RESEARCH ARTICLE

Significance of HPV Infection and Genic Mutation of APC and K-ras in Patients with Rectal Cancer

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Abstract

Background: Significance of HPV infection and genic mutation of APC and K-ras in rectal cancer has been investigated but not clarified. The objective of our study was to investigate these parameters in patients with rectal cancer to analyze correlations with biological behaviour, to determine relationships among the three, and also to demonstrate survival prognosis effects. Methods: From December 2007 to September 2008, 75 rectal cancer cases confirmed by histopathology in the Tumor Hospital of Xinjiang Medical University were enrolled. The control group consisted of normal rectal mucous membrane taken simultaneously, a least 10 cm distant from the carcinoma fringe. HPV DNA, the MCR of APC and exon-1 of K-ras were detected by PCR and PCR-SSCP. All results were analyzed in relation to clinical pathological material, using chi-square and correlation analysis via SPSS.13 and Fisher's Exact Probability via STATA. 9.0. All 75 patients were followed up for survival analysis using Kaplan-Meier and Log-rank tests. Results: 55 out of 75 cases demonstrated gene HPV L1 while it was notdetected in normal rectal mucosa tissue. HPV infection was correlated with age and lymphatic metastasis (P<0.05) but not other characteristics, such as ethnicity, tumor size, histological type, tumor type, Duke's stage and infiltration depth. Some 43 cases exhibited APC genic mutation (57.3%) and 34 K-ras genic mutation (45.3%). APC genic mutation was correlated with gender(P<0.05), but not age, histological type, infiltration depth, lymphatic metastasis and Duke's stage. In 55 cases of rectal cancer with HPV infection, there were 31 cases with genic mutation of APC (56.4%) and 24 with genic mutation of K-ras (43.6%). For the 20 cases of rectal cancer with non-HPV infection, the figures were 12 cases (60%) and 10 (50.0%), respectively, with no significant relation. Survival analysis showed no statistical significance for K-ras genic mutation, APC genic mutation or HPV infection (P>0.05). However, the survival time of the patients with HPV infection was a little shorter than in cases without HPV infection. Conclusions: Our results suggest that HPV infection might be an important factor to bring about malignant phenotype of rectal cancer and influence prognosis. Genic mutation of APC and K-ras might be common early molecular events of rectal cancer, but without prognostic effects on medium-term or early stage patients with rectal cancer.

Keywords: Human papillomavirus - rectal carcinoma - gene APC - gene K-ras - mutation

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Introduction

Colorectal cancer is one of the most common malignancies, but its pathogenesis hasn't been elucidate so far. Recently, most researchers consider that occurrence and evolution of colorectal cancer refer to mutiple factors, more procedures and complex sequential change of polygenes in different segments. Meanwhile, In different regions virus infection involved in it, of which human papilloma virus (HPV) has occupied the most for being researched (Cimino-Mathews et al., 2012), while its detection positive incidence exists more difference (Kirgan et al., 1990; Zhou et al., 2004; Yavuzer et al., 2010). And gene oncogene APC and P53 have been researched most, meanwhile, protooncogene Ras has been studied much. Although genovariation research of gene APC and K-ras of colorectal cancer were common occurance, rectal carcinoma together with colon caner were included in the study scope, in which sample capacity of rectal cancer occupied less and rare research with objects suffered from only rectal cancer.

What's more, it's very difficult to find discussing HPV infection, genic mutation of gene APC and K-ras, and coexistion among the three. In the study, we discussed relation between HPV infection, MCR region of gene APC, and codon of gene K-ras respectively, and clinical pathologic factors for rectal cancer, and also probed into the relevance among the three.

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Materials and Methods

Patients and tissue samples

The study included 75 newly diagnosed cancer cases. The cases of primary rectal cancer got resection and were confirmed by histopathology in Tumor Hospital of Xinjiang Medical University, from December 2007 to September 2008. Cases with secondary, recurrent tumors, other tumors, chemoradiotherapy or biotherapy were excluded. The control group consisted of normal rectal mucous membrane taken simultaneously, 10cm apart from carcinoma fringe. Normal rectal mucous membrane in Control group matched with tumor tissue in the same case in Test group. In the research, for age, the range was from 26 to 79 years old, Median age 60; for gender, male 37 cases and female 38 cases; for peoples, ethnic Han 56 cases, ethnic Uyghur 14 cases and ethnic Hui 5 cases; for size of tumor, ≤10cm 35 cases and >5cm 40 cases; for pathology, Mass type or Fungating type 22 cases and ulcerative type 53 cases; for degrees of differentiation, high differentiation 27 cases, moderately differentiation 42 cases and poorly differentiation 6 cases; for Duke's stage, stage A 18 cases, stage B 28 cases and stage C 29 cases; for lymphatic metastasis (LM), with LM 29 cases and no LM 46 cases; for Invasion degree, invasion of full layer of Intestinal wall 53 cases, the others 22 cases. All the cases signed the formed consent. The ethics committee of Tumor Hospital of Xinjiang Medical University reviewed and approved the study

Specimen were in cryostorage (Negative 80°C) in vitro. Genome DNA DNA extraction was performed strictly accordingly to individual operating steps of Sangon Clinical sample-DNA extraction kit, by agarose gel electrophoresis (AGE) with consistence of 1%. 0.1μg was taken out for PCR. And clinical materials were judged by pathological report by two experienced pathology expert.

Primers

Globulin β (inner reference) was used to identify DNA quality. The design of Globulinβ and primer of HPV L1 referred to the treatise reported by Brennan (Brennan et al., 2001), and gene K-ras primer referred to the dissertation reported by K Servomaa (Serbomaa et al., 2000). All primers were provided by Sangon Biologicals Corp.

PCR amplification

Amplification of gene segment of Globulinβ, HPV L1 and K-ras by PCR was carried out according to the operating steps of Sango PCR kit. Amplification products were analysed by 2% agar gel electrophoresis. Positive control of HPV was positive DNA of HPV from cervical squamous cancer, provided by Endemic Key Laboratory of Xinjiang Medical University, and negative control was saline without DNA.

Single-strand conformation polymorphism (SSCP)

8% and 12% non-Modified polypropylene gel electrophoresis were used respectively to separate amplification products and identify mutational site of gene APC and K-ras. PCR amplification products 2μl and degeneration sample liquid 8μl were put into centrifuge tube, made instantaneously centrifugal blending, put into PCR Amplifier to degenerate in 99°C for 10 minutes, and then made in Ice bath quenching immediately for 10 minutes. Degeneration sample 4μl was absorbed put into Gel pores, using micropipettor. Electrophoresis chamber was placed in refrigerator in 4°C, preliminary electrophoresis in 250 volts for 5minutes, then electrophoresis in 3 watts for about 4 hours, till that Bromphenol Blue moved to the bottom of gel. After electrophoresis, unhinge gel to silver stain as followings, firstly, rinse: twice, using ddH_2O; secondly, fixed: took down gel carefully with plastic wedge, put into stationary liquid containing 10% alcohol and 0.5% acetic and fixed for 5 minutes; thirdly, silver staining: took out gel, put into Silvering Solution containing 0.15% silver nitrate and 0.056% formaldehyde, then dyed for 7 minutes, and rinse fast twice usingdeionized water (not more than 15 seconds each time); Fourthly, developing: put gel into TMB Substrate containing 1.5%sodium hydroxide and 0.185% formaldehyde and took out in time when band became clear (from 3 to 5 minutes); finally, put gel into stop buffer containing 10% alcohol and 0.5% acetic and took photos after 2 minutes. Each sample after electrophoresis compared with electrophoresis band type of PCR products of colon mucosa own matched, to analytically judge difference of product single configuration on the basis of electrophoretic band of SSCP, Indirectly reflecting sequence differences of templateDNA.

Follow-up

All the patients were followed up (for 52 months) and divided into three groups, HPV (infection, +) and HPV (-), APC (mutation, +) and APC (-) and K-ras (mutation, +) and K-ras (-). Chemotherapy scheme FOLFOX6 was carried out to the patients with rectal cancer of stage DUKE'S C, as followings, oxaliplatin (L-OHP) 130 mg/m^2, intravenously infused for 3 hours, on the first day, calcium folinate (CF) injection, 300 mg/m^2, intravenously infused, on the first day, 5-FU injection, 400 mg/m^2, intravenously injected, on the first day, and 5-FU 2400 mg/m^2, Continuously intravenously infusion by micro pump for 48 hours. 14 days a cycle, 12 cycles in total. For the patients with drug resistance, chemotherapy scheme XELOX was applied, as followings, oxaliplatin (L-OHP) injection, 130 mg/m^2, intravenously infused for 3 hours, on the first day, Capecitabine tablets, 1000 mg/m^2, po., twice a day, from first to fourth day, 3 weeks a cycle.

Statistical analysis

The comparison of Variety rate were performed with chi-square and correlation analysis was made with spearman correlation analysis, which above were carried out via SPSS for Windows Version 13 (SPSS Inc., Chicago, IL, USA). Disease-free survival (DFS) and Overall survival (OS) were analysed by the method of Kaplan—Meier, and Log-rank test were used for comparation between groups. The procedure Fisher's Exact Propability from the statistical package STATA 9.0 (Stata Corp, College Station, TX, USA) was used for the calculations. P were considered statistically significant

Table 1. The Relation Between HPV Infection, Genic Mutation of APC and k-ras, and Clinical Pathology of Rectal Cancer

Clinicopathologic index	N	HPV		χ^2	APC		χ^2	K-ras		χ^2
		+	—	*P*	+	—	*P*	+	—	*P*
Peoples										
Han	56	42	14	—	30	26	—	27	29	—
Uygur	14	10	4		8	6		5	9	
Hui	5	3	2	0.731*	5	0	0.134*	2	3	0.718*
Gender										
male	37	25	12	1.241	26	11	4.996	16	21	0.129
female	38	30	8	0.265	17	21	0.025	18	20	0.720
Age										
≥50	55	36	19	6.547	31	24	0.079	26	29	0.313
<50	20	19	1	0.011	12	8	0.778	8	12	0.576
Tumor size (cm)										
≤5	35	24	11	0.761	19	16	0.249	14	21	0.753
>5	40	31	9	0.383	24	16	0.618	20	20	0.385
Tumor general type										
mass/fungating	22	17	5	0.247	13	9	0.039	12	10	1.066
ulcerative	53	38	15	0.619	30	23	0.843	22	31	0.302
Differentiated degree										
well	27	23	4	—	16	11	—	14	13	—
moderately	42	29	13		24	18		18	24	
poorly	6	3	3	0.113*	3	3	0.941*	2	4	0.721*
Duke's staging										
stage A	18	16	2	—	10	8	2.998	10	8	3.203
stage B	28	22	6		13	15		9	19	
stage C	29	17	12	0.068*	20	9	0.224	15	14	0.202
Lymph follicle metastasis;										
no	46	38	8	5.234	23	23	2.615	19	27	0.779
yes	29	17	12	0.022	20	9	0.106	15	14	0.377
Infiltrate depth										
full-thickness	53	36	17	2.703	29	24	0.506	24	29	0.000
part-thickness	22	19	3	0.100	14	8	0.477	10	12	0.989

*value *P* of precise probability ratio

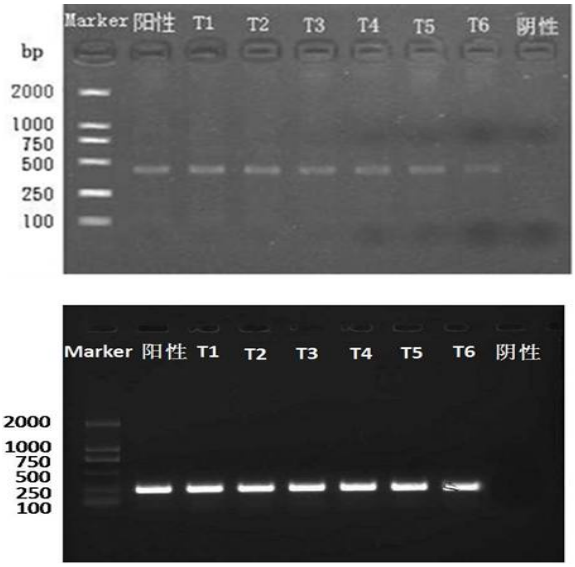

Figure 1. PCR Amplification Product Electrophoresis Result of HPV. M: DNA Marker, Positive control; Negative control; T1-T6 of rectal cancer specimen. (Upper photo: HPV primer; Lower photo: β-actin primer with the same template as the Upper)

which was less than or equal to 0.05.

Results

HPV DNA detection

Through making PCR amplification of 75 cases of colorectal cancer samples and normal rectal mucosa tissue own matched, gene segments of Globulinβin each group were detected, which confirmed all the tissue specimens were extracted successfully. 55 cases out of 75 cases of colorectal cancer were detected gene HPV L1 while it didn't detected in normal rectal mucosa tissue. Which were shown in Figure 1.

In 75 cases of rectal cancer, there were 55 cases getting HPV infection (occupied 73.3%), 43 cases detected APC genic mutation (occupied 57.3%, Figure 2) and 34 cases detected K-ras genic mutation (occupied 45.3%, Figure 3). Relation between the three above and the clinical pathological characteristic, such as peoples, gender, age, tumor size, pathological type, differentiated degree, Duke's staging, Lymph follicle metastasis and Infiltrate depth, were shown in Table 1.

Relativity between HPV infection and genic mutation of APC and k-ras respectively

In 55 cases of rectal cancer with HPV infection, there were 31 cases with genic mutation of APC (occupied 56.4%) and 24 cases with genic mutation of K-ras (occupied 43.6%). Meanwhile, in 20 cases of rectal cancer with non-HPV infection, there were 12 cases with genic mutation of APC (occupied 60%) and 10 cases with genic mutation of K-ras (occupied 50.0%). And there were no relativity between HPV infection and genic mutation of APC or k-ras (detailed shown in Table 2).

Survival analysis of the groups of the patients with

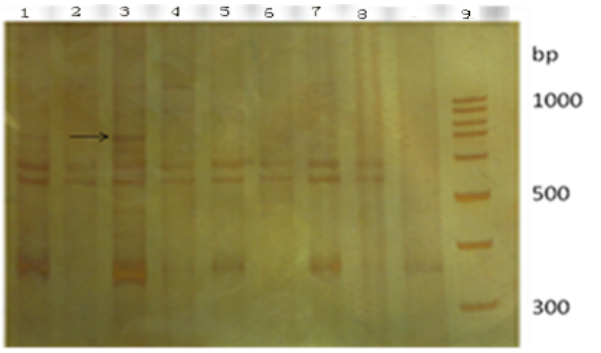

Figure 2. The SSCP Graph of Gene APC. M: DNA Marker, 1, 3, 5, 7: Tumor tissue specimens; 2, 4, 6, 8: Normal tissue; 9: Marker. Arrowhead displayed catastrophe band

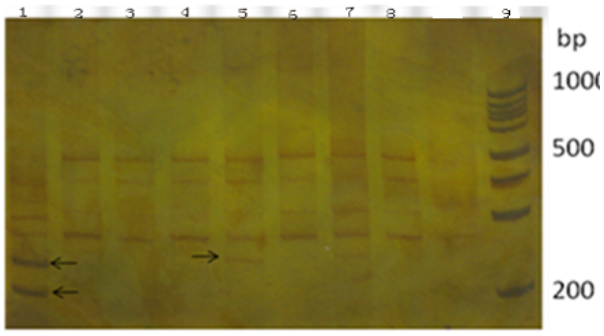

Figure 3. The SSCP Graph of Genic Mutation of K-ras. M: DNA Marker, 1, 3, 5, 7: Tumor tissue specimens; 2, 4, 6, 8: Normal tissue; 9: Marker. Arrowhead displayed catastrophe band

Table 2. Relativity Between HPV Infection and Genic Mutation of APC, K-ras

HPV	APC Gene mutation		K-ras Gene mutation	
infection	+	-	+	-
+	31	24	24	31
-	12	8	10	10
χ^2	0.079		0.240	
P	0.778		0.624	

HPV infection, APC genic mutation or K-ras genic mutation

For 75 cases followed up, Survival analysis of Kaplan-Meier and Log-rank indicated, there were no statistical significance in the groups of whether HPV infection or not, APC genic mutation or not or K-ras genic mutation or not ($P>0.05$). Nonetheless, from above we could find that the patients without HPV infection had lived a little longer than the patients with HPV infection, compared with the other group comparations.

Discussion

Rectal cancer is one of the most serious cause of cancer related death in both men and women, while its exact pathogenesis keeps unknown till now. Very recently, as is known to us, morbidity pattern of rectal caner says that tumor related gene, such as gene APC, K-ras and P53, got abnormally activation or mutation, which results in normal mucous epithelium hyperplasia to form tumor at last (Pan et al., 2004; Zhan et al., 2005; Weijenberg et al., 2007; Estrada et al., 2009; Lea et al., 2009). In this study, the method of PCR-SSCP was performed to research gene APC and K-ras. In 1989, a Japanese called Orita discovered that single-stranded DNA (ssDNA) segments appear complex space fold conformation. The stereochemical structure mainly sustains by intramolecularly interaction force from interior base pairing, and when some basic group change, space conformation will be more or less influenced, which makes conformation transform. Consequently ssDNA molecules with space conformation difference get different resistance in non-degeneration polyacrylamide gel, which causes electrophoresis speed different and the results could be gotten by detection in gel. And this method is called Single Stranded Conformational Polymorphism (SSCP) analysis, whose susceptiveness is very high and 0.2% basic group mutation can be detected out theoretically. However, it is impossible to point out exact mutation site and mutation type (Orita et al., 1989). Genital cell mutation of APC gene is Molecular pathology foundation of Familial Adenomatous Polyposis (FAP), meanwhile somatic mutation plays an important role to cause sporadic rectal cancer(Kámory et al., 2008; Kittiyod Poovorawan et al., 2012). Most of somatic mutation is nonsense mutation, and more than 60% of somatic mutation called codon 1286~1513, whose area is named MCR (Tang et al., 2006; Hinoi et al., 2007). In the study, 43 cases were detected out MCR area mutation of gene APC out of 75 cases with rectal cancer, which was consistent with researcher Zhan' report(Zhan et al., 2005). Not only this, MCR area mutation of gene APC had Correlation with gender in the research($P<0.05$), while there are no similar reports recently. The reason might be that there exit the difference of life custom, hormone level and so on between men and women, which caused this (De Voge et al., 2006); It also may be correlation with the similar number between male and female in the study. And to know the detail reason needs further study. 43.6% of all the rectal cancer (75 cases in total) exited genic mutation of K-ras, which was in accordance to Georgieva' report (Georgieva et al., 2009). Therefore, genic mutation of K-ras was considered to be early molecular events of rectal cancer.

There are also some cofactors to participate canceration procedure of rectal cancer, which can unite to bring about cell transform and to make cell immortalized finally. Cheng and some other researchers revealed that there were Correlation between HPV infection and anal epidermoid cancer, cervical cancer, the upper respiratory tract cancer, breast carcinoma, and so on, respectively (Cheng et al., 1991; Jira Chansaenroj et al., 2012). In recent years, there were many studies on correlation between HPV infection and colorectal cancer (Palefsky et al., 2010). Kirgan detected up to 29 cases got HPV infection out of 30 cases (occupied 97%) of rectal cancer (Kirgan et al., 1990). And Cheng detected DNA segments of HPV in three rectal cancer cell line. However, Yavuzer failed to get the evidence of HPV infection of rectal cancer(Yavuzer et al., 2010). In this study, the rate of HPV infection was 73.3% (55/75) in 75 cases of rectal cancer, the positive rate of which was higher than that of Młynarczyk's results (Mlynarczyk et al., 2009), and there were correlation between HPV infection and age or lymphatic metastasis. The reason why there were difference between our study

and some others, maybe were the followings, first of all, we only used Universal primer which could amplify more than 30 types of HPV; what's more, the objects of the study were only the cases with rectal cancer, while domestic and overseas related reports showed the closer to anus HPV infection. the higher the positive rate of HPV infection was (Von Knebel Doeberitz et al., 2010). As for the correlation between HPV infection and age, lymphatic metastasis, which coincide d with Pochylski's report (Pochylski et al., 2003), it could prognose that HPV infection might be involved in rectal cancer happening and malignant biological behavior, because HPV infection mostly via sexual transmission. Consequently, the reason why HPV infection correlated with age may be that sexual life of throng less than 50 years old were more active than the older ones, nonetheless, its detailed mechanism needs further research

Pochylski showed that protein ras and HPV maybe participate jointly formation cervical cancer(Pochylski et al., 2003; Shukla et al., 2009; Chaturvedi et al., 2010). Mazurek reported that HPV E7 protein and gene ras took part in cellular transformation together and directly bonded protein M2-PK and induce its induced second polymers change, in recover the function of the nucleic acid synthesis and cell proliferation(Mazurek et al., 2001). In our study, there were no relativity between HPV infection and genic mutation of APC and K-ras ($P>0.05$), which told that HPV infection didn't improve the genic mutation rate and vice versa. Yet, it needs more further research on their function mechanism of formation and evolution of rectal cancer and HPV oncogene inducing vicious transformation of colorectum.

Relatively fewer research about prognosis effect of HPV infection, APC genic mutation and K-ras mutationon on the patients with rectal cancer were carried out. Gene K-ras is the key factor of epidermal growth factor receptor (EGFR) pathway. The relativity of between its genic mutation and drug curative effect and prognosis of patients with rectal cancer were reported by some researchers at home and abroad. Karapetis indicated that for the patients with advanced rectal cancer, curative effect could obviously be improved by Cetuximab therapy applied on the patients received chemotherapy simultaneously (Karapetis et al., 2008). Meanwhile there were few studies on prognosis effect of K-ras genic mutation on patients with rectal cancer of median-early stage. In the trial, for the patients with rectal cancer of Duke's C stage, received chemotherapy, no prognosis effect of K-ras mutation was indicated, which was consistent with Bleeker and so on (Bleeker et al., 2001; Westra et al., 2005; Ogino et al., 2009). APC genic mutation inactivation may be early molecular events of patients suffering from rectal cancer, while it stably exists in the whole process of tumor occurrence and development (Yuan P et al., 2001). Presently there were also fewer trials on prognosis effect of APC genic mutation for patients with rectal cancer. Our research showed that there was no statistical significance between the groups of whether APC genic mutation or not. Relatively more researches on effect of HPV infection on rectal cancer occurrence and its clinicopathologic material association were carried out, while prognosis effect on patients with rectal cancer was not clear and studied relatively less. In our study, like K-ras and APC genic mutation there was no statistical significance between groups of whether HPV infection or not. However, figure 3a showed that the patients with rectal rectal and HPV infection lived relatively shorter than those without no HPV infection. For the phenomenon above, maybe it needs to combine with clinical reality to demonstrate HPV infection effect on patients with rectal cancer. In brief, it is of necessity to conduct more large sample studies and combine with clinical reality to confirm the prognosis effect of K-ras genic mutation and genic mutation and HPV infection on patients with rectal cancer.

In summary, there exists some correlation between HPV infection and happening of rectal cancer and its malignant biological behavior (Mlynarczyk et al., 2009). Genic mutation of K-ras could be concerned as early molecular events (Onozato et al., 2011; Beliaeva et al., 2012); However, koinonia of HPV infection and gene K-ras might play a certain part in formation of rectal cancer. However, is HPV infection one of Initiating factors or accompanied infection after rectal cancer formation? Therefore, more further study on detailed synergistic effect of HPV infection, gene APC and K-ras, and prognosis influence on patients with rectal cancer are necessary to be applied. Maybe they can be combined with Cancer related gene, tumor-suppressor genes and apoptosis related gene for deeper research.

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