Normal and Modified Urinary Nucleosides as Novel Biomarkers for Colorectal Cancer

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Abstract: Background: Up till now, there is still no ideal tumour marker for early diagnosis and effective monitoring, especially for patients who undergo surgical resection of colorectal cancer (CRC). Objective: to evaluate the clinical utility of normal and modified urinary nucleosides as diagnostic biomarkers to be used for the purpose of screening for CRC, in addition to assessment of the correlation between their preoperative levels, tumour size and modified Duke's staging, as well as their role in monitoring of surgery, as compared to CEA, the routinely used serum marker**. Subjects and Methods:** This study was conducted on 30 patients with CRC (Group I), 30 patients with benign colorectal diseases (Group II) and 30 apparently healthy subjects (Group III). Morning urine and serum samples were collected before surgery and on day 7 postoperative, for the assay of urinary nucleosides (adenosine, cytidine, guanosine, uridine, 1-methyladenosine, 7-methylguanosine and N4-acetylcytidine) by reversed phase high-performance liquid chromatography, and serum CEA by chemiluminescent sequential immunometric assay**. Results:** The levels of the measured urinary nucleosides in group I were significantly higher than those of group II or group III. Moreover, the elevated levels of the urinary nucleosides significantly decreased after curative resection of CRC. A significant positive correlation was found between the preoperative levels of some nucleosides and the tumour size, as well as the modified Duke's staging of CRC. **Conclusion:** Urinary nucleosides are satisfactory diagnostic biomarkers of CRC. Moreover, they are apparently of value in the postoperative monitoring of CRC patients.

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1. Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide after lung and breast cancers with two-thirds of all CRCs occurring in the more developed regions of the world. In Egypt, it contributes for 5.0 % of all cancers. Each year there are nearly one million new cases of CRC diagnosed worldwide and half a million deaths **(**Elsabah and Adel, 2013; Gado *et al.,* 2013).

Although being an invasive procedure, colonoscopy-guided biopsy is considered till now the gold standard for cancer colon diagnosis, in spite of its high cost and inconvenience (Atkin *et al.,* 2013; van Dam *et al.,* 2013). On the other hand, the nowadays applied tumour markers, carcinoembryonic antigen (CEA) and CA19-9 have shown poor sensitivity and specificity for early diagnosis of CRC, as well as in judging the effectiveness of the surgical resection of the tumour **(**Hawk and Levin, 2005; Struck *et al.,* 2011). These facts have prompted the search for other non-invasive reliable markers for the disease.

Normal nucleosides play an important role in a variety of fundamental biological processes. They are essential building blocks of biomembranes, DNA and RNA structures, in addition to their role in the transport of chemical energy in the form of phosphate groups (Rossi *et al.,* 2007). Modified nucleosides are formed at the post-transcriptional stage by chemical modification of normal nucleosides within the ribonucleic acid (RNA). These modified nucleosides cannot be reutilized or further degraded, but they are excreted in the urine as intact molecules (Jiang and Ma, 2009). The elevated levels of urinary nucleosides have served as potential cancer biomarkers in many cancers such as cancer lung and ovary (Siedel *et al.,* 2006), urogenital tract cancer (Szymańska *et al.,* 2010), breast cancer (Hsu *et al.,* 2011) and hepatocellular carcinoma (Chen *et al.,* 2013). The aim of this study is to evaluate the clinical utility of normal and modified urinary nucleosides as diagnostic biomarkers to be used for the purpose of screening for colorectal cancer, in addition to assessment of the correlation between their preoperative levels, tumour size and modified Duke's staging, as well as their role in monitoring of surgery, as compared to CEA, the routinely used serum marker.

2.Subjects and Methods:

Subjects

This study was conducted at the General Surgery Department of Ain Shams University Hospitals. The study was performed according to Helsinki declaration. All participants granted their consent to share in this study.

Group I: Patients with CRC (n = 30):

This group included 30 CRC patients (17 males and 13 females), aged 53±12.5 years. The diagnosis of CRC was based on histopathologic examination of a colonoscopy-guided biopsy. Group I was further subdivided according to the modified Duke′s staging system into three subgroups; subgroup Ia which consisted of 10 patients with modified Duke′s stages A and B, subgroup Ib which consisted of 10 patients with modified Duke′s stage C, and subgroup Ic which included 10 patients with Duke′s stage D.

Group II: Patients with Benign Colorectal diseases (n= 30):

This group included 30 patients, (20 males and 10 females; aged 41 ± 22 years), with benign colorectal diseases. These included ulcerative colitis (n=18), diverticular disease (n=5), rectal ulcer (n=5) and benign villous adenoma (n=2).

Group III: Healthy Control (n= 30):

This group included 30 apparently healthy subjects serving as a healthy control group (15 males and 15 females, aged 43 ± 10.5 years).

All participants in this study were subjected to complete history taking, clinical examination, radiological investigations including CT scan of the abdomen and pelvis, total body bone scan (performed for groups I and II, only); in addition to the assay of urinary normal and modified nucleosides and serum CEA.

SAMPLING:

Three milliliters (3 mL) of venous blood were withdrawn in sterile dry vacutainers under complete aseptic conditions before any surgical or medical intervention was done. Serum was then separated by centrifugation and was used for immediate assay of serum CEA. Any hemolyzed or lipemic sample was discarded.

Random morning urine samples (10 mL) were collected in clean dry polyethylene cups and divided into two aliquots. The first aliquot was used for the immediate estimation of urinary creatinine, while, the second aliquot was stored at −20ºC for subsequent assay of the urinary nucleosides; adenosine, cytidine, guanosine, uridine, N4-acetylcytidine, 7-methylguanosine, and 1-methyladenosine.

As regards CRC patients, an additional 2 mL-venous blood samples and 10 mL-urine samples were collected on the 7th postoperative day for estimation of serum CEA and urinary nucleosides, respectively.

METHODS:

Analytical Methods:

-serum CEA assay was performed by chemiluminescent sequential immunometric assay applied on the IMMULITE (Diagnostic Product Corporation: 5700 West 96 Street, Los Angeles) (Wu *et al.,* 2011)

-Urinary creatinine analysis was performed on Synchron CX-9 autoanalyser (Beckman Instruments Inc., Scientific Instruments Division, Fullerton, CA92634-3100, USA) by a modified rate Jaffé method (Lamb and Price, 2008).

- Urinary nucleosides (normal and modified) assay was performed by a reversed-phase HPLC technique according to the method described by Feng *et al.* (2005) and Hsu *et al.* (2009). All used chemicals were purchased from Sigma (Sigma-Aldrich, Inc. 3050 Spruce Street, St Louis, MO 63103, USA).

a) Working external standards: Stock solutions of the 7 nucleoside standards was prepared by the reconstitution of the individual lyophilized standards with 25 mmol/L potassium dihydrogen phosphate buffer (prepared by dissolving 3.4 g of lyophilized potassium dihydrogen phosphate in 1 liter HPLC-grade water). The prepared solutions were quite stable at −20 ºC for 6 months.

b) Urine sample preparation: The urine samples were acidified using 2 mol/L hydrochloric acid. The acidified urine was then centrifuged at 10,000 xg for 10 minutes. A cation-exchange cartridge MCX column (Waters Corporation Milford, Mass, Burnsville, MN 55337 – 0336, USA) was conditioned and equilibrated with 1 mL methanol and 1 mL water. After that, each urine sample was loaded into the MCX column directly and then washed with 1 mL 0.1% formic acid (in H_2O). Finally, each urine sample was eluted with 1 mL of 2.8% ammonium hydroxide in methanol. The elute (1 mL) was evaporated to dryness in a vacuum system at 37˚C and reconstituted in 100 µL solution of the mobile phase before being subjected to HPLC analysis.

c) Chromatographic analysis: It was performed by reversed-phase HPLC system (Beckman Coulter, USA). The system consisted of a reversed- phase octadecyl silica (ODS) C18-column (4.6 x 250 mm; 5µm particle size) and an ultraviolet detector, adjusted at 254 nm. The mobile phase consists of 25 mmol/L potassium dihydrogen phosphate buffer (pH 5.0; Solvent A) and methanol-water (3:2, v:v; Solvent B). The gradient started with 100% Solvent A, which reached 40% at 25 minutes. The flow rate was maintained at 0.7 mL/min throughout the

chromatographic run (25 minutes). The chromatographic profile corresponding to the elution pattern of urinary nucleosides is shown in **figure (1)**, where peaks at 7.1, 8.1, 11.4, 13.2, 14.4, 16.5 and 22.7 minutes correspond to cytidine, uridine, 1-methyladenosine, 7-methylguanosine, guanosine, N4 acetylcytidine and adenosine, respectively.

d) Calculation of results: The calculation of the final concentrations of samples was based on the external standard method, using the peak area. Finally, the results were expressed as ng/µg creatinine.

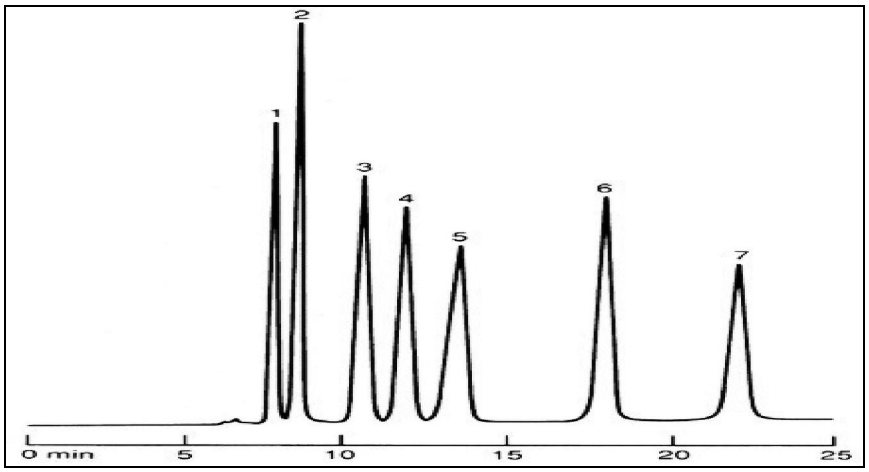


Figure (1): Chromatographic separation of a standard mixture of cytidine (peak 1), uridine (peak 2), 1-methyladenosine (peak 3), 7-methylguanosine (peak 4), guanosine (peak 5), N4- acetylcytidine (peak 6) and adenosine (peak 7) standards using reversed-phase HPLC with an ultraviolet detector.

Statistical Methods: Statistical analysis was performed using statistical software program SPSS version 9.02. Non-parametric data were expressed as median and interquartile range (Q1-Q3). Comparative statistics were done using Kruskall Wallis test, Mann–Whitney U-test and Wilcoxon′s signed-rank test. Correlation analysis was performed using Spearman′s rank correlation coefficient (rs). $p<0.05$ was considered significant and $p<0.01$ was considered highly significant. Receiver operating characteristic curve (ROC) analysis was applied to assess the overall diagnostic performance of each test in the study.

3.Results:

The results of the present study are shown in tables (1-5) and figures (2 – 5).

As regards the measured urinary nucleosides (adenosine, cytidine, guanosine, uridine, 1-methyladenosine, 7-methylguanosine and N4-acetylcytidine), a highly significant increase was found in group I when compared to either group II or group III ($p<0.01$, respectively). However, a non-significant difference was found in urinary nucleosides levels when comparing group II with group III ($p>0.05$), with the exception of N4-acetylcytidine that was significantly higher in the former group ($p<0.01$). As regards serum CEA, a highly significant increase was recorded in group I and group II as compared to group III levels ($p<0.01$, respectively). However, there was no statistically significant difference between both groups ($p>0.05$) (Tables 1 and 2).

Descriptive and comparative statistics of the different patients′ subgroups when classified according to the modified Duke's staging system are shown in tables (3 and 4). A statistically significant difference between the various stages was only recorded in case of guanosine ($p<0.05$), 1-methyladenosine and 7-methylguanosine ($p<0.01$, respectively; Table 3). The highest levels were recorded in modified Duke's D stage (subgroup Ic) as compared to the other stages (subgroups Ia & Ib), where p was <0.01, respectively in case of guanosine and 7-methylguanosine, and <0.01 in case of 1-methyladenosine (Table 4). However, the other urinary nucleosides and CEA did not show any statistically significant difference between these three subgroups ($p>0.05$, respectively; Table 3).

In the context of tumour size, no statistically significant difference was recorded between serum CEA and urinary nucleosides levels of CRC patients with tumour size <5 cm and those with tumour size ≥5 cm ($p>0.05$, respectively). However, a statistically significant positive correlation was recorded between the tumour size and 7-methylguanosine and 1-methyladenosine levels ($r_s=0.38$, $p<0.05$, respectively).

On comparing the preoperative and postoperative levels of the studied markers, both urinary nucleosides and serum CEA showed a highly significant decrease postoperatively (p <0.01, respectively; Table 5). The recorded values were insignificantly different from healthy control group levels in case of cytidine, 1-methyladenosine, 7-methylguanosine and N4-acetylcytidine, only (p >0.05, respectively; Figure 2).

Assessment of the diagnostic performance of the studied markers in CRC patients versus the healthy and benign colon disease groups using ROC curve analysis is shown in figures (3 and 4). CEA showed a very poor discriminatory power at the manufacturer's recommended cutoff level of 5.0 ng/mL as evidenced by an AUC of 0.51, a diagnostic sensitivity of 20%, specificity 90%, and 55% efficacy. Urinary nucleosides showed a much better diagnostic performance, with the best performance being achieved by adenosine at a cutoff level of 7.1 ng/µg creatinine (AUC =0.91, diagnostic efficacy 94%, sensitivity 87%, specificity 98%, PPV 96% and NPV 94%), followed by N4-acetylcytidine (cutoff level 7.8 ng/µg creatinine, AUC= 0.86, diagnostic efficacy 91%, 77% sensitivity, 98% specificity, 95% PPV, and 89% NPV). Both cytidine (cutoff level 5.4 ng/µg creatinine, AUC= 0.82) and uridine (cutoff level 6.3 ng/µg creatinine AUC= 0.82) had 90% efficacy, 77% sensitivity and 89% NPV, respectively; however their specificities were 95% and 97%, while their PPVs were 89% and 92%, respectively. Guanosine (2.8 ng/µg creatinine) showed an AUC=0.69, 88% efficacy with 73% sensitivity, 95% specificity, 88% PPV and NPV, respectively. 7-methylguanosine (6.5 ng/µg creatinine) showed an AUC=0.64, 80% diagnostic efficacy with only 63% sensitivity, 88% specificity, 73% PPV and 83% NPV. Finally, 1-methyladenosine at a cutoff level of 5.2 ng/µg creatinine had an AUC=0.61, 78% efficacy with a sensitivity of 57%, specificity 88%, PPV 71% and NPV 80%.

ROC curve analysis showing the diagnostic performance of the studied urinary nucleosides in modified Duke's stage D versus modified Duke's stage C is shown in figure 5. The AUCs of 7-methylguanosine, guanosine and 1-methyladenosine were 0.75, 0.81, and 0.86, respectively. The best cutoff level of 7-methylguanosine was 16.1 ng/µg creatinine (diagnostic efficacy 95 %, sensitivity 100%, specificity 90%). The best cutoff level of guanosine was 8.0 ng/µg creatinine (diagnostic efficacy 85 %). This had the same 90% diagnostic specificity with a lower diagnostic sensitivity of 80%. Finally, in case of 1-methyladenosine, the best cutoff level was 8.8 ng/µg creatinine. This had 90% diagnostic efficacy, sensitivity and specificity, respectively.

Table 1. Descriptive and Comparative Statistics of the Measured Parameters in the Three Studied Groups

Parameter	Group I (n=30)	Group II (n=30)	Group III (n=30)	H	p
	Median (Q_1-Q_3)	Median (Q_1-Q_3)	Median (Q_1-Q_3)		
CEA (ng/mL)	3.1 (1.7-4.3)	2.5 (2.0-4.0)	0.6 (0.3-1.6)	23.2	**<0.01**
Adenosine (ng/µg creatinine)	23.8 (10.5-39.1)	2.8 (1.7-4.8)	2.6 (1.7-4.1)	38.8	**<0.01**
Cytidine (ng/µg creatinine)	15.8 (5.3-27.8)	2.2 (0.8-3.3)	1.6 (1.0-2.3)	32.3	**<0.01**
Guanosine (ng/µg creatinine)	6.4 (1.8-16.5)	0.7 (0.4-1.3)	0.8 (0.4-1.0)	20.6	**<0.01**
Uridine (ng/µg creatinine)	13.3 (7.0-26.5)	3.3 (1.6-4.4)	3.1 (1.5-4.4)	34.4	**<0.01**
1-methyladenosine (ng/µg creatinine)	6.5 (2.0-23.0)	1.5 (0.2-3.6)	1.6 (0.7-4.5)	14.7	**<0.01**
7-methylguanosine (ng/µg creatinine)	9.3 (3.1-29.7)	2.9 (0.9-4.8)	2.9 (0.9-4.8)	14.7	**<0.01**
N4-acetylcytidine (ng/µg creatinine)	18.6 (7.58-30.4)	2.2 (1.2-3.7)	1.0 (0.7-2.7)	26.5	**<0.01**

Group I: Colorectal cancer group; Group II: Benign colorectal disease group
Group III: Healthy control group; CEA: Carcinoembryonic antigen
Q_1: 25th Percentile; Q_3: 75th Percentile; P<0.01: Highly significant difference

Table 2. Between-Two Group Comparison of Serum CEA and Urinary Nucleosides Levels in the Various Studied Groups, Using the Mann-Whitney U-Test

Parameter	Group I vs Group II		Group I vs Group III		Group II vs Group III	
	Z	*p*	Z	*p*	Z	*p*
CEA (ng/mL)	0.53	>0.05	4.43	**<0.01**	4.32	**<0.01**
Adenosine (ng/µg creatinine)	6.08	**<0.01**	6.23	**<0.01**	0.53	>0.05
Cytidine (ng/µg creatinine)	5.30	**<0.01**	5.69	**<0.01**	0.84	>0.05
Guanosine (ng/µg creatinine)	4.43	**<0.01**	4.55	**<0.01**	0.09	>0.05
Uridine (ng/µg creatinine)	5.77	**<0.01**	5.87	**<0.01**	0.36	>0.05
1-Methyladenosine (ng/µg creatinine)	4.09	**<0.01**	3.84	**<0.01**	0.53	>0.05
7-Methylguanosine (ng/µg creatinine)	3.66	**<0.01**	3.84	**<0.01**	0.04	>0.05
N4-acetylcytidine (ng/µg creatinine)	5.85	**<0.01**	5.15	**<0.01**	3.05	**<0.01**

Group I: Colorectal cancergroup; Group II: Benign colorectal disease group; Group III: Healthy control group; CEA: Carcinoembryonic antigen; *p*> 0.05: Non- significant difference; *p*<0.01: Highly significant difference

Table 3. Statistical Comparison between Serum CEA and Urinary Nucleosides Levels in the Different Modified Duke's Stages, Using the Kruskall Wallis Test

Parameters	Duke's A and B (Subgroup Ia, n=10)	Duke's C (Subgroup Ib, n=10)	Duke's D (Subgroup Ic, n=10)	H	*p*
	Median (Q_1-Q_3)	Median (Q_1-Q_3)	Median (Q_1-Q_3)		
CEA (ng/mL)	3.2 (1.7-7.5)	3.2 (2.2-3.7)	2.9 (1.4-4.4)	0.51	>0.05
Adenosine (ng/µg creatinine)	28.0 (16.3-42.3)	18.2 (7.6-38.6)	29.7 (8.3-43.4)	0.99	>0.05
Cytidine (ng/µg creatinine)	15.4 (3.9-29.3)	15.8 (6.8-21.0)	17.6 95.1-44.0)	0.56	>0.05
Guanosine (ng/µg creatinine)	4.9 (1.1-11.0)	5.1 (0.2-6.8)	17.7 (7.8-29.6)	8.24	**<0.05**
Uridine (ng/µg creatinine)	12.4 (5.5-17.5)	13.3 (5.5-31.4)	14.5 (8.5-45.3)	1.22	>0.05
1-methyladenosine (ng/µg creatinine)	2.0 (1.3-3.5)	5.9 (2.3-7.5)	24.0 (19.8-44.2)	18.9	**<0.01**
7-methylguanosine (ng/µg creatinine)	7.1 (4.8-10.9)	2.8 (1.3-11.0)	36.7 (23.2-48.6)	17.2	**<0.01**
N4-acetylcytidine (ng/µg creatinine)	18.3 (11.7-23.7)	22.6 (16.5-55.0)	11.7 (2.3-43.8)	3.05	>0.05

p>0.05: Non significant difference; *p*<0.05: significant difference; *p*<0.01: Highly significant difference
Q_1: 25th Percentile; Q_3: 75th Percentile CEA: Carcinoembryonic antigen

Table 4. Between-Two Group Comparison of Urinary Guanosine, 1-Methyladenosine and 7-Methylguanosine in the Different Modified Duke's Stages, Using the Mann-Whitney U-Test

Parameter	Duke's A and B vs Duke's C		Duke's C vs Duke's D		Duke's A and B vs Duke's D	
	Z	*p*	Z	*p*	Z	*p*
Guanosine (ng/µg creatinine)	0.53	>0.05	2.72	**<0.01**	2.75	**<0.01**
1-Methyladenosine (ng/µg creatinine)	1.85	>0.05	3.52	**<0.01**	3.63	**<0.01**
7-Methylguanosine (ng/µg creatinine)	1.59	>0.05	3.48	**<0.01**	3.40	**<0.01**

p>0.05: Non significant difference ; *p*<0.05: significant difference;
p<0.01: Highly significant difference

Table 5. Statistical Comparison between Preoperative and Postoperative Levels of Serum CEA and Urinary Nucleosides of CRC Patients, Using the Wilcoxon's Signed Rank Test

Parameter	CRC Group (n=30)		Z	*p*
	Preoperative	Postoperative		
	Median (Q_1-Q_3)	Median (Q_1-Q_3)		
CEA (ng/mL)	3.1 (1.7-4.3)	2.0 (1.2-3.4)	4.79	**<0.01**
Adenosine (ng/µg creatinine)	23.8 (10.5-39.1)	6.1 (2.2-9.8)	4.78	**<0.01**
Cytidine (ng/µg creatinine)	15.8 (5.3-27.8)	2.7 (1.2-6.7)	4.77	**<0.01**
Guanosine (ng/µg creatinine)	6.4 (1.8-16.5)	2.0 (0.7-3.8)	0.46	**<0.01**
Uridine (ng/µg creatinine)	13.3 (7.0-26.5)	5.2 (2.3-7.8)	4.78	**<0.01**
1-methyladenosine (ng/µg creatinine)	6.5 (2.05-23.03)	2.0 (0.7-6.3)	4.70	**<0.01**
7-methylguanosine (ng/µg creatinine)	9.3 (3.1-29.7)	2.3 (1.3-9.3)	4.79	**<0.01**
N4-acetylcytidine (ng/µg creatinine)	18.6 (7.5-30.4)	4.4 (1.4-8.5)	4.78	**<0.01**

Q_1: 25th Percentile; Q_3: 75th Percentile *p*<0.01: Highly significant difference;
CRC: Colorectal cancer CEA: Carcinoembryonic antigen

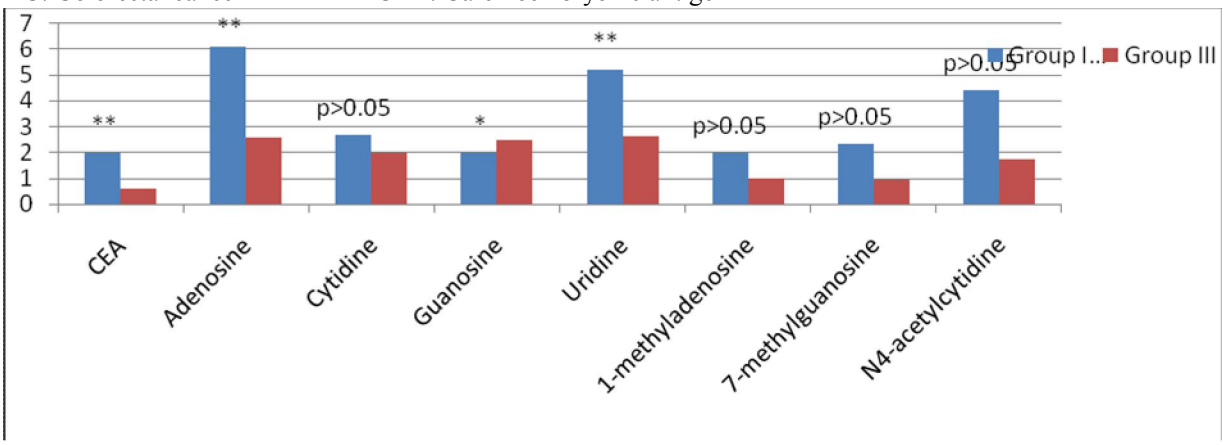


Figure 2. Median values of postoperative serum CEA and urinary nucleosides in CRC patients as compared to the healthy control group.
Group I: CRC group; Group III: Healthy control group; * p<0.05; ** p<0.01

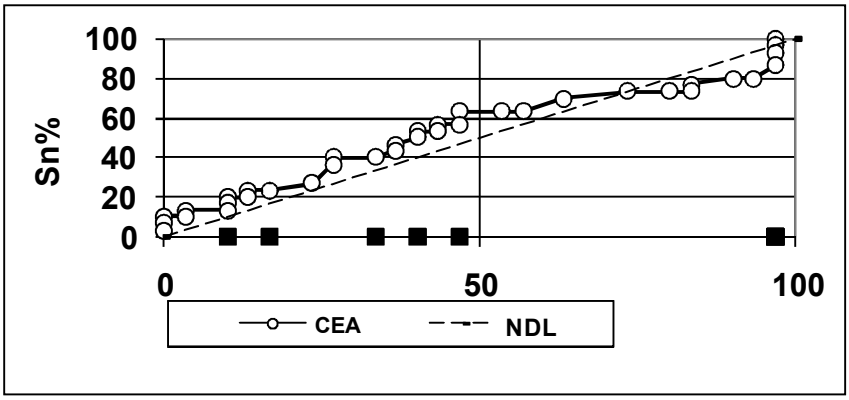
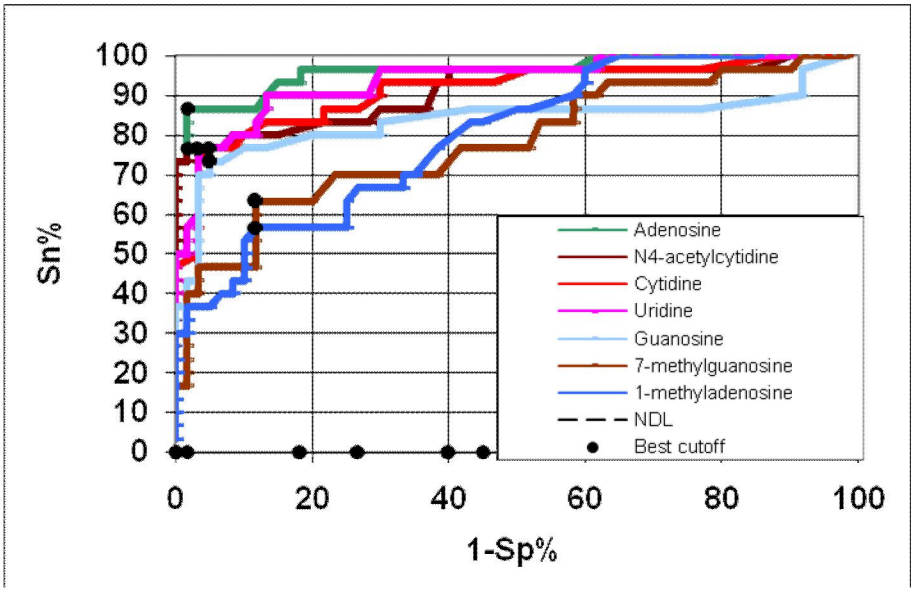
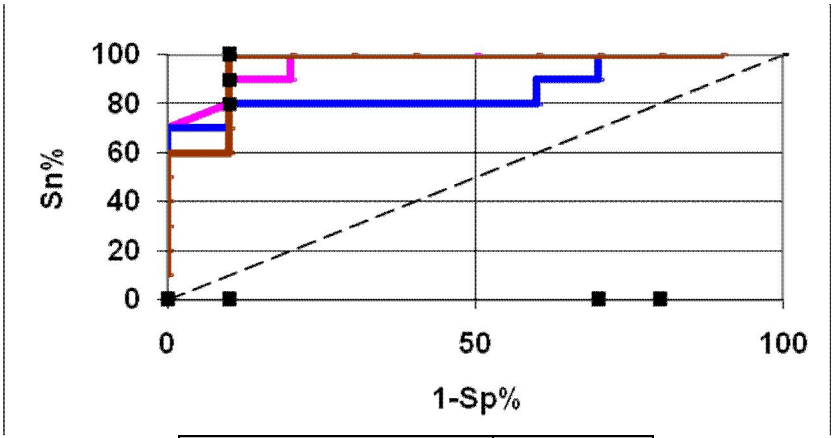


Figure 3. ROC curve analysis showing the diagnostic performance of the serum CEA in CRC patients versus the benign colorectal disease group.



Nucleoside	AUC
Adenosine	0.91
N4-acetylcytidine	0.86
Cytidine	0.82
Uridine	0.82
Guanosine	0.69
7-Methylguanosine	0.64
1-methyladenosine	0.61

Figure 4. ROC curve analysis showing the diagnostic performance of the measured urinary nucleosides in CRC patients versus the healthy and benign colorectal disease groups.



Nucleoside	AUC
1-methyladenosine	0.86
Guanosine	0.81
7-methylguanosine	0.75

Figure 5. ROC curve analysis showing the diagnostic performance of guanosine, 1-methyladenosine, and 7-methylguanosine in Duke's stage D versus stage C.

4.Discussion

Data of the present study revealed that CEA exhibited a statistically highly significant increase in either patients with CRC or those with benign colorectal disease as compared to healthy subjects. However, there was no statistically significant difference between the recorded levels of the former two groups. Moreover, CEA revealed a very poor discriminatory ability between these former groups even at the manufacturer's recommended cutoff level of 5.0 ng/mL which showed a very low diagnostic sensitivity of 20% with 90% specificity and 55% diagnostic efficacy. Previous research studies carried out by Marelli *et al.* (2011) and Wu *et al.* (2011) have pointed out to the very low diagnostic sensitivity of CEA in CRC patients (36.4%, and 39.4%, respectively). The increased levels of the marker in benign colorectal conditions confirm the lack of its diagnostic specificity, as has been previously reported by other investigators (Lumachi *et al.*, 2012).

Concerning the studied urinary nucleosides, these showed highly significant elevations in CRC patients with median levels ranging between 3-4 times the median levels observed in healthy subjects in case of 7-methylguanosine, 1-methyladenosine, and uridine. Higher levels (8-9.6 x the median) were recorded in case of guanosine, adenosine, and cytidine. The highest elevation was recorded in case of N4-acetylcytidine (18.7 x the median level of healthy control subjects). The previous findings are in accordance with those of Hsu *et al.* (2009) who reported a significant rise in the urinary excretion of adenosine, cytidine, uridine, 1-methyladenosine and N4-acetylcytidine in their studied CRC patients. This elevation was attributed to the increased activity and capacity of tRNA methyltransferase from cancer tissue as compared to the enzyme derived from the corresponding normal tissue of origin, hence leading to elevated levels of both normal and modified nucleosides. Accordingly, tRNA from neoplastic tissue is suggested to have a much more rapid turnover rate than the tRNA from the corresponding normal tissue (Struck *et al.*, 2011).

In contrast to these findings, Feng *et al.* (2005) and Zheng *et al.* (2005) found no statistically significant increase in urinary uridine and guanosine levels in their studied CRC patients. This difference may be attributed to the unique characteristics of CRC in Egypt, which differ from that reported in the Western society, as the disease usually presents at an advanced stage, with the prevalence of the dysplasia-carcinoma sequence rather than the adenoma-carcinoma sequence (Soliman *et al.*, 1997; Gado *et al.*, 2013).

Fortunately, all studied nucleosides were significantly higher in CRC patients than those with benign colorectal diseases as well, where the folds of increase in median levels were similar to the above mentioned values in case of comparison to the healthy control group. This was obviously attributed to absence of a statistically significant difference between the levels of normal and modified nucleosides in healthy subjects and benign colorectal conditions. The only difference was in case of N4-acetylcytidine which showed a lesser degree of elevation in comparison to the pathological control group (8.3 x the median compared to 18.7 x the median of healthy controls). This is explained by its significantly higher level in patients with benign

colorectal diseases as compared to the healthy controls. In this respect, Zheng *et al.* (2005) have previously reported that cytidine concentrations of patients with benign colorectal disease were higher than those of healthy adults. This was attributed to the protective and direct anti-inflammatory functions of some nucleosides in tissues under different kinds of cellular stress or pathological conditions (Alsafarjalani *et al.*, 2001).

Concerning the preoperative urinary nucleoside levels in the different modified Duke′s stages, the only statistically significant increase was recorded in Duke′s D stage as compared to Duke′s C in case of guanosine, 1-methyladenosine and 7-methylguanosine. Similarly, Feng *et al.* (2005) and Zheng *et al.* (2005) recorded a statistically significant difference between the various modified Duke′s stages in case of 1-methyladenosine. This may be attributed to its immunosuppressive effect, as has been suggested by Masuda *et al.* (1993). In contrast to our results, Hsu *et al.* (2009) reported no statistically significant difference between urinary nucleosides levels in various modified Duke′s stages of the disease.

In the context of the tumour size, data of the present study showed no statistically significant difference between serum CEA or urinary nucleosides levels in CRC patients with tumour size < 5cm and those with tumour size ≥5 cm. However, our correlation study revealed a highly significant positive correlation between the size of the resected tumours and each of 1-methyladenosine and 7-methylguanosine. This is in accordance with the findings of Feng *et al.* (2005) and Zheng *et al.* (2005). A plausible explanation is that progressive larger tumours contain greater amounts of hyperactive methylase enzyme, thus producing larger amounts of modified nucleosides in cancer cells (Schram, 1998 ; Nagaraju and El-Rayes, 2013).

Concerning the postoperative levels of the studied markers, both CEA and urinary nucleosides showed a highly significant decrease after surgical resection, with cytidine, 1-methyladenosine, 7-methylguanosine and N4-acetlycytidine returning to the healthy control group values. This is in accordance with the finding of Zheng *et al.* (2005) and Lee *et al.* (2012) where the former added that although CEA significantly decreased after surgical resection, CRC monitoring by CEA is not effective because of its relatively poor sensitivity, specificity and longer half-life (3-13 days) as compared to the urinary nucleosides (10 seconds in case of adenosine). Accordingly, it was concluded that urinary nucleosides show a faster response to therapy and disease recurrence.

On assessment of the diagnostic performance of the studied markers using ROC curve analysis in CRC patients versus the healthy and benign colorectal disease groups, the best diagnostic performance was recorded in case of adenosine whose AUC was 0.915. Its best cutoff level (7.1ng/µg creatinine) had a diagnostic efficacy of 94%, sensitivity 87%, specificity 98%, PPV 96% and 94% NPV. N4-acetylcytidine, cytidine, uridine, and guanosine had smaller AUCs of 0.867, 0.829, 0.824 and 0.692, respectively. Their corresponding best cutoff levels had 10-14% lower diagnostic sensitivity with 95-98% specificity. The least AUCs were recorded in case of 1-methyladenosine and 7-methylguanosine (0.614 and 0.642, respectively) whose best cutoffs had a very much lower diagnostic sensitivity of 57 and 63%, with a further 10% decrease in diagnostic specificity. The latter results are comparable to those of Feng *et al.* (2005) and Zheng *et al.* (2005) who demonstrated that the sensitivity of their studied, namely cytidine, guanosine and uridine, urinary nucleosides in patients with CRC were 77% and 71%, respectively. Unexpectedly, they also recorded a similarly low sensitivity in case of adenosine.

Concerning the ROC curve analysis of the three markers showing a statistically significant difference between Duke's D verses Duke's C stages (7-methylguanosine, guanosine and 1-methyladenosine), the recorded AUCs were 0.750, 0.811 and 0.867, respectively. The best diagnostic cutoff level with the highest diagnostic efficacy was that of 7-methylguanosine (95% compared to 85% in case of guanosine and 90% in case of 1-methyladenosine). All three nucleosides had the same 90% specificity, with the best achievable diagnostic sensitivity being recorded in case of 7-methylguanosine (100%) compared to 80% in case of guanosine and 90 % in case of 1-methyladenosine. These findings highlight the great discriminatory power of 7-methylguanosine with positive and negative predictive values of 91% and 100%, respectively.

In conclusion, urinary nucleosides are satisfactory diagnostic markers of CRC. They are blessed by their higher sensitivity and specificity compared to CEA. Moreover, they do not need any patient preparation prior to sampling. Further large-scale studies are needed to confirm the value of adenosine in early diagnosis and screening of high-risk patients or/populations. Moreover, the prognostic significance of nucleosides especially guanosine, 1-methyladenosine and 7-methylguanosine and their role in postoperative monitoring and assessment of patients response to therapy is worth investigation.

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