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Original research article

Correlation of serum exosomal miRNA-1290 levels with stage of disease in colorectal cancer patients

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Abstract

miRNAs are a class of small non-coding, naturally occurring RNA molecule 19-25 nucleotides in length They were first discovered in 1993 by Ambrose *et al*. when they discovered a small RNA which exerted regulatory functions on a specific mRNA resulting in suppression of its action. This small RNA was subsequently discovered to be a member of an abundant family of tiny regulatory RNAs called miRNAs. Blood samples were collected from 30 colorectal cancer patients admitted at department of surgical gastroenterology. One sample from each collected during preoperative period another sample on post-operative day 7.All the blood samples were collected in EDTA coated blood collection tubes. Plasma Expression values of miRNA-1290 between stage 1 & 2 vs 3&4 among pre-operative blood samples of patients showed no statistically significant relation in our Indian patients. This suggests that in our Indian patient's plasma miRNA-1290 levels will not correlate with stage of disease.

Keywords: Serum exosomal miRNA-1290, stage of disease, colorectal cancer

Introduction

Colorectal cancer is one of the major causes of cancer related deaths worldwide in both men and women [1]. It is the fourth most common cancer in the world and in India, it is the fifth most common cancer [1]. Primary diagnosis of colorectal cancer is based on clinical findings,colonoscopic examination and confimed by histopathological examination. Flexible colonoscopy and biopsy is the gold standard method for diagnosis.The TNM staging system is the most widely used and recommended system for colorectal cancer staging [2]. TNM stage is based on the extent of the disease at diagnosis, which provides an important estimation of prognosis in colorectal cancer.Carcino ebryonic antigen(CEA)in serum is the most widely used biomarker in Colorectal cancer. Elevated levels at diagnosis are associated with increased tumor stage and poor prognosis [3] but the test is compromised by low sensitivity and specificity and high rates of false positive. CA (carcinoma antigen) 19-9 tumor marker is often measured in addition to CEA. Elevated levels are associated with advanced-stage disease and adverse prognosis, but the test is limited by low specificity and sensitivity and its clinical usefulness is not clear [4, 5]. Therefore, there is a need for the development of colorectal cancer-specific diagnostic and prognostic markers which are non-invasive, highly sensitive and specific. Currently circulating tumour markers such as circulating tumour cells, tumour DNA, miRNA and long RNAs being investigated.

miRNAs are a class of small non-coding, naturally occurring RNA molecule 19-25 nucleotides in length [6]. They were first discovered in 1993 by Ambrose *et al.* [7] when they discovered a small RNA which exerted regulatory functions on a specific mRNA resulting in suppression of its action. This small RNA was subsequently discovered to be a member of an abundant family of tiny regulatory RNAs called miRNAs. The importance of miRNAs as regulatory molecules has become increasingly obvious as more miRNAs are discovered and their regulatory targets are elucidated. Functional studies have shown miRNAs to participate in almost every cellular process including apoptosis, proliferation and differentiation [8]. In fact, single miRNAs may regulate multiple target genes acting as a master control of gene expression [9]. Although miRNAs constitute only 1-3% of the human genome, it is suggested that they regulate up to 30% of human genes miRNAs regulate gene expression by inhibiting or inactivating target messenger RNAs (mRNAs) [10, 11]. Several miRNAs showing high levels of expression in cancer tissues have been reported as suitable diagnostic or prognostic markers [12].

Exosomes are vesicles 40-100nm in size, containing characteristic tetraspanin proteins originating from endosomes, and released by multivesicular endosome fusion with the plasma membrane [13]. Exosomal miRNA signatures appear to mirror pathological changes of colorectal cancer patients [14]. Recent studies demonstrated that miRNAs are secreted from various cells, including cancer cells, into body fluids such as blood, urine, breast milk, and saliva, via exosomes. Exosomes embed protein, lipids, mRNAs, and

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miRNAs, depending on the origin of the secreting cells. Therefore, exosomal miRNAs in body fluids may be useful diagnostic biomarkers for the detection of cancer. Such studies are essential because increased expression of circulating miRNAs would be indicative of miRNAs secreted from tumor tissue, raising the overall diagnostic specificity and usefullness for clinicopathological correlation of the biomarker. However, currently there are only few studies regarding the relationship between exosomal miRNA profiles in blood and the pathological condition of cancer patients.

Methodology

Inclusion criteria

- Patients diagnosed with colon or rectum cancer attending department of surgical gastroenterology were included.
- Patients more than 12 years less than 75 years were included.

Exclusion criteria

- Patients having any other synchronous malignancy.
- Patients less than 12 years and more than 75 years old.

Study design: prospective case control study

Sample size: To detect correlation of 0.5(assumed) between serum exosomal miRNA-1290 levels and staging the required sample size is 30 per group with 80% power and 0.5 as type 1 error. MediCal C software was used.
The sample size was estimated using the following formula

$N = [(Z\alpha + Z\beta)/ C]/ + 3,$

$Z\alpha$ factor corresponding to type 1 error and is assumed as 1.96 for two tail test $Z\beta$ is factor corresponding to type 2 error and was assumed as 80% power and the value of it is 0.842
The correlation coefficient was 0.5
By substituting the values in the above formula
C = 0.5X LN [(1.05) / (1 -0.5)] + 3
= 0.5 X LN (3) + 3
= 0.5 X 1.0986 =0.5493
= [(1.96 +0.842)/0.5493] X [(1.96 + 0.842)/0.5493]+3
= (5.1010 X 5.1010) + 3=26 + 3= 29 rounded of 30 cases

Sample collection

Blood samples were collected from 30 colorectal cancer patients admitted at department of surgical gastroenterology. One sample from each collected during preoperative period another sample on post-operative day 7. All the blood samples were collected in EDTA coated blood collection tubes.
Blood samples were also collected from 10 matched healthy controls who are staff of institute of gastroenterology. They were centrifuged at 1500 rpm for 15 min. for plasma separation. Plasma was stored at -80 °C for long time preservation.
To validate that miRNA-1290 arising from colorectal cancer cells 10 sub samples colorectal cancer tissue and normal tissue were also analyzed for miRNA-1290 expression.

Results

Plasma miRNA-1290 expression: miRNA-1290 expression profile in our patients in pre-operative blood samples showed following patteren, two fold change in miRNA-1290 compared to healthy controls was considered as significant.in our study 6(20%) patients showed up regulation (possitive 2 fold change), 8 (26.6) patients down regulation, (negative 2 fold change), remaining 16 (53.3%) patients showed no significnant change, showing different expression profile in our Indian patients compared with other parts of world.

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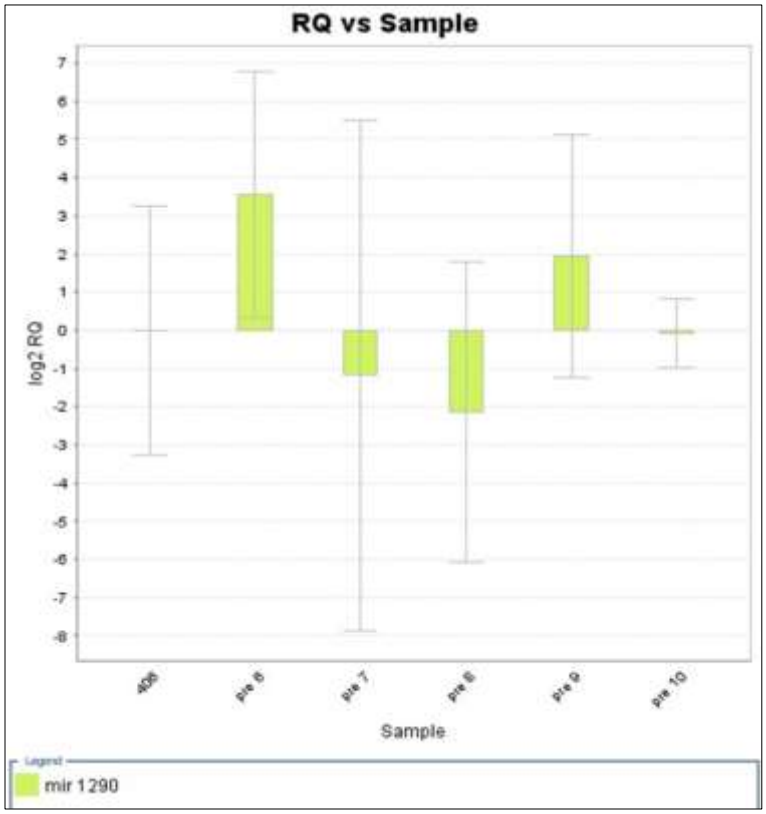
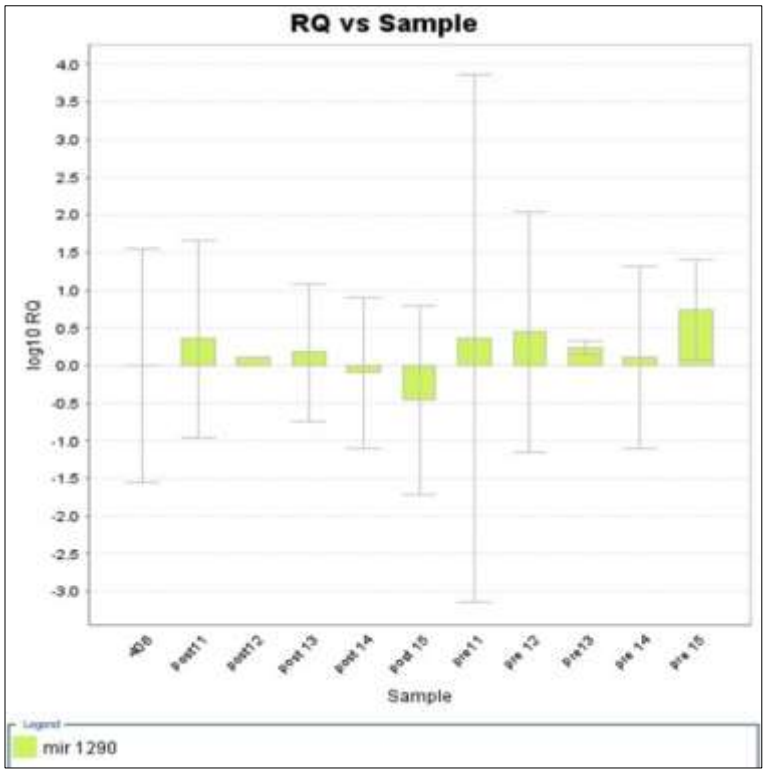


Graph 1: Representative picture of plasma miRNA-1290 in pre-operative samples



Graph 2: Representative picture of plasma miRNA-1290 in pre and postoperative samples

Tissue miRNA-1290 expression: We collected 10 tissue samples of colorectal cancer tissues and normal tissues among them good quality RNA was isolated in 4. Out of 4 colorectal cancer tissues 2 showed miRNA-1290 upregulation another two showed downregulation, which is consistent with our plasma exosomal miRNA-1290 expression cohort showing varying expression.

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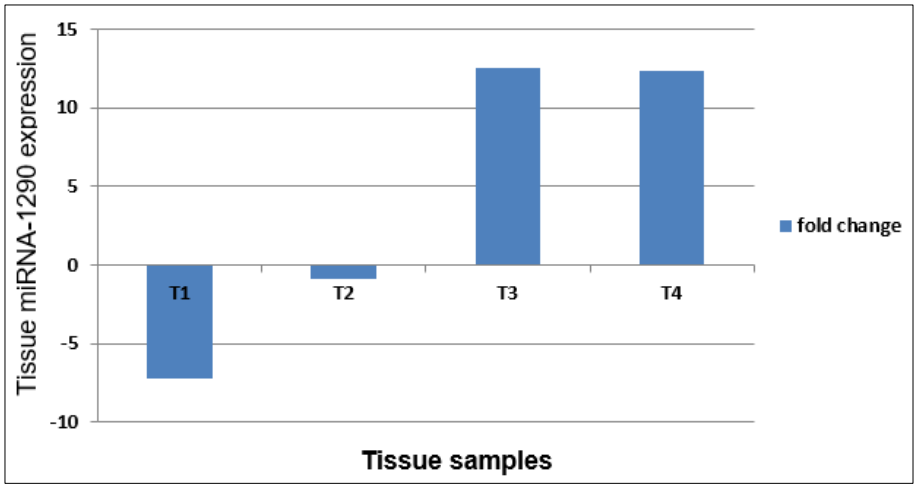
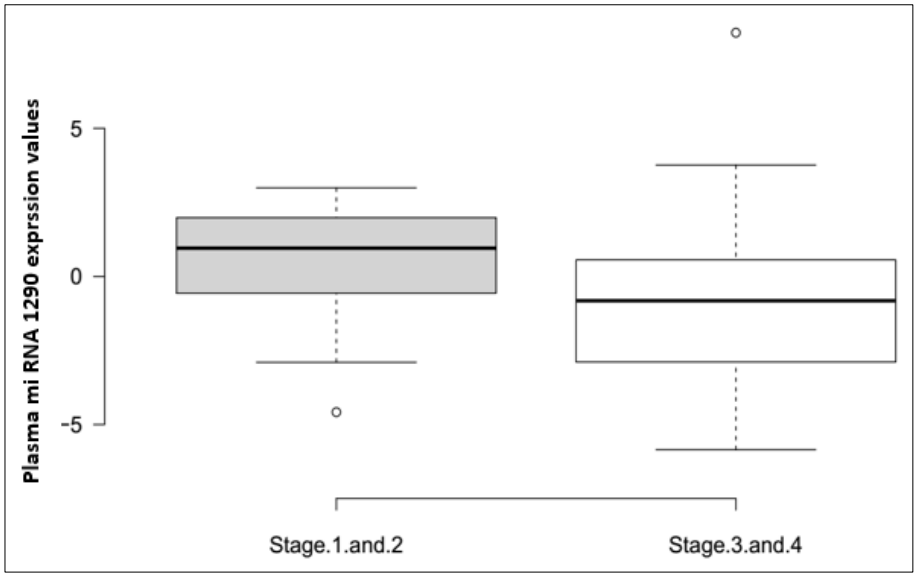


Graph 3: Representative picture of tumour tissue miRNA-1290 expression

Stage wise correlation of plasma exosomal miRNA-1290 expression: plasma Expression values of miRNA-1290 between stage 1 & 2 vs 3&4 among pre-operative blood samples of patients showed no statistically significant relation in our Indian patients. This suggests that in our Indian patient's plasma miRNA-1290 levels will not correlate with stage of disease.

Table 1: Association between Plasma Expression levels of miRNA-1290 and stage

Mi RNA 1290 expression values	Stage (I+II)	Stage (III+IV)	p
n	12	18	0.264
Medain	0.95	-2.91	
IQR	-0.56 -2.24	-2.91 – 0.68	



Graph 4: Association between Plasma Expression levels of miRNA-1290 and stage of Disease P=0.264

Post-operative change in plasma exosomal miRNA-1290 levels: In our study we studied change in plasma expression value of miRNA-1290 on post-operative day 7 (POD 7) sample, 19 patients showed upregulation, 8 patients showed down regulation remaining 3 no significant change, Two patients who underwent non curative resection showed up regulation (100%) compared to who underwent curative resection (60%).

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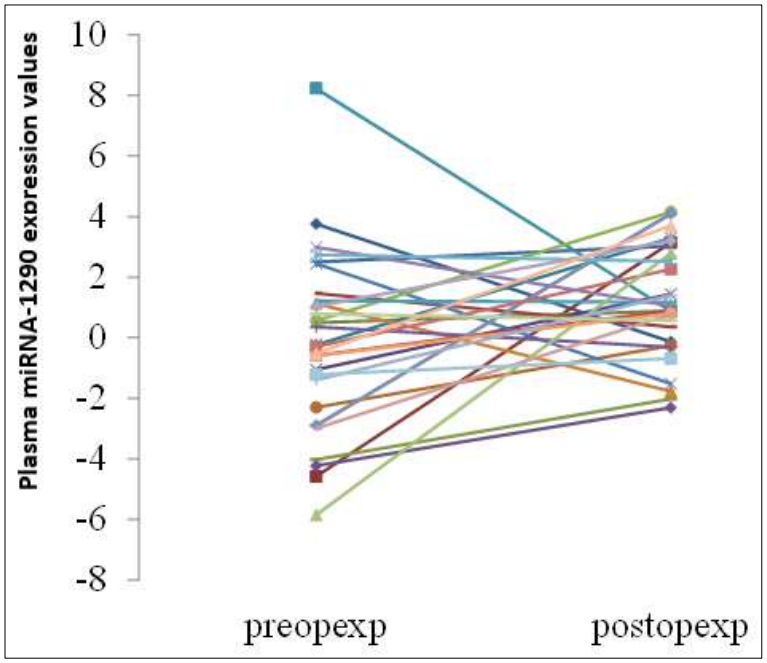


Graph 5: Post-operative change in miRNA-1290 Levels in curative resection patients

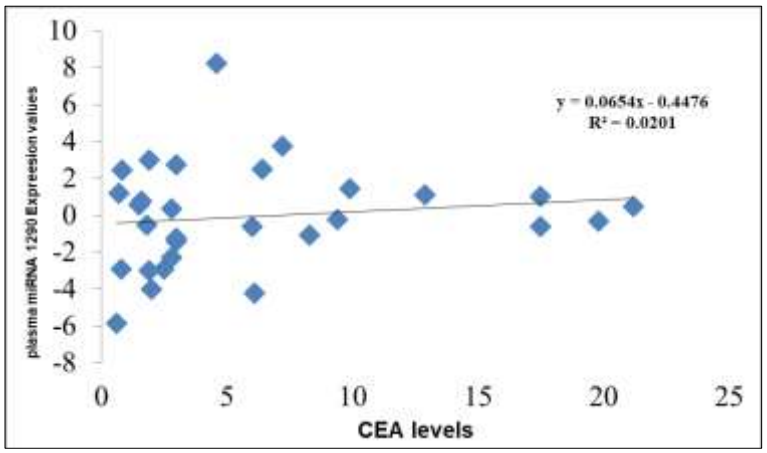
Preoperative CEA levels: In present study considering CEA<4ng/ml as normal (our laboratory value).46.7%patients had elevated levels and 53.3% had normal levels.

Table 2: Distribution of patients by preoperative CEA levels

CEA levels	no of patients	Percentage of patients
>4ng/ml	16	46.67%
<4ng/ml	15	53.33%

Correlation between CEA and plasma exosomal miRNA-1290 levels

We studied relation between plasma exosomal expression level of mi RNA 1290 and CEA levels in our patients there was no direct correlation.



Graph 6: Association between CEA and plasma exosomal miRNA-1290 levels

Lymph node metastasis: In present study 70% patients had regional lymph node metastasis. There was no statistically significant (p=0.247) correlation between plasma exosomal levels of miRNA-1290 and lymph node positivity on histological examination in our Indian patients.

Table 3: Correlation between plasma miRNA-1290 levels and lymph node metastasis

miRNA-1290 expression values	Lymph node metastasis		
	Yes	No	p
n	21	9	0.247
Median	0.56	0.78	
IQR	-2.74-1.36	-1.7-1.97	

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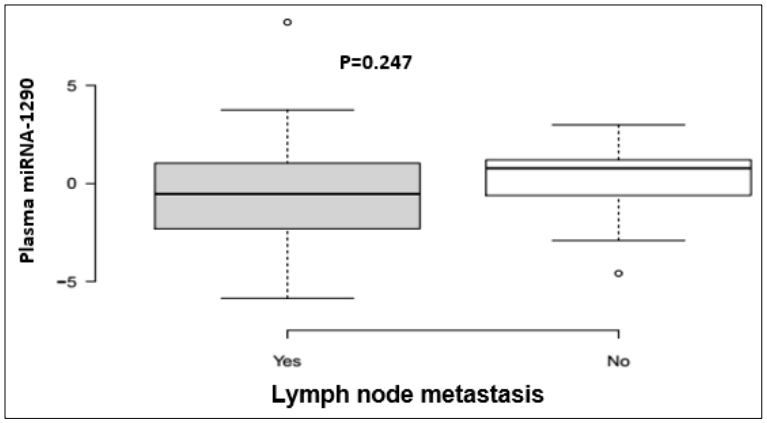

Graph 7: Correlation between plasma miRNA-1290 levels and lymph node metastasis

Tumor size: we studied relation between plasma expression of exosomal miRNA-1290 levels and tumour size observed that there is no significant correlation between plasma expression of miRNA-1290 levels and tumour size among our Indian patients.

Table 4: Correlation between plasma miRNA-1290 levels and Tumour size

Plasma miRNA-1290 expression values	Tumour size(max DM in cm)		p
	<6	>6	
n	22	8	
Medain	0.03	-0.64	1.000
IQR	-1.16 - 1.71	-3.74 - 0.92	

Vascular invasion: In this study relation between plasma expression of exosomal miRNA-1290 levels and vascular invasion showed no significant correlation.

Table 5: Correlation between plasma miRNA-1290 levels and Tumour size

Plasma miRNA-1290 expression values	Vascular invasion		P
	Yes	No	
n	23	7	P =0.080
median	0.59	1.13	
IQR	-2.9 -1.04	0.50 -2.50	

Discussion

Plasma miRNA-1290 expression levels in our Indian colorectal cancer patients showed that positive 2 fold ie significant up regulation was seen in 20% patients only compared to 100%, in study by H. Imoaka *et al.* [15] among Japanese patients this can be explained by observation that most Indian colorectal cancer patients do not follow typical adenoma-carcinoma sequence, it is being observed in only 15 to 20%. Genetic alterations involved in Indian colorectal cancer patients differ from other parts of world.

In study by H. Imoaka *et al.* [15] they have shown statistically significant correlation between plasma levels miRNA-1290 and stage of disease and also shown down regulation MiRNA-1290 levels in post-operative day 7 samples in patients who have undergone curative resection, however similar results were not observed in our study both patients who underwent non curative resection showed up regulation of plasma miRNA-1290 on post-operative day 7 and among patients who underwent curative resection post-operative down regulation of miRNA-1290 was observed in 21%.

CEA was elevated in 46.6% of our patients it is comparable to study by Wang JY *et al.* [16].

In present study 77.3% had tumor less than 6cm, 26.6% had tumour more than 6cm.Relation between plasma expression of exosomal miRNA-1290 levels and tumour size showed that there is no statistically significant correlation between plasma expression of miRNA-1290 levels and tumour size among our Indian patients. H. Imoaka *et al.* reported statistically significant correlation between serum miRNA-1290l evels and tumour size in Japanese patients.

Lymph node metastasis was present in 70% patients of study group.there was no statistically significant correlation between plasma exosomal miRNA-1290 leves and lymph node metastasis in our study group. H. Imoaka *et al.* reported statistically significant correlation between serum miRNA-1290levels and lymph node metastasis.

Vascular invasion by tumour was present 76.6% patients. There was no statistically significant correlation between plasma exosomal miRNA-1290 levels and vascular invasion in present study group. H. Imoaka *et al.* reported significant correlation between serum miRNA-1290 levels and vascular invasion by rumour.

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Recent Meta analysis by Song Gao *et al.* reported that In general, blood miR141 and tissue miR21, miR181a, miR224, and miR126 have significant prognostic value. Among these, blood miR141 and tissue miR224 are strong biomarkers of prognosis in CRC.

In our study we were proposed to do univariate and multivariate analysis as there was no correlation between plasma exosomal of mi RNA1290 expression levels and stage of disease, vascular invasion and lymph node matastasis we couldn't do it. Plasma miRNA levels didn't show significant correlation with stage of disease so we have not compared it with CEA levels for staging of disease.

Among 28 patients who underwent curative resection none had recurrence during follow up ranging from 3 to 7 months.

In literature Limit the clinical application of circulating miRNAs is also reflected in the inconsistent results. Tracing it to the cause of these conflicting conclusions of circulating miRNAs in diagnosis, early screening, TNM stage and prognosis, may be attributed to the following factors: (1) genetic variations among different region, ethnic groups and different environmental and dietary factors, (2) difference of sample collection procedures or processing conditions, the source choice of plasma, serum or whole blood (3) uniform inclusion and exclusion criteria of subjects (i.e., early or late stages of cancers) to the same research purpose (diagnosis or TNM stage), (4) usage of pooled samples, (5) different miRNA expression levels between tissue and plasma, (6) sample size, screening method. Another limitation with the use of circulating miRNAs as biomarkers is that they are not unique for CRC, but act as broad spectrum biomarker for many other cancers or non-oncological diseases, such as lung, gastric, ovarian, pancreatic cancers and ulcerative colitis. Therefore, an effective and special diagnostic method should be considered for the clinical application target CRC.

There are few limitations to present study, first in this study we have not done serum micro array analysis for global miRNA expression profiling in Indian colorectal cancer patients, second this study was conducted in small number of patients.

Conclusion

Present study shows that Plasma exosomal miRNA-1290 upregulated in 20% of Indian colorectal cancer patients. Plasma exosomal miRNA-1290 levels didn't show significant fall after curative resection on post-operative day 7 limiting its use for assessing prognosis. There is no statistically significant correlation between Plasma exosomal miRNA-1290 levels and tumour size, vascular invasion, lymph node metastasis. Its necessary to do micro array analysis for global miRNA expression profiling in Indian colorectal cancer patients, which will help us choosing proper miRNAs for early diagnosis and prognosis in Indian colorectal cancer patients.

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