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Guanylyl Cyclase C as a tumor marker for detection of circulating tumor cells in the peripheral blood of colorectal cancer patients

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Original Article

Abstract

Purpose: Guanylyl cyclase C (GCC) is one of the most frequent tumor markers to detect the circulating tumor cells (CTCs) in peripheral blood of colorectal cancer (CRC) patients. It has been proposed as a new marker for the molecular staging of CRC. The level of GCC mRNA expression in peripheral blood of CRC patients was evaluated to explore its probable correlations with the clinicopathological features. **Methods:** Relative quantitative expression analysis of GCC mRNA was performed on 80 blood samples (40 patients and 40 normal) using the Real-time RT-PCR. **Results:** GCC mRNA expression was detected in 70% of the CRC blood samples. The level of GCC mRNA expression in peripheral blood of patients was significantly higher than that in the normal cases ($p = 0.031$). Moreover, there was a significant correlation between the GCC copy number and advanced stages of tumor ($p = 0.041$). Furthermore, we have observed a significant correlation between tumor sizes and GCC copy numbers ($p = 0.050$). **Conclusion:** GCC can be a useful marker not only for detection of CTCs in CRC blood samples, but also for the molecular staging of colorectal cancer.

Keywords: Guanylyl cyclase C, Colorectal cancer, Peripheral blood, Tumor marker, Circulating tumor cells

1. Introduction

Colorectal cancer (CRC) is the fourth common cancer in the world with almost one million new cases annually and mortality rate of almost 50%.[1,2] CRC incidence is varied in different areas in which there is a higher prevalence in Western countries (North America, Northern and Southern Europe) in comparison with the other regions. Although, CRC is more common in developed countries, its prevalence is growing in developing countries.[3] The age-standardized rate (ASR) of CRC in Iran is 7.5/105 in females and 8.1/105 in males, ranking the third and fourth common cancers in Iran, respectively.[4-7] More than 30% of cases have developed metastasis and approximately 20% of patients have unresectable tumors. Although, Surgery has the highest impact on survival, it only removes efficiently the tumors, which are in the early stages. Rate of tumor relapses ranges from approximately 10% to more than 60% for the tumors in stages of I and III,

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respectively.[8, 9] CRC is poorly diagnosed in the initial stages of tumor and the symptoms usually appear in the advanced stages of tumorigenesis. The first sign of CRC progression appears in the lymph nodes, which is an important criterion for the histopathological staging. The efficiency of different therapeutic modalities after the surgery is directly related to the proper tumor staging.[10] Since circulating free tumor cells can cause micro metastasis [11, 12], detection of such cells in the patients' blood can be an important way for the metastasis prediction to provide a non-invasive, faster, and accurate diagnostic method in comparison with the histopathological analysis. Molecular staging of CRC includes analysis of tumor cells in lymph nodes, peripheral blood, and bone marrow of patients to get the complete and accurate information[13]. Although, Carcino-embryonic antigen (CEA) and carbohydrate antigen19-9 (CA19-9) are currently applied as clinical markers for the diagnosis and screening of CRC[2, 14], CEA expression is reported in a variety of cancers and is not specific for the CRC patients[15-17]. One of the main approaches to identify and evaluate the presence of blood CTCs is assessment of tissue-specific biomarkers such as Guanylyl cyclase C (GCC) in CRC patients by RT-PCR based methods [18-20]. GCC is a receptor for the paracrine intestinal hormones such as guanylyl, uroguanylylin, and heat-stable entrotoxin (ST). It is expressed specifically in the apical surface of intestinal epithelial cells from duodenum to rectum, and was introduced as a special marker for the CRC[10, 21]. Despite of GCC expression in CRC tissues and its role in tumor metastasis, it has been shown that GCC protein and mRNA were not found in extra gastrointestinal tissues and tumor microenvironments[22, 21]. Moreover, this receptor is continuously expressed during and after neoplastic transformation of normal epithelial cell to CRC cell. Since GCC expression has been identified in all primary and metastatic colorectal tumors, it may be deserved as a suitable prognostic and predictive marker to identify the CRC micro metastasis[21, 23]. Due to the lack of specific and universal markers for CRC detection[24], in the present study we assessed the diagnostic efficiency and importance of GCC copy number variation in the peripheral blood samples of Iranian CRC patients using a quantitative real time PCR technique.

2. Methods and Materials

2.1. Study subjects

Forty consecutive histologically confirmed CRC patients were enrolled in the present study who were undergone the tumor resection between 2009 to 2011 in Imam Reza and Omid Hospitals (Mashhad, Iran) and Baqiatallah Hospital (Tehran, Iran). Clinicopathological features of tumors were defined according to the American Joint Commission on Cancer (AJCC) criteria[25]. In addition, forty healthy volunteers who had normal colonoscopy reports were served as the control cases. Peripheral blood of selected cases was collected before the surgery and any chemo-radio therapeutic treatments. All the patients and healthy volunteers were informed about the study and approved the consent forms, which were confirmed by the ethic committee of Mashhad University of Medical Sciences.

2.2. RNA extraction and cDNA synthesis

The peripheral mononuclear cells (PBMCs) were isolated from the 5 ml of whole blood using a lymphocyte separation medium (Lymphodex, Quest Biomedical, UK). Total RNA was extracted from the CRC cell line (HT29) and PBMCs using a Trizol reagent (Roche, Applied Sciences, and Indianapolis, IN). Extracted RNA was analyzed by the agarose gel electrophoresis and its concentration was determined in an absorbance of 260 and 280 nm using a spectrophotometer. cDNA synthesis from the total RNA was performed using the cDNA synthesis kit and oligo (dT)18 as amplification primer (Fermentas, Vilnius, Lithuania).

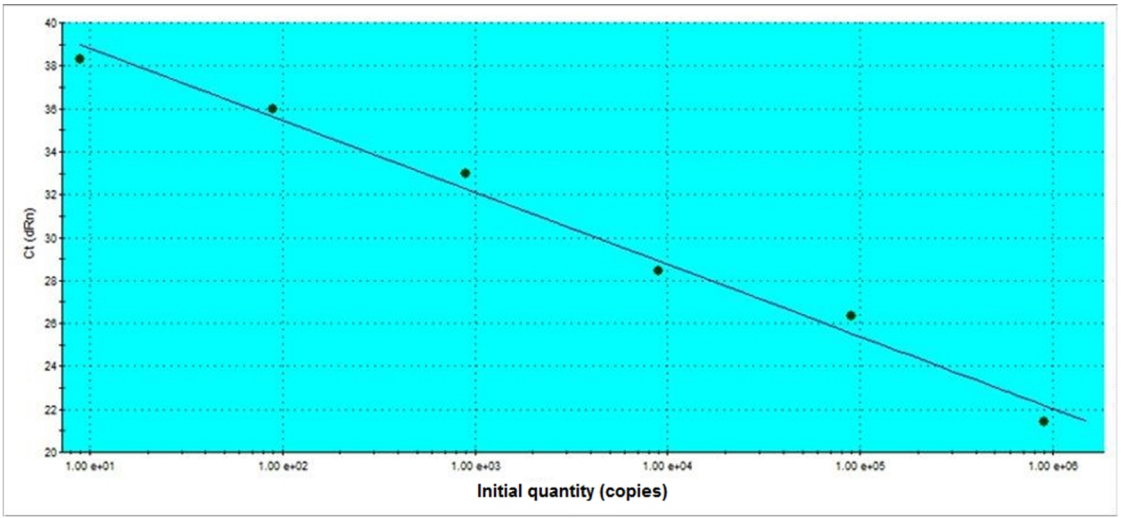

Figure 1: Standard curve of the GCC mRNA copy numbers.

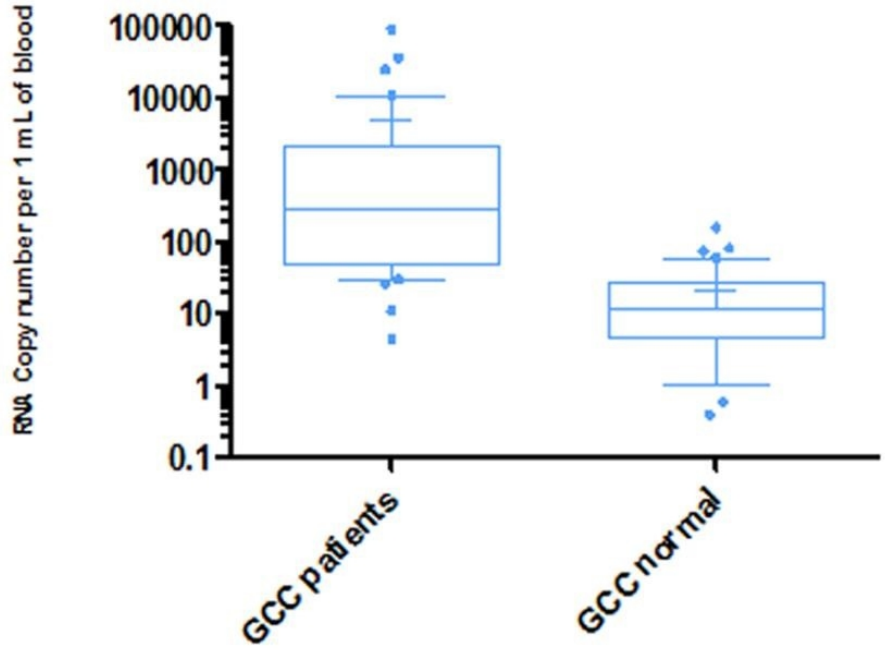

Figure 2: Quantification of GCC mRNA in blood of patients and healthy donors. Copy number is shown in 1 ml of the peripheral blood.

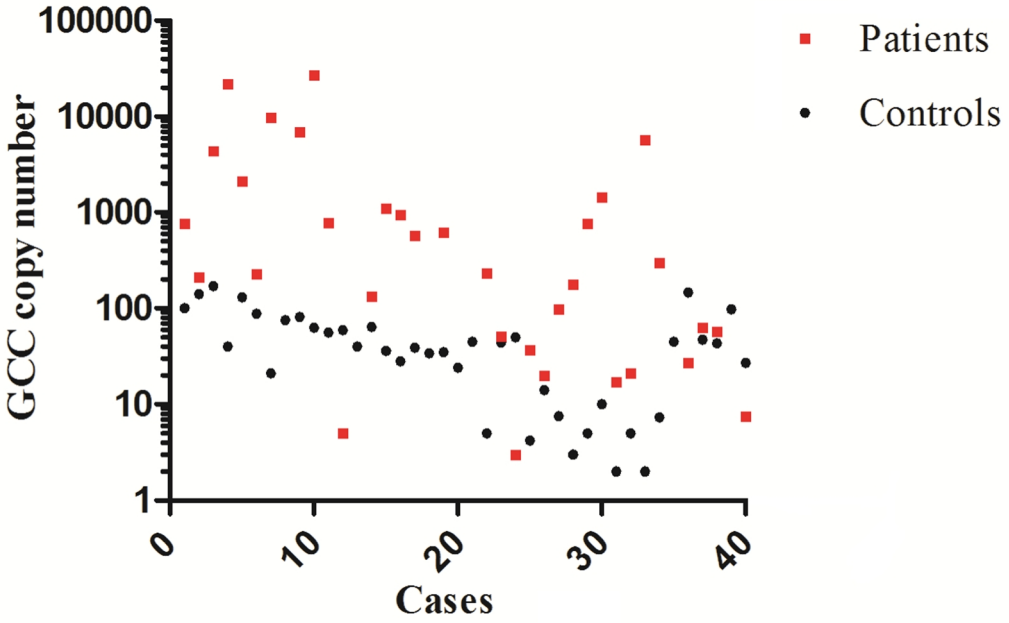

Figure 3: Scatter plot represents descriptive analysis of the GCC quantitative gene expression in CRC patients and controls.

2.3. Construction of standards

To prepare the standard curve, the GCC mRNA fragment was amplified using the HT29 cDNA and primer sequences which are presented in Table.1. PCR product was purified using the DNA purification kit (Fermentas, USA), and inserted into the pTZR/57 vector (Fermentas, Vilnius, Lithuania). The recombinant vectors were extracted and linearized with Mava I and diluted serially. The concentration of linearized vector was determined spectrophotometrically, and the copy numbers were calculated as described before[26, 27]. A standard curve was prepared from 10-fold serial dilutions of linearized plasmid containing the GCC gene fragment. Seven standards (serial dilution) were included in each run of real-time PCR. Each reaction was prepared and performed in duplicate along with triplicates of 10-fold serial dilutions of the plasmid. The GCC mRNA value was calculated with a reference to the standard curve (Figure 1), whose slope was -3.04 indicating high PCR amplification efficiency (98.4%).

2.4. Quantitative reverse transcription-polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed in duplicate reactions based on SYBR green (absolute quantitative Δct) method (Invitrogen, Carlsbad, CA, USA) in Stratagene Mx-3000P real-time thermo cycler (Stratagene, La Jolla, CA, USA). Each reaction mix was prepared by adding 10 µl SYBR Green, 0.04 µl ROX, 0.6 µl of each primer (10 pmol/µl), 3 µl cDNA, and 5.76 µl DEPC water in a total volume of 20 µl. The thermal profile was applied as 10 min of initial denaturation at 95ºC, followed by 45 cycles at 95ºC for 30s, 60ºC for 30s, and 72ºC for 30s. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous control to normalize the data [28, 29].

2.5. Statistical analysis

The statistical analysis was performed using the SPSS 16.0 (SPSS, Chicago, IL, USA). The associations between categorical data (lymph node involvement, tumor size, location, grade, and stage) and GCC copy numbers were examined using the Spearman, Mann–Whitney U, and the Kruskal–Wallis H-tests. A value of $p \leq 0.05$ was considered statistically significant.

3. Results

A total of 40 CRC patients consisting of 30 males and 10 females were recruited for the present study. The total mean age was 59.1±17.6 years ranging between 25 to 86 years. The sex ratio for 40 control cases was 1.4 (23 males, 17 females) with mean age of 57.2 ± 7.3 years. Clinicopathological features of the patients are summarized in Table.2. Most of cases were moderately differentiated (22/40, 55%) and located in distal colon (26/40, 65%). Only 9 out of 40 cases (22.5%) had lymph node metastasis. Range of tumor sizes were between 1 to 10 cm with mean size of (4.90 ± 1.98 cm). Majority of cases were in tumor stages of B2 (18/40, 45%) and C2 (11/40, 27.5%). Real-time PCR analysis of the circulating GCC mRNA revealed that the levels of GCC mRNA in the peripheral blood of the patients was significantly higher than that in the controls (70% vs. 53%, $p = 0.031$) (Figures 2 and 3). The mean copy number of GCC mRNA in peripheral blood of the patients and normal cases were 2.17E3 ± 5624.20 and 47.15 ± 42.95 copies, respectively. The level of GCC copy number had significantly increased in advanced stages of tumor ($p = 0.041$). This marker produced an overall sensitivity of 70% and a specificity of 46%. Although there was not any significant correlation between the tumor grade and levels of GCC mRNA expression, moderately differentiated tumors had higher levels of GCC expression in comparison with the well differentiated cases (2.52E3 ± 1.29E3 Vs. 1.96 ± 1.37E3, copy numbers). In the case of lymph node status, there was not also any significant correlation with the GCC copy numbers. However, as we expected, levels of GCC mRNA expression in patients with metastatic lymph nodes were higher than that in the other cases (4.38E3 ± 2.48E3 vs. 1.53E3 ± 8.89E2, copy numbers). Moreover, patients with proximal tumors had shown higher GCC copy numbers in their peripheral blood in comparison with the cases with distal tumors (3.23E3 ± 2.00E3 vs. 1.59E3 ± 8.60E2, copy numbers). While, there was a significant correlation between the GCC copy numbers and tumor sizes ($p = 0.050$), there was not any significant correlation between GCC copy number and age of patients ($p = 0.731$).

Table 1: Primer Sequences which are used in cloning and qRT-PCR.

Primer	Sequence (5' to 3')	Size (bp)
GCC(Real time PCR)	F:GACCACAACAGGAAAAGCAATC R:AGGCAAGACGAAAGTCTCGTTT	262
GCC(Cloning)	F:AAACTGAGGACTGGTTCTGGTACC R: GTCACTGGACCGTCATACCCT	510
GAPDH	F: GGAAGGTGAAGGTCGGAGTCA R:GTCATTGATGGCAACAATATCCACT	101

Table 2: Clinicopathological features of CRC patients.

	Total	GCC mRNA (mean copy number)	*P Value*
Patients	40	2.17E3±5624.20	
Mean age (mean ± SD)	59.05±17.61		0.731
Size (mean ± SD)	4.90±1.98		0.050
			0.390
Sex			
Male	30(75.0%)	2.62E3±1.17E3	
Female	10(25.0%)	8.25E2±4.25E2	
			0.387
Location			
Proximal	14(35.0%)	3.23E3±2.00E3	
Distal	26(65.0%)	1.59E3±8.60E2	
			0.594
Grade			
P.D	2(5.0%)	13.50±0.00	
M.D	22(55.0%)	2.52E3±1.29E3	
W.D	16(40.0%)	1.96E3±1.37E3	

4. Discussion

Despite different diagnostic methods, CRC is still one of the leading cause of cancer related deaths in the world and most of cases are detected in advanced stages of tumor leading to an inefficient therapy[30]. Therefore finding a specific tumor marker is essential for the early detection[31]. Although, surgery is one of the best treatment strategies in early stages of CRC, between 30-50% of such patients face with the tumor relapse and metastasis[32, 27, 33]. Micro metastasis plays a key role in tumor relapse in which, the tumor cells spread from the primary site to the other organs through the lymphatic circulation or bloodstream[34]. Molecular staging of CRC using the specific molecular techniques allows detection of CTCs which cannot be achieved by the histological staging. Accuracy and fidelity of a tumor staging marker is related to its frequency of expression and preferential expression in a specific tumor when compared to the other types of normal or tumor tissues[10]. Although, CEA is one of the main tumor markers for CRC detection, it has some limitations such as poor specificity and sensitivity. We have recently reported that Sall4 and CEA can be used as sensitive serum markers for the early CRC detection and screening[26, 27]. However, SALL4 had higher sensitivity in comparison with the CEA (96.1% vs. 80%). Indeed, introducing more specific markers prepares a complete panel of specific markers for the CRC screening. Therefore, following our recent studies, in the present study we assessed the levels of GCC mRNA expression using the quantitative Real-time PCR method in peripheral blood of CRC patients. The GCC copy numbers were significantly higher in peripheral blood of CRC patients in comparison with the healthy control group. The results showed that there is a significant correlation between the high copy numbers of blood GCC mRNA and tumor stage and size. Low levels of GCC expression has been reported in normal intestinal mucosa cells, while it is over expression has been identified in colorectal cancer cells and all primary and metastatic colorectal tumors[22, 35, 36, 37]. GCC may improve the criteria of a suitable prognostic and predictive marker to identify the colorectal micro metastasis[21]. It has been demonstrated that GCC over expression in CRC patients is highly associated with tumor relapse and may enhance the accuracy of staging. GCC is also introduced as the most sensitive and specific tumor marker for detection of metastatic CRC in extra-intestinal tissues[21, 37]. It can be used as a noninvasive tool for the micro metastasis diagnosis in clinical testing and as an indicator of therapeutic response. Therefore, GCC can be used as a suitable marker for the monitoring of circulating tumor cells in peripheral blood of CRC patients[38, 39]. Our data confirmed the previous studies and suggested copy number variation of GCC to improve monitoring methods of prognosis in CRC patients.

5. Conclusion

In conclusion, regarding our recent reports and present study, a panel of multiple specific markers such as Sall4, CEA, and GCC can be a more accurate method in comparison with the single markers for the identification of CTCs in CRC peripheral bloods. Moreover, there was a significant correlation between the GCC copy numbers and stage of tumors which introduced GCC as a selective non-invasive marker not only for the measuring of free tumor cells in the peripheral blood of CRC patients, but also for the molecular tumor staging.

Conflict of Interest

The authors declare that they have no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Mashhad University of Medical Sciences research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

References

1. Nugent M, Miller N, Kerin MJ. MicroRNAs in colorectal cancer: function, dysregulation and potential as novel biomarkers. Eur J Surg Oncol. 37(8):649-54.
2. Soreide K, Nedrebo BS, Knapp JC, *et al.* Evolving molecular classification by genomic and proteomic biomarkers in colorectal cancer: potential implications for the surgical oncologist. Surg Oncol. 2009;18 (1):31-50.
3. Boyle P, Langman JS. ABC of colorectal cancer: Epidemiology. Bmj. 2000;321(7264):805-8.
4. Abdifard E, Ghaderi S, Hosseini S, *et al.* Incidence trends of colorectal cancer in the West of Iran during 2000-2005. Asian Pac J Cancer Prev. 2000;14(3):1807-11.
5. Azadeh S, Moghimi-Dehkordi B, Fatem SR, *et al.* Colorectal cancer in Iran: an epidemiological study. Asian Pac J Cancer Prev. 2008;9(1):123-6.
6. Mousavi SM, Gouya MM, Ramazani R, *et al.* Cancer incidence and mortality in Iran. Ann Oncol. 2009;20(3):556-63.
7. Roya N, Abbas B. Colorectal cancer trends in Kerman province, the largest province in Iran, with forecasting until 2016. Asian Pac J Cancer Prev. 2016;14(2):791-3.
8. Compton CC, Greene FL. The staging of colorectal cancer: and beyond. CA Cancer J Clin. 2004;54 (6):295-308.
9. Cunningham D, Atkin W, Lenz HJ, *et al.* Colorectal cancer. Lancet. 375(9719):1030-47.
10. Bustin SA, Mueller R. Real-time reverse transcription PCR and the detection of occult disease in colorectal cancer. Mol Aspects Med. 2006;27(2-3):192-223.
11. Bustin SA, Dorudi S. Molecular assessment of tumour stage and disease recurrence using PCR-based assays. Mol Med Today. 1998;4(9):389-96.
12. Calaluce R, Miedema BW, Yesus YW. Micrometastasis in colorectal carcinoma: a review. J Surg Oncol. 1998;67 (3):194-202.
13. Baisse B, Bouzourene H, Saraga EP, *et al.* Intratumor genetic heterogeneity in advanced human colorectal adenocarcinoma. Int J Cancer. 2001;93(3):346-52.
14. Yamashita K, Watanabe M. Clinical significance of tumor markers and an emerging perspective on colorectal cancer. Cancer Sci. 2009;100(2):195-9.
15. Chevinsky AH. CEA in tumors of other than colorectal origin. Semin Surg Oncol. 1991;7(3):162-6.
16. Munjal DD, Picken J, Pritchard J. Combined measurement and significance of lipid-bound sialic acid and carcinoembryonic antigen in detection of human cancer. Diagn Immunol. 1984;2(1):36-43.
17. Okada M, Nishio W, Sakamoto T, *et al.* Prognostic significance of perioperative serum carcinoembryonic antigen in non-small cell lung cancer: analysis of 1,000 consecutive resections for clinical stage I disease. Ann Thorac Surg. 2004;78(1):216-21.
18. Bustin SA, Gyselman VG, Williams NS, *et al.* Detection of cytokeratins 19/20 and guanylyl cyclase C in peripheral blood of colorectal cancer patients. Br J Cancer. 1999;79(11-12):1813-20.
19. Carrithers SL, Barber MT, Biswas S, *et al.* Guanylyl cyclase C is a selective marker for metastatic colorectal tumors in human extraintestinal tissues. Proc Natl Acad Sci USA. 1996;93(25):14827-32.
20. Vlems FA, Diepstra JH, Cornelissen IM, *et al.* Investigations for a multi-marker RT-PCR to improve sensitivity of disseminated tumor cell detection. Anticancer Res. 2003;23(1A):179-86.
21. Mejia A, Waldmana SA. Previstage GCC test for staging patients with colorectal cancer. Expert Rev Mol Diagn. 2008;8(5):571-8.
22. Frick GS, Pitari GM, Weinberg DS, *et al.* Guanylyl cyclase C: a molecular marker for staging and postoperative surveillance of patients with colorectal cancer. Expert Rev Mol Diagn. 2005;5(5):701-13.
23. Waldman SA, Cagir B, Rakinic J, *et al.* Use of guanylyl cyclase C for detecting micrometastases in lymph nodes of patients with colon cancer. Dis Colon Rectum. 1998;41(3):310-5.
24. Ohlsson L, Hammarstrom ML, Israelsson A, *et al.* Biomarker selection for detection of occult tumour cells in lymph nodes of colorectal

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cancer patients using real-time quantitative RT-PCR. Br J Cancer. 2006;95 (2):218-25.
25. Edge SB, Compton CC. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. Ann Surg Oncol. 17(6):1471-4.
26. Ardalan Khales S, Abbaszadegan MR, Abdollahi A, *et al.* SALL4 as a new biomarker for early colorectal cancers. J Cancer Res Clin Oncol. 141(2):229-35.
27. Raeisossadati R, Farshchian M, Ganji A, *et al.* Quantitative analysis of TEM-8 and CEA tumor markers indicating free tumor cells in the peripheral blood of colorectal cancer patients. Int J Colorectal Dis. 26(10):1265-70.
28. Forghanifard MM, Moghbeli M, Raeisossadati R, *et al.* Role of SALL4 in the progression and metastasis of colorectal cancer. J Biomed Sci. 20:6.
29. Raeisossadati R, Abbaszadegan MR, Moghbeli M, *et al.* Aberrant expression of DPPA2 and HIWI genes in colorectal cancer and their impacts on poor prognosis. Tumour Biol. 35(6):5299-305.
30. Siegel R, Naishadham D, Jemal A. Cancer statistics. CA Cancer J Clin. 2012;62(1):10-29.
31. Newton KF, Newman W, Hill J. Review of biomarkers in colorectal cancer. Colorectal Dis. 14(1):3-17.
32. Lurje G, Schiesser M, Claudius A, *et al.* Circulating tumor cells in gastrointestinal malignancies: current techniques and clinical implications. J Oncol. 2010:392652.
33. Silva JM, Rodriguez R, Garcia JM, *et al*. Detection of epithelial tumour RNA in the plasma of colon cancer patients is associated with advanced stages and circulating tumour cells. Gut. 2002;50(4):530-4.
34. Shen C, Hu L, Xia L, *et al*. Quantitative real-time RT-PCR detection for survivin, CK20 and CEA in peripheral blood of colorectal cancer patients. Jpn J Clin Oncol. 2008;38(11):770-6.
35. Carrithers SL, Parkinson SJ, Goldstein SD, *et al*. Escherichia coli heat-stable enterotoxin receptors. A novel marker for colorectal tumors. Dis Colon Rectum. 1996;39(2):171-81.
36. Schulz S, Hyslop T, Haaf J, *et al*. A validated quantitative assay to detect occult micrometastases by reverse transcriptase-polymerase chain reaction of guanylyl cyclase C in patients with colorectal cancer. Clin Cancer Res. 2006;12(15):4545-52.
37. Waldman SA, Hyslop T, Schulz S, *et al*. Association of GUCY2C expression in lymph nodes with time to recurrence and disease-free survival in pN0 colorectal cancer. Jama. 2009;301(7):745-52.
38. Camci C, Sahin A, Sevinc A, *et al.* Peripheral blood guanylyl cyclase c (GCC) expressions are associated with prognostic parameters and response to therapy in colorectal cancer patients. Tumour Biol. 32(6):1265-70.
39. Liu Y, Qian J, Feng JG, *et al.* Detection of circulating tumor cells in peripheral blood of colorectal cancer patients without distant organ metastases. Cell Oncol (Dordr). 36(1):43-53.

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