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Analysis of the role of hMLH1 hypermethylation and microsatellite instability in meningioma progression

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ABSTRACT. We investigated a possible role of hMLH1 hypermethylation and microsatellite instability in meningioma progression. Fifty meningomas were examined for methylation of hMLH1 using a methylation-specific PCR; 43 of them were analyzed for microsatellite instability using nine microsatellite markers. Loss of heterozygosity on chromosome 22q was detected using two markers. Two atypical meningiomas showed microsatellite instability at four loci; one was methylated on hMLH1 and the other was unmethylated. Nine meningiomas were found to have methylated hMLH1; the frequencies in the different grades of meningioma were one of 20, two of 16, and six of 14, respectively. We concluded that the methylation status of hMLH1 is associated with the meningioma grade but not with microsatellite instability. Loss of heterozygosity was detected in 22 cases in at least one marker. The frequency of loss of heterozygosity increased with meningioma

grade, but the tendency was not significant. The correlation between loss of heterozygosity and methylation of the hMLH1 gene was also not significant. We conclude that hypermethylation of the promoter of hMLH1 is an epigenetic change in meningiomas and is associated with the tumor grade, while microsatellite instability is an uncommon event in meningiomas.

Key words: Meningioma; Methylation; Microsatellite instability; hMLH1

INTRODUCTION

Meningiomas are the second most common primary tumors of the central nervous system, accounting for 20-25% of primary central nervous system tumors (Longstreth Jr. et al., 1993). According to the criteria of the World Health Organization, meningiomas are classified to three grades. Grade I is a benign tumor that can be surgically cured and has a 31% chance of recurrence within 5 years if subtotally resected. Grade II is an atypical meningioma displaying a higher risk of local recurrence and a 40% likelihood of 5-year recurrence even after gross total resection is performed. Grade III is an anaplastic tumor with a median survival of 1.5 years (Perry et al., 1997, 1999; Radner et al., 2002).

Considerable genetic investigations of meningiomas have been undertaken to understand their development and progression. The most common alteration is loss of heterozygosity (LOH) on chromosome 22q and mutation of neurofibromatosis type 2, with the frequency of 50-70 and 20-30%, respectively (Ng et al., 1995; Wellenreuther et al., 1995; Ueki et al., 1999). Chromosome 1p is the second most frequently seen region of LOH in meningiomas, whereas $p18^{INK4C}$, located at 1p32, is rarely altered.

Recent studies have indicated that in addition to genetic alterations, epigenetic changes may also play important roles in the progression of human tumors. Microsatellite instability (MSI), a genomic instability induced by somatic alteration within microsatellites, is believed to act as a pathway for carcinogenesis. MSI is caused by deficient DNA mismatch repair. Recent studies have revealed that hypermethylation of a promoter gene for DNA mismatch, hMLH1, is related to MSI in a wide variety of human cancers, including colorectal cancer, gastric cancer, and endometrial carcinomas (Veigl et al., 1998; Simpkins et al., 1999; Fleisher et al., 2001). These studies have demonstrated that hMLH1 promoter hypermethylation is associated with the loss of hMLH1 expression and the MSI phenotype in colorectal carcinomas and gastric cancers (Cunningham et al., 1998; Herman et al., 1998; Deng et al., 1999; Leung et al., 1999; Baylin and Herman, 2000). The correlation between the methylation of hMLH1 and the MSI phenotype has also been found in uterine endometrial carcinomas (Esteller et al., 1999). These findings suggest that hMLH1 promoter hypermethylation is an early event in human tumorigenesis; however, the role of MSI in meningiomas is still controversial. Pykett et al. (1994) have reported that 25% of meningiomas exhibit MSI, whereas Alvino et al. (2000) found only sporadic MSI in meningiomas. Until now, no reports on methylation of hMLH1 in meningiomas have been published. The current study was carried out to investigate the roles of the methylation of hMLH1, MSI, and LOH on chromosome 22q in meningiomas, as well as their correlation with one another.

MATERIAL AND METHODS

Specimens and DNA preparation

Fifty tumor samples and 5 normal control specimens were obtained from meningiomas in our brain tumor archives (West China Hospital, Sichuan University). All cases were single masses without other systematic diseases or genetic lesions. The 50 cases were reviewed and graded according to World Health Organization criteria and previously published criteria (Perry et al., 1997, 2001). Twenty cases were graded as classic meningiomas, 16 as atypical meningiomas, and 14 as anaplastic meningiomas. Normal samples were obtained from meningeal cells during tumor resection. Patient age ranged from 17 to 84 years (50.04 ± 13.57), and the ratio of female to male patients was 2.33:1. Tumor-matched blood samples were obtained from 43 meningiomas. The extraction of genomic DNA from frozen tissues and blood was performed using conventional proteinase K digestion and phenol/chloroform extraction (Sambrook et al., 1989).

DNA bisulfite modification

The bisulfite treatment of DNA converts all unmethylated cytosine to uracil, leaving methylated cytosine intact. The DNA is then amplified through subsequent polymerase chain reaction (PCR) with unmethylated and methylated specific primers differentially. Five micrograms of original DNA was modified using the CpGenome DNA Modification Kit (Intergen) according to manufacturer recommendations. Modified DNA was dissolved in 25 μL buffer (10 mM Tris, 0.1 mM ethylenediaminetetraacetic acid, pH 7.5).

Methylation-specific PCR assay

Primer sequences of hMLH1 for the unmethylated reaction were 5'-TTT TGA TGT AGA TGT TTT ATT AGG GTT GT-3' (sense) and 5'-ACC ACC TCA TCA TAA CTA CCC ACA-3' (antisense); for the methylated reaction, they were 5'-ACG TAG ACG TTT TAT TAG GGT CGC-3' (sense) and 5'-CCT CAT CGT AAC TAC CCG CG-3' (antisense) (Dong et al., 2001). PCR was performed in a 20-μL reaction volume containing bisulfite-modified DNA (50 ng), 0.3 μM primers, 0.2 mM deoxyribonucleotide triphosphate, 2.5 mM $MgCl_2$, 1X PCR buffer II, and 0.4 U AmpliTaq Gold DNA polymerase (Applied Biosystems) under the following conditions: 95°C for 10 min to active the enzyme, 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 50 s, and a final extension of 72°C for 10 min. Five microliters of each PCR product was applied for electrophoresis. All PCRs were performed with CpGenome Universal DNA (Intergen) as a positive control and no DNA-loaded control.

Microsatellite analysis

Forty-three of the 50 meningiomas with matched blood DNA were analyzed using 9 microsatellite markers: BAT25, BAT26, D2S123, D5S346, D17S250, *hum*AR, CTT16, D19S210, and DHFRP2. Two polymorphism markers on chromosome 22q - D22s280, located at 22q12.2-12.3, and D22s929, located at 22q12.2 - were selected for the LOH analysis. Primers were labeled in the 5'-end with the fluorochromes HEX (green) or FAM (blue) (Tech Dragon

Limited). PCR was carried out in a 20-μL reaction volume containing genomic DNA (50 ng), 0.3 μM primers, 0.2 mM deoxyribonucleotide triphosphate, 2.5 mM $MgCl_2$, 1X PCR buffer II, and 0.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems) under the following conditions: 95°C for 10 min to activate the enzyme, 35 cycles of 94°C for 40 s, 52°-60°C (depending on the specific marker) for 40 s, and 72°C for 1 min, and a final extension at 72°C for 10 min. Two microliters of each PCR product was mixed with 3 μL loading buffer (formamide:dextran blue:ethylenediaminetetraacetic acid, 5:1:1, ROX 400 size standard). Samples were loaded onto 36-lane gels and electrophoresed on an ABI Prism 377 automated DNA sequencer (Perkin-Elmer). The gel image was analyzed using Genescan and Genotyper (Applied Biosystems).

The presence of MSI was defined according to previously reported criteria (Kulke et al., 2001). LOH was scored with a semi-quantitative expression of the degree of allelic imbalance: $Q^{LOH} = (t1/t2) / (n1/n2)$, where *t1*, *t2*, *n1*, *n2* are the peak height of 2 alleles in tumor and blood samples. When this ratio gives a value greater than 1, Q^{LOH} is set to inversion. Thus, Q^{LOH} ranging from 0 to 1 indicates total loss to retained heterozygosity (Skotheim et al., 2001). If Q^{LOH} was less than 0.4, LOH was identified.

Statistical analysis

Statistical analysis was performed using SPSS version 13.0. The correlation of frequency of methylation of hMLH1 among the 3 tumor grades was evaluated with the Pearson chi-square test, and the correlation between 2 parameters was evaluated using the Fisher exact test.

RESULTS

Methylation analysis

Fifty meningiomas and 5 normal samples were examined in this study. We found that the 5 normal DNA samples were unmethylated, whereas 18% (9 of 50) of meningiomas exhibited aberrant promoter hypermethylation in hMLH1. The rates of methylation of hMLH1 in classic, atypical, and anaplastic meningiomas were 5 (1 of 20), 12.5 (2 of 16), and 42.86% (6 of 14), respectively. Statistical analysis showed that the incidences of methylation was significantly correlated with grade (P = 0.014, Pearson chi-square test; Figure 1, Table 1).

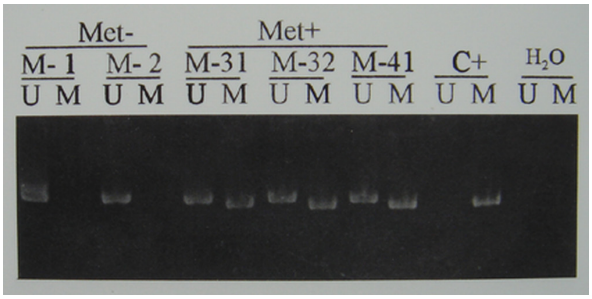

Figure 1. Representative methylation-specific PCR results of hMLH1 for meningiomas. The case numbers are indicted on top. Met- = unmethylated case; Met+ = methylated case; U = unmethylation; M = methylation; C+ = CpGenome Universal Methylated DNA as methylation control; H_2O = water blanks.

Table 1. Summary of methylation-specific PCR (MSP) and microsatellite analysis.

Cases	Grade	MSP of hMLH1	Microsatellite analysis					
			CTT16	D17S250	D18S210	DHFRP2	D22S280	D22S929
M1	I	-					R	NI
M2	I	-					R	L
M3	I	-					ND	ND
M4	I	-					R	R
M5	I	-					L	L
M6	I	-					L	L
M7	I	M					L	R
M8	I	-					R	L
M9	I	-					R	R
M10	I	-					R	R
M11	I	-					R	R
M12	I	-					R	NI
M13	I	-					R	R
M14	I	-					ND	ND
M15	I	-					R	R
M16	I	-					ND	ND
M17	I	-					L	NI
M18	I	-					ND	ND
M19	I	-					ND	ND
M20	I	-					R	R
M21	II	-					L	L
M22	II	-					R	R
M23	II	-					L	NI
M24	II	-					L	L
M25	II	-					R	R
M26	II	-					L	R
M27	II	-					R	R
M28	II	-	MSI	MSI		MSI	MSI	L
M29	II	-					ND	ND
M30	II	-					NI	R
M31	II	M			MSI		L	NI
M32	II	M					R	R
M33	II	-					ND	ND
M34	II	-					R	NI
M35	II	-					NI	R
M48	II	-					L	L
M36	III	M					L	L
M37	III	-					R	R
M38	III	-					L	NI
M39	III	-					R	R
M40	III	-					L	NI
M41	III	M					L	L
M42	III	-					L	L
M43	III	-					NI	L
M44	III	-					L	L
M45	III	-					L	L
M46	III	M					R	R
M47	III	M					R	R
M49	III	M					R	R
M50	III	M					L	L

M = methylated; - = unmethylated; MSI = microsatellite instability; L = loss of heterozygosity; R = retention of heterozygosity; NI = non-informative; ND = not done.

Microsatellite analysis

Forty-three meningiomas with matched blood DNA were evaluated for microsatellite analysis, including 15 classic, 14 atypical, and 14 anaplastic meningiomas. Nine microsatellite markers, which were identified for examining MSI in meningiomas or other tumors in previous studies (Alvino et al., 2000), were selected to detect MSI. We found only 2 atypical meningiomas that displayed MSI in 4 markers. M28 showed MSI on D17S250, CTT16, and DHFRP2, and M31 showed MSI on

D19s210. D17S250 localized at chromosome 1711.2-12, CTT16 at 17pter-qter, DHFRP2 at 6pter-qter, and D19s210 at 19q13.4. Our findings were consistent with those of Pykett et al. (1994). M31 was methylated, whereas M28 was unmethylated on a promoter of hMLH1. Statistical analysis showed no difference in MSI between methylated and unmethylated samples (Figure 2; see Table 1).

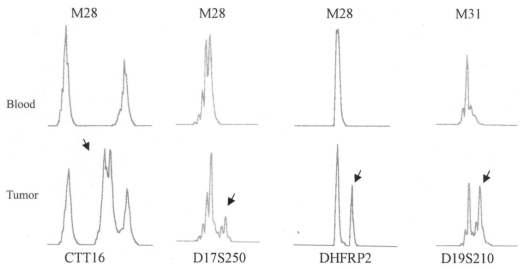

Figure 2. Two meningiomas showed microsatellite instability at 4 loci. Blood samples are at the first row and tumors are at the second row. Cases are on the top and markers are given at the bottom. Arrows indicate the new alleles.

LOH analysis

Two markers, D22s280 and D22s929, were used to examine LOH on chromosome 22q in the series of 43 meningiomas. All 43 pairs of specimens were informative for at least 1 marker. We detected LOH on at least 1 marker in 22 (51.2%) cases, and the frequencies of LOH among classic, atypical, and anaplastic meningiomas were 40, 50, and 64.3%, respectively. Eighteen of 40 informative meningiomas exhibited LOH on D22s280. M28, the case showing MSI at 3 loci, also displayed MSI on D22S280. Fifteen of 35 informative meningiomas showed LOH on D22s929, and 11 cases showed LOH on both markers. Statistical analysis showed no association between LOH on chromosome 22q and grade of meningiomas ($P > 0.05$, Fisher exact test) and no correlation between LOH and methylation of hMLH1 ($P > 0.05$, Fisher exact test; Figure 3; see Table 1).

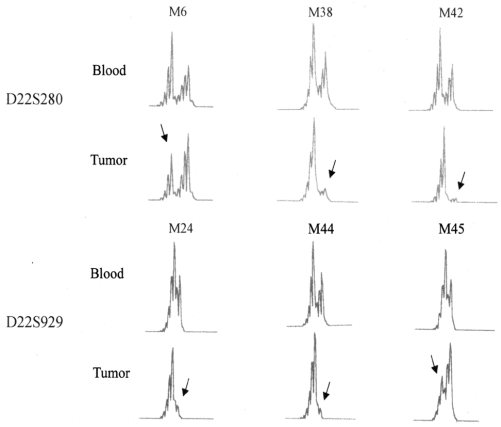

Figure 3. Representative loss of heterozygosity (LOH) of two markers on chromosome 22q. Markers are given at left. Blood samples are at the first row and tumors are at the second row for each marker. Cases are on the top of the peaks. LOH are indicated by arrows.

DISCUSSION

MSI is thought to act as a pathway for carcinogenesis. It was first reported in colorectal carcinomas (Thibodeau et al., 1993) and later described in a wide variety of human tumors, including gastric and endometrial carcinomas (Wirtz et al., 1998; Salvesen et al., 2000). However, the role of MSI in brain tumors is still controversial. Dams et al. (1995) have reported that MSI in glioblastoma can be regarded as an evolution in tumor progression, whereas Lundin et al. (1998) have shown that MSI is infrequent in sporadic adult gliomas. Pykett et al. (1994) examined 2 meningioma cell lines and 14 primary meningiomas (no grade information given) with 15 pairs of primers. They found 1 cell line and 3 primary meningiomas that displayed MSI with a frequency of 25%. The main microsatellite markers showing MSI were *hum*AR, CTT16, D19S210, and DHFRP2. However, Sobrido et al. (2000) have reported that only 6.3% (2 of 32) of meningiomas show MSI at 2 or 3 loci, which is consistent with the findings of Zhu et al. (1996). In our study, 43 meningiomas - including 15 classic, 14 atypical, and 14 anaplastic meningiomas - were examined with 9 microsatellite markers, 5 of which were considered to be the most sensitive and specific for detecting MSI. Four of the markers have shown a high frequency of MSI in meningiomas in previous reports (Pykett et al., 1994; Dietmaier et al., 1997; Boland et al., 1998). We found that only 2 cases exhibited MSI in 4 different loci with an incidence of 4.66%, which is consistent with the results of Sobrido et al. (2000). These findings suggested that MSI is uncommon in meningiomas.

It has been reported that hypermethylation of a promoter gene for DNA mismatch, hMLH1, is correlated with MSI in a variety of human cancers (Veigl et al., 1998; Simpkins et al., 1999; Fleisher et al., 2001). However, no reports have examined the methylation of hMLH1 in meningiomas thus far. In our study, 18% (9 of 50) of meningiomas showed hypermethylation of the hMLH1 promoter, and the frequency in the 3 grades was 5, 12.5, and 42.86%, respectively. Statistical analysis revealed that the methylation of hMLH1 was associated with the grades of the meningiomas. This finding supports those of Bello et al. (2004), who concluded that aberrant DNA methylation of promoter-associated CpG islands contributes to the development of meningiomas. Our study has also uncovered methylation in other multiple genes (Liu et al., 2005).

To investigate the relationship between methylation and MSI, we correlated the methylation status of hMLH1 with MSI and found that 1 of the 2 cases of MSI showed methylation of hMLH1, whereas the other was unmethylated. No significant difference was found for MSI between the groups with or without hMLH1 methylation. Our results showed that methylation of hMLH1 was correlated with the grade of meningioma but not with MSI, suggesting a role for hMLH1 hypermethylation in meningioma progression. Conversely, MSI was unrelated to the development of meningiomas, although it has been found in the tumorigenesis of colorectal and gastric carcinomas (Thibodeau et al., 1993; Wirtz et al., 1998).

We found LOH on chromosome 22q with a frequency of 51.2% and no significant difference among grades in this study, suggesting that LOH on chromosome 22 is an event in the tumorigenesis of meningiomas. However, no significant correlation was found between methylation of hMLH1 and LOH, indicating that methylation of hMLH1 is independent from LOH on chromosome 22q.

In summary, hypermethylation of a promoter of hMLH1 is an epigenetic change in meningiomas and is associated with tumor grade; however, it is not correlated with MSI,

which is an uncommon event in meningioma tumorigenesis. Our data suggested a role for hMLH1 hypermethylation in meningioma progression.

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