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Mutational Alterations of DNA Methylation-related Genes *CTCF, ZFP57,* and *ATF7IP* Genes in Colon Cancers

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Abstract: Deregulations of DNA-methylation-related genes are common in cancers, but frameshift mutation status in colon cancer (CC) is unknown. Our study aims to assess whether *CTCF, ZFP57,* and *ATF7IP* genes in this category are mutated in CC. *CTCF, ZFP57,* and *ATF7IP* genes have repeat coding sequences, which are frequently deleted or duplicated in CC, harboring the phenotype of unstable or high microsatellite instability (MSI-H). We studied 140 CCs [95 MSI-H CCs and 45 stable MSI (MSS) CCs], and found 7 CCs with MSI-H (6/95: 6.3%) harbored frameshift mutations within the repeats, whereas those with MSS did not. Of note, the *CTCF* frameshift mutations showed the regional difference in the 2 (12.5%) of 16 MSI-H CCs, indicating there was intratumoral heterogeneity. In the immunohistochemistry for ATF7IP, the MSI-H CC showed low intensity compared to MSS CC. Together, *CTCF, ZFP57,* and *ATF7IP* genes, despite the low incidence of the mutations, are altered in several ways (mutation, expression, and intratumoral heterogeneity) and could contribute to MSI-H CC development.

Key Words: *CTCF, ZFP57, ATF7IP,* mutation, colon neoplasm

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D NA methylation is a common epigenetic regulation tool for eukaryotic gene expression.[1] DNA methylation directly hinders transcription factor binding or regulates gene transcription through methyl-binding proteins (MBPs) that attract chromatin remodeling complex for transcriptional regulation.[1–3] MBPs function as readers of the epigenome and MBP mutations can lead to loss of methylation-associated gene repression. When mutations within methyl binding domains (MBDs) occur, it leads to loss of MBD binding specificity to methylated sites, gene deregulation, and therefore causing diseases, including cancer.[1–3] The MBPs are classified into methyl-CpG-binding, MBD-containing, and SRA domain-containing proteins.[1–3] Alterations of DNA methylation are frequently found in cancer.[4–7] The methyl-CpG-binding proteins included 8 genes, including *CTCF* and *ZFP57* genes.[8] ZFP57 expression enhances tumor growth in high-grade glioblastomas.[1,9] The CTCF is known to possess either tumor suppressor gene or oncogene activity in the cancer type context.[10,11] Expression of the *CTCF* gene is increased and associated with poor outcomes in ovarian cancer patients,[10] while its depletion decreases the proliferation of breast cancer cells.[11] ATF7IP, another methylation-related protein, interacts with MBD1 and performs transcriptional repression and heterochromatin formation.[12] ATF7IP also increases for many cancers and is necessary for cancer proliferation by activating telomerase gene transcription.[12] Depletion of cellular ATF7IP promotes premature induction of senescence with cell cycle arrest.[13] However, alterations of *CTCF, ZFP57,* and *ATF7IP* genes in gastrointestinal cancers remain unexplored.

DNA mismatch repair is a system for repairing erroneous insertion, deletion, and misincorporation of bases that can arise during DNA replication and recombination. Repairing the mismatches is essential for cells because failure to do so results in microsatellite instability (MSI) and an elevated spontaneous mutation rate (mutator phenotype).[14] In sporadic CC, somatic mutations frequently occur in the mismatch repair pathway, producing frameshift mutations within repetitive DNA sequences in the genes that would truncate the affected protein synthesis.[14] *CTCF* has a poly-adenine repeat (A7), *ZFP57* has a poly-thymine repeat (T7), and *ATF7IP* has a poly-adenine repeat (A7) in the coding exons, which are potential mutation targets in CCs. In this study, we analyzed *CTCF, ZFP57,* and *ATF7IP* genes to detect somatic mutations and their intratumoral heterogeneity (ITH) in CC. We also studied the ATF7IP expression by immunohistochemistry.

MATERIALS AND METHODS

Tumor Tissues

We used archival patient specimens previously fixed in 10% formalin-fixed and paraffin-embedded tissues of 140 CCs [95 CCs with high MSI (MSI-H) and 45 CCs with

microsatellite stable (MSS) phenotypes] (Supplementary Table 1, Supplemental Digital Content 1, http://links.lww.com/AIMM/A323). The MSI-H CCs are overrepresented compared with the prevalence of the ratio for MSS and MSI-H CCs. We evaluated the MSI using 5 mono-nucleotide repeats (BAT25, BAT26, NR-21, NR-24, and MONO-27) according to the earlier documentation.[14] From the formalin-fixed and paraffin-embedded tissues, cancer and normal cells were microdissected as described previously.[15,16]

Single Strand Conformation Polymorphism (SSCP) and Sequencing Analyses

We focused the mutation analyses on the coding sequences in the A7 in *CTCF*, T7 in *ZFP57*, and A7 in *ATF7IP*. They were amplified by polymerase chain reaction (PCR), visualized on SSCP gels, and sequenced by Sanger DNA sequencing as described previously.[15,16] Genomic DNA was amplified by PCR with primers (*CTCF* gene 5′-CTGTCTGTGCTCTTCTTTGCC-3′ and 5′-TGGGCCTCCTCTTCCTCT-3′, *ZFP57* gene 5′-TGGGACTATGGACAGGACCC-3′ and 5′-CAGCTTGGGATTGTGAAC, *ATF7IP* gene 5′-AAAGTCTGTACCAGTTTGTG-3′ and 5′-ATCCAATGAGTCTTTACTCTG-3′). [^{32}P]dCTP was incorporated to the PCR products for visualization in autoradiogram. We determined aberrant gel motility in the SSCP (FMC Mutation Detection Enhancement system; Intermountain Scientific, Kaysville, UT) using visual inspection, which subsequently sequenced by Sanger DNA sequencing (3730 DNA Analyzer, Applied Biosystem, Carlsbad, CA). Among the 140 CCs, multiregional acquisition of samples was performed for 39 CCs (16 MSI-H and 23 MSS CCs) to study the ITH, while a single-regional study was for 101 CCs (79 MSI-H and 22 MSS CCs). For the 39 CCs, we analyzed the *CTCF*, *ZFP57*, and *ATF7IP* frameshift mutations in 4 to 7 different areas per tumor. All the samples were collected anonymously and were waived the need for informed consent.

Immunohistochemistry

For the expression, the ATF7IP immunohistochemistry with anti-ATF7IP antibody (Atlas Antibodies, Stockholm, Sweden; 1/400 dilution) was performed as described in our earlier studies using ImmPRESS System (Vector Laboratories, Burlingame, CA).[16] After deparaffinization, heat-induced epitope retrieval was conducted by immersing the slides in Coplin jars filled with 10 mmol/L citrate buffer (pH 6.0) and boiling the buffer for 30 minutes in a pressure cooker (Nordic Ware, Minneapolis, MN) inside a microwave oven at 700 W; the jars were then cooled for 20 minutes. The chromogen for immunohistochemistry was diaminobenzidine (brown) with hematoxylin counterstaining (blue). The resulting intensity was scored as follows: 0, negative; 1+, weak staining in nucleus; 2+, moderate; and 3+, intense. The extent was scored as follows: 0, 0% t0 10%; 1, 10% to 39%; 2, 40% to 69%; 3, >70% positivity of cells. Immunohistochemistry score (IS) was calculated by multiplication of the intensity and extent scores (IS 0 or1 as negative, 2 or 3 or 4 as +, and 6 or 9 as ++). Replacement of primary antibody with the blocking reagent was a negative control of the immunostaining.

Statistical Analysis

The Fisher exact test was used to analyze the statistical analysis of the mutation and immunohistochemical data (http://www.physics.csbsju.edu/stats/exact.html). The level of significance was set at $P < 0.05$.

RESULTS

Somatic Mutations

Through the PCR-SSCP, we found 6 frameshift mutations (duplication or deletion within the repeats) in CCs (*CTCF*: 2 CCs, *ZFP57*: 1 CC, and *ATF7IP*: 3 CCs) (Table 1). We detected the variants in the CCs, but not in corresponding normal tissues and interpreted them as somatic variants. All 6 CCs with the mutations were MSI-H CCs (6/95, 6.3%), but CCs with MSS phenotype did not have the mutations (0/45, 0%). DNA sequences for the mutations display wild-type sequences and the mutation sequences (heterozygous mutations; Fig. 1). The 6 CCs with the frameshift mutations were negative for MLH1 immunostainings. Besides, we analyzed 39 CCs with multiregion sampling (16 MSI-H and 23 MSS CCs). We found that 2 (12.5%) CCs with MSH-H had regional differences in the frameshift mutation of *CTCF* (c.1086delA mutation). The 23 CCs with MSS did not show the frameshift mutations in any of the multiregions. However, we did not find any statistical difference either in patients' clinical outcomes between the cases with and without the ITH (Fisher exact test, $P > 0.05$).

Immunohistochemistry

Protein expression of ATF7IP was analyzed by immunohistochemistry (Fig. 2). ATF7IP is well stained in normal colonic epithelial cells, by the immunohistochemistry

TABLE 1. Summary of *CTCF, ZFP57* and *ATF7IP* Mutations in Colon Cancers

Gene	Wild Type	Mutation	MSI Status of the Mutation Cases (n)	Incidence in MSI-H Colon Cancers, n/N (%)	Nucleotide Change (Predicted Amino Acid Change)
ATF7IP	A7	A6	MSI-H (1)	1/76 (1.3)	c.959delA (p.Asn320MetfsX7)
		A8	MSI-H (2)	2/76 (2.6)	c.959dupA (p.Asn320LysfsX4)
CTCF	A7	A6	MSI-H (2)	2/76 (2.6)	c.1086delA (p.Glu363SerfsX30)
ZFP57	T7	T6	MSI-H (1)	1/76 (1.3)	c.313delT (p.Cys105AlafsX25)

MSI indicates microsatellite instability; MSI-H, high MSI.

(Figs. 2A–C). In the CCs, the MSS (39/45, 86.7%) and MSI-H (70/95, 73.7%) CCs exhibited positive immunostaining (+ or ++) with no statistically different prevalence between MSS and MSI-H cases (Fisher exact test, *P* = 0.062) (Figs. 2A–E). The IS of the positive cases, however, was different between MSS and MSI-H cases (MSI-H: ++ in 49 CCs, + in 21 CCs; MSS: ++ in 36 CCs, + in 3 CCs) (Fisher exact test, *P* = 0.004). Importantly, all the MSI-H CCs harboring the *ATF7IP* mutations displayed either − (n = 1) or + (n = 2) (Figs. 2F, E). Lymphoid cells were well stained as an internal control (Fig. 2), while the negative control showed no immunostainings in the cells. There was no significant difference in patients' clinical outcomes between negative (n = 31) and positive (n = 109) CCs (Fisher exact test, *P* > 0.05). The positive staining was in the nucleus (Fig. 2), in which ATF7IP protein functions the roles for transcription and heterochromatin formation.[12,13]

DISCUSSION

Currently, the epigenetic pathway is considered a target for cancer therapy, which needs to study more alterations for the target development. In this analysis, we discovered frameshift mutations of DNA methylation-associated genes *CTCF*, *ZFP57*, and *ATF7IP* in MSI-H CCs. The *CTCF* frameshift mutation shows ITH well; and ATF7IP immunostaining in MSI-H CCs is scanty compared with MSS CCs, suggesting that alterations of DNA methylation-associated genes occur by various mechanisms in CCs.

At present, ATF7IP is known to have pro-cancer activities such as cell proliferation in many cancers.[12,13,17] However, its biological significance remains largely unknown in CCs. The frameshift mutation typically exhibits loss-of-function for the affected genes. The *ATF7IP* frameshift mutations would produce premature stop codons, resulting in truncated ATF7IPs, and leading wild-type ATF7IP to an inactive form. Our observation in MSI-H CCs is not in agreement with the oncogenic activities of ATF7IP in other cancers.[12,13,17] The MSI-H CCs showed decreased ATF7IP immunostaining and increased *ATF7IP* frameshift mutation compared with MSS CCs. ATF7IP in MSI-H CCs might have different activities from those in MSS CCs. However, it remains unclear that ATF7IP has a causative role in MSI-H CC, or it is the result that the MSI-H CC has increased mutation frequency. All of the 6 cases with the frameshift mutations revealed MLH1 protein loss in the cancer cells, suggesting that the latter hypothesis was most likely to be the cases. Decreased ATF7IP in the nonframeshift MSI-H tumors were also identified, suggesting that ATF7IP expression is affected not only by frameshift mutations, but also by other mechanisms such as transcriptional and epigenomic regulation. CTCF exhibits cancer-promoting activity in glioblastoma and cancer-suppressing activity in breast cancer.[10,11] *CTCF* frameshift mutation in MSI-H CCs, but not in MSS CCs, suggests that *CTCF* alteration might be different even in the CC concerning the MSI status.

The epitope of ATF7IP antibody adopted for our study is amino acids 320-519 (NP_060649). As the mutations would delete this area in the mutants (p.Asn320-MetfsX7 and p.Asn320LysfsX4), the truncated ATF7IP protein might not be immunostained in the CCs. Heterozygous *ATF7IP* mutations (Fig. 1) suggest that the second (wild-type) alleles might be intact. Negative (−) or mildly positive (+) ATF7IP immunostaining in *ATF7IP*-mutated CCs seemed to result from a deleted allele.

Our data show that a regional difference in *CTCF* frameshift mutation is evident in MSI-H CC. ITH in cancer is a potential mechanism of therapeutic resistance and therefore, a significant clinical challenge.[18] Although not common, the ITH identified in our study could further
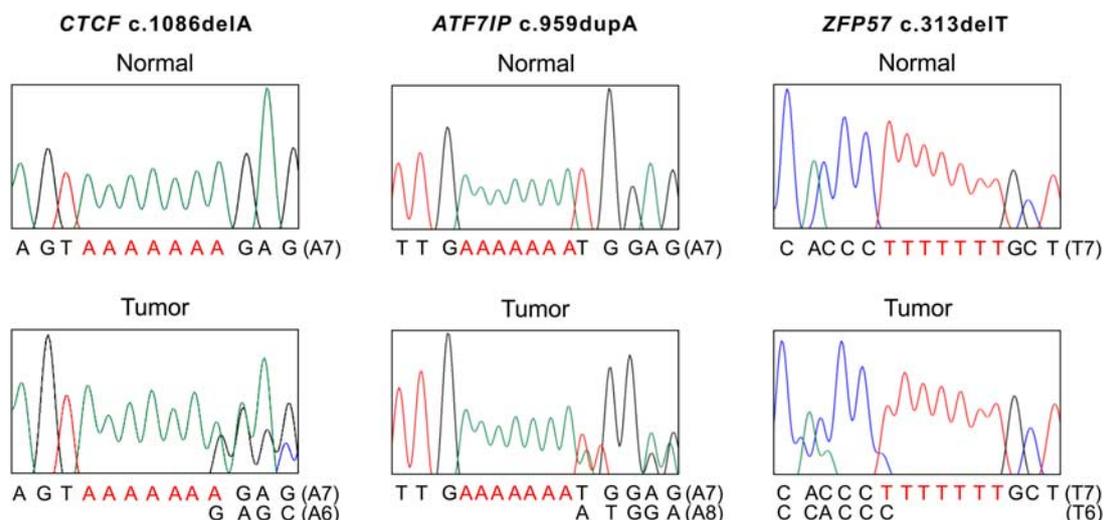
FIGURE 1. DNA sequencings of *CTCF*, *ZFP57*, and *ATF7IP* mutations in colon cancers. DNA sequencing analyses of the A7 repeat of *CTCF* (left), the A7 repeat of *ATF7IP* (middle), and the T7 repeat of *ZFP57* (right) from normal (upper) and tumor tissues (lower). Sanger DNA sequencing analyses reveal heterozygous deletion or duplication of a base within the repeats in the tumor tissues as compared with normal tissues.
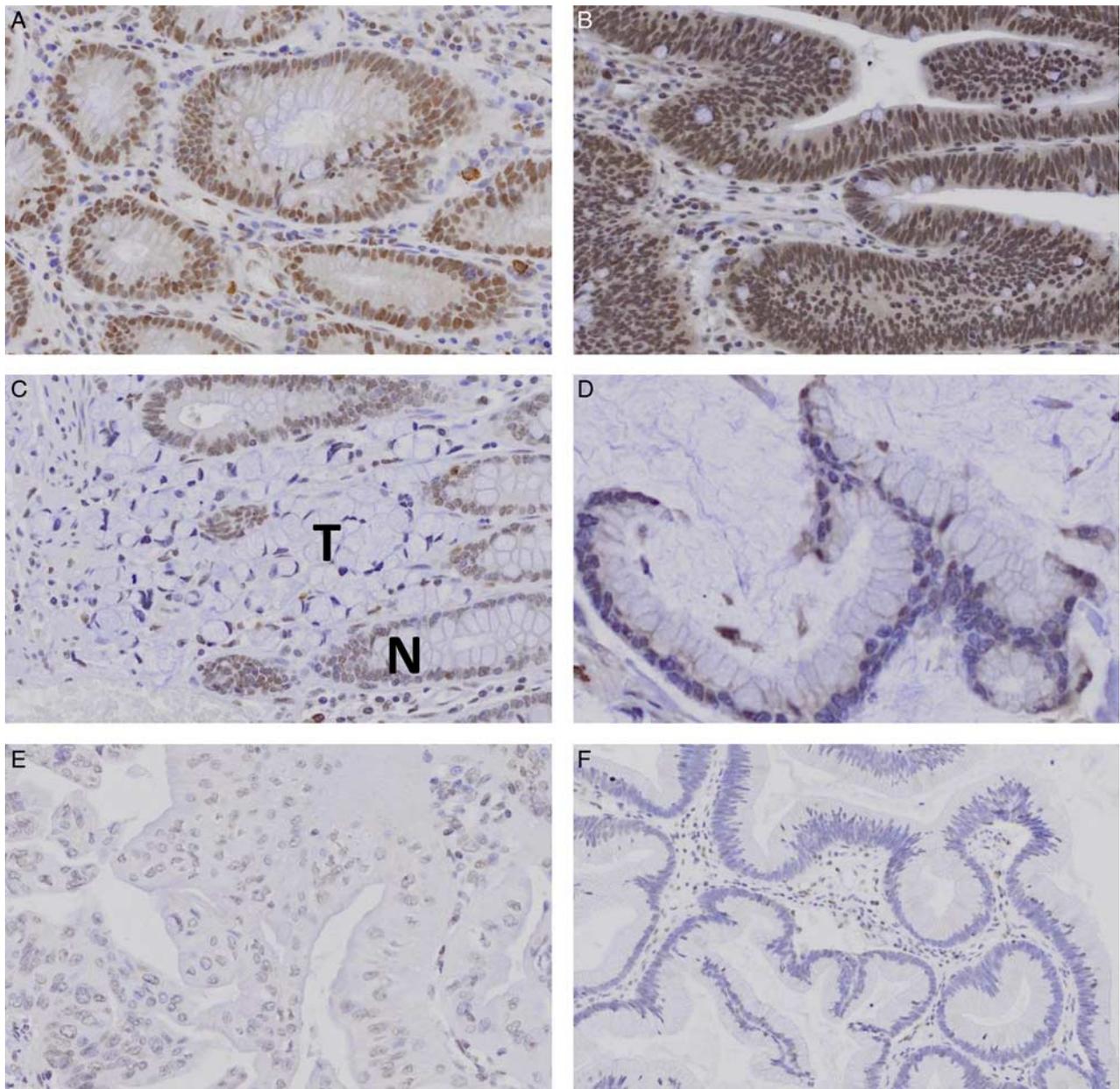
FIGURE 2. ATF7IP expression in colon cancers. Normal colonic mucosal epithelial cells show positive ATF7IP immunostaining (A, C). Colon cancer cells show strong (A, B) or mild (D, E) positive ATF7IP immunostaining. In some colon cancers (C, F), cancer cells show negative ATF7IP immunostaining. N indicates normal colon epithelial cells; T, colon cancer cells.

change the *CTCF* gene alteration. Our previous reports also indicated that MSI-H CCs might be prone to harbor mutational ITH.[15,19] DNA methylation-related genes are overexpressed in many cancers, and manipulation of their activities would be helpful to anti-cancer therapies.[1,4,6] In this sense, our findings on genetic and expression alterations of DNA methylation-related genes in MSI-H CCs may provide useful information.

REFERENCES

1. Allis CD, Jenuwein T. The molecular hallmarks of epigenetic control. *Nat Rev Genet*. 2016;17:487–500.
2. Hake SB, Xiao A, Allis CD. Linking the epigenetic "language" of covalent histone modifications to cancer. *Br J Cancer*. 2007;96(suppl): R31–R39.
3. Klose RJ, Bird AP. Genomic DNA methylation: the mark and its mediators. *Trends Biochem Sci*. 2006;31:89–97.
4. Mahmood N, Rabbani SA. DNA Methylation readers and cancer: mechanistic and therapeutic applications. *Front Oncol*. 2019;9:489.
5. Bachman KE, Park BH, Rhee I, et al. Histone modifications and silencing prior to DNA methylation of a tumor suppressor gene. *Cancer Cell*. 2003;3:89–95.
6. Esteller M. Relevance of DNA methylation in the management of cancer. *Lancet Oncol*. 2003;4:351–358.
7. Laird PW. The power and the promise of DNA methylation markers. *Nat Rev Cancer*. 2003;3:253–266.

8. Hudson NO, Buck-Koehntop BA. Zinc Finger Readers of Methylated DNA. *Molecules*. 2018;23:2555.
9. Cirillo A, Di Salle A, Petillo O, et al. High grade glioblastoma is associated with aberrant expression of ZFP57, a protein involved in gene imprinting, and of CPT1A and CPT1C that regulate fatty acid metabolism. *Cancer Biol Ther*. 2014;15:735–741.
10. Zhao L, Yang Y, Yin S, et al. CTCF promotes epithelial ovarian cancer metastasis by broadly controlling the expression of metastasis-associated genes. *Oncotarget*. 2017;8:62217–62230.
11. Lee JY, Mustafa M, Kim CY, et al. Depletion of CTCF in breast cancer cells selectively induces cancer cell death via p53. *J Cancer*. 2017;8:2124–2131.
12. Liu L, Ishihara K, Ichimura T, et al. MCAF1/AM is involved in Sp1-mediated maintenance of cancer-associated telomerase activity. *J Biol Chem*. 2009;284:5165–5174.
13. Sasai N, Saitoh N, Saitoh H, et al. The transcriptional cofactor MCAF1/ATF7IP is involved in histone gene expression and cellular senescence. *PLoS One*. 2013;8:e68478.
14. Imai K, Yamamoto H. Carcinogenesis and microsatellite instability: the interrelationship between genetics and epigenetics. *Carcinogenesis*. 2008;29:673–680.
15. Mo HY, An CH, Choi EJ, et al. Somatic mutation and loss of expression of a candidate tumor suppressor gene TET3 in gastric and colorectal cancers. *Pathol Res Pract*. 2020;216:152759.
16. An CH, Je EM, Yoo NJ, et al. Frameshift mutations of cadherin genes DCHS2, CDH10 and CDH24 genes in gastric and colorectal cancers with high microsatellite instability. *Pathol Oncol Res*. 2015;21:181–185.
17. Ichimura T, Watanabe S, Sakamoto Y, et al. Transcriptional repression and heterochromatin formation by MBD1 and MCAF/AM family proteins. *J Biol Chem*. 2005;280:13928–13935.
18. McGranahan N, Swanton C. Biological and therapeutic impact of intratumor heterogeneity in cancer evolution. *Cancer Cell*. 2015;27:15–26.
19. Moon SW, Son HJ, Mo HY, et al. Mutation and expression alterations of histone methylation-related NSD2, KDM2B and SETMAR genes in colon cancers. *Pathol Res Pract*. 2021;219:153354.