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Original Article

High Frequency of HES1 Loss in Colorectal Adenocarcinomas with *RAS/BRAF* Mutations

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Abstract

Background and objectives: Hes1 is the downstream target of the canonical Notch-signaling pathway, which plays an essential role in maintaining intestinal proliferative crypts and regulating enterocyte differentiation. Loss of Hes1 expression is frequently observed in right-sided colon cancers. This study aims to present the relationship between the dysregulated Notch pathway and the status of *RAS* or *BRAF* mutations. ***Methods:*** Forty-three cases of primary colorectal adenocarcinomas were collected in a tertiary teaching hospital. Hes1 expression was assessed by the immunohistochemical stain. The *RAS (KRAS* and *NRAS)* and *APC* status were determined by the next-generation sequencing study. In addition, *BRAF V600E* was tested by PCR-based mutation analysis. ***Results:*** Overall, loss of Hes1 expression was observed more frequently in colorectal cancer specimens with either *RAS* or *BRAF* mutations than in the wild type (78.6% *vs.* 40.0%, $p < 0.05$). All the right-side tumors with *RAS* or *BRAF* mutations showed loss of Hes1 expression (12/12, 100%) ($p < 0.05$), compared to only 62.5% (10/16) of left-sided tumors. In addition, patients with Hes1 loss in tumor tissue were less likely to have immediate metastasis (59.1%, 13/22) compared to those with preserved Hes1 expression (83.3%, 5/6) ($p = 0.37$). ***Conclusion:*** The high frequency of Hes1 loss in colorectal adenocarcinoma is associated with either *RAS* or *BRAF* mutations, suggesting that synergistic effects by dysregulated Notch and *RAS/BRAF* mutation might play a vital role in colon carcinogenesis in some forms, especially the right-sided tumors. This finding might help guide future treatments for a subset of colon cancers.

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Keywords: Notch; Hes1; Colon adenocarcinoma; RAS; BRAF; Pathology.

Abbreviations: CRC, colorectal carcinoma; HES1, Hairy enhancer of split-1; NGS, next generation sequencing; Notch1, Neurogenic locus notch homolog protein 1.

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Introduction

Colorectal cancer (CRC) is a lethal disease. Globally, CRC is the third most commonly diagnosed cancer in males and the second in females. Approximately 151,030 new cases of CRC and 52,580 deaths are reported annually in the United States, accounting for approximately 9% of all cancer deaths.[1]

Molecular studies over the past decades have considerably improved our understanding of CRC carcinogenesis, leading to significant advances in treating CRC by introducing novel chemotherapies and targeted agents. *RAS* and *BRAF* participate in the MAPK-ERK pathway, mediating cellular responses to many extracellular signals regulating programmed cell death, cell growth, and differentiation. *KRAS* mutations, found in up to 50% of sporadic CRCs, result in continuous gene activation, leading to uncontrolled autonomous cell proliferation.[1–3] Previous reports demonstrated that it is more common in proximal (right-sided) colon cancers than in distal (left-sided) colorectal primaries.[4–6] *KRAS* mutations have also been implicated in the process of tumor invasion and metastasis.[7]

BRAF and RAS are components of the MAPK-ERK pathway. The most commonly identified *BRAF* mutation in human CRC is a V600E amino acid substitution, seen in sporadic CRCs with a high degree of microsatellite instability (MSI-H). They are *KRAS* wild-type and appear to abrogate a favorable prognosis.[8,9] *BRAF*-mutated sporadic CRCs with MSI-H are widely considered to develop from serrated polyps, commonly seen in the right-sided colon as *KRAS*-mutated colon cancer, although *KRAS* and *BRAF* mutations are mutually exclusive to each other.

The hairy enhancer of split-1 (HES1) is a downstream target of the Notch signaling pathway, which plays an essential role in promoting cell survival.[10] Activation of the Notch pathway leads to the release of the Notch intracellular domain, which translocates to the nucleus and activates transcription of numerous downstream target genes, including *HES1*, *HES2, HEY1, HEY2, and DTX1*. HES1 is expressed in the nuclei of normal intestinal epithelial cells. It has been associated with maintaining intestinal proliferative crypts and regulating enterocyte differentiation. We reported previously that loss of HES1 expression is frequently observed in the right-sided colon cancers in mice, which commonly harbors *RAS* mutations and *BRAF* mutation.[11] Very recently, Jackstadt *et al.* showed that enforced Notch signaling in the mu-

rine intestinal epithelium leads to highly penetrant metastasis (100% metastasis; with >80% liver metastases) in *KRAS* mutation-driven serrated colon cancer.[10] Based on the above findings, it is exciting to explore the relationship between the dysregulated Notch pathway and the status of *RAS* and *BRAF* mutations in human CRC, which, to our best knowledge, has never been studied and reported.

Materials and methods

Patients and clinicopathological data

This study was approved by the Institutional Review Board of University Hospitals Cleveland Medical Center/Case Western Reserve University. HIPPA waiver was approved as this is a discarded tissue and chart review study with no patient-identifiable data collected or stored after the screening. The protocol conformed to the ethical guidelines of the latest version of the Declaration of Helsinki. A total of 43 patients with primary CRC who underwent tumor resection or biopsy between 2/23/2009 and 5/2/2017 and received molecular workup between 1/1/2016 and 12/31/2017 at the University Hospitals Cleveland Medical Center were enrolled in this study. All specimens were formalin-fixed and paraffin-embedded tissue sections. Hematoxylin and eosin-stained slides were reviewed. All the cases were screened by the junior pathologists and then independently reviewed by two experienced gastrointestinal pathologists. Only the cases with a consensus of CRC diagnosis among three pathologists were included in the study.

Detailed clinicopathological data were retrieved from the respective pathology reports/clinical records, including tumor diagnosis, staging, locations, and patient follow-up status. Survival time was correlated with the medical record chart in the data analysis.

DNA extraction

Formalin-fixed, paraffin-embedded tissues were retrieved for DNA extraction. Unstained slides were accompanied by an H&E-stained slide to allow the identification of cancer cells (>20% viable cancer cell nuclei) by a pathologist. QIAamp FFPE DNA Isolation Kit (Qiagen, Valencia, CA) was used for genomic DNA isolation following the suggested procedures.

RAS (KRAS and NRAS) and APC status evaluation

Gene status of *RAS* (*KRAS* and *NRAS*) and *APC* were determined by the next-generation sequencing (NGS) at University Hospitals Cleveland Medical Center's Clinical Laboratory Improvement Amendments-certified Translational Laboratory (UHTL). Ion AmpliSeqTM Cancer Hotspot Panel v2 (CHPv2) library kit was used to establish DNA library preparation following the standard protocol (Thermo Fisher, Carlsbad, CA) with the amount of 30 ng of genomic DNA. The template amplification and enrichment were prepared using the One Touch 2 system. The OT2 200 sequencing kit was used for the sequencing, which was performed on Ion Torrent PGM with 8–12 samples per 318-Chip.

BRAF V600E status evaluation

The c.1799T>A (V600E) point mutation was detected by the allele-specific hybridization of PCR amplification. The reagents used for the Applied Biosystems 7500 Fast Real-Time PCR System allele-specific Taqman® probes, fluorescently labeled with VIC-(wild-type) or FAM-(V600E mutant). Delta Ct value was calculated as the difference in Ct value between wild-type and mutant *BRAF* allele. Results were interpreted based on the patient delta Ct value of V600E relative to the corresponding 5% sensitivity controls.

Immunohistochemistry (IHC) evaluation of HES1

Protein expression of HES1 was evaluated by IHC on all cases included in the study, as described in our previous paper.[12] The IHC was performed by the Immunohistochemistry Diagnostic Laboratory of University Hospitals Cleveland Medical Center. Tissue slides were processed using a BenchMark Ultra automated immunostainer (Ventana, Tucson, AZ). Slides were deparaffinized, antigen retrieved with standard Cell Conditioning 1 (Ventana Medical Systems, AZ), a tris-based buffer pH 8.3 solution for 64 minutes at 95°C, then incubated at 37°C with the primary antibody HES1 rabbit monoclonal (1:200 dilutions, clone EPR4226 from Epitomics, CA, part of Abcam, Cambridge, MA) for 24 minutes. Detection was performed with Ultraview-DAB from Ventana and subsequently counterstained.

Statistical analysis

Statistical analyses were performed with SPSS 16.0 (SPSS Inc., Chicago, IL). Categorical data were analyzed by the Fisher exact test (2-tailed). Cohen's Kappa (κ) analysis was used to examine the agreement for HES1 loss and RAS/BRAF mutations. p value less than 0.05 was considered statistically significant.

Results

Patients

A total of 43 cases with primary CRC were enrolled in our study, with a mean patient age of 65.7 years (ranging from 31 to 83 years) and a male-to-female ratio of 1.9:1. The ethnic distribution was as follows: Caucasian 21 (48.8%), African American 18 (41.9%), Asian 2 (4.7%) and others/unknown 2 (4.7%). The majority were resection specimens (35/43, 81.4%), while 8 were biopsies of the primary colon masses. Fourteen specimens (32.6%) were from the right colon (cecum and ascending colon), and 29 specimens (67.4%) were from the left colon (transverse colon, descending colon, sigmoid colon, and rectum).

RAS, BRAF V600E, and APC mutations of CRC

RAS, BRAF V600E, and APC gene status were identified by chart review or examined using the same methods if they were not performed previously. Successful molecular evaluations were acquired from all 43 specimens by NGS.

Mutations of either *KRAS* or *NRAS* were considered *RAS* mutation-positive, which account for 55.8% of all tested specimens (24/43). Among them, twenty-two (22/24, 91.7%) had *KRAS* mutation, and the other two (2/24, 8.3%) had NRAS mutation. Right-side tumors harbored a higher rate of RAS mutations (10/14, 71.4%) compared to left-side tumors (14/29, 48.3%) ($p < 0.05$, chi-square test). The most frequent mutation observed was a *KRAS* point mutation in codon 12 (16/24, 66.7%), including *KRAS* p. G12V (c.35G>T) (6/24, 25.0%), followed by *KRAS* p. G12D (c.35G>A) (5/24, 20.8 %). A KRAS point mutation in codon 13 [p. G13D (c.38G>A)] was observed in four cases (4/24, 16.7%).

BRAF V600E mutation was identified in four cases (4/43, 9.3%), which were mutually exclusive to *KRAS* mutations. Two specimens (50.0%) were from the right-side colon, and the other two (50.0%) were from the left side. In addition, DNA mismatch repair (MMR) gene deficiency was detected by IHC and confirmed by NGS in two cases harboring *BRAF*

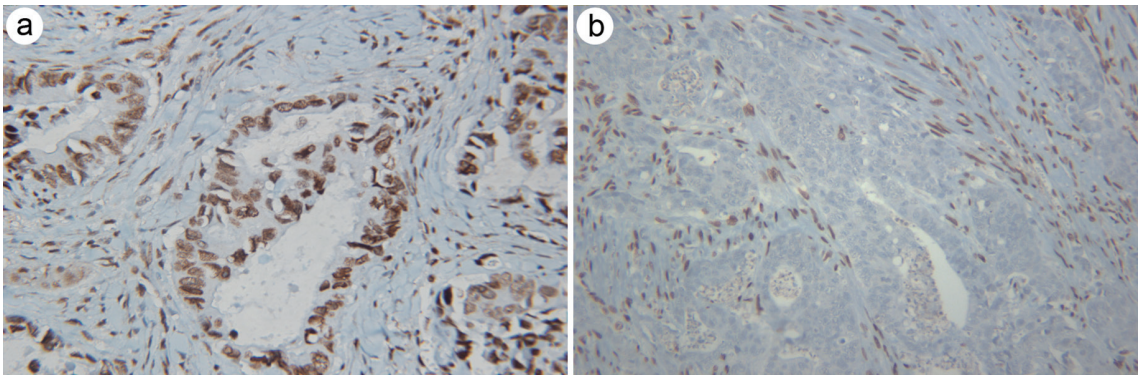

Fig. 1. HES1 expression by IHC. (a) Nuclei expression of HES1 is identified in one case with *RAS* and *BRAF* wide type (200×); (b) It is usually lost in cases with mutated *RAS* or *BRAF* gene. Lymphocytes serve as an internal control in negative cases (200×).

V600E mutation and one case without *BRAF V600E* mutation.

APC mutation was found in 12 specimens (12/43, 27.9%), with eight from the left-sided colon and four from the right-sided colon.

HES1 expression, RAS/BRAF V600E, and APC mutations

Overall, loss of HES1 nuclear expression was found in two-thirds of the cancer specimens (28/43, 65.1%), with a much higher frequency in right-sided tumors (13/14, 92.9%), compared to the left-sided (15/29, 51.7%) ($p < 0.05$) (Fig. 1). We also noticed that HES1 absence was more frequently identified in tumor tissue with either *RAS* or *BRAF V600E* mutations than cases that remained in wild-type (78.6% *vs.* 40.0%, $p < 0.05$) (Table 1). Importantly, further analysis with tumor location and *RAS/BRAF V600E* mutations showed that all the specimens from the right-side colon with *RAS* or *BRAF* mutations had a loss of HES1 expression (12/12, 100%) ($p < 0.05$), while only 62.5% (10/16) of left-sided tumors with *RAS* or *BRAF* mutation had lost HES1 expression (Table 1). The concordance of HES1 loss and *RAS/BRAF V600E* mutations was 92.8% (13/14, κ = 0.89; $p < 0.01$) in right-sided tumors.

Among 3 MMR deficiency cases, HES1 loss was identified in one case (1/3, 33.3%) and preserved in the other two cases (2/3, 66.7%), including one LYNCH patient. However, there is no reliable conclusion due to the minimal case number here.

HES1 loss was found in 83.3% (10/12) cases with APC mutations and 58.1% (18/31) cases without APC mutations. However, our studies observed no statistically significant differences between HES1 expression and APC mutations.

HES1 expression and distant metastasis

A clinical chart review for distant metastasis was performed on all the cases. More than a half of our patients presented with stage IV disease (24/43, 55.8%), including metastasis to liver (n = 19), lung (n = 3), ovary (n = 1) and peritoneum (n = 1). Twelve patients (12/43, 27.9%) lived without metastasis in our observation window. One case (1/43, 2.3%) developed local recurrence. The rest cases (6/43, 14.0%) developed metastasis during the follow-up after initial diagnosis with time-to-metastasis ranging from 2 months to 48 months. The relationship between HES1 loss and distant metastasis was also analyzed in patients harboring *RAS/BRAF V600E* mutations. Patients with HES1 loss in tumor tissue were less likely to have immediate metastasis (at initial diagnosis or within six months) (59.1%, 13/22) compared to patients with preserved HES1 expression (83.3%, 5/6) ($p = 0.37$) (Table 2).

Discussion

HES1 protein is expressed in the nuclei of epithelial cells of the intestine in humans and mice. However, the expression of HES1 in CRC shown in the literature is inconsistent. In a study of Notch effectors in intestinal tumorigenesis, HES1

Table 1. *RAS* or *BRAF V600E* mutation and HES1 expression in the right/left colorectal adenocarcinoma

HES1	Right side (n = 14)			Left side (n = 29)		
	*RAS/BRAF**		*p*	*RAS/BRAF**		*p*
	Wild-type	Mutated		Wild-type	Mutated	
Present	1 (50.0%)	0 (0)	0.01	8 (61.5%)	6 (37.5%)	0.19
Loss	1 (50.0%)	12 (100%)		5 (38.5%)	10 (62.5%)	

**RAS/BRAF* wild-type refers to cases with no mutations detected in *RAS* or *BRAF*; while Mutated refers to cases with either *RAS* or *BRAF* mutation.

Table 2. HES1 mutation and distance metastasis in patients with *RAS/BRAF V600E* mutations

HES1	Immediate metastasis (within 6 months)		*p*
	Yes (n = 18)	No (n = 10)	
Absent (n = 22)	13 (59.1%)	9 (40.9%)	0.37
Present (n = 6)	5 (83.3%)	1 (16.7%)	

expression was identified by IHC on all 14 random CRC specimens.[13] Fre *et al.* reported that HES1 was strongly expressed in adenomas but not expressed in 14 cases of human colon adenocarcinomas, indicating that loss of HES1 could be associated with the carcinogenic transformation of adenoma.[14] Some studies showed the heterogeneous nature of HES1 expression in cancer tissue. Gao *et al.* reported that HES1 was associated with the differentiation of the adenocarcinoma, with a stronger expression in poorly differentiated adenocarcinoma.[15] Our previous study of an animal model with genetic defects in Notch signaling indicated that suppressed HES1 expression was associated with adenocarcinoma development in mouse colon.[11]

Previous reports have shown differences between the right-side colon (including the caecum, ascending colon, and transverse colon) and the left-side colon (including the descending colon, sigmoid colon, and rectum) in epidemiologic incidence, morphology, and molecular alterations.[16–18] Our study, for the first time, showed that the loss of HES1 expression was highly associated with right-sided CRC (92.9%) and was absent in all the right-sided cases with either *RAS* or *BRAF V600E*-activated mutations (100.0%). This finding highlights the question, "does HES1, or the Notch pathway, have a role in carcinogenesis with the MAPK-ERK pathway?" Activation mutations of *RAS* and *BRAF* would lead to upregulation of the MAPK-ERK pathway, which was the most common oncogenic event in colorectal tumorigenesis.[19] Suppression of HES1 in the tumor tissue with activated MAPK-ERK pathway suggested a possible role of HES1 in the carcinogenesis mediated by MAPK-ERK pathway. Very recently, HES1 was reported to be involved in the initiation and progression of *KRAS*-driven pancreatic tumorigenesis in mice.[20] In their study, activation of MAPK signaling via mutant KRAS activation induced sustained HES1 expression in pancreatic acinar cells. Interestingly, our finding indicates HES1 loss, rather than retained expression in the above study, associated with *RAS*-mutant CRC, which enhances the complexity of the role of HES1 in the MAPK-ERK pathway.

Although patients diagnosed with localized early-stage tumors can be treated with surgery and adjuvant regimens, most patients diagnosed with distant metastasis succumb to cancer.[21] It is, therefore, critical to understand the mechanisms that enable CRC to metastasize and develop effective strategies to prevent metastasis. So far, there is little evidence for activating Notch gene mutations in human CRC metastasis. Recently, Jackstadt *et al.*[10] showed that enforced Notch signaling led to highly penetrant metastasis in *KRAS*-G12D mutation-driven serrated colon cancer in the murine intestinal epithelium, which is consistent with our finding that patients with HES1 loss were less likely to have immediate metastasis, compared to patients with preserved HES1 expression. In our study, the most frequent mutation of KRAS is p. G12V (c.35G>T) (6/24, 25.0%), followed by *KRAS* p. G12D (c.35G>A) (5/24, 20.8 %). Unfortunately, due to the limitation of the case number, our study did not reach statistical significance. However, it would be interesting to further explore the topic with a larger sample volume.

RAS and *BRAF* mutation status were essential markers closely related to patient management and prognosis prediction, which clinicians frequently requested. Currently, IHC can only detect *BRAF V600E* in limited organizations, while the RAS status exam needs molecular diagnostic study, which is usually expensive and takes a longer turn-around time compared to IHC. Therefore, finding markers that could predict the status and be easily examined with IHC is demanded. Based on our data, it is reasonable to propose HES1 as one of the candidates with excellent concordance in the right-sided tumor cases. While NGS is the current "golden standard" method, it would be helpful if a quick IHC test could serve as a predictor in local labs.

Conclusions

High frequency of HES1 loss in colorectal adenocarcinoma is associated with either *RAS* or *BRAF* mutations, suggesting synergistic effects by dysregulated Notch and *RAS/BRAF* mutation may play an important role in colon carcinogenesis in some forms, especially the right-sided tumors. This finding may help guide future treatments for a subset of colon cancers. Although the loss of Hes1 in RAS or BRAF mutations showed increased immediate metastasis compared to those with the normal Hes1 expression cases, the case number is not large enough to warrant a statistical difference. To explore the possibility, a large-scale study may be warranted to establish the relationship.

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Conflict of interest

The authors have no conflicts of interest related to this publication.

Author contributions

Performance of experiments, analysis and interpretation of data, manuscript writing, statistical analysis (WC); study design, analysis and interpretation of data, critical revision, statistical analysis, critical funding, administration, technical or material support (LZ); study design, experimentation, analysis and interpretation of data, critical revision, statistical analysis, critical funding, administration, technical or material support (WX). All authors have contributed significantly to this study and approved the final manuscript.

Ethical statements

This study was approved by the Institutional Review Board of University Hospitals Cleveland Medical Center/Case Western Reserve University. HIPPA waiver was approved as this is a discarded tissue and chart review study with no patient-identifiable data collected or stored after the screening. The protocol conformed to the ethical guidelines of the latest version of the Declaration of Helsinki. The project was finished before both authors left the original institutions. The IRB is still active under Dr. Lan Zhou as the new PI.

Data sharing statement

All data used to support the findings of this study are included in the article.

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