p300 gene alterations in intestinal and diffuse types of gastric carcinoma

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Abstract

Background. *p300* is a transcriptional coactivator, and bi-allelic mutations of the *p300* gene have been demonstrated in human cancers.

Methods. In 80 gastric carcinoma tissues, loss of heterozygosity (LOH) was analyzed by polymerase chain reaction (PCR) analysis, using 14 primer pairs at the 22q11–13 locus containing *NF2* and *p300*. The mutations of the *p300* gene were examined by reverse transcriptase-PCR (RT-PCR) and single-stranded conformation polymorphism (SSCP) analysis.

Results. LOH was frequently observed at the 22q13 locus containing the *p300* gene in gastric carcinomas, with an equal frequency in the intestinal (21/34) and diffuse (30/46) types. However, LOH was significantly correlated with advanced stage and lymph node metastasis only in the intestinal type. Using RT-PCR-SSCP analysis, mutations of the *p300* gene were identified in the intestinal (6/15) and in the diffuse (4/18) types, but all of the mutations were accompanied by LOH at the 22q13 locus only in the intestinal type.

Conclusion. The *p300* gene behaves as a tumor suppressor gene in the intestinal, but not in the diffuse type of gastric carcinoma. The significance of frequent gene alteration in the diffuse type should be further investigated by the clarification of other mechanisms of *p300* inactivation and by clarification of the functional role of *p300* in cell adhesion/spreading.

Key words *p300* · Chromosome 22q · Gastric carcinoma · Tumor suppressor gene · Loss of heterozygosity

Introduction

Gastric carcinoma shares common chromosomal losses with colorectal carcinoma, such as that of 5q (30% to 40% at or near *APC*), 17p (over 60% at *TP53*), and 18q (over 60% at *DCC*) [1]. Loss of heterozygosity (LOH)

on chromosome 22q was also observed in both carcinomas [2,3], but the involved gene has not yet been clarified. Candidate suppressor genes on chromosome 22q are the *neurofibromatosis 2* (*NF2*) gene [4] and the *p300* gene [5]. *NF2* encodes a 595-amino-acid protein, schwannomin or merlin (moesin-ezrin-radixin-like-protein), which exhibits significant homology to a highly conserved family of proteins involved in connecting the cytoskeleton to components of plasma membranes. *NF2* is considered to be a tumor suppressor gene for tumors associated with *NF2* disorders (schwannoma and meningioma) and for *NF2*-unrelated tumors (mesothelioma, colon cancer, and gastrointestinal stromal tumor) [6–8]. On the other hand, *p300* is a transcriptional coactivator that facilitates gene expression through several mechanisms [9], including molecular scaffolds coupling many transcriptional factors [10], chromatin remodeling by histone acetyltransferase (HAT) activity [11], and activation of transcription factors, such as *p53* and *GATA-1*, by acetylation (FAT activity) [12]. *p300* is a target for the adenoviral E1A oncoprotein, and bi-allelic mutations have been demonstrated in some human cancers, including colorectal carcinomas [13,14]. Furthermore, in regard to its highly homologous protein, CBP (cAMP response element binding protein [CREB]-binding protein), it is known that patients with one mutant germline copy of CBP develop a rare, multisystem disorder, Rubinstein Taybi syndrome, one of the characteristics of which is a heightened cancer risk [15]. In the present study, we focused on the alterations on chromosome 22q to clarify its significance in the development and progression of gastric carcinoma. Detailed mapping of LOH and subsequent polymerase chain reaction (PCR)-single-stranded conformation polymorphism (SSCP) analysis demonstrated that *p300* gene alteration occurs frequently in gastric carcinoma. However, the significance of the abnormalities is different in the intestinal and diffuse types of gastric carcinoma. Each subtype of gastric carcinoma exhibits

its own clinicopathological features and is considered to develop through a different genetic pathway [1].

Patients, materials, and methods

Tumor samples

Tumor samples consisted of 60 advanced (invasion in and beyond the muscular layer) and 20 early gastric carcinomas (intramucosal carcinoma or invasion of the submucosal layer), which were resected from 80 patients at Tokyo Metropolitan Komagome Hospital and Jichi Medical School Hospital. The corresponding normal tissues, which were located at a site remote from the carcinoma, were also obtained simultaneously. All samples were taken immediately after resection and frozen in dry ice-hexane. The resected stomach was then routinely processed with hematoxylin-eosin staining for histopathological analysis. Tumor location, depth of invasion, and lymphatic or vascular invasion were determined with reference to the *Japanese classification of gastric carcinoma* of the Japanese Research Society for Gastric Cancer [16]. Histological subtypes of the gastric carcinoma were classified as diffuse type or intestinal type, using Laurén's criteria [17]. The presence of Epstein-Barr virus (EBV) was determined by an in situ hybridization (ISH) method targeting EBV-encoded small RNA (EBER) [18], using formalin-fixed paraffin-embedded sections of the carcinoma.

LOH analysis using microsatellite markers

High-molecular weight DNA was extracted from frozen tissues, using phenol-chloroform extraction, as reported previously [19]. The DNA was then subjected to the PCR-based analyses, as described below. PCR-based analysis of LOH was performed using microsatellite markers. The primers targeted 14 microsatellites; D22S264 (22q11), D22S446 (22q11), D22S315 (22q11–12), D22S300 (22q12), NF2CA3 (22q12), D22S268 (22q12), D22S275 (22q12), D22S280 (22q12), D22S304 (22q12–13), D22S277 (22q13), IL2RB (22q12–13), D22S423 (22q13), D22S279 (22q13), and D22S274 (22q13). The sequences of the primers were obtained from the Genome Database (<http://www.gdb.org/gdb/>) and Ensembl Human (http://www.ensembl.org/Homo_sapiens/) on the Internet. PCR was performed under the same conditions as those outlined in a previous study [18]. The reaction product was then denatured and electrophoresed on a 6% polyacrylamide gel containing 7M urea. The gel was exposed to X-ray film for 48–72 h at -80°C . By comparing the electrophoresis patterns of the PCR products of the paired DNA samples of the same patient, LOH was defined as the

loss of any band for the normal tissue in the tumor sample.

Reverse transcriptase-PCR (RT-PCR) and RT-PCR-SSCP

Total RNA was extracted, using the acid guanidium method, from 33 advanced gastric carcinomas and five normal tissues of the stomach mucosa. All carcinomas were advanced ones, because of the limitation of the amount of the tissue available for RNA study. Isolated RNA was reverse-transcribed to cDNA, using the Ready To GO kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The coding region of the *p300* gene between codons 111 and 1832 (71% of the *p300* gene) was divided into 16 regions, each of which was amplified by each specific primer pair, as described previously [13]. Then, the amplified products were separated overnight at 6–8 W on 6% non-denaturing polyacrylamide gels containing 6% glycerol.

Sequencing analysis

The shifted bands were excised from the SSCP gel and subjected to DNA extraction. The extracted DNA was amplified by PCR, using the same conditions as for the RT-PCR-SSCP. DNA sequencing of PCR products was carried out using an ABI PRISM™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Chiba, Japan) and an ABI PRISM™ 377 DNA Sequencer.

Statistical analysis

Statistical analysis was performed using Fisher's exact probability test, the χ^2 test for independence, the Kruskal-Wallis rank test, and the Mann-Whitney *U*-test. A *P* value of <0.05 was considered significant.

Results

PCR evaluation of LOH using microsatellite markers

Fourteen microsatellite markers were first used to evaluate the LOH on chromosome arm 22q in gastric carcinoma. Sixty cases of advanced gastric carcinoma (36 diffuse type and 24 intestinal type) were examined, because only a limited amount of DNA was available from the early carcinomas.

All of these microsatellite loci showed LOH at various frequencies (Fig. 1), but the loci that showed LOH of more than 30% were D22S275 (14 of 43 informative cases; 33%), D22S277 (21/59; 36%), D22S304 (21/56; 38%), and IL2RB (27/59; 46%). Thus, the most fre-

Fig. 1A,B. Evaluation of loss of heterozygosity (LOH) on chromosome 22q in gastric carcinoma. **A** LOH analysis using microsatellite markers. LOH was evaluated by comparing the electrophoresis patterns of the amplified fragments of microsatellite markers, using DNA samples of carcinomas (T) and corresponding normal tissue (N). LOH was defined as the loss of any band of non-neoplastic tissue in the carcinoma, such as LOHs (arrowheads) on D22S304, D22S277, and IL2RB. Cases 3, 8, 10, and 37, advanced carcinomas. Case 61, early carcinoma. **B** The frequency of loss of heterozygosity (LOH) on chromosome arm 22q in advanced carcinomas. A high frequency of LOH, of more than 30%, is observed on D22S275 (33%), D22S304 (38%), D22S277 (36%), and IL2RB (46%). The location of D22S304, D22S277, and IL2RB is close to the *p300* gene locus on chromosome arm 22q13

quent LOH was located at the 22q13 locus containing the *p300* gene, which was telomeric to the *NF2* gene.

Clinicopathological significance of 22q13-LOH

To clarify the significance of 22q13-LOH in the development and progression of gastric carcinoma, we further examined 20 more cases of early carcinoma

(10 diffuse and 10 intestinal type). Using three microsatellite markers, D22S304, D22S277, and IL2RB (Fig. 1), the frequency of LOH on these loci was significantly lower in the early carcinomas, at 26% (5/19 informative cases), 15% (3/20), and 0%, respectively, than in the advanced cases (36%, 38% and 46%, respectively).

When the data for the early and advanced gastric carcinomas were combined, we could evaluate the clinicopathological characteristics of the patients whose specimens showed LOH on any of the three loci of 22q13 (Table 1). The frequency of 22q13-LOH was the same in the diffuse (30/46) and intestinal types (21/34). In the diffuse type, 22q13-LOH was not correlated with any of the clinicopathological factors. On the other hand, in the intestinal type, 22q13-LOH was more frequent in the carcinomas of advanced stage, showing vascular and lymphatic invasion and lymph node metastasis. As for the association with EBV, a positive signal for EBER was much more frequently observed in the diffuse type, but the frequency of 22q13-LOH was not significantly different in the carcinomas with or without EBV in either the intestinal or the diffuse types.

Sequence analysis of *p300* cDNA

Because *p300* is located on chromosome 22q13, we evaluated the somatic mutation of the *p300* gene by RT-PCR and SSCP techniques. The specimens examined consisted of 27 carcinomas showing 22q13-LOH (14 diffuse type and 13 intestinal type), and 6 carcinomas without LOH (4 diffuse and 2 intestinal type). All carcinomas were advanced ones, because of the limitation of the amount of tissue available for RNA study.

Twelve mutations were identified in ten samples (Fig. 2), consisting of four cases of diffuse type and six cases of intestinal type of gastric carcinoma (Table 2). There was no significant correlation of the mutation with clinicopathological factors such as age, sex, or location, although there was a significant correlation with the histological type.

Nine mutations were point mutations: seven were transition and two were transversion type mutations. The other three mutations were frameshift mutations, which resulted in the generation of a stop codon. Five point mutations and one frameshift mutation in cases 8, 25, 39 and 47 were within the first and second Cys/His-rich regions (C/H-1 and -2) encoded by the *p300* gene. One deletion mutation, in case 37, occurred in the bromodomain (Br) and the two point mutations in cases 41 and 57 were within the CREB-binding domain.

Regarding alteration of the other allele, mutation of the *p300* gene was only identified in 1 of the 14 cases of the diffuse type of gastric carcinoma with 22q13-LOH

Table 1. Loss of heterozygosity on chromosome 22q13 and clinicopathological factors in diffuse and intestinal types of gastric carcinoma

Clinicopathological factors	Diffuse type			Intestinal type		
	LOH (+)	LOH (-)	<i>P</i> value	LOH (+)	LOH (-)	<i>P</i> value
Age	65 ± 14	64 ± 13	NS	64 ± 7	63 ± 7	NS
Sex (male/female)	22/8	9/7	NS	15/6	9/4	NS
Location (cardia/body/antrum)	10/12/8	2/9/5	NS	4/11/6	1/6/6	NS
Early/advanced	5/25	5/11	NS	3/18	7/6	0.0042
Depth of invasion (m/sm/mp/ss, se, si)	2/3/5/20	3/2/2/9	NS	2/1/0/18	5/2/3/3	0.0003
Venous invasion (+/-)	26/4	11/5	NS	19/2	6/7	0.0044
Lymphatic invasion (+/-)	26/4	12/4	NS	19/2	8/5	0.0426
Lymph node metastasis (+/-)	20/10	10/6	NS	17/4	4/9	0.0034
EBV (+/-) ^a	10/20	4/12	NS	4/17	2/11	NS

LOH, loss of heterozygosity; NS, not significant; m, intramucosal carcinoma; sm, carcinoma invading to the submucosa; mp, carcinoma invading to the submucosa; ss, carcinoma invading to the subserosa; se, carcinoma exposed on the serosal surface; si, carcinoma infiltrating to other organs

^a Positive or negative signal by Epstein-Barr virus-encoded small RNA (EBER) in situ hybridization in advanced carcinoma

Fig. 2. Reverse transcriptase-polymerase chain reaction (RT-PCR)-single-stranded conformation polymorphism (SSCP) analysis of the *p300* gene. A fragment of *p300* gene cDNA was amplified and subjected to PCR-SSCP analysis; 300M corresponds to the region between codons 1378 and 1477 in case 3; 300C, region between codons 327 and 443 in case 8; and 300J, region between codons 1060 and 1173 in case 37. The sequencing of the abnormally shifted bands (arrowheads) demonstrates 1 base-pair deletion on codon 1468 in case 3, a missense mutation, GGG to GAG (Gly to Glu), on codon 359 in case 8, and 25 base-pair deletion on codon 1084 to 1092 in case 37. T and N as in Fig. 1

(7%), whereas it was observed in 6 of the 13 cases of the intestinal type with LOH (46%). In the cases without 22q13-LOH, three mutations were identified, and all of the cases with the mutation were of the diffuse type histologically. Two of the three mutations were frame-shift mutations, and two of the three cases were associated with EBV infection.

Discussion

Gastric carcinoma has the same alterations of tumor suppressor genes as colorectal carcinoma, such as *APC* on chromosome 5q, *TP53* on 17p, and *DCC* on 18q. In the present study, we confirmed that LOH on chromosome 22q was also observed with the same considerable

Table 2. *p300* mutations in diffuse and intestinal types of gastric carcinomas

Case no.	Diffuse/ Intestinal	Nucleotide	Codon	Domain	Alteration	Nucleotide change	Nature of the mutation	22q13-LOH		
								IL2RB	D22S304	D22S277
8	Diffuse	2275	359	C/H-1	Transition	G <u>G</u> G → G <u>A</u> G	Gly → Glu	LOH	LOH	Hete
3	Diffuse	5601	1468		Deletion	One base-pair deletion	Frameshift	Hete	Hete	Hete
18	Diffuse	1804	202		Transversion	C <u>G</u> A → C <u>T</u> A	Arg → Leu	Hete	Hete	Hete
25	Diffuse	2353–2366	385–389	C/H-1	Deletion	14 base-pair deletion	Frameshift	Hete	Hete	Hete
37	Intestinal	4451–4475	1084–1092	Br	Deletion	25 base-pair deletion	Frameshift	LOH	Hete	LOH
39	Intestinal	5535	1446	C/H-2	Transition	T <u>A</u> T → C <u>A</u> T	Tyr → His	LOH	Hete	LOH
41	Intestinal	2718	507	CREB	Transition	G <u>A</u> G → G <u>G</u> G	Glu → Gly	LOH	Hete	NI
46	Intestinal	1705	169		Transition	A <u>C</u> G → A <u>T</u> G	Thr → Met	LOH	Hete	NI
		1809	204		Transition	G <u>A</u> T → A <u>A</u> T	Asp → Asn			
47	Intestinal	2247	350	C/H-1	Transition	A <u>A</u> G → G <u>A</u> G	Lys → Glu	LOH	LOH	Hete
		2315	372	C/H-1	Transversion	A <u>A</u> G → A <u>A</u> T	Lys → Asn			
57	Intestinal	2722	508	CREB	Transition	A <u>A</u> C → A <u>G</u> C	Asn → Ser	LOH	LOH	LOH

LOH, loss of heterozygosity; hete, heterozygous; NI, not informative; C/H-1,2,3; Cys/His rich regions; Br, bromodomain; GRR, glutamine-rich region; CREB, cAMP response element binding protein (CREB) binding domain; HAT, histone acetyltransferase domain

Schematic drawing of *p300* molecule, showing the positions of the mutations, is represented below the table. *Arrows*, altered codons; *hatched boxes*, regions homologous to CREB-binding protein (CBP)

frequency in gastric carcinomas as in colon carcinomas [2]. A candidate for the tumor suppressor gene on chromosome 22q is the *p300* gene, because the most frequent LOH was located at the 22q13 locus. When we directly analyzed the *p300* gene mutations by RT-PCR-SSCP, mutations were observed in 6 of the 13 gastric carcinomas of the intestinal type with 22q13-LOH. One mutation was a frameshift mutation at codon 1084, which resulted in truncation of the encoded product before the C/H-2 (Table 2). C/H-2 is located within the histone acetyltransferase (HAT) domain, where *p300*/CBP also interacts with *p53* functioning as a coactivator for *p53*-mediated transcription [12]. The other five mutations were missense mutations at the C/H-1 and -2, and CREB-binding domains. Thus, mutation within a functionally important region in combination with loss of another allele fits the two-hit theory of the inactivation of tumor suppressor genes. Therefore, the *p300* gene is the tumor suppressor gene in the intestinal type of gastric carcinoma, and this finding also illustrates that common genetic alterations underlie colorectal carcinoma and the intestinal type of gastric carcinoma.

LOH on chromosome 22q was observed mainly in the late stage of colorectal carcinomas, correlating with lymph node metastasis [20]. In the intestinal type of gastric carcinoma, 22q13-LOH was more frequently observed in the advanced stage, accompanied by vascular and lymphatic invasion, and lymph node metastasis.

Thus, the inactivation of *p300* may be involved in the progression of gastric carcinoma, facilitating metastasis via vascular and lymphatic invasion. This may be related to the function of *p300* other than that of histone acetyl transferase, that is, the activation of a major adhesion molecule, $\beta 1$ integrin, by interacting with the nuclear protooncoprotein SYT [21]. It is also interesting to note that there were two cases that showed two different mutations in the same tumor tissue. This also suggests that the inactivation of *p300* is a late genetic event in the subpopulation of the intestinal type of gastric carcinoma.

In the diffuse type of gastric carcinoma, the frequency of 22q13-LOH was similar to that in the intestinal type, but *p300* gene mutation was rare in the carcinomas with LOH. Furthermore, in contrast to findings in the intestinal type, 22q13-LOH was not correlated with any of the clinicopathological factors. Therefore, the significance of *p300* gene alteration in the diffuse type is apparently different from that in the intestinal type. Because two of the three mutations were frameshift mutations in the diffuse type without 22q-LOH, the alteration on at least one allele of the *p300* gene may be more ubiquitous in the diffuse type. Therefore, when combined with other alterations, such as homozygous deletion, promoter methylation, and EBV infection, inactivation of *p300* may occur along the carcinogenesis of the diffuse type of gastric carcinoma. Mouse embryonic cells (MEC) with a *p300*+/- genotype showed defec-

tiveness in adhesion to fibronectin-coated surfaces in comparison with the wild type, suggesting that failure to form a sufficient quantity of SYT/p300 complexes leads to a major defect in cell adhesion/spreading [21]. In addition to the example of Rubinstein Taybi syndrome in humans, mice with monoallelic inactivation of the *CBP* gene show an increased incidence of hematological malignancies [22]. Thus, *p300* gene alteration, even on one allele (haploinsufficiency), may be involved in the development of the carcinoma. Knock-down experiments, using RNA interference, may clarify the role of p300 in gastric cancer.

In conclusion, *p300* gene alteration may play an important role in both the intestinal type and the diffuse type of gastric carcinoma, but the significance of the alterations in each type is different. Mutation within a functionally important region, in combination with loss of one allele, suggests that the *p300* gene is the tumor suppressor gene in the intestinal type of gastric carcinoma, as it is in colorectal carcinoma. On the other hand, in the diffuse type, the *p300* gene is not a classical suppressor gene, but further study is necessary to find other mechanisms of p300 inactivation and to clarify the functional role of p300 in cell adhesion/spreading.

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