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Colorectal Cancers in Patients With the (9A/6A) Polymorphism of *TGFBR1* Exhibit Lesser Inter-(simple sequence repeat) PCR Genomic Instability and Present Clinically at Greater Age

Smitha S. Dutt^a, Neng Chen^b, Huferesh K. Darbary^a, Helen Swede^c, Nicholas J. Petrelli^d, Daniel L. Stoler^e, and Garth R. Anderson^{a*}

^aDepartment of Cancer Biology, Roswell Park Cancer Institute, Buffalo, NY

^bDepartment of Dermatology, Stanford University School of Medicine, Stanford, CA

^cDepartment of Community Medicine and Health Care and Neag Cancer Center, University of Connecticut Health Center, Farmington, CT

^dHelen F. Graham Cancer Center, Newark, DE

^eDepartment of Head and Neck Surgery, Roswell Park Cancer Institute, Buffalo NY

Abstract

TGFβ is involved in the response to DNA damage and signaling the cell cycle checkpoint response, in large part achieved by modulating the activity of the ATM kinase. We have investigated if the presence of a common polymorphism in the TGFβ receptor *TGFBR1* might impact genomic instability in human colorectal cancer. In order to obtain statistically significant numbers of patients with the lesser polymorphism, one hundred seventy-seven colorectal cancer patients were genotyped for either the major form of the *TGFBR1* receptor gene, homozygous for an internal segment of 9 alanines (9A/9A), or the lesser form, heterozygous for the polymorphism containing 6 alanines (9A/6A). Intrachromosomal genomic instability in the tumors was then quantified by the robust inter-(simple sequence repeat) PCR method. Tumors from all twenty-six patients heterozygous with the (9A/6A) polymorphism in *TGFBR1* exhibited significantly lower genomic instability than from a randomly selected set [the first identified] of thirty-seven patients with the (9A/9A) polymorphism ($p=0.0002$, Mann-Whitney). The median age of onset for the (9A/6A) patients was 70 years, compared with a median age of onset of 63 years for the patients carrying the (9A/9A) form ($p=0.031$, Mann-Whitney). These results are consistent with the model wherein genomic instability facilitates tumor progression, with lesser instability associated with later disease presentation. Clinically, our findings may be developed into improved screening guidelines with respect to the age at which colonoscopy is initiated in carriers of the *TGFBR1**6A allele.

Keywords

TGFBR1; 9A/6A polymorphism; genomic instability; colorectal cancer

*Corresponding Author: Garth R. Anderson PhD, Department of Cancer Biology, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263. Office:(716) 845-4529, Fax (716) 845-8126, E-mail: garth.anderson@roswellpark.org.

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1. Introduction

Colorectal cancer genomes contain many mutations, with the recent report by Sjoblom et al finding around 90 mutated genes per tumor cell, and an earlier report by Stoler et al estimating around one hundred times that many events in the combined coding and noncoding sequences of the genome [1,2]. The ongoing accumulation of this large number of mutations during tumor progression is believed to arise as a consequence of the multiple independent genomic instabilities that appear during the course of tumor progression [3–5].

TGF β is a pleiotropic regulator of cell proliferation, and plays an important role in the maintenance of genomic integrity [6,7]. The primary transducer of a cell's responses to DNA damage is the nuclear protein kinase ATM, which activates and recruits a program activating checkpoint and repair responses essential for enabling the DNA damage itself to be efficiently repaired [8]. The activity of this kinase is modulated by TGF β [9].

Components of the TGF β signaling pathway are often found mutated in cancers. The receptor subunit TGFBR2 is a major mutation site in mismatch repair deficient, microsatellite unstable colorectal cancers [10]. Cigarette smoking is associated with an increased frequency of mutations in the TGF β signal transducer *SMAD4*, and with a decreased frequency of mutations in *TGFBR2* in colorectal cancers [11]. Germline mutations in *SMAD4* and *BMPRI1A*, encoding an alternate receptor in the TGF- β pathway, are found in juvenile polyposis [12,13]. Polymorphisms in the *TGFBR1* receptor subunit gene have been modestly associated with certain hereditary colorectal cancers [14].

Many genes have been identified which when knocked out or mutated in model systems can give rise to genome instability and tumor formation. In order to evaluate if TGF β signaling significantly impacts genomic instability in naturally occurring human colorectal cancer, we have exploited the presence of a relatively common polymorphism in the TGF β RI receptor. By comparing tumors from patients carrying each form of the receptor, we can test if a subtle variation in the TGF β pathway impacts the overall level of genomic instability, and can further evaluate if the patients exhibiting more unstable tumors have experienced earlier onset of disease.

The gene for *TGFBR1* encodes a 53 kD protein of 503 amino acids. The common polymorphism involves an internal GCG repeat coding for alanine. The major form of the repeat element encodes nine alanines (9A), and the variant present heterozygously in about one-sixth of the population encodes six alanines (6A). Cells with the variant TGF- β receptor do not respond as effectively with known TGF β growth-inhibitory effects [15,16]. *TGFBR1**6A and *TGFBR1* encode identical mature receptor proteins; the 9 bp deletion is within the *TGFBR1* signal sequence [17]. The frequency of *TGFBR1*(9A/6A) heterozygotes is similar in healthy individuals and in patients with a diagnosis of colon cancer; although earlier studies proposed *TGFBR1**6A to be a tumor susceptibility allele in colon cancer, a more recent large-scale study has shown this variant is associated with no increased risk for the disease [15,18,19]. Similarly, no association exists with lung cancer, but the picture remains unclear for breast cancer [20–22].

In this report we describe how genomic instability, age of onset of disease, and the polymorphism in *TGFBR1* are interrelated. Patients with the variant *TGFBR1* (9A/6A) exhibited a lower degree of genomic instability in their tumors, and were significantly older at diagnosis than patients with the 9A/9A form.

2. Materials and Methods

2.1. Patients and Tissues

DNA was isolated from 177 sporadic colorectal cancers and corresponding normal colonic mucosa from patients who underwent surgical resections at Roswell Park Cancer Institute from 1992–1998 (Table 1). All patients had given written consent for obtaining tissue specimens, as approved by the Roswell Park Cancer Institute Internal Review Board. DNA was prepared from frozen tumor tissue (stored in the Anderson laboratory at -70°C) and the adjacent normal mucosa several centimeters beyond the tumor margin, as reported previously [2,23,24].

2.2 Genomic Instability Measurements

Inter-(simple sequence repeat) PCR is a simple, robust genomic sampling technique able to quantify alterations in genomic sequences occurring between those repeat sequences present within about two kilobases of one another [23]. This assay methodology as used detects intrachromosomal genomic instability manifested between pairs of microsatellite sequences, but not within the microsatellite sequences themselves. It is independent of microsatellite instability, fractional allelic loss rate, and copy number change rate as revealed by microarray comparative genomic hybridization [23,24].

The assays were performed as originally described in Basik et al [23]. The genomic instability index (GII) represents the percent of altered bands observed in the PCR products amplified from the tumor DNA, as compared with the total number of products generated from corresponding normal tissue DNA from each patient.

2.3 Loss Of Heterozygosity Assays

Loss of heterozygosity assays were performed by electrophoretic analysis of specific PCR amplification products, with autoradiographic visualization [24].

2.4 PCR Determination of TGF β R-I Isoforms

Primers for the TGFBR1 gene were designed around the region encompassing the 9 GCG repeat sequence. P2: CGTCGCCCCCGGGAGCAGCGCCGC $T_m = 81^{\circ}\text{C}$; P7: CGCGTCCCCGGCTGCTCCTCCTCGTGCTG $T_m = 80.1^{\circ}\text{C}$. Primers were designed using the Vector NTI software. PCR analysis was carried out at annealing temperature 75°C .

The PCR products were electrophoresed on a 12% polyacrylamide gel, which reveals the established size differences in PCR products resulting from the GCG repeat sequence variations. The wild type TGFBR1 gene yielded a 74 bp PCR product. The variant with 6 GCG repeats yielded a 65 bp product. Patients showing bands of either or both the sizes were identified. 145 patients exhibited the (9A/9A) genotype, 26 exhibited the heterozygous (9A/6A) genotype and 2 exhibited the homozygous (6A/6A) genotype. Of the remaining 3 patients, one was heterozygous (9A/5A), one was heterozygous (9A/10A), and one was indeterminate. Our finding of 2 out of 177 patients with the (6A/6A) genotype is consistent with a previous report finding this genotype in 9 out of 851 patients [18].

2.5 Statistical Analyses

The nonparametric Mann Whitney U-Test and the t-Test for independent samples were used to compare genomic instability levels in tumors according to *TGFBR1* genotype (9A/9A, 9A/6A). We report median as well as mean values for illustration. We further stratified the Mann-Whitney analyses according to smoking history of the patients, which was obtained either from the medical record or by self-report from an epidemiological questionnaire administered at the time of hospital admission.

3. Results

***TGFBR1* Polymorphism Determination**

Both tumor DNA and corresponding normal colonic mucosal tissue DNA from each of 177 sporadic colon cancer patients were analyzed for the *TGFBR1* gene polymorphism, as illustrated in Figure 1A; the PCR primers were selected so as to amplify the region containing the GCG repeat sequence encoding the polymorphic segment of multiple alanines. All 26 patients in the set found with the 9A/6A genotype were analyzed further for genomic instability since this genotype was limiting; this was done in order to maximize the statistical significance of comparisons between wild type 9A/9A patients and the 9A/6A patients and the corresponding effect of the polymorphism on genomic instability. In order to have a comparably sized group of patients for further genomic analyses, the first thirty-seven patients with the wild type *TGFBR1* (9A/9A) gene [out of the 145 so identified] were chosen for detailed genomic instability comparisons. The number of 9A/6A patients found was similar to the 15 percent incidence of this polymorphism that has been reported previously [15,18]. None of the 9A/6A tumors were found to have arisen from somatic mutations in the *TGFBR1* gene, as indicated by the identical presence of the polymorphism in each case in tumor and in corresponding normal tissue. This is in slight contrast to the report of Pasche et al, where two percent of the colorectal cancers were found to have undergone somatic mutation to create the polymorphism [17].

Two of the 177 patients were found to be homozygous for the 6A genotype in both their tumor and normal tissue DNAs. This value is consistent with the Hardy-Weinberg equilibrium for an infrequent allele, and is also consistent with the frequencies detected by Pasche et al [15,18] and by Stefanovska et al [25].

Assessment of Genomic Instability

In order to test for a relationship between the polymorphism and genomic instability, we chose the robust method of inter-(simple sequence repeat) PCR, which is able to detect a broad range of intrachromosomal instabilities [2,23,24,26]. The genomic instability index (GII) represents the percentage of major bands found altered when the genomic sample is analyzed on nondenaturing gel electrophoresis, as compared to the profile of bands generated from normal tissue DNA from the same patient [23]. Faint bands reflecting alterations present only in small subpopulations of tumor cells were ignored for further analysis (Figure 1B) [27]. The group of patients heterozygous for *TGFBR1* (9A/6A) exhibited GII values ranging from 0.0 to 3.9, while those homozygous with the variant (9A/9A) showed GII values that ranged from 0 to 13.0 (Table 1). Tumors from patients with the 9A/9A polymorphism exhibited a substantially higher mean level of GII compared to those who were heterozygous with the 9A/6A alleles (4.53 versus 1.37, $p < 0.0001$, t-test for independent samples), as shown in Table 2. This significant difference was maintained when comparing distributions of GII values between these two groups of patients ($p = 0.0002$, by Mann-Whitney U non-parametric test.)

The two patients homozygous with 6A/6A each had tumors with genomic instability indices of 0.0, which is consistent with the 6A polymorphism being associated with lesser genomic instability as revealed by inter-(simple sequence repeat) PCR. These two patients' ages at presentation were 51 and 71 years old. However this very small set is not statistically significant.

Smoking had little impact on the difference in GII values between patients classified as (9A/9A) and patients classified (9A/6A); among smokers, the median GII value observed in tumors from patients with the (9A/9A) genotype was 2.6 compared to 0.0 from tumors of patients

classified as (9A/6A) ($p = 0.0029$); among non-smokers, the median GII values were 3.9 (9A/9A group) compared to 1.25 (9A/6A group) ($p = 0.0029$).

Although genomic evaluation of the 9A/9A and 9A/6A forms of the *TGFBR1* gene was performed in both tumor and normal DNA from each patient, we independently tested if loss of heterozygosity in the region including the gene itself might have occurred in the tumors. We selected two polymorphic markers, D9S272 which is 116 kbps to the left of the *TGFBR1* gene and D9S176 which is 146 kbps to the right of the gene. These highly polymorphic markers were selected based on their high frequency of detectable heterozygosity, making informative allelotyping assays for at least one marker very likely and with any loss of heterozygosity very likely to be revealed. Marker D9S272 normally shows a 79.24% heterozygous rate, and the corresponding primers amplify a sequence of 211 bps. Marker D9S176 normally shows 82.3% heterozygosity, and the corresponding primers amplify a sequence that is 147 bps long (GDB database). In our tumor DNA samples we found no losses of heterozygosity in the *TGFBR1* region in any of the 65 tumors (including the two 6A/6A) as compared with their normal tissue DNA controls.

Disease Age of Onset

At the clinical level, patients homozygous with the 9A/9A form of the *TGFBR1* gene presented with colorectal cancer at an age significantly younger than patients heterozygous with the 9A/6A forms (Figure 2). As detailed in Table 2, the mean age difference was 6.1 years [61.1 years versus 67.2, respectively, $p=0.04$], and the median age difference was 7.5 years [63.0 years versus 70.5, respectively, $p=0.05$]. Compared to patients with one 6A allele, patients who were homozygous for the 9A allele tended to present with later stage disease, and have malignant lesions on the right versus left colon, but these differences did not reach statistical significance. The median survival time was greater among those who were homozygous for the 9A allele compared to those with one 6A allele (86.4 months versus 65.6 months); this reflects in part that the generally younger patients who were homozygous for 9A were able to have significantly longer median follow-up time (141.9 months versus 92.9 months, $p=0.004$, among those alive at the end of the study).

The median age at diagnosis for all patients in our entire group, including those with 9A/9A and 9A/6A, was 65 years. For those patients at or below the median age, 23 tumors were of the 9A/9A genotype and 10 were 9A/6A, while for those later onset patients above the median age, 14 were 9A/9A and 16 were 9A/6A. Statistical analysis using Fisher's Exact Test revealed a trend between the 9A/6A polymorphism and later onset of colorectal carcinoma that was nearly statistically significant ($p=0.055$). The 9A/6A genotype thus appears to be a substantial determinant for later age of onset colorectal cancers.

4. Discussion

Our studies show that different polymorphisms in the *TGFBR1* gene are associated with different degrees of intrachromosomal genomic instability in colorectal cancers arising in patients carrying each polymorphism, with the 9A/9A form associated with greater genomic instability than seen with the 9A/6A form. Furthermore, individuals with the 9A/9A form tend to present with disease at a significantly younger age than individuals with the 9A/6A form.

Our findings are consistent with the known role of TGF β in modulating ATM. With ATM playing a key role in coordinating the cellular responses to DNA damage, particularly by activating repair systems and cycle checkpoints, it follows that altering the modulation of this system would impact the degree of genomic instability [8,9].

Our studies have examined in detail one assay for genomic instability, measurement by inter- (simple sequence repeat) PCR. Although this assay is robust and detects many different forms of genomic alterations, additional studies using other measures of instability such as with high-density SNP microarray analyses or BAC-array CGH may provide useful additional insights; limited comparisons of fractional allelic loss rates showed no difference.

The 9A/9A patient tumors which exhibit greater genomic change may present at a generally younger age because they progress more rapidly to clinically significant disease; alternatively, initiation might be occurring earlier in the 9A/9A patients and progression rates are unchanged. Since considerable evidence indicates genomic instability facilitates the many genomic events associated with tumor progression, the latter possibility appears unlikely [5,28].

Our work appears to have useful clinical implications. By being able to identify through a simple test of the *TGFBR1* gene which individuals are more or less likely to develop colorectal cancer at an earlier or later age, it may be possible to more closely tailor guidelines for the age at which colonoscopy first becomes appropriate [29]. With the 9A/6A polymorphism present in fifteen percent of the population and around a seven year later age of onset, the rather simple genetic test for the 9A/6A gene polymorphism may be able to spare the fifteen percent of the population with this polymorphism the expense and unpleasantness of one or more of these colorectal examinations. Presumably additional genes will be found which will also impact the age of onset of colorectal cancer, and together with our findings may be able to further refine screening guidelines. Our results further suggest an interesting approach to tumor prevention. By modulation of the activity of the TGF β signaling pathway, optimal DNA repair and genomic stabilization may be achieved; in turn, the onset of cancer might be delayed beyond the normal human lifespan [9].

Acknowledgement and Conflict of Interest Statement

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Figure 1.

Detection of the *TGFBR1* gene polymorphism and measurement of genomic instability. (A), Electrophoretic analysis of PCR products of the region in the *TGFBR1* gene encoding the 9A or 6A polymorphism, as detailed in the Methods. Both tumor (T) and normal (N) tissues from each patient revealed identical results in the polymorphism assay. (B) Inter-(simple sequence repeat) PCR comparisons of tumor and normal DNAs from the same patient, to assess genomic instability. The (>) indicates a major band alteration specific to the tumor DNA of patient 3153, reflecting genomic instability.

Figure 2.

Age of colorectal cancer presentation in patients carrying the two different polymorphisms of the *TGFBR1* receptor. The y-axis shows the number of tumors that had appeared by the age shown on the x-axis, divided by the total number of tumors that eventually appeared in patients with the given genotype.

() 9A/9A; () 9A/6A.

Table 1
Features of patients and tumors analyzed in this study: TGF β R1 polymorphism compared with genomic instability indices (GII).

9A/6A Tumor #	AGE	Sex	Stage	GII	9A/9A Tumor #	AGE	Sex	Stage	GII
2811	68	M	1	3.75	3120	37	F	1	0
656	71	M	1	1.25	3929	43	M	1	2.6
6376	72	F	1	3.9	3201	53	M	1	0
696	76	M	1	0	3118	63	F	1	3.9
3157	76	F	1	3.9	3023	64	M	1	7.8
14118	76	F	1	0	3211	64	M	1	3.9
677	78	M	1	0	3155	70	M	1	2.6
3621	41	M	2	0	3041	75	M	1	2.6
3486	51	M	2	0	3135	75	F	1	11.7
6388	59	M	2	2.6	3027	81	F	1	2.6
3122	60	M	2	2.6	3205	83	M	1	0
3116	63	M	2	0	3199	45	M	2	6.5
381	73	F	2	3.75	3180	58	F	2	1.3
2095	74	M	2	0	6394	63	M	2	6.5
3127	80	M	2	0	3151	66	F	2	1.3
1885	61	M	3	0	3936	79	M	2	6.5
712	53	F	4	0	6368	39	M	3	2.6
3463	55	F	4	1.25	6384	47	F	3	1.3
3414	59	M	4	3.75	3187	54	F	3	3.9
14035	65	M	4	0	3195	54	M	3	2.6
3215	68	M	4	1.3	3035	56	F	3	6.5
14044	70	M	4	2.5	6380	58	F	3	0
264	71	F	4	5	3182	61	M	3	5.2
668	71	F	4	0	6392	65	F	3	0
542	77	M	4	0	3153	66	M	3	10.4
2251	78	F	4	0	6370	67	M	3	7.8
					3143	68	M	3	11.7
					3043	69	M	3	2.6
					3029	72	M	3	3.9
					3209	77	F	3	0
					6382	44	F	4	3.9
					6386	45	M	4	5.2
					3203	48	F	4	1.3
					3125	49	F	4	6.5
					3033	61	M	4	10.4
					3207	65	F	4	1.3
					3177	76	F	4	9.1

Table 2
Clinicopathological Characteristics of 63 Patients with Invasive Colorectal Cancer, Roswell Park Cancer Institute

	<i>TGFBRI</i> Polymorphism		<i>p</i> -value
	9A/9A (n = 37)	9A/6A (n = 26)	
Age			
Mean	61.1 yrs	67.2 yrs	$p^a = 0.04$
Median	63.0 yrs	70.5 yrs	$p^c = 0.05$
GII			
Mean	4.53	1.37	$p^b < 0.0001$
Median	3.9	0.0	$p^c = 0.0002$
Sex			
Female	17 (45.9%)	9 (34.6%)	$p^a = 0.37$
Male	20 (54.1%)	17 (65.4%)	
Stage			
I and II	16 (43.2%)	15 (57.7%)	$p^a = 0.26$
III and IV	21 (56.8%)	11 (42.3%)	
Sub-site			
Rectum/rectum sig. junction	20 (54.1%)	14 (53.8%)	$p^a = 0.77$
Left	4 (10.8%)	5 (19.2%)	
Transverse	3 (8.1%)	2 (7.7%)	
Right	10 (27.0%)	5 (19.2%)	
Vital Status			
Alive	16 (43.2%)	11 (42.3%)	$p^a = 0.94$
Dead	21 (56.8%)	15 (57.7%)	
Median Survival	86.4 months	65.6 months	$p^c = 0.31$
Median Follow-up (among alive)	141.9 months	92.9 months	$p^c = 0.004$

^a Chi² test of proportions

^b t-test for independent samples

^c Mann-Whitney U non-parametric test