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Original Article

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

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Abstract

Aim: The level of free DNA is used as a cancer biomarker due to having several advantages related to lower cost, easy applicability, and rapid results compared to invasive methods.

Materials and Methods: This study investigated whether or not free DNA functions as intended when stored. The free DNA isolated for this purpose was kept at –80 degrees for four years. Then, the level of free DNA was measured in patients with bladder cancer by both photometric and fluorometric methods, and it was investigated whether this biomarker was effective in determining Single Nucleotide Polymorphisms (SNP). The gene names and the reference numbers of the locations where polymorphism was expected were as follows: *CCAT2* (rs6983267), *HRAS* (rs12628), and *RET* (rs1799939).

Results: According to the results, having the T allele at rs12628 increases the risk of disease by 2.6 times (OR: 2.60; 95% CI: 1.10–6.10) and having the G allele at rs1799939 increases the risk of disease 2.7 times (OR: 2.70; 95% CI: 1.12–6.69).

Conclusions: The general findings showed that fluorometric measurements were more advantageous when considering the sensitivity of free DNA measurements. It was also concluded that, if treated rapidly and conserved in appropriate laboratory conditions, free DNA can be effectively used in SNP studies. This study provides important data to demonstrate the quality of free DNA.

Keywords: Free DNA, SNP, *CCAT2, HRAS, RET*

INTRODUCTION

Urine produced in the kidney is transported through the ureter to the bladder; a storage organ with a flexible structure composed of irregular and tightly connected muscle tissues. The most frequent cases of malignant tumors in the excretory system occur in the bladder, and the prevalence of bladder cancer is increasing day by day. This disease is more commonly seen in men than in women. In a study published in 2010, it was stated that the number of people diagnosed with bladder cancer in the world was 380,000 and that of those who died due to this disease was around 150,000 (1). In addition to the primary factor of smoking that leads to DNA damage, bladder cancer can occur due to the effects of chemical substances, including aromatic amine-containing dye; fuel products, such as 4-nitrobenzyl, 4-aminobibenzyl, and benzidine; minerals; materials related to chopping, lubrication, asphalting, plastics, and welding; and chlorination by-products in drinking water, such as inorganic arsenic, nitrate, and trihalomethane. It is also known that processed food containing N-nitroso compounds, heterocyclic amines, and 4-aminobiphenyl MX; animal fats; and certain drugs; e.g., cyclophosphamide and phenacetin can be inducers of bladder cancer (2).

The most commonly used method for the diagnosis of bladder cancer is cystoscopy, which is invasive and expensive. In this method, the internal part of the bladder is scanned by the physician based on observation using an optic instrument called a cystoscope. Urine cytology has limited value, especially in low-grade disease. In recent years, many molecular markers have been developed for the diagnosis of bladder cancer. Although these markers provide better results than urine cytology, they are still not utilized in clinical diagnosis. Some of these markers

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target complement factor H-associated protein, *NMP22* and *BLCA-4* nucleus proteins, and *C8, C18* and C19 cytoskeletal proteins (3).

Free DNA in the bloodstream is generally considered to be from DNA fragments remaining from cells that have been destroyed by mechanisms, such as natural cell senescence, apoptosis, and necrosis. It is assumed that these particles, which have a half-life of approximately two hours and are destroyed by macrophages, are generally increased in cancer cases (4). In addition to research on the quantification of free DNA, there are also studies screening cancer markers using isolated free DNA. Free DNA has been used to screen mutations in the *N-RAS* gene in patients with myelodysplastic syndromes and acute myelogenous leukemia and those in the *K-RAS* gene in patients with pancreatic carcinoma and cystic fibrosis (4,5). In addition, using free DNA, microsatellite instability has been determined in patients with head and neck squamous cell carcinoma and small cell lung cancer (6,7).

Today, free DNA obtained from serum or plasma without invasion is used in the diagnosis and treatment of many genetic diseases, especially many types of cancers, and research on this subject rapidly continues. In the liquid biopsy method, in order to remove blood cells, first, blood samples taken from patients are immediately centrifuged. The following step is to isolate free DNA in the plasma or serum, which may also include DNA that may be formed from tumor tissues as a result of apoptosis or necrosis. The obtained DNA is an important material for the diagnosis of diseases when genetic tumor markers are used (8).

When the literature is examined, it can be seen that there are many commercial ready-made kits or procedures used for free DNA extraction and quantification from past to present, and they provide widely varying results. There is almost no study on the required storage conditions for free DNA. In our study, free DNA, which was rapidly isolated from the plasma of 55 patients and healthy individuals, was kept at –80 degrees for four years and used for quantification and SNP scanning. In the first stage of our study, photometric and fluorometric measurements were performed for the free DNA extraction and quantity analysis from the plasma samples taken in three different periods from patients with bladder cancer using a current commercial kit. By comparing these measurements, it was aimed to determine the most ideal method for quantification. In the second stage of the study, three different single nucleotide polymorphisms (SNPs) were screened using the free DNA samples. This was also important to demonstrate the quality of the free DNA obtained.

MATERIALS and METHODS

Blood Sample Collection

This study was approved by the Ethics Committee of the Rectorate of Erzincan Binali Yildirim University (Number: 44495147/52-7690). Blood was collected from 25 patients, 21 male and four female (mean age 68.73, minimum 37, maximum 84 years), who presented to the Urology Clinic of Erzincan Mengucek Gazi Training and Research Hospital and were diagnosed with bladder cancer, and 30 healthy controls (mean age 67.03, minimum 36, maximum 85 years). The disease level was high in 13 patients and low in 12 patients with bladder cancer. The disease progression was invasive in 16 patients and non-invasive in nine patients. In the patient group, blood samples were obtained three times: before the surgical operation (pre-op), immediately after the operation (post-op1) and one month after the operation (post-op2). In the control group, blood collection was performed once.

Immediately after collection, the peripheral blood samples were centrifuged at 2,500 xg for 10 minutes in a non-gel tube containing EDTA (around 20 ml). The supernatant was removed and transferred to a sterile tube and centrifuged at 16,000 xg for a further 10 minutes. The resulting new supernatant was transferred to a new sterile tube and stored at –80°C.

DNA Isolation and Quantification

After four years, a plasma/serum cell-free circulating DNA purification micro kit (obtained from Norgen, Cat. 55500) was used for the isolation of free DNA and the indicated kit procedure was followed. The free DNA in the samples was fluorometrically quantified using a Qubit™ ssDNA assay kit (Thermo Fisher, Catalog number Q10212) was used for single-stranded DNA measurements and a Qubit™ dsDNA HS assay kit (Thermo Fisher, Catalog number Q32851) for double-stranded DNA measurements. The fluorometric measurements were conducted with a Qubit 4 fluorometer (Thermo Fisher, Catalog No. Q33226) and spectrophotometric measurements with the µDrop™ plate (Thermo Fisher, Catalog number N12391) integrated into the Multiskan GO device (Thermo Fisher, Catalog number N10588).

SNP Analysis

In the study, the primers and probes of SNPs rs6983267, rs12628, and rs1799939 belonging to the genes *CCAT2*, *HRAS*, and *RET,* respectively were obtained from Applied Biosystems (TaqMan SNP Genotyping Assays), and genotyping was performed using Thermo Fisher Quantstudio™ 3 (Catalog number A28566).

Statistical Analysis

Descriptive statistics for continuous variables were summarized as mean ± standard deviation and median (minimum - maximum) values. The chi-square test was used in the analysis of categorical variables where appropriate, and the odds ratio (OR) was calculated for disease risk. The assumption of the normal distribution of continuous variables was checked by the Shapiro-Wilk test. Since the DNA amounts were not normally distributed, the Kruskal-Wallis analysis of variance was used in the analysis, and Dunn's test was undertaken for multiple comparisons. Corrected p values were presented in multiple comparisons. A p value of <0.05 was considered statistically significant. The Statistical Package for the Social Sciences v. 19 (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp.) was used for all statistical analyses.

RESULTS

The fluorometric comparisons of the free DNA levels between the groups showed statistically significant differences in ssDNA (single-stranded DNA) and dsDNA (double-stranded DNA). In the photometric comparison, the Kruskal-Wallis test was used because the assumption of normal distribution in the free DNA level was not provided; however, the results revealed no significant difference; thus, paired comparisons were not undertaken for the free DNA level (Table 1).

When paired group comparisons were examined for ssDNA, the differences in the DNA amount were significant only between the control and pre-op groups, and between the control and post-op1 groups (p = 0.014 and p = 0.019, respectively). For dsDNA, only the difference between the control and post-op1 groups and that between the control and post-op2 was significant (p = 0.019 and p = 0.019, respectively) (Table 1).

The allele frequencies and genotype ratios of the *CCAT2* gene were compared between the patients with bladder cancer and healthy controls, and no significant difference was detected (p = 0.205 and p = 0.154, respectively). Similarly, within the *CCAT2* gene, GG vs. GT + TT and GG + GT vs. TT comparisons did not reveal any significant difference (p = 0.234 and p = 0.355, respectively) (Table 2).

Table 1. Descriptive statistics of the DNA amounts in the control, preoperative (Pre–op) and postoperative samples (Post–op1 and Post–op2)

	Control	Pre–op	Post–op1	Post–op2	p
ssDNA	0.137±0.064	0.224±0.125	0.236±0.147	0.144±0.081	
	0.130	0.188	0.196	0.130	0.002
	(0.030–0.250)	(0.080–0.680)	(0.100–0.680)	(0.050–0.320)	
dsDNA	0.092±0.094	0.072±0.035	0.057±0.020	0.046±0.017	
	0.053	0.051	0.050	0.050	0.002
	(0.050–0.470)	(0.050–0.160)	(0.050–0.120)	(0.010–0.060)	
Total Free DNA	49.51±11.23	60.24±27.43	50.94±9.96	50.59±16.49	
Measurement with	48.75	53.50	50.94	50.13	0.343
µDrop Plate	(30.25–73.50)	(37.50–146.30)	(36.50–70.50)	(35.50–109.50)	

DNA concentrations were calculated in ng/µL. Data are presented as mean ± standard deviation and median (minimum–maximum) values. ssDNA: Single stranded DNA, dsDNA: Double Stranded DNA, p: p value

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

CCAT2 Gene			Group			
			Control	Bladder	p	OR (95% CI)
Allele	G	n	26	15	0.205	Ref.
		%	44.8	32.6		
	T	n	32	31		1.68 (0.75–3.75)
		%	55.2	67.4		
Codominant	GG	n	6	2	0.154	Ref.
		%	20.7	8.7		
	GT	n	14	11		2.40 (0.40–14.00)
		%	48.3	47.8		
	GT	n	9	10		3.30 (0.50–20.90)
		%	31.0	43.5		
Dominant	GG	n	6	2	0.234	Ref.
		%	20.7	8.7		
	GT + TT	n	23	21		2.74 (0.50–15.09)
		%	79.3	91.3		
Recessive	GG + GT	n	20	13	0.355	Ref.
		%	69.0	56.5		
	TT	n	9	10		1.71 (0.54–5.34)
		%	31.0	43.5		

OR: odds ratio, CI: confidence interval, Ref.: reference, p: p value

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

HRAS Gene			Group			
			Control	Bladder	p	OR (95% CI)
Allele	T	n	31	35	0.029	Ref.
		%	55.4	76.1		
	C	n	25	11		0.39 (0.16–0.91)
		%	44.6	23.9		
Codominant	TT	n	6	15	0.022	Ref.
		%	21.4	65.2		
	TC	n	19	5		0.11 (0.03–0.41)
		%	67.9	21.7		
	CC	n	3	3		0.40 (0.06–2.60)
		%	10.7	13.0		
Dominant	TT	n	6	15	0.002	Ref.
		%	21.4	65.2		
	TC + CC	n	22	8		0.15 (0.04–0.51)
		%	78.6	34.8		
Recessive	TT + TC	n	25	20	0.797	Ref.
		%	89.3	87.0		
	CC	n	3	3		1.25 (0.23–6.88)
		%	10.7	13.0		

OR: odds ratio, CI: confidence interval, Ref.: reference, p: p value

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

RET Gene			Group			
			Control	Bladder	p	OR (95% CI)
Allele	G	n	36	37	0.024	Ref.
		%	60.0	80.4		
	A	n	24	9		0.36 (0.15–0.89)
		%	40.0	19.6		
Codominant	GG	n	14	15	0.115	Ref.
		%	46.7	65.2		
	GA	n	8	7		0.82 (0.23–2.85)
		%	26.7	30.4		
	AA	n	8	1		0.12 (0.01–1.10)
		%	26.7	4.3		
Dominant	GG	n	14	15		Ref.
		%	46.7	65.2		
	GA + AA	n	16	8		0.47 (0.15–1.43)
		%	53.3	34.8		
Recessive	GG + GA	n	22	22		Ref.
		%	73.3	95.7		
	AA	n	8	1		0.13 (0.01–1.09)
		%	26.7	4.3		

OR: odds ratio, CI: confidence interval, Ref.: reference, p: p value

When the allele frequencies and genotype ratios of the *HRAS* gene were compared between the two groups, the differences were found to be significant (p = 0.029 and p = 0.022, respectively). Having the T allele in the *HRAS* gene increased the risk of the disease by 2.6 times [OR: 2.60; 95% confidence interval (CI): 1.10–6.10]. In contrast, having the C allele had a 2.6-fold protective effect (OR: 0.39; 95% CI: 0.16–0.91) and having the TC genotype had a 9.10-fold protective effect (OR: 0.11; 95% CI: 0.03–0.41) against the disease. TT vs. TC + CC and TT + TC vs. CC comparisons were also performed (p = 0.002 and p = 0.797, respectively). When the TT genotype was taken as reference, having the TC or CC genotype was found to provide 6.66 times greater protective effect against the disease (OR: 0.15; 95% CI: 0.04–0.51) (Table 3).

The comparison of the allele frequencies of the *RET* gene between the bladder cancer group and the control group revealed statistically significant differences (p = 0.024). Having the G allele in the *RET* gene increased the risk of the disease by 2.7 times (OR: 2.70; 95% CI: 1.12–6.69) while carrying the A allele had a 2.7-fold protective effect against the development of the disease (OR: 0.36; 95% CI: 0.15–0.89). There was no significant result for genotype frequencies (p = 0.115) or the comparisons of GG vs. GA + AA and GG + GA vs. AA (p = 0.179 and p = 0.061) (Table 4).

DISCUSSION

Research on whether quantification of free DNA will have a quantitative diagnostic value is still ongoing. In the light of the findings obtained from many different studies to date, it can be concluded that free DNA levels increase in diseases compared to healthy individuals in general. Some of these studies showed that the amount of free DNA in the urine of cancer patients was significantly higher compared to healthy individuals (9) while others did not report a significant increase in the free DNA level (10). Another example of the latter was from Zancan et al. (11), who did not detect a significant increase in free DNA in the urine of bladder cancer cases despite the use of specific purification kits.

Our main goals were to perform the quantification of free DNA and to perform a study of SNP to show that gene structure still maintains its integrity after being quickly isolated and then held at –80 degrees. In this study, the analyses were conducted on the plasma level, which is more disadvantageous than serum in free DNA isolation, and quantification was performed both photometrically and fluorometrically as in previous studies (12,13). This difference in measurements shows that although the 260/230 and 260/280 ratios are taken into consideration, protein, RNA, and other contaminants in the samples significantly affect the measurement values, and thus photometry is not adequate to quantify free DNA. There was no statistically significant difference between the groups in the photometric measurement. However, in the fluorometric analysis, the comparison between the groups for ssDNA, and dsDNA was found to be statistically significant. ssDNA was significantly higher in the bladder cancer group compared to the control group, and the free DNA level significantly increased immediately after the operation compared to the pre-op group and was significantly reduced in the post-op2 group. The fact that the DNA level in the post-op2 group did not statistically differ from that of the control group and had a close value indicates that the course of ssDNA is parallel to the disease recovery process. The Qubit® ssDNA Kit is used for quantitating single-stranded DNA or oligonucleotides. However, it is known that this kit is not specific for single-stranded DNA. It also detects dsDNA and RNA but does not detect contaminating protein or nucleotides. The inter-group comparison of the dsDNA level provided significant differences. When paired comparisons were undertaken, there were significant differences only between the control and post-op1 groups and control and post-op2 groups; however, all the bladder groups had lower levels of dsDNA compared to the control group. Nevertheless, the decrease in the free DNA level in the bladder groups throughout the measurement period (from pre-op to post-op1 to post-op2) and the parallelism of the disease course to the healing process can be considered as important findings. Unlike the Qubit® ssDNA Kit, the Qubit dsDNA HS Assay Kit is highly selective for dsDNA. Free DNA can originate from cell death through senescence, apoptosis, and necrosis. It is found naturally in the form of fragments in the blood. Free DNA does not consist of only dsDNA. Also, it is necessary to include breaks in DNA in plasm that has been preserved for a long time. These are the main reasons why the amount of ssDNA in each group is higher than the amount of dsDNA. Also, is the reason that the dsDNA measurements from different periods of patients are low compared to the control group the fact that the dsDNA in patients with cancer suffers more diffraction? This issue could be decided by future studies on individuals with bladder cancer. The measurement methods we used have been studied before and it has been seen that fluorometric measurements give more suitable results for free DNA (12).

The transcription of the *CCAT2* gene results in a long non-encoded RNA that is upregulated in colon cancer and in some other cancer types, such as intrahepatic cholangiocarcinoma and cell renal carcinoma. This transcript promotes cell division and suppresses apoptosis, and plays a role as a negative regulator on biogenesis of miRNA (microRNA) 145 (mir-145). *CCAT2* gene expression was investigated in bladder cancer, and it was proposed that this gene might be an oncogene in bladder cancer and can be used for therapeutic purposes (14). Adopting a different approach, the inhibition of *CCAT2* gene expression in in vitro conditions was shown to lead to the suppression of glioma cell development and cell migration and invasion and induce the early apoptosis of glioma cells (15). Investigating SNP rs6983267 (G>T), located on chromosome 8q24 in the *CCAT2* gene, it was reported that when the TT genotype was taken as reference, the GG genotype could be a risk factor primarily for prostate cancer, as well as colon, kidney, thyroid and laryngeal cancers, and similar to our results, it was noted that this SNP did not create a statistically significant risk for bladder cancer (16,17).

The three most known members of the *RAS* gene family are *HRAS* with a 3-kb length located on 11p15.5, 35-kb KRAS on 1p13, and 7-kb NRAS on 12p12.1. In mammals, at least these three *RAS* proteins that are closely related to each other are expressed. Mutations in the *RAS* protooncogenes can occur in codons 12, 13 or 61, and *RAS* oncogenes can trigger human cancers. Fifty percent of *HRAS* tumors originate from codon 12 in the first exon and forty percent from the mutations of codon 61 in the second exon (18-20). In the *HRAS* gene, SNP rs12628 is located at the 81 T→C position in codon 27. According to the results obtained from our study, having the T allele in the *HRAS* gene increases the risk of bladder cancer by 2.6 times. Furthermore, when the TT genotype is taken as reference, having the TC genotype has a 9.10-fold protective effect and having the TC or CC genotype has a 6.66-fold protective effect against the development of the disease. In line with previous studies, it can be stated that the presence of the T allele and TT genotype in SNP rs12628 in *HRAS* is a risk factor for bladder cancer (21). Pandith et al. (22) found that the CC variant was more common in individuals with bladder cancer compared to the healthy individuals in the Kashmir populations. Similar results were reported by Johne et al. (23), but the authors found no mutation in codons 12, 13 and 61, which change the structure of *RAS* protein in bladder cancer cases, and therefore it was concluded that this polymorphism seen in codon 27 could not be individually associated with bladder cancer.

The *RET* protein is a transmembrane tyrosine kinase receptor protein encoded by the *RET* protooncogene on the 10q11.2 locus and plays an important role in the endocrine, enteric nervous and excretory systems (24). It is known that somatic line mutations in the *RET* gene are associated with papillary thyroid carcinoma (25). One of these mutations is SNP rs1799939 (G>A, G691S) found in exon 11. In addition, the presence of G691S polymorphism has been reported in various types of disease, including hereditary and sporadic medullary thyroid carcinoma (26, 27), multiple endocrine neoplasia type 2A (28), pancreatic cancer (29), and skin malignant melanoma (30). However, there are no studies in the literature investigating G691S mutation in bladder cancer. Contrary to expectations, our results show that in bladder cancer cases, guanine, a wild-type allele, is increased and a mutant allele adenine is decreased. The greatest factor in the formation of these results can be seen as a high rate of AA genotype reaching 26.7% in the control group. Therefore, to fully establish a relationship between any disease and SNP and obtain more objective data, it is of great importance to conduct studies with larger numbers of individuals both in the control and patient groups.

In this study, in the control group, the number of individuals with an undetermined genotype was one for the *CCAT2* gene and two for the *HRAS* gene. In the patient group, the number of individuals without primary amplification was two for each of the *CCAT2, HRAS*, and *RET* genes.

CONCLUSION

The commercial kits used for the isolation and fluorometric determination of free DNA have been successful. This study showed that free DNA, which is already fragmented and available in small amounts, can be preserved for long periods under suitable laboratory conditions. The current study shows that fluorometric measurements are more advantageous considering their sensitivity in the quantification of free DNA. It has also been concluded that if rapidly treated, free DNA has sufficient efficacy to allow an SNP analysis, and this can provide important data for the demonstration of the quality of free DNA.

Competing Interests: The authors declare that they have no competing interest.

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Ethical Approval: This study was approved by the Ethics Committee of the Rectorate of Erzincan Binali Yildirim University (Number: 44495147/52-7690).

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