Can hypoxia-inducible factor-1α overexpression discriminate human colorectal cancers with different microsatellite instability?

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Clinicopathological features of high-frequency microsatellite instability (MSI-H) colorectal cancers (CRCs) are different from low-frequency MSI (MSI-L) and microsatellite stable (MSS) CRCs. The clinical features of MSI-L cases are unknown, and although the tumors usually show instability for dinucleotide markers, evaluation based on dinucleotides alone could lead to the misclassification of MSI-L or MSS as MSI-H. In this research, we investigated the usefulness of hypoxia-inducible factor-1α (HIF-1α) expression to discriminate MSI-L from MSS and MSI-H in human CRC. Tumor tissue from 94 CRC patients was used to determine the expression level of HIF-1α mRNA and HIF-1α protein using quantitative real-time PCR and immunohistochemistry analyses, respectively. The results indicated that HIF-1α mRNA and HIF-1α protein levels were upregulated in CRC patients compared with controls (P < 0.0001). Average HIF-1α expression in tissues with advanced stages and grades was also higher than that in earlier stages and grades. Expression of HIF-1α mRNA varied between CRC patients with different types of microsatellite instability (MSS, MSI-L and MSI-H). Taken together, our findings provide preliminary evidence that HIF-1α expression level in CRC tumors correlates with different MSI categories. HIF-1α expression may therefore represent a novel marker to separate the MSI-L group from the MSS and MSI-H groups.

Key words: colorectal cancer (CRC), microsatellite instability (MSI), HIF-1α, MSI-L, MSI-H

INTRODUCTION

The incidence of colorectal cancer (CRC), the third leading cause of cancer death worldwide, is rising among young adults (Bhandari et al., 2017; Bray et al., 2018). Numerous factors are important in colorectal carcinogenesis, including genetic and epigenetic alterations (Zoratto et al., 2014). Molecular genetic changes that occur in CRC may be determined based on three features: DNA microsatellite instability (MSI), chromosomal instability and CpG island methylator phenotype (Nazemalhosseini Mojarad et al., 2013). MSI is a hypermutable phenotype, which is the result of a deficiency in DNA mismatch repair (MMR). MSI is responsible for 12% of sporadic and 3% of hereditary CRC (Boland and Goel, 2010). The Bethesda panel for diagnosing MSI in CRC includes five microsatellite loci: two mono-
nucleotides (BAT25 and BAT26) and three dinucleotides (DSS346, D2S123 and D17S250). Based on this panel, three categories of MSI have been recognized: MSI-High (MSI-H), representing instability at > 30% of loci; MSI-Low (MSI-L), indicating instability at 10–30% of loci, and microsatellite stable (MSS), demonstrating instability at < 10% of loci (Vilar and Gruber, 2010).

MSI occurs in CRC patients by two different mechanisms. In sporadic cases, hypermethylation of the MLH1 promoter and sometimes sporadic mutations are the main causes, while in hereditary nonpolyposis colorectal cancer the main cause is a germline mutation in the MMR genes (Nazemalhosseini Mojarrad et al., 2016). Determination of MSI is important in prognosis, prediction, therapeutic implications and classification of CRC (Sinicrope and Sargent, 2012; Alwers et al., 2019; Hu et al., 2019). Recently, MSI status testing has been introduced for identifying hereditary and detecting sporadic CRC patients (Oh et al., 2012). Furthermore, a cDNA microarray expression study has presented MSI-L as a distinct phenotype from MSS and MSI-H (Mori et al., 2003). MSI testing has the potential for predicting patient outcomes in response to chemotherapy agents (Jo and Carethers, 2006; Kawakami et al., 2015). The clinical features of MSI-L cases are unknown and tumor cells usually show instability for dinucleotide markers, but the evaluation of dinucleotides alone could lead to the misclassification of MSS or MSI-L as MSI-H (Mori et al., 2003; Vilar and Gruber, 2010).

Hypoxia-induced factors (HIFs) are transcription factors that play a major role in controlling hypoxia during carcinogenesis (Zhong et al., 1999; Momin and Nagaraju, 2020). HIFs are composed of two subunits, α and β. Numerous cancer phenotypes, such as genomic instability, angiogenesis, treatment resistance, cell invasion and metastasis, are influenced by the interaction of hypoxia and HIFs (Wigerup et al., 2016). Hypoxia-inducible factor-1α (HIF-1α) is responsible for genetic instability in cancer cells, which correlates with lower DNA repair activity by inhibition of the MMR genes MSH2 and MSH6. MSI can therefore occur in tumors under hypoxic conditions (Rodríguez-Jiménez et al., 2008).

The main goal of this study was to determine the HIF-1α expression level in CRC tumors with different MSI categories and thus to identify a marker that can separate the MSI-L group from the MSS and MSI-H groups.

RESULTS AND DISCUSSION

We observed overexpression of HIF-1α in patients compared with controls (P < 0.0001). Furthermore, different mRNA expression of HIF-1α was observed between patients with different types of microsatellite instability (MSS, MS-L and MS-H) (Fig. 1A). Our results showed that patients with MSI-L display HIF-1α downregulation compared with other groups (MSI-H and MSS) (P < 0.0054). The average HIF-1α expression in tissues with advanced stages (III/IV) was higher than that in tissues with earlier stages (I/II) (P < 0.0002) (Fig. 1B). Moreover, the average HIF-1α expression in high-grade tumors was greater than that in tumors of low or moderate grades (P < 0.0332) (Fig. 1C). Our immunohistochemistry protein expression results confirmed the HIF-1α mRNA alteration (Fig. 2). The diagnostic and prognostic value of HIF-1α was assessed by plotting a ROC (receiver operating characteristic) curve. The area under the curve was 94% with sensitivity 85.11% and specificity 90% (Fig. 3).

Microsatellite instability plays an important role in the occurrence, progression and prognosis of many cancers, including CRC (Yang et al., 2019). HIF-1α upregulation has been reported in many different tumor types compared with normal tissues, including colon, breast, gastric and pancreatic (Cao et al., 2009). In this study, we focused on HIF-1α gene expression in relation to microsatellite instability in CRC patients. CRC tumors were divided into MSS, MSI-L and MSI-H, which have different responses to chemotherapy (Jo and Carethers, 2006). Overall, our results show HIF-1α upregulation in patients compared with controls. The expression ratio of the HIF-1α gene increased at advanced stages and high grades compared with earlier stages and low or moderate grades, respectively (Fig. 1B and 1C). Furthermore, we have shown that HIF-1α mRNA expression is correlated with protein expression: samples with up- and downregulated mRNA levels showed increased and decreased levels of protein expression, respectively. As CRC progresses, the oxygen balance of tumors is disrupted and the expression of genes that promote tumor development subsequently increases. This process can play the main role in increasing tumor grades and stages (Hohenberger et al., 1998; Zhou et al., 2006; Galanis et al., 2008; Assi, 2017). Consistent with our study, a growing body of research indicates that the HIF-1α gene is upregulated in colorectal adenocarcinomas at both the mRNA and protein levels, and the expression of HIF-1α is frequently correlated with disease stage (Ioannou et al., 2015). Jiang et al. (2003) have shown that HIF-1α expression level was significantly higher in Dukes stages C and D than in Dukes stages A or B. Baba et al. (2010), by examining 731 CRC specimens, demonstrated that HIF-1α overexpression is independently associated with poor prognosis, and proposed HIF-1α as a biomarker with potentially important therapeutic implications. Chen et al. (2013) analyzed a total of 23 studies, including 2,984 colorectal cancer patients, and reported that upregulation of HIFs was significantly correlated with increased risk of mortality, including overall survival and disease-free survival. They showed that HIF-1α upregulation was significantly associated with poor overall survival, par-
HIF-1α gene expression in CRC

particularly in Asian countries. Moreover, they showed that HIF-1α upregulation was associated with clinicopathological features including Dukes’ stages, depth of invasion, lymph node status and metastasis. Koshiji et al. (2005) demonstrated that HIF-1 induces genetic instability by transcriptionally downregulating MutS expression; furthermore, they proposed a molecular mechanism underlying hypoxia-induced genetic instability. In contrast to our findings, the results of Khatibi et al. (2018) indicated that there is no relationship between HIF-1α expression in adenoma and hyperplastic polyps compared to the control group. These authors also found no significant correlation between clinicopathological features and HIF-1α expression in colorectal polyps.

The main result of our research is that HIF-1α expression may represent a novel marker to separate the MSI-L group from the MSS and MSI-H groups. We have shown downregulation of HIF-1α in the MSI-L group compared with MSS and MSI-H groups. Furthermore, the data obtained from the ROC curve (Fig. 3) demonstrated that HIF-1α can be used as a valuable biomarker to distinguish MSI-L patients from MSS and MSI-H patients. Hypoxia is one of the main features of solid tumors, and leads to resistance to radiotherapy and anticancer chemotherapy as well as predisposing for increased tumor metastases (Brown, 2007). Hypoxia-inducible factors play an important role in drug resistance, and MSI has different responses to chemotherapy (Jo and Carethers, 2006; Lv et al., 2015). HIF-1α mediates chemoresistance by activating the multidrug resistance (MDR) 1 gene, which encodes a glycoprotein that belongs to the ABC transporter family and functions as a drug efflux pump. Therefore, HIF-1α
can act in hypoxia-induced drug resistance (Comerford et al., 2002). By upregulation of MDR genes, the chemoresistance response occurs. Expression of \( HIF-1\alpha \) probably causes the upregulation of MDR genes due to resistance to the therapy. Therapy involving 5-fluorouracil may not useful for patients whose tumors display MSI-H compared to patients with MSS tumors (Jo and Carethers, 2006).

Fig. 2. Immunohistochemical staining of CRC tissues with antibody against HIF-1\( \alpha \). (A) Negative HIF-1\( \alpha \) control. (B) Detectable HIF-1\( \alpha \) in MSI-L specimen. (C) Detectable HIF-1\( \alpha \) in MSI-H specimen. (D) Detectable HIF-1\( \alpha \) in MSS specimen. Magnification in all images is ×40.

Fig. 3. ROC curve showing sensitivity and specificity of \( HIF-1\alpha \) gene expression between MSI-L and MSS+MSI-H groups. The area under the curve (AUC) was 94% with 90% specificity and 85.11% sensitivity.
In summary, our data demonstrated that low expression of HIF-1α can distinguish MSI-L from MSS in CRC. This finding is important in the context of therapeutic intervention programs with chemotherapy agents. To understand the response to treatment and prognosis, it may be useful to distinguish the MSS group from the MSI-L group. To the best of our knowledge, this is the first study to evaluate the relationship between HIF-1α and MSI. Although HIF-1α upregulation and MSI-L are both correlated with poor prognosis in CRC, further larger-scale studies are required to validate the significance of this finding.

MATERIALS AND METHODS

Tissue samples Formalin-fixed, paraffin-embedded (FFPE) tumor tissue specimens were obtained from 94 colorectal cancer patients diagnosed in the Gastroenterology and Liver Diseases Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran from 2004 to 2016. Informed consent was given by the donor or a trusted family member. None of the patients underwent radiotherapy or chemotherapy before surgery. MSI information was collected from a previous study using five mononucleotide repeat microsatellite targets, namely BAT25, BAT26, NR21, NR24 and NR27 (Nazemalhosseini-Mojarrad et al., 2020). Among the 94 patients, 45 tissue samples were categorized as MSS, 21 as MSI-L and 28 as MSI-H. Table 1 describes the clinicopathological status of the patients studied.

RNA isolation, cDNA synthesis and quantitative real-time PCR (qRT-PCR) Total RNA was extracted from FFPE tissue sections using the RNeasy FFPE kit (QIAGEN, Germany) according to the manufacturer’s instructions. RNA concentration was estimated using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). Total RNA was reverse-transcribed using the Prime Script RT Reagent Kit (Takara, Dalian, China). qRT-PCR was performed using SYBR Premix Ex Taq II (Takara) with the Applied Biosystems 7500 Real-Time PCR System (ABI, USA). The β2M gene was used as a normalization control and fold change was calculated according to the 2−ΔΔct formula. DNA sequences of primers are shown in Table 2.

Immunohistochemistry Tissue samples were sectioned at 4-μm thickness and mounted on polylysine-coated slides. Specimens were deparaffinized and rehydrated with xylene and graded alcohols. Endogenous peroxidase was inactivated with 3% H2O2, and antigen retrieval was then carried out in citrate buffer. Primary antibody Anti-HIF-1 alpha antibody (Abcam, UK) was incubated at room temperature for 20 min. Slides were then incubated with secondary mouse antibody (Abcam) at 4°C for 1 h. Staining was performed with 3,3’-diaminobenzidine and counter-staining with hematoxylin.Negative controls lacked the primary antibody.

Statistical analysis We analyzed the experimental data using GraphPad Prism (GraphPad Software, USA). The data were examined for normality using the Kolmogorov–Smirnov test. We used one-way ANOVA and t-test analysis for quantitative data. P < 0.05 was considered statistically significant.

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REFERENCES

Alwers, E., Bläker, H., Walter, V., Jansen, L., Kloor, M., Arnold, A., Sieber-Frank, J., Herpel, E., Tagscherer, K. E., Roth, W., et al. (2019) External validation of molecular subtype classifications of colorectal cancer based on microsatellite instability, CIMP, BRAF and KRAS. BMC Cancer 19, 681.
Assi, M. (2017) The differential role of reactive oxygen species in early and late stages of cancer. Am. J. Physiol. Regul. Integr. Comp. Physiol. 313, R646–R653.

Baba, Y., Nosho, K., Shima, K., Irahara, N., Chan, A. T., Meyerhardt, J. A., Chung, D. C., Giovannucci, E. L., Fuchs, C. S., and Ogino, S. (2010) HIF1α overexpression is associated with poor prognosis in a cohort of 731 colorectal cancers. Am. J. Pathol. 176, 2292–2301.

Bhandari, A., Woodhouse, M., and Gupta, S. (2017) Colorectal cancer is a leading cause of cancer incidence and mortality among adults younger than 50 years in the USA: a SEER-based analysis with comparison to other young-onset cancers. J. Investig. Med. 65, 311–315.

Boland, C. R., and Goel, A. (2010) Microsatellite instability in colorectal cancer. Gastroenterology 138, 2073–2087. e3.

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., and Jemal, A. (2018) Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J. Clin. 68, 394–424.

Brown, J. M. (2007) Tumor hypoxia in cancer therapy. Methods Enzymol. 435, 295–321.

Cao, D., Hou, M., Guan, Y.-s., Jiang, M., Yang, Y., and Gou, H.-f. (2009) Expression of HIF-1alpha and VEGF in colorectal cancer: association with clinical outcomes and prognostic implications. BMC Cancer 9, 432.

Chen, Z., He, X., Xia, W., Huang, Q., Zhang, Z., Ye, J., Ni, C., Wu, P., Wu, D., Xu, J., et al. (2013) Prognostic value and clinicopathological differences of HIFs in colorectal cancer: evidence from meta-analysis. PLoS One 8, e68033.

Comerford, K. M., Wallace, T. J., Karhausen, J., Louis, N. A., Montalto, M. C., and Colgan, S. P. (2002) Hypoxia-inducible factor-1-dependent regulation of the multidrug resistance (MDR1) gene. Cancer Res. 62, 3387–3394.

Galanis, A., Pappa, A., Giannakakis, A., Lanitis, E., Dangaj, D., and Sandilzopoulos, R. (2008) Reactive oxygen species and HIF-1 signalling in cancer. Cancer Lett. 266, 12–20.

Hohenberger, P., Felgner, C., Haensch, W., and Schlag, P. M. (1998) Tumor oxygenation correlates with molecular growth determinants in breast cancer. Breast Cancer Res. Treat. 48, 97–106.

Hu, W., Yang, Y., Qi, L., Chen, J., Ge, W., and Zheng, S. (2019) Subtyping of microsatellite instability-high colorectal cancer. Cell Commun. Signal. 17, 79.

Joannou, M., Paraskeva, E., Baxevanidou, K., Simos, G., Papamichali, R., Papacharalambous, C., Samara, M., and Koukoulis, G. (2015) HIF-1α in colorectal carcinoma: review of the literature. J. BUON 20, 680–689.

Jiang, Y.-A., Fan, L.-F., Jiang, C.-Q., Zhang, Y.-Y., Luo, H.-S., Tang, Z.-J., Xia, D., and Wang, M. (2003) Expression and significance of PTEN, hypoxia-inducible factor-1 alpha in colorectal adenoma and adenocarcinoma. World J. Gastroenterol. 9, 491–494.

Jo, W.-S., and Carethers, J. M. (2006) Chemotherapeutic implications in microsatellite unstable colorectal cancer. Cancer Biomark. 2, 51–60.

Kawakami, H., Zaanan, A., and Sinicrope, F. A. (2015) Microsatellite instability testing and its role in the management of colorectal cancer. Curr. Treat. Options Oncol. 16, 30.

Khattib, S., Nazemalhosseini Mojarad, E., Forouzesh, F., Pezeshkian, Z., Asadzadeh Aghdaei, H., and Zali, M. R. (2018) HIF-1 alpha gene expression is not a suitable biomarker for evaluating malignancy risk in colorectal polyps. WCRJ 5, e1128.

Koshiji, M., To, K. K.-W., Hammer, S., Kumamoto, K., Harris, A. L., Modrich, P., and Huang, L. E. (2005) HIF-1α induces genetic instability by transcriptionally downregulating MuttS expression. Mol. Cell 17, 793–803.

Lx, Y., Zhao, S., Han, J., Zheng, L., Yang, Z., and Zhao, L. (2015) Hypoxia-inducible factor-1α induces multidrug resistance protein in colon cancer. OncoTargets Ther. 8, 1941–1948.

Momin, S., and Nagaraju, G. P. (2020) The Role of Hypoxia-Inducible Factor 1-Alpha in Colorectal Cancer. In Therapeutics Approaches to Gastric and Colon Cancer. pp. 61–68. Springer, Singapore.

Mori, Y., Sato, F., Sato, F., Y., Simms, L. A., Xu, Y., Olaru, A., Deacu, E., Wang, S., Taylor, J. M., et al. (2003) The impact of microsatellite instability on the molecular phenotype of colorectal tumors. Cancer Res. 63, 4577–4582.

Nazemalhosseini Mojarad, E., Farahani, R. K., Haghhi, M. M., Aghdai, H. A., Kuppen, P. J. K., and Zali, M. R. (2013) Clinical implications of BRAF mutation test in colorectal cancer. Gastroenterol. Hepatol. Bed Bench 6, 6–13.

Nazemalhosseini Mojarad, E., Khashi, S. M. H., Mirtalebi, H., Taleghani, M. Y., Azimzadeh, P., Savabak, S., Pourhoseingholi, M. A., Jalaekookhoo, H., Asadzadeh Aghdai, H., Kuppen, P. J. K., et al. (2016) Low level of microsatellite instability correlates with poor clinical prognosis in stage II colorectal cancer patients. J. Oncol. 2016, 2196703.

Nazemalhosseini-Mojarad, E., Khoshali Farahani, R., Mehrizi, M., Baghaei, K., Yaghoob Taleghani, M., Golmohammedi, M., Peyravian, N., Ashtari, S., Pourhoseingholi, M. A., Asadzadeh Aghdai, H., and et al. (2020) Prognostic value of BRAF and KRAS mutation in relation to colorectal cancer survival in Iranian patients: correlated to microsatellite instability. J. Gastrointest. Cancer 51, 53–62.

Oh, J. R., Kim, D.-W., Lee, H. S., Lee, H. E., Lee, S. M., Jang, J.-H., Kang, S.-B., Ku, J.-L., Jeong, S.-Y., and Park, J.-G. (2012) Microsatellite instability testing in Korean patients with colorectal cancer. Fam. Cancer 11, 459–466.

Rodriguez-Jiménez, P. J., Moreno-Manzano, V., Lucas-Dominguez, R., and Sánchez-Puelles, J. M. (2008) Hypoxia causes down-regulation of mismatch repair system and genomic instability in stem cells. Stem Cells 26, 2052–2062.

Sinicrope, F. A., and Sargent, D. J. (2012) Molecular pathways: microsatellite instability in colorectal cancer: prognostic, predictive, and therapeutic implications. Clin. Cancer Res. 18, 1506–1512.

Vilar, E., and Gruber, S. B. (2010) Microsatellite instability in colorectal cancer—the stable evidence. Nat. Rev. Clin. Oncol. 7, 153–162.

Wigerup, C., Pählman, S., and Bexell, D. (2016) Therapeutic targeting of hypoxia and hypoxia-inducible factors in cancer. Pharmacoel. Ther. 164, 152–169.

Yang, G., Zheng, R.-y., and Jin, Z.-s. (2019) Correlations between microsatellite instability and the biological behaviour of tumours. J. Cancer Res. Clin. Oncol. 145, 2891–2899.

Zhong, H., De Marzo, A. M., Laughner, E., Lim, M., Hilton, D. A., Zagzag, D., Buechler, P., Isaacs, W. B., Senzema, G. L., and Simons, J. W. (1999) Overexpression of hypoxia-inducible factor 1α in common human cancers and their metastases. Cancer Res. 59, 5830–5835.

Zhou, J., Schmid, T., Schnitzer, S., and Brüne, B. (2006) Tumor hypoxia and cancer progression. Cancer Lett. 237, 10–21.

Zorzato, F., Rossi, L., Vernaco, M., Papa, A., Basso, E., Zullo, A., Tomao, L., Romiti, A., Russo, G. L., and Tomao, S. (2014) Focus on genetic and epigenetic events of colorectal cancer pathogenesis: implications for molecular diagnosis. Tumor Biol. 35, 6195–6206.