Original Article

Ring Finger 43 Hot-spot Frameshift Mutation G659V in Colorectal Cancer Patients: Report from a Tertiary Cancer Care Hospital in North India

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Abstract

Background: The Ring Finger 43 (*RNF43*) is a tumor suppressor gene that negatively regulates the Wnt/β-catenin signaling. The p.G659fs is a recurrent RNF43 C-terminal truncating variant frequent in colorectal cancer (CRC) patients. We aimed to identify this hotspot variant in CRC patients and assessed the relationship between the mutation, clinical characteristics, and tumor β-catenin localization. **Materials and Methods:** Formalin-fixed, paraffin-embedded tissue samples of upfront, surgically resected, sporadic colorectal adenocarcinoma cases were selected. The p.G659fs mutation was determined by capillary sequencing with sequence-specific primers. Tissue microarray and immunohistochemistry were employed to analyze nuclear β-catenin expression and the expression of mismatch repair (MMR) proteins, respectively. In addition, clinical details were retrieved from the hospital medical records and data were analyzed. **Results:** The RNF43 p.G659fs mutation was observed in 8% of CRC patients. In total, 25% of tumors showed a loss of immunostaining for one or more MMR proteins and 14.6% of tumors showed positive nuclear β-catenin staining. The p.G659fs variant was significantly enriched in MMR-deficient tumors (P = 0.04). Importantly, no correlation was observed between the variant and nuclear β-catenin localization (P = 0.48), indicating a Wnt-independent role of this variant in CRC tumors. **Conclusions:** To the best of our knowledge, this is the first study from North India to show the involvement of RNF43 p.G659fs variant in CRC patients. The mutation correlated with MMR protein deficiency and seems to be conferring tumorigenicity independent of the Wnt pathway.

Keywords: *Colorectal adenocarcinoma, microsatellite instability, ring finger 43 gene mutations, Wnt signaling, β-catenin*

Introduction

The tumor suppressor gene Ring Finger 43 (*RNF43*) encodes a RING-type E3 ubiquitin ligase that ubiquitinates the frizzled receptors and targets them for endocytosis and lysosomal degradation. *RNF43* is a direct Wnt target gene, and it negatively regulates Wnt/β-catenin signaling. Loss-of-function mutations in *RNF43* activate Wnt signaling by increasing the frizzled receptors on the cell surface, which results in stabilization and subsequent abundance of β-catenin in the cytoplasm. The accumulated β-catenin translocates to the nucleus, complexes with the lymphoid enhancer factor/T-cell factor DNA-binding transcription factors and activates target genes involved in cell proliferation. Inactivating mutations in *RNF43* lead to constitutive activation of the Wnt pathway and tumorigenesis.[1,2]

Mutations in *RNF43* have been reported in many solid tumors, including colorectal cancer (CRC), endometrial, pancreatic, ovarian, and stomach cancer.[3-7] Among the various genetic aberrations in *RNF43*, the frameshift mutation p.G659fs (c.1976delC) is the most recurrent hot-spot variant that results in a truncated protein.[6,8] It involves a single nucleotide deletion/insertion in the $(G)_7$ homopolymeric tract, around RNF43 codon 659, that changes the reading frame to add a stop codon after 41 amino acids. There are contradictory reports on this RNF43-truncating variant in Wnt regulation. Some studies suggest that the RNF43 p.G659fs mutation is fully capable of attenuating Wnt signaling and gives this variant a passenger status, likely resulting from error-prone replication of the $(G)_7$ repeat tract in microsatellite-instable tumors.[8,9] However, a recent study by Fang *et al*. shows that the RNF43 p.G659fs variant is able to promote tumor development and has a Wnt-independent oncogenic role.[10]

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To date, the RNF43 p.G659fs variant has not been explored in Indian patients. We aimed to study the occurrence and clinicopathology of CRC patients carrying this variant. Further, we wanted to understand whether the RNF43 p.G659fs mutation has a role in activating the canonical Wnt signaling in CRC tumors.

Materials and Methods

Patient selection and clinicopathological data collection

The study was initiated following Ethical Approval from the Institutional Review Board (RGCIRC/IRB-BHR/64/2021) and carried out in accordance with the Declaration of Helsinki. Since the study was retrospective and used archived formalin-fixed, paraffin-embedded (FFPE) tissue blocks, a waiver of informed consent was granted by the Board.

The FFPE tumor tissue blocks of 50 treatment naïve CRC patients who underwent surgical resection between 2012 and 2017 were included in this study. We selected consecutive CRC cases with adenocarcinoma histology and no hereditary history of cancer. Patients with multiple cancers or suffering from inflammatory bowel disease were excluded from the study.

Pathological information, clinical stage (staged according to the 7th edition guidelines of the American Joint Committee on Cancer)[11] and treatment details were retrieved from the hospital medical records. Patient follow-up was maintained until February 20, 2023, by referring to hospital visits or through telephonic contact. The patients belonged to northern parts of India including Delhi, Haryana, Punjab, Uttarakhand, and Jammu and Kashmir.

Tissue sectioning, staining, and Sanger sequencing

As described previously,[12] the FFPE blocks were sectioned, Hematoxyin and Eosin (H and E) stained, and the tumor area was manually macrodissected. Total RNA was extracted using the Promega ReliaPrep FFPE Total RNA kit (Promega, USA) following the manufacturer's guidelines, and quantitated on Qubit® 3.0 Fluorometer (Invitrogen Life Technologies, USA). cDNA was synthesized using the SuperScript VILO cDNA synthesis kit (ThermoFisher Scientific, USA) and polymerase chain reaction was carried out with Thermo Scientific (PCR) Mastermix (K0171) on the Bio-Rad C1000 Touch Thermal Cycler with Exon 9 sequence-specific *RNF43* (p.G659fs) primers 5'-CCAGTACCAGCAGTCTGTTCAACTT-3' (sense) and 5'-TGGGGACCAAGGATATGCCACACT-3' (antisense) to amplify 180 bp product.[13] The thermocycling conditions were 95°C for 2 min (initial denaturation 1 cycle), 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min (denaturation, annealing, and extension repeated for 35 cycles), and 72°C for 8 min (final extension 1 cycle). ExoSAP-IT Express PCR Product Cleanup (ThermoFisher Scientific) was used to purify the amplified product, followed by cycle sequencing (Big Dye Terminator version 3.1 sequencing kit, ThermoFisher Scientific), and bead purification (BigDye XTerminator Purification Kit, ThermoFisher Scientific). The protocol followed for each step was according to the manufacturers' insert. The product was sequenced by capillary electrophoresis on SeqStudio Genetic Analyser (Applied Biosystems). Sequencing chromatograms were viewed on FinchTV software (https://digitalworldbiology.com/FinchTV) and analyzed using the Indigo: Rapid InDel Discovery web tool (https://www.gear-genomics.com/indigo).[14]

Tissue microarray and immunohistochemistry

Tissue microarrays were constructed from the FFPE blocks of the selected patients. Briefly, the representative tumor region on the corresponding H and E slide was overlaid on the donor block. The marked area was punched out using a Quick-Ray Manual Tissue Microarrayer and inserted into the recipient block. In one recipient block, about 25 cores were arrayed, thereafter, heated at 60°C for 10 min, cooled, sectioned, and stained with anti-β-catenin antibody along with chromogen diaminobenzidine. Two experienced pathologists (AM and DK) evaluated the nuclear β-catenin and determined the expression positive when the staining intensity was moderate or strong.

The expression of the mismatch repair (MMR) proteins mutL homolog 1 (MLH1), mutS homolog 2, mutS homolog 6, and postmeiotic segregation (PMS2) increased was evaluated in all the FFPE samples by an immunohistochemistry marker panel as described previously.[15] Automated staining was performed on VENTANA BenchMark XT following the standard laboratory protocol. The MMR protein expression was interpreted by an experienced pathologist (AM). Tumors that showed a loss of staining in one or more MMR proteins were classified as MMR deficient. Tumors with intact nuclear expression of all four MMR proteins were considered MMR proficient.

Data analysis

The data were summarized using descriptive statistics. Pearson's Chi-square test or Fisher's exact test was applied to determine the association between RNF43 p.G659fs mutation status and clinicopathological variables. Overall survival was defined as the time from the date of diagnosis until death due to any cause or last contact. Survival analysis was performed by the Kaplan–Meier method, and the difference between the outcomes was assessed by the Log-Rank test. A $P < 0.05$ was considered statistically significant. All the statistical analyses were performed using IBM SPSS® version 23.0 (SPSS Inc., Chicago, IL, USA).

Results

Of the fifty CRC cases selected, forty-eight FFPE samples were of adequate quality and were further studied.

The clinicopathological features of the study group are tabulated in Table 1. Majority were male (68.7%), colon cancer (83.3%), and Stage II (45.8%) cases with moderate tumor differentiation (83.3%). After surgical resection, 20 cases (41.7%) were treated by adjuvant chemotherapy (FOLFOX/FOLFIRI regimen) and/or

Table 1: Clinicopathological features according to ring finger 43 p.G659fs mutation status (*n*=48, colorectal cancer cases)

Variables	Total (*n*=48), *n* (%)	RNF43-659 wild (*n*=44), *n* (%)	RNF43-659 mutant (*n*=4), *n* (%)	*P*
Age (years), median (range)	57 (20–77)	56 (20–74)	65 (45–77)	
≤50	18 (37.5)	17 (38.6)	1 (25)	0.52
>50	30 (62.5)	27 (61.4)	3 (75)	
Gender				
Male	33 (68.7)	29 (65.9)	4 (100)	0.29
Female	15 (31.3)	15 (34.1)	0	
Tumor location				
Colon	40 (83.3)	37 (84.1)	3 (75)	0.53
Rectum	8 (16.7)	7 (15.9)	1 (25)	
Tumor laterality[#]				
Left-sided	22 (45.8)	21 (47.7)	1 (25)	0.61
Right-sided	26 (54.2)	23 (52.3)	3 (75)	
Anatomic site of primary tumor				
Cecum	4 (8.3)	4 (9.1)	0	0.85
Ascending colon	11 (22.9)	9 (20.5)	2 (50)	
Hepatic flexure	5 (10.4)	4 (9.1)	1 (25)	
Transverse colon	6 (12.5)	6 (13.6)	0	
Splenic flexure	3 (6.3)	3 (6.8)	0	
Descending colon	3 (6.3)	3 (6.8)	0	
Sigmoid colon	8 (16.7)	8 (18.2)	0	
Rectum	8 (16.7)	7 (15.9)	1 (25)	
Tumor differentiation				
Well	3 (6.3)	2 (4.5)	1 (25)	0.12
Moderate	40 (83.3)	38 (86.4)	2 (50)	
Poor	5 (10.4)	4 (9.1)	1 (25)	
Tumor stage				
I	5 (10.4)	5 (11.4)	0	0.24
II	22 (45.8)	18 (40.9)	4 (100)	
III	11 (22.9)	11 (25)	0	
IV	10 (20.8)	10 (22.7)	0	
Metastasis*				
Absent	38 (79.2)	34 (77.3)	4 (100)	0.57
Present	10 (20.8)	10 (22.7)	0	
Nuclear β-catenin expression				
Positive	7 (14.6)	6 (13.6)	1 (25)	0.48
Negative	41 (85.4)	38 (86.4)	3 (75)	
MMR protein IHC				
MMR proficient	36 (75)	35 (79.5)	1 (25)	0.04
MMR deficient	12 (25)	9 (20.5)	3 (75)	
Comorbidity[¥]				
Absent	21 (43.7)	20 (45.5)	1 (25)	0.62
Present	27 (56.3)	24 (54.5)	3 (75)	
Treatment details				
Surgery	28 (58.3)	25 (56.8)	3 (75)	0.70
Surgery and chemotherapy	18 (37.5)	17 (38.6)	1 (25)	
Surgery and radiotherapy	1 (2.1)	1 (2.3)	0	
Surgery, chemotherapy and radiotherapy	1 (2.1)	1 (2.3)	0	

*The metastatic site includes liver, lung, or peritoneum; [#]Left-sided: Distal CRC tumors located in splenic flexure, descending colon, sigmoid colon, or rectum. Right sided: Proximal CRC tumors located in the cecum, ascending colon, hepatic flexure, or transverse colon; [¥]Comorbid conditions include COPD, diabetes mellitus, hypertension, hypothyroidism, or seizures in CRC patients. MMR: Mismatch repair; CRC: Colorectal cancer; COPD: Chronic obstructive pulmonary disease; IHC: Immunohistochemistry; RNF43: Ring finger 43

radiotherapy. Ten patients (20.8%) had metastatic disease. Four (8%) cases harbored the truncating RNF43 p.G659fs mutation [Table 1 and Figure 1a and b]. Altogether, 12 (25%) tumors showed a loss of immunostaining for one or more MMR proteins [Table 1] and 7 (14.6%) tumors showed positive nuclear β-catenin staining [Table 1 and Figure 1c and d]. Importantly, no association was found between the RNF43 p.G659fs mutation and nuclear β-catenin expression (P = 0.48) [Table 1]. This mutation was observed to be significantly associated with MMR protein-deficient tumors (75% vs. 20.5%, P = 0.04). The individual expression of the four MMR proteins in the RNF43 659 mutant tumors or those showing positive nuclear β-catenin staining is summarized in Table 2. The co-loss of PMS2 and MLH1 was found in two of the four RNF43 p.G659fs-mutated cases. Among the seven patients who showed positive nuclear β-catenin expression, 4 (57.1%) were MMR deficient [Table 2].

Next, we assessed the impact of the mutant genotype on the overall survival of the patients. Three of four patients harboring RNF43 p.G659fs mutation were alive and doing well on the last date of follow-up and one case was lost to follow-up. The overall survival of the cohort was 53.7% at 106 months. The survival outcome of the RNF 659 mutant group was better than the RNF 659 wild-type group, but the difference failed to achieve statistical significance ($P_{Log\text{-}Rank}$ = 0.137) [Figure 1e].

Discussion

The Wnt signaling pathway is frequently activated in sporadic CRCs. RNF43 is expressed in the intestinal

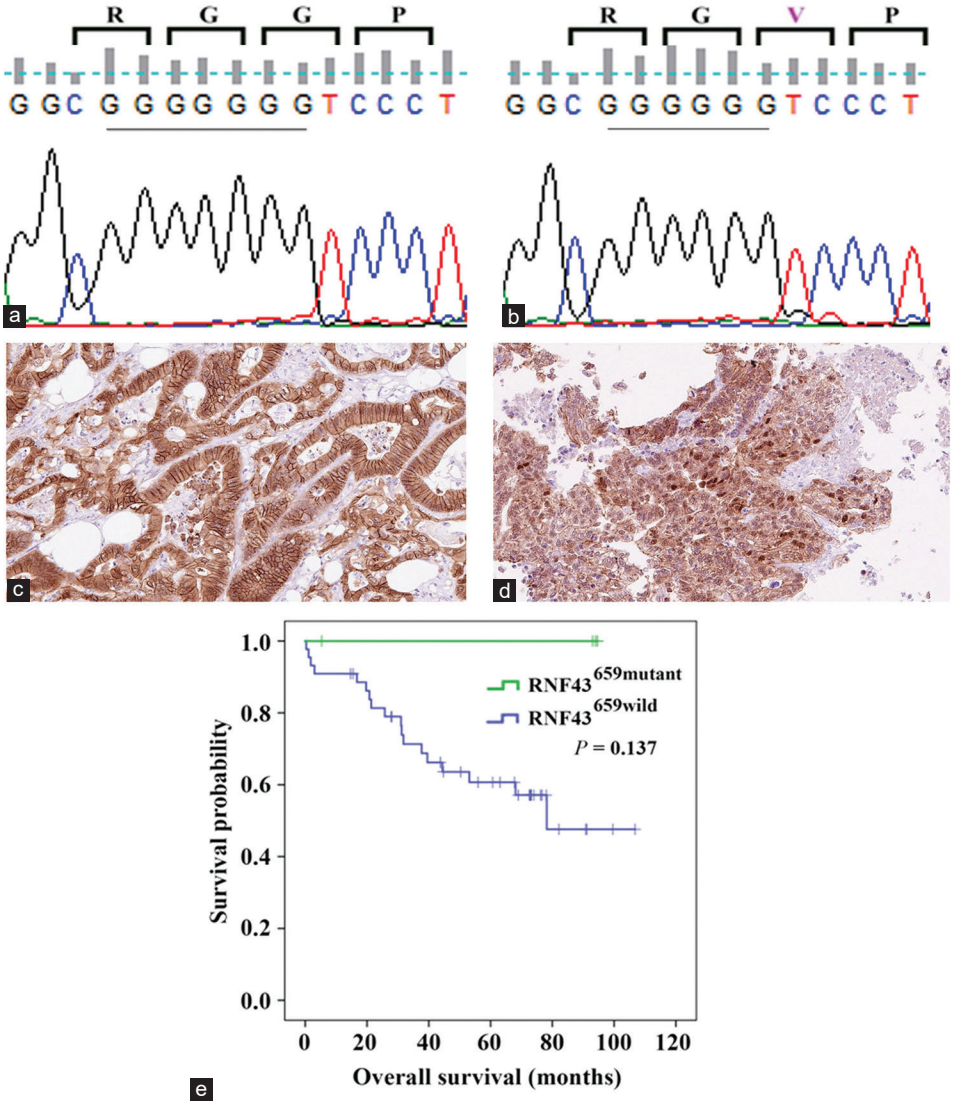

Figure 1: Ring finger 43 (RNF43)-659 mutation, β-catenin expression and patient survival. Representative sequencing chromatogram of (a) RNF43-659 wild (b) RNF43-659 mutant harboring a single base (G) deletion in $(G)_7$ tract. Immunohistochemical staining for β-catenin showing (c) absence (d) positive nuclear expression (×20) (e) Kaplan–Meier survival curve showing overall survival according to RNF43 p.G659fs mutation status (n = 48, colorectal cancer patients). RNF43: Ring finger 43

Table 2: Mismatch repair protein status of the patients harboring ring finger 43 p.G659V mutation or showing nuclear β-catenin expression on tissue microarray (*n*=10, colorectal cancer cases)

Case	RNF43 p.G659fs mutation status	Nuclear β-catenin expression	MLH1	MSH2	MSH6	PMS2	MMR status
1	Mutated	Negative	Loss	Intact	Intact	Loss	Deficient
2	Mutated	Negative	Loss	Intact	Intact	Loss	Deficient
3	Mutated	Negative	Intact	Intact	Intact	Intact	Proficient
4	Mutated	Positive	Intact	Loss	Loss	Intact	Deficient
5	Wild	Positive	Intact	Loss	Loss	Intact	Deficient
6	Wild	Positive	Intact	Intact	Intact	Loss	Deficient
7	Wild	Positive	Intact	Intact	Intact	Intact	Proficient
8	Wild	Positive	Intact	Intact	Intact	Intact	Proficient
9	Wild	Positive	Intact	Intact	Intact	Intact	Proficient
10	Wild	Positive	Loss	Intact	Loss	Intact	Deficient

RNF43: Ring finger 43; MMR: Mismatch repair; MLH1: Mutl homolog 1; PMS2: Postmeiotic segregation increased; MSH2: MutS homolog 2; MSH6: MutS homolog 6

epithelia, where, along with its paralog zinc and ring finger 3, it represses the Wnt signaling and cell proliferation. Inactivating mutations in *RNF43* prevent the degradation of the frizzled receptors, which causes hyperactive Wnt signaling and promotes colorectal adenoma development. Recently, various mutations in *RNF43* have gained recognition because tumors carrying these mutations are sensitive to anti-frizzled blocking antibodies or porcupine inhibitors and are clinically actionable.[16] Variants in *RNF43* gene are diverse and functionally heterogeneous depending on the mutation type and genomic location.[17,18] The p.G659fs mutation is a recurrent, C-terminal truncating RNF43 variant and its role in activating Wnt signaling is not well understood. In this study, we have gained insights into the RNF43 p.G659fs mutation in a sporadic CRC patient cohort.

First, about 8% of the patients carried the truncating p.G659fs mutation. The rate is similar to earlier studies that reported the frequency of this variant at around 8%.[6,10] The frequency is more than expected by mere chance alone and it seems that the mutation is getting positively selected during tumorogenesis, possibly conferring a fitness advantage to the tumor.[6,10] The mutation was enriched in MMR-deficient tumors, which is consistent with previous reports that show a strong association between microsatellite instability and the RNF43 p.G659fs mutation.[6,13,19] MMR proteins are nuclear enzymes that correct mismatched bases that arise during replication in proliferating cells by binding to the incorrect base and facilitating its removal. A loss of MMR proteins introduces replication errors, mostly in the microsatellites region.[15,19,20] The seven G tandem repeats involving codon 659 of the *RNF43* gene make this region highly error-prone and more reliant on proficient MMR protein functioning, and so, as anticipated, we observed a greater frequency of the p.G659fs alteration in tumors showing loss in MMR protein staining. In our study, a co-loss of MLH1 and PMS2 was observed in two of the four patients harboring the p.G659fs variant. Since MLH1 stabilizes PMS2 by forming a heterodimer complex, a co-loss of MLH1 and PMS2 indicates a defect in MLH1.[21]

Second, we found no relation between the mutation and the patient's age, gender, tumor sidedness, tumor differentiation, or stage. Previous studies have associated RNF43 p.G659fs mutations with right-sided CRC and an aggressive phenotype.[19,22,23] In our study, three of the four mutant cases had right-side tumor location and favorable survival outcomes. Ethnic disparities and differences in the patient inclusion criteria, as well as the small sample size of our study, could be possible reasons for these discrepancies in results. In our cohort, all the RNF43 659 mutated cases had Stage II disease and therefore had a relatively indolent disease course.

Third, if the RNF43 p.G659fs mutation had a role in activating canonical Wnt signaling, then we would have expected more stabilization and nuclear β-catenin staining. However, in our study, we found no correlation between tumors harboring the p.G659fs mutation and high nuclear β-catenin accumulation, so it seems unlikely that this mutation has a major role in activating the canonical Wnt signaling. Our results are in agreement with former immunohistochemistry-based studies by Siraj *et al.*, and Elez *et al*., which reported no correlation between nuclear β-catenin expression levels and the RNF43 p.G659fs mutation.[19,24] A functional study by Tu *et al*. further confirms that the RNF43 p.G659fs truncating variant is able to inhibit canonical Wnt signaling completely.[8] Supportingly, a study by Cho *et al.* shows that the RNF43 p.G659V variant retains the ability to suppress Wnt signaling.[25] Adding to the study, Fang *et al*. have shown that the RNF43 659 mutant is able to suppress Wnt signaling and potentially confers tumorigenicity independent of Wnt regulation. According to the authors, the mutant protein degrades the regulatory subunit p85 and stimulates PI3 kinase signaling, which contributes to CRC carcinogenesis.[10] Another possibility is that the RNF43 p.G659fs mutation could be involved in activating the β-catenin independent, noncanonical Wnt pathway in CRC.[26] Future studies on the effect of this variant

on noncanonical Wnt regulation may shed light on this notion.

The limitations of this study are the small sample size and the lack of information on the mutation status of other genes that are associated with CRC.

Conclusion

Taken together, we conclude that the RNF43 p.G659fs mutation is involved in our CRC patient cohort and merits further investigation in CRC pathogenesis.

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Conflicts of interest

There are no conflicts of interest.

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