GD, Pribish DM, Valone FH, Memoli VA, Bzik DJ, Yao SL. Soluble normal and mutated DNA sequences from single-copy genes in human blood. Cancer Epidemiol Biomarkers Prev 1994; 3:67–71.
10. Yolcu M, Dogan A, Kurtoglu N, Hancer VS, Gürbüzel M. New indicator of cellular ischemia in coronary slow-flow phenomenon: cell-free DNA. Turk Kardiyol Dernegi Arsivi 2020; 48:558–65.
11. Paweletz CP, Sacher GS, Raymond K, Alden RS, Oconnell A, Mach SL, et al. Bias-corrected targeted next-generation sequencing for rapid, multiplexed detection of actionable alterations in cell-free DNA from advanced lung cancer patients. Clin Cancer Res 2016;22:915–22.
12. Volik S, Alcaide M, Morin RD, Collins C. Cell-free DNA (cfDNA): clinical significance and utility in cancer shaped by emerging technologies. Mol Cancer Res 2016;14:898–908.
13. Ponti G, Maccaferri M, Micali S, Manfredini M, Milandri R, Bianchi G, et al. Seminal cell free DNA concentration levels discriminate between prostate cancer and benign prostatic hyperplasia. Anticancer Res 2018;38:5121–5.
14. Ponti G, Maccaferri M, Manfredini M, Micali S, Torricelli F, Milandri R, et al. Quick assessment of cell-free DNA in seminal fluid and fragment size for early non-invasive prostate cancer diagnosis. Clin Chim Acta 2019;497:76–80.
15. Jung K, Carsten S, Lewandowski M, Klotzek S, Jung M, Glen K, et al. Increased cell-free DNA in plasma of patients with metastatic spread in prostate cancer. Cancer Lett 2004;205:173–80.
16. Baydeniz S, Gurbuzel M, Sayar I, Bozkurt A, Arslan YK. Quantification of cell-free DNA from bladder cancer patients after long-term storage and its use in determining CCAT2, HRAS, and RET polymorphism: a pilot study. Ann Med Res 2021;28: 1926–32.
17. Ponti G, Maccaferri M, Maccaferri M, Kaleci S, Mandrioli M, Pellacani G, et al. The value of fluorimetry (Qubit) and spectrophotometry (NanoDrop) in the quantification of cell-free DNA (cfDNA) in malignant melanoma and prostate cancer patients. Clin Chim Acta 2018;479:14–9.
18. Pomerantz MM, Ahmadiyeh N, Jia L, Herman P, Verzi MP, Doddapaneni H, et al. The 8q24 cancer risk variant rs6983267 shows long-range interaction with MYC in colorectal cancer. Nat Genet 2009;41:882–4.
19. Ling H, Spizzo R, Atlasi Y, Nicoloso M, Shimizu M, Redis RS, et al. CCAT2, a novel noncoding RNA mapping to 8q24, underlies metastatic progression and chromosomal instability in colon cancer. Genome Res 2013;23:1446–61.
20. Terada N, Tsuchiya N, Ma Z, Shimizu Y, Kobayashi T, Nakamura E, et al. Association of genetic polymorphisms at 8q24 with the risk of prostate cancer in a Japanese population. Prostate 2008;68: 1689–95.
21. Branković AS, Brajušković GN, Mirčetić JD, Nikolić ZZ, Kalaba PB, Vukotić VD, et al. Common variants at 8q24 are associated with prostate cancer risk in Serbian population. Pathol Oncol Res 2013;19:559–69.
22. Ho CKM, Halley L, Wei J, Habib FK. Analysis of prostate cancer association with four single-nucleotide polymorphisms from genome-wide studies and serum phyto-estrogen concentrations. Prostate Cancer Prostatic Dis 2012;15:365–8.
23. Bos JL. Ras oncogenes in human cancer: a review. Cancer Res 1989;49:4682–9.
24. Macaluso M, Russo G, Cinti C, Bazan V, Gebbia N, Russo A. Ras family genes: an interesting link between cell cycle and cancer. J Cell Physiol 2002;192:125–30.
25. Prior IA, Lewis PD, Mattos C. A comprehensive survey of Ras mutations in cancer. Cancer Res 2012;72:2457–67.
26. Nikiforov YE. Molecular analysis of thyroid tumors. Mod Pathol 2011;24:S34–43.
27. Taparowsky E, Suard Y, Fasano O, Shimizu K, Goldfarb M, Wigler M. Activation of the T24 bladder carcinoma transforming gene is linked to a single amino acid change. Nature 1982;300: 762–5.
28. Castro P, Soares P, Gusmão L, Seruca R, Sobrinho-Simões M. H-RAS 81 polymorphism is significantly associated with aneuploidy in follicular tumors of the thyroid. Oncogene 2006;25: 4620–7.
29. Chen JB, Zhang M, Cui Y, Liu PH, Qi YW, Li C, et al. Association between 12 polymorphisms of VEGF/hypoxia/angiogenesis pathway genes and risk of urogenital carcinomas: a meta-analysis based on case-control studies. Front Physiol 2018;9: 715.
30. Sanyal S, Festa F, Sakano S, Zhang Z, Steineck G, Norming U, et al. Polymorphisms in DNA repair and metabolic genes in bladder cancer. Carcinogenesis 2004;25:729–34.
31. Pandith AA, Shah ZA, Khan NP, Baba KM, Wani MS, Siddiqi MA. HRAS T81C polymorphism modulates risk of urinary bladder cancer and predicts advanced tumors in ethnic Kashmiri population. Urol Oncol 2013;31:487–92.

32. Pasini B, Hofstra RM, Yin L, Bocciardi R, Santamaria G, Grootscholten PM, et al. The physical map of the human RET proto-oncogene. Oncogene 1995;11:1737–43.
33. Pandey A, Duan H, Di-Fiore PP, Dixit VM. The Ret receptor protein tyrosine kinase associates with the SH2-containing adapter protein Grb10. J Biol Chem 1995;270:21461–3.
34. Takahashi M, Kawai K, Asai N. Roles of the RET proto-oncogene in cancer and development. JMA J 2020;3:175–81.
35. Sosonkina N, Hong SK, Starenki D, Park JI. Kinome sequencing reveals RET G691S polymorphism in human neuroendocrine lung cancer cell lines. Genes Genomics 2014;36:829–41.

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