Associations between Markers of Colorectal Cancer Stem Cells, Mutations, Mirna, and Clinical Characteristics of Ulcerative Colitis

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Abstract

Background: Several factors have been implicated in the pathogenesis of colorectal cancer (CRC) associated with ulcerative colitis (UC). We investigated markers of cancer cell pluripotency, including CD44 and CD166, microRNA-21 (miR-21) and microRNA-215 (miR-215), and APC, K-ras and DCC mutations in biopsy specimens from patients with UC to evaluate any correlations with clinical risk factors.

Methods: We observed 18 patients with UC and collected two biopsy specimens from each patient at diagnosis and at a follow-up endpoint. We examined the expression of CD44, CD166, miR-21 and miR-215 as well as APCK-ras and DCC mutations. We compared these markers from the two points in time and assessed their associations with clinical characteristics, including colitis duration, histological alterations, and patient age at UC onset.

Results: Most patients (16/18) had attenuated colonic lesions or remained stable during follow-up, except one patient who developed dysplasia, and one who had severe lesion aggravation during follow-up. Enhanced expression of CD44, CD166 and miR-21 with miR-215 was found in the specimens obtained at follow-up, despite attenuation of mucosal lesions. Coherence of cancer stem cell markers and microRNAs was seen in patients who had significant aggravation of mucosal alteration, dysplasia, and long duration of colitis. APC mutation occurred in only one patient, who had the longest duration of UC (23 years).

Conclusion: Enhanced markers of CRC in the follow-up colon samples support our conclusion that UC duration plays the most important role in UC-related carcinogenesis.

Introduction

Ulcerative colitis is thought to greatly increase the risk of colorectal cancer (CRC). A predisposition to CRC has been attributed to a long-standing inflammatory environment and the immune mediators associated with ulcerative colitis (UC) [1,2]. Clinical factors such as a family history of CRC, early onset and duration of UC more than 10 years, dysplasia in the colon, co-existing primary sclerosing cholangitis, and disease extent are thought to independently increase the relative risk of neoplasia related to inflammatory bowel disease (IBD) [1,3-8]. Although thought to have potential as a surrogate marker for the early detection of colitis-associated cancer [9,10], colonic cell dysplasia is rare in individuals with colitis and often has a normal-looking appearance. Therefore, studies are needed to identify biomarker(s) for colitis-associated carcinogenesis that correlate with clinical characteristics of IBD.

This area of research has implicated cancer stem cells (CSCs), microRNA (miRNA) and genetic mutations as markers of CRC development. Studies have identified CD44 as a specific CSC marker and have also found several CD44 splice variants, some of which correlated well with colon cancer progression [12-14]. Our previous study reported increased expression of CD166 with CD44 to be associated with colon carcinogenesis. Recent advances in molecular biology have also revealed the potential role of miRNA in tumor initiation, progression and metastasis [15]. Our recent research [16] suggested that miR-21 plays an important role in regulating pluripotency by modulating TGFbR2 signaling in colon cancer cells. Genetic alterations, including mutations in APC, K-ras, and DCC genes, are thought to be the most important factors facilitating sporadic colorectal carcinogenesis.

The present investigation was undertaken to examine changes in CSCs miRNAs and mutations of APC, K-ras, and DCC genes in patients with UC and to determine whether a relationship exsits between any of these markers and clinical characteristics of colitis-associated carcinogenesis. From 18 patients with UC under our observation, we collected colon specimens at two points in time: at diagnosis or before starting medical treatments, and later during follow-up. For each colon specimen, we evaluated histological changes

together with changes in miR-21, miR-215 and CSC markers CD44 and CD166, and also mutations of APC, K-ras and DCC genes.

Method

Patients and tissue samples

The study protocol was approved by the Institutional Review Board of the Veterans Administration Medical Center in affiliation with Wayne State University in Detroit, Michigan, U.S.A., and the Ethics Committee of the Shanghai Ninth People's Hospital in Shanghai, the People's Republic of China. We included in the study 18 patients with UC who were consecutively enrolled at either the Veterans Administration Medical Center or at the Shanghai Ninth People's Hospital. The clinical diagnosis of UC was confirmed by routine radiological, endoscopic, and histological criteria. There were 8 women and 10 men in the study sample, with a mean age at disease onset of 37.22 ± 9.14 years (range, 22–54 years; median, 38). The mean duration of UC was 11.22 ± 7.51 years (range, 1–23 years; median, 10.5). None of the patients had a family history of CRC or familial adenomatous polyposis. All patients underwent colonoscopy at least 2 times during the follow-up, and colon specimens were taken from inflamed and non-inflamed mucosa of each patient. The general characteristics of the patients are shown in Table 1.

Endoscopic examination and histological assessment of disease activity

Sufficient biopsy specimens were taken from the lesions. All the slides were stained with haematoxylin-eosin and histologically assessed by two assigned pathologists who were unaware of the clinical data.

The assessment of the level of clinical disease activity in the colon was determined according to previously described criteria for UC [17,18].

Immunofluorescence double staining for CD44 and CD166 with p-EGFR

Most tumors express multiple epidermal growth factor receptors (EGFRs). Activated EGFR (phosphorylated EGFR [p-EGFR]), which plays important roles in various cellular processes, such as cell proliferation, differentiation, adhesion, migration and apoptosis [19, 20], shows high expression in colon carcinogenesis. Therefore, we detected double staining of CD44 and CD166 with p-EGFR, respectively, to assess the coherence of CSC biomarkers with p-EGFR. Paraffin-embedded tissues were cut into 5-μm sections, and subsequently analyzed for the expression of CD44 and CD166 with p-EGFR. The primary antibodies used were anti-mouse CD44 (Cell Signaling Technology, USA) and anti-mouse CD166 (Santa Cruz Biotechnology, USA). The slides were first deparaffinized and hydrated, and then steamed in target retrieval solution (Dako) for 30 min. After cooling to room temperature (RT), endogenous peroxidase activity was blocked with 3% hydrogen peroxide at RT for 15 min, followed by incubation in blocking serum for 30 min (Vectastain, USA) to block nonspecific endogenous proteinases. The slides were then incubated with primary antibody overnight at 4°C in a humidified chamber and then treated with secondary antibody TRITC (anti-mouse, Sigma) for 1.5 hr at RT. Next, the anti-human-specific p-EGFR (Abcam Inc., USA) antibodies were used for 2 hr at RT. The slides were incubated with FITC (anti-rabbit, Sigma) for another 1.5 hr at RT. Finally, they were rinsed, mounted and prepared for observation under a fluorescence microscope. Slides treated with phosphate buffered saline instead of primary antibody served as the negative control.

Characteristic	Cases	%
Gender		
Male	10	55.56
Female	8	44.44
Age at onset(yrs)		
Median	38	
Range	22-54	
Smoking		
Ever	4	22.22
Never	14	77.78
Drinking		
Ever	3	16.67
Never	15	83.33
PSC		
Duration of disease (yrs)		
Median	10.5	
Range	1-23	

Extension			
Proctosigmoiditis		4	22.22
Left colitis		10	55.56
Pancolitis		4	22.22
Family history of CRC		No*	
UC activity level			
At diagnosis	Low	4	22.22
	Low-moderate	4	22.22
	Moderate	6	33.34
	Severe	4	22.22
At follow-up	Low	11	61.11
	Low-moderate	1	5.56
	Moderate	2	11.11
	Moderate-severe	3	16.67
	Severe	1	5.56
Dysplasia	1 at follow-up point		
Medication			
Regular		16	88.89
Irregular or never		2	11.11

Table 1: General characteristics of patients with ulcerative colitis. *2 patients could not provide information on family history of CRC. PSC, primary sclerosing cholangitis.

Gene	Sense primer(5'-3')	Anti-sense primer(5'-3')
CD44	5'-ACTGCAATGCAAACTGCAAG-3'	5'-AAGGTGGAGCAAACACAACC-3'
CD166	5'-CGCAGACATAGTTTCCAGCA-3'	5'-TAGCAGGAATGCAACTGTGG-3'
β-actin	5'-ACATCTGCTGGAAGGTGGAC-3'	5'-CCCAGCACAATGAAGATCAA-3'
miR-21	5'-TGAGACTGATGTTGACTGTTGAA-3'	5'-TGTCAGACAGCCCATCGAC-3'
miR-215	5'-ATCATTCAGAAATGGTATACAG-3'	5'-TTGAAGTAGCACAGTCATACAG-3'
Rnu6B	5'-GTGCTCGCTTCGGCAGCACATA-3'	5'-CACGAATTTGCGTGTCATCCTTGCG-3'

Table 2: Primers for PCR.

RNA isolation, reverse transcription polymerase chain reaction (RT-PCR), and quantitative real-time PCR

RNA was isolated from the formalin-fixed paraffin-embedded (FFPE) tissue samples using the corresponding commercial kit (Qiagen, USA). Isolated total RNA was subjected to reverse transcription-PCR (RT-PCR) following the manufacturer's protocol. PCR was performed using the appropriate primers purchased from Invitrogen (USA). The primers we used are listed in Table 2, with β-actin as the internal reference for message RNA, and Rnu6B as the internal reference for microRNAs. Quantitative real-time PCR was performed using the SYBR Green Master Mix Kit (Applied Biosystems, USA) in an Applied Biosystems 7500 Real-Time PCR System. The conditions for running real-time PCR were as follows: DNA polymerase activation required a hot start for 10 min at 95°C, and then the reaction mixture was cycled at 95°C for 15 s and at 60°C for 1 min, for a total of 40 cycles. The threshold cycles (CTs) were recorded for all samples for both the target gene and the β-actin control. Melt curve analysis was done for each run. Relative gene expression of the target gene was calculated as ΔCT, and this was determined by subtracting the CT of the reference gene from the CT of the target gene.

Genomic DNA extraction

Genomic DNA was extracted from the FFPE tissue samples by using DNAzol Reagent (Invitrogen, USA), and re-suspended in 8Mm NaOH.

After adjusting the pH and detecting the concentration and purification of DNA at 260nm and 280nm, we stored the DNA at -20°C for further use.

Analysis of molecular alteration through PCR

Mutations in the APC, K-ras and DCC genes were identified through agarose gel of PCR products. 100ng of genomic DNA served as a template in a reaction volume of 20µl containing 10µl AmpliTaq Gold® PCR Master Mix (Applied Biosystems, USA) Table 3. PCR reactions containing sample DNA were processed at 95°C for 10 min and then through 40 cycles at 95°C for 30s, annealing for 30s at 50°C (for APC Mut2), 55°C (for APC Mut1, APC Con and K-ras), 59°C (β-actin), or 61°C (for DCC), followed by 12 min of extension at 72°C. Mutations in the APC gene were identified by checking the PCR product on 2.5% agarose gel [21]. HCT-116 cells were used as the positive control.

Name	Sequence	Product size (bp)	TM (°C)
APC Con F	5'-GTGAACCATGCAGTGGAATG-3'	119	50
APC Con R	5'-AGCTGTTTGAGGAGGTGGTG-3'		
APCMut1 F	5'-CTGCAGGGTTCTAGTTTATC-3	174	55
APCMut1 R	5'-ATCAAGTGAACTGACAGAAG-3'		
APCMut2 F	5'-GACCCCACTCATGTTTAGC-3'	179	50
APCMut2 R	5'-TTACTTCTGCTTGGTGGCAT-3'		
K-ras F	5' ACTGAATATAAACTTGTGGTAGTTGGACCT 3'	135	55
K-ras R	5' TAATATGTCGACTAAAACAAGATTTACCTC 3'		
DCC F	5' CCCAGACTAACTGCATCATCATGAAG 3'	135	61
DCC R	5' CCTCTCAATGGAATAATATCGCTGC 3'		
β-actin F	5'-CCCAGCACAATGAAGATCAA-3'	103	59
β-actin R	5'-ACATCTGCTGGAAGGTGGAC-3'		

Table 3: PCR Pairs for gene detection, size of the respective amplicons and annealing temperatures (TM). F: Forward primer; R: Reverse primer; Con: conserved sequence; Mut: mutation.

Statistics

The experimental data are presented as the mean value ± standard derivation. The SPSS software package was used for statistical analysis. Where applicable, the results were compared by Wilcoxon signed rank sum test. Values of $p < 0.05$ are considered statistically significant. The relationships between expression of detectors and duration of disease, age at onset of disease, and extent of colitis were analyzed by Pearson's and partial correlation analyses.

Results

Assessment of clinical activity and histological analysis

According to previously described criteria for analyzing the clinical activity of UC, 4 patients had low disease activity, 4 had low to moderate disease activity, 6 had moderate disease activity, and 4 had severe disease. Four had proctosigmoiditis, 10 had left side colitis, and 4 had pancolitis. Most of the patients did not smoke or ingest any alcohol (77.78% and 83.33%, respectively). Sixteen patients with UC (16/18, 88.89%) were receiving regular medication when the biopsies were collected or during the entire follow-up. Histopathologic results were confirmed in all biopsy samples collected from patients with UC. Most of the patient samples (15/18) showed alleviation of mucosal lesions following treatment. In only one patient did the UC progress to dysplasia (after 6 years). One patient had severe aggravation of UC after 1 year of follow-up. Table 1 shows the clinical characteristics of the patients with UC.

Immunofluorescence and co-expression of biomarkers

Positive staining was seen for CD166 and CD44. The expression frequency of CD166 in membranous staining was significantly higher in the follow-up samples compared to the samples obtained at diagnosis, with p=0.003. The same trend was seen in membranous staining of CD44. In addition, we observed high co-expression of CD44 with p-EGFR and CD166 with p-EGFR. Figure 1 shows the typical expressions observed in this analysis.
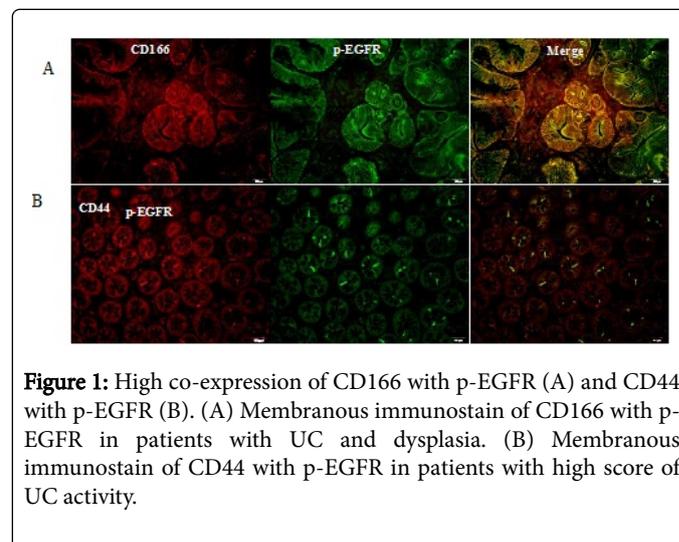

Figure 1: High co-expression of CD166 with p-EGFR (A) and CD44 with p-EGFR (B). (A) Membranous immunostain of CD166 with p-EGFR in patients with UC and dysplasia. (B) Membranous immunostain of CD44 with p-EGFR in patients with high score of UC activity.

Relative expression of CD44, CD166, miR-21 and miR-215 in real-time PCR

Most of the patients showed higher expression of CD44 (55.6%, 10/18), CD166 (77.8%, 14/18), miR-21 (66.7%, 12/18) and miR-215 (88.9%, 16/18) in the follow-up samples than in those obtained at diagnosis or before medication was administered. For 6 patients (numbered as 1, 6, 9, 15, 17, and 18 in Figure 2), we found all markers to show increased expression at the follow-up endpoint. These 6 patients also showed moderate–severe and severe disease activity: 4 patients had progressive disease (numbered 1, 15, 17, and 18), one patient had dysplasia (number 6), and one patient had the longest duration of disease (number 9). For another patient (number 3), we

found all markers to show decreased expression in conjunction with a great level of alleviation of the mucosal lesion. The overall differences in all biomarkers when comparing the samples obtained at diagnosis and at follow-up were significant. The results of our study agree with those reported by other researchers [1,2,8]. Figure 2 shows the details of these markers.
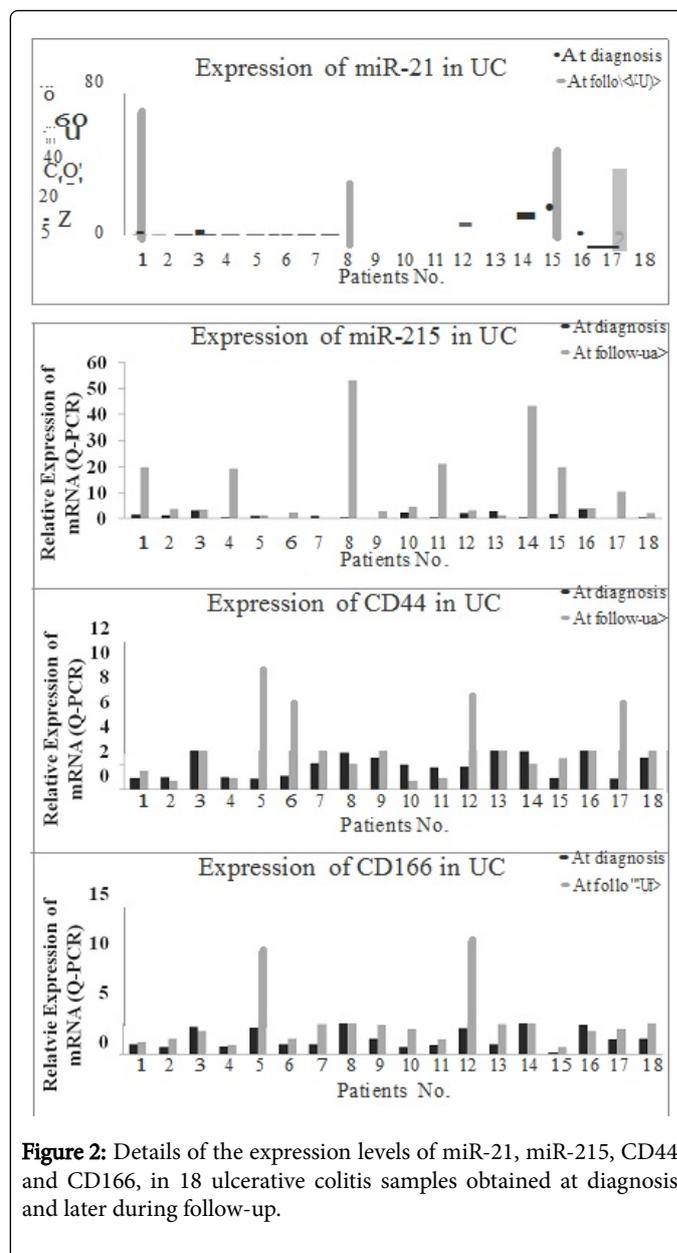
Analysis of genetic mutations

We investigated possible associations between mutations of APC, K-ras and DCC genes in all 18 UC patients (Figure 3). The analysis of mutations around codon 1337 (as mutation 1 Mut1), but not around codon 1380 (as mutation 2, Mut2) of the APC gene showed that one patient (number 9) presented with a genetic mutation of APC (Figure 4). The patient with this mutation had endured UC for more than 20 years and had pancolitis, with moderate–severe disease activity even when given regular medication. No alterations or mutations in K-ras or DCC genes were detected in this study.

Discussion

In our study, the expression levels of the biomarkers at the follow-up endpoint were higher than at the starting point, with statistical significance ($p < 0.05$). A total of 52 among the 72 markers we analyzed (72.2%) were elevated at the follow-up point despite the attenuation of UC lesions following medical treatment, which suggests that long-standing UC is a risk factor for CRC.

Our findings support that the duration of colitis as the most important clinical risk factor for CRC. We detected APC mutation in only one patient, an individual who had endured UC for 23 years, experiencing continuous, moderate to severe inflammation, even with the use of consecutive therapeutic agents (azathioprine with mesalamine, followed by infliximab with prednisone). The other biomarkers (CD44, CD166, miR-21 and miR-215) were also augmented in that patient. No genetic mutations in either Kras or DCC were detected in patient samples obtained at the time of diagnosis or prior to treatment for UC, indicating neither Kras nor DCC is affected at the initial stage of UC. However, earlier studies have reported that several other factor(s) may be involved in increasing the risk of CRC in patients with IBD, and that one of the most important risk factor is the duration of colitis. A large meta-analysis estimated that the risk of CRC in UC patients increases by 0.5%-1.0% per year, beginning 8-10 years after diagnosis [1].

The use of regular medication for UC appears to help prevent progression to CRC, even though we concur that the duration of colitis plays a crucial role in carcinogenesis. The APC/β-catenin pathway is known to play a crucial role in sporadic colorectal carcinogenesis. Alterations of this pathway are mostly caused by inactivation of the APC gene, which is a frequent and early genetic event in sporadic colorectal carcinogenesis. Studies of UC-associated dysplasia and cancer in humans have reported various degrees of loss of function of APC [22-26]. The reported frequency of APC mutations in UC-related neoplasia has varied from 0% to 6% [27] and up to 50% [23] in several small studies. The APC genetic mutation rate in the present study was only 5.67% (one patient), which was much lower than that reported in other studies. As we had detected only 2 codons of mutations for the APC gene, more codons of potential mutations in APC should be investigated in addition to more cases of UC with longer follow-up. Another important factor is that most of our patients received regular medication for UC.

Figure 2: Details of the expression levels of miR-21, miR-215, CD44 and CD166, in 18 ulcerative colitis samples obtained at diagnosis and later during follow-up.

MiRNAs are noncoding RNAs that are 21 to 25 nucleotides in length and which induce the degradation of the target mRNA or repress mRNA translation by imperfectly binding to their 3'-untranslated region [28-34]. Functional studies of dysregulated miRNAs indicate that they regulate molecular pathways in cancer via targeting different oncogenes and/or tumor suppressors. MiR-21 is one of the most studied miRNAs due to its involvement in cancer progression. A published study revealed a putative role of miR-21 in regulating the growth of CSCs [35]. Our previous study also investigated the influence of miR-21 on the pluripotency of CSCs and the Wnt signaling pathway by generating miR-21 overexpressed HCT-116 cells through stable transfection. Further evidence showed that cells that overexpressed miR-21 induced an increase in the size and frequency of the formation of spheres, indicating a role for miR-21 in the proliferation of CSCs [16]. MiR-215 is highly expressed in various cancers, but has low expression in colonic cancer [36-39]. Yet

our data indicate that miR-215 is highly expressed in the follow-up colon specimens, which is in contrast to the findings of some other studies. However, Olaru et al. [40], who observed an up-regulation of miR-215 in colitis-associated neoplasia, are in agreement what we have noted as were Yu et al. [41]. Although the cause of this discrepancy is unclear, we reason that miR-215 may act as a double-edged sword, modulating multiple targets and pathways. Karaayvaz et al. reported that a high level of miR-215 suppresses tumor cell proliferation and increases cell cycle arrest [42]. The differential expression of miR-215 is reflected by the stage of transformation, dysplasia versus carcinoma.

Figure 3: Relative expression of four markers. CD44, CD166, miR-21, and miR-215 showed higher levels of expression in follow-up samples than in those obtained at diagnosis. The significance levels of the values were 0.03, 0.01, 0.02 and 0.045, respectively. Good coherence is seen in CD44 with CD166 (14/18, 77.8%) and in miR-21 with miR-215 (14/18, 77.8%).

Figure 4: APC gene mutation. Amplification of APC gene in conserved sequence of all patients is shown in Row 1; distilled water is shown as the blank reference. APC genetic mutation is seen only at number 9, which shows a band at 174bp; HCT-116 cells are shown as a positive reference (shown in Row 2). No other patients had APC mutations.

In our study, most of the biomarkers were found to be enhanced at the follow-up endpoint, and APC genetic mutation occurred in only the patient with the longest duration of disease, which suggests that the duration of disease plays a pivotal role in colonic carcinogenesis. Although no direct relationship was shown between the duration of the disease and the biomarkers we studied, this finding may result from the small size of our study.

Conclusions

The biomarkers selected in our study have all been associated with the pathogenesis of colitis-associated cancer. Based on the evidence of enhanced expression of these indicators, our study shows that multiple alterations occur during ulcerative colitis, and that inflammation of the colon associated with persistently severe lesion activity, dysplasia, and long duration of disease are among the most important factors for increased risk of CRC. Surveillance and interventional treatments should be provided for patients with ulcerative colitis in order to reverse or retard the progression of colorectal cancer.

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Author Contributions

LY and APM designed and ran the study, analysed the results and wrote the manuscript, EL, JHD, HHZ and RM were involved in the running of the project, formed the steering group and revised the manuscript.

Competing Interests

The authors have no conflicts of interest to declare.

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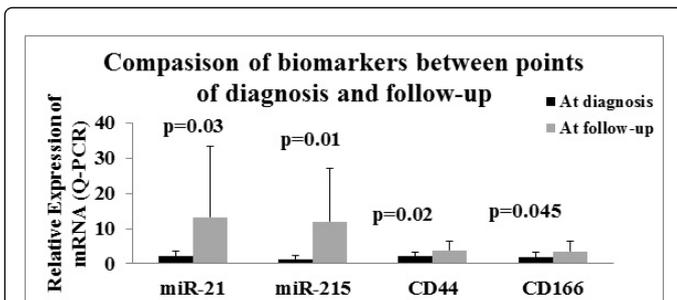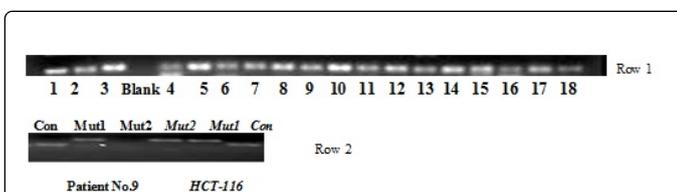
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