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Mutational analysis of the *hCDC4* gene in gastric carcinomas

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ABSTRACT

hCDC4, a ubiquitin ligase, plays a role in the control of cell cycle and chromosome stability. The *hCDC4* gene is considered a tumour suppressor gene and is mutated in several human neoplasias, including colorectal and endometrial tumours. Data on the *hCDC4* mutation in gastric cancer is, however, lacking. This study explored the possibility that *hCDC4* mutation is involved in the development of gastric cancer. The *hCDC4* gene in 162 gastric adenocarcinoma tissues was analysed for somatic mutations using a polymerase chain reaction-single strand conformation polymorphism assay. Overall, six *hCDC4* mutations were found (3.7%), comprising four missense, one frameshift deletion and one nonsense mutation(s). It is notable that the *hCDC4* mutations were found in early as well as in advanced gastric carcinomas. These data indicate that *hCDC4* mutation occasionally occurs in gastric carcinomas and suggest that it might play a role in the development of some gastric carcinomas.

1. Introduction

Human cancers may arise as the result of an accumulation of genetic mutations and subsequent clonal selection of variant progeny with increasingly aggressive behaviours.[1] Proto-oncogenes and tumour suppressor genes are the principal targets of mutations in cancer development.[1] hCDC4 (also known as Fbw7, Archipelago or Sel-10) functions as a phosphor-epitope-specific substrate recognition component of the SCF ubiquitin ligase complex that regulates ubiquitination and degradation of cellular regulators, including cyclin-E, c-Jun, c-Myc and Notch.[2–5] In *Drosophila*, cells with an inactivating mutant of *Archipelago* (the *hCDC4* orthologue) have a growth advantage over wild-type cells.[6] Suppression of hCDC4 function in mammalian cells also caused various characteristics of tumours, such as chromosomal instability,[7] cyclin-E accumulation[2] and c-Myc's growth promoting function,[3] thus being considered as a candidate tumour suppressor. In a mouse model, loss of one *hCDC4* allele promoted tumour transformation, suggesting that the *hCDC4* gene is a haplo-insufficient tumour suppressor gene.[8]

The *hCDC4* gene was reported to be mutated in both cancer cell lines and primary human cancers. *hCDC4* mutations were found in breast, ovary and leukaemia cell lines, and functionally the mutations were associated with increased levels of cyclin-E protein.[2,6] In primary tumour tissues, *hCDC4* mutations were detected in different tumours, including endometrial, colorectal and ovarian tumours (2.9–16% of the endometrial carcinomas, 13% of the colorectal tumours and 2% of the ovarian carcinomas).[7,9–12]

Gastric cancer occurs with a high incidence in Asia and is one of the leading causes of cancer deaths worldwide. However, little is known about the molecular genetic event in the development and progression of gastric cancer. Previous reports on the occurrence of *hCDC4* mutations in several cancer tissues raised the possibility that the *hCDC4* gene might be mutated widely in other human cancers. To explore this possibility, this study analysed 162 gastric adenocarcinoma

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tissues and found that the *hCDC4* gene is somatically mutated in some gastric cancers.

2. Materials and methods

2.1. Tissue samples and microdissection

Methacarn-fixed tissues of 162 gastric carcinomas were randomly selected for polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) of the *hCDC4* gene. All of the patients were Asian (Korean). Approval for this study was obtained from the Catholic University of Korea, College of Medicine's institutional review board. Informed consent was provided according to the Declaration of Helsinki. The gastric carcinoma samples consisted of 70 diffuse-type, 55 intestinal-type and 37 mixed-type gastric adenocarcinomas by Lauren's classification, and 40 early gastric carcinomas and 122 advanced gastric carcinomas according to the depth of invasion. The tumour-node-metastasis (TNM) stages of the gastric cancers were 15 stage 0, 57 stage I, 40 stage II, 35 stage III and 15 stage IV. As a positive control for the hCDC4 mutation,[6] we also analysed the SKOV3 cell line.

Malignant and normal cells were selectively procured from haematoxylin and eosin (H&E) stained slides using a 301/2 gauge hypodermic needle (Becton Dickinson, Franklin Lakes, NJ, United States of America (USA)) affixed to a micromanipulator, as described previously.[13] In this study, primary lesions, but not the metastatic lesions, were analysed for mutations. DNA extraction was performed by a modified single-step DNA extraction method, as described previously.[13]

Table 1 – Primer sequences of *hCDC4* gene used in this study

Gene	Sequences	Size (bp)
hCDC4 Exon 3	F: 5′-TGTAGTACCACTGGGCTTGT-3′ R: 5′-GCAATTAAGTGAGGCATTTC-3′	191
hCDC4 Exon 4-1	F: 5′-AAGCCTGTAATTTGGGACATCT-3′ R: 5′-AAACTGGGGTTCTATCACTTGC-3′	160
hCDC4 Exon 4-2	F: 5′-CAGAGAAATTGCTTGCTTTAG-3′ R: 5′-ATAACACCCAATGAAGAATGTA-3′	159
hCDC4 Exon 5-1	F: 5′-TTTATCAAGTATCTCATCCTGTG-3′ R: 5′-CAGCCAAAATTCTCCAGTAG-3′	182
hCDC4 Exon 5-2	F: 5′-CTGGAACCCAAAGACCT -3′ R: 5′-ACTTGTTTTCAGAATCACTCT-3′	167
hCDC4 Exon 6	F: 5′-GTGAAGGCAATTTACTCTTGA-3′ R: 5′-AACACTGATTAACGGTTTCTG-3′	218
hCDC4 Exon 7	F: 5′-ATTAACATATTTCTAATCTGCAC-3′ R: 5′-ACTTTGTGAAGTGTAGGAAG -3′	181
hCDC4 Exon 8-1	F: 5′-AAATCACTTTTCCTTTCTACC-3′ R: 5′-TATACATTCTCCAGTCTCTGC-3′	177
hCDC4 Exon 8-2	F: 5′-GGAGTATGGTCATCACAAATG-3′ R: 5′-TTCACCAATAATAGAGGAAGAAG-3′	203
hCDC4 Exon 9-1	F: 5′-TTTTGTTTTTCTGTTTCTCCC-3′ R: 5′-TACCATAAAATCATATGCTCCAC-3′	179
hCDC4 Exon 9-2	F: 5′-TCAATATGATGGCAGGAG -3′ R: 5′-TGAGTAAAACAACCTTATGATTG-3′	185
hCDC4 Exon 10-1	F: 5′-AATTGATAGGAAGAGTATCCATA-3′ R: 5′-ATTGTCTTTGAGTTCCATTC-3′	186
hCDC4 Exon 10-2	F: 5′-TTTGGGATGTGGAGACAG-3′ R: 5′-CAACAAAACGAAAGGTGAGTA-3′	191
hCDC4 Exon 11-1	F: 5′-TTTTGGTTTTTGTCTAGGTCC-3′ R: 5′-CACAACTCCCCCACTCC-3′	178
hCDC4 Exon 11-2	F: 5′-CCTAGTCACATTGGAGAGTGG-3′ R: 5′-TTGGACAAATTCATCTTTTCTG-3′	174

2.2. PCR-SSCP analysis

Most of the *hCDC4* mutations in the tumour tissues have been reported within the exon 3–11.[7,9–12] Thus, we analysed the *hCDC4* mutation in these nine exons. Genomic DNAs from tumour cells and normal cells from the same patients were amplified with 15 primer pairs (Table 1) covering the exon 3–11 of human *hCDC4* gene. Numbering of cDNA of *hCDC4* was carried out with respect to the ATG start codon (Genbank NM_033632).

Radioisotope ([^{32}P]dCTP) was incorporated into the PCR products for detection by autoradiogram. The PCR reaction mixture was denatured for 1 min at 94 °C and incubated for 30 cycles (denaturing for 30 s at 94 °C, annealing for 30 s at 50–60 °C, and extending for 30 s at 72 °C). Other procedures of PCR and SSCP analysis were performed as described previously.[14,15] After SSCP, DNAs showing mobility shifts were cut out from the dried gel, and re-amplified for 30 cycles using the same primer sets. Sequencing of the PCR products was carried out using a cyclic sequencing kit (Perkin–Elmer, Foster City, CA, USA) according to the manufacturer's recommendation. The experiments were repeated twice, including PCR, SSCP and sequencing analysis to ensure the specificity of the results.

3. Results

Genomic DNAs isolated by microdissection from the gastric adenocarcinomas and normal tissues of the same patients were analysed by PCR-SSCP for mutations in the exons and the exon-intron junctions in exon 3–11 of the *hCDC4* gene. DNA sequence analysis of aberrantly migrating bands on the SSCP led to the identification of six *hCDC4* mutations (3.7%) in the 162 gastric cancers (Table 2 and Fig. 1). None of the normal samples from the same patients showed evidence of mutations using SSCP (Fig. 1), indicating that the mutations had risen somatically. The *hCDC4* mutations consisted of four missense, one frameshift deletion and one nonsense mutation(s) in the coding sequences. Three mutations (D440N, R465C and W649X) were detected in the WD40 repeats, while the other three (S668fsX706, R674C and E693K) were found at the C-terminal portion next to the WD40 repeats. In all of the six mutations SSCP patterns at the mutation sites showed both aberrant bands and wild-type ones (Fig. 1), indicating heterozygous mutations. The bands of wild-type alleles in the mutation cases could result from normal tissue contamination. However, because a micrometrically precise microdissection technique was used[13] and the same results were obtained through two separate microdissections, the microdissected tumour samples should be nearly devoid of normal tissue contamination.

According to the depth of the tumour invasion, two *hCDC4* mutations (S668fsX706 and W469X) were detected in early gastric carcinomas, and the remaining four mutations were found in advanced gastric carcinomas (Table 2). Regarding the histological subtypes and staging of the gastric cancers,

Table 2 – Summary of the *hCDC4* mutations of gastric carcinomas

Case	Sex/age (years)	TNM stage	Primary tumour*	Mutation type	Mutation site	Nucleotide change	Predicted amino acid changes
91	M/63	II	AGC	Missense	Exon 8	1318G > A	D440N
203	F/69	IB	AGC	Missense	Exon 8	1393C > T	R465C
177	M/67	0	EGC	Nonsense	Exon 11	1947G > A	W649X
270	F/70	IA	EGC	Frameshift deletion	Exon 11	1996delG	S668fsX706
83	M/62	IIB	AGC	Missense	Exon 11	2021G > A	R674Q
189	M/67	III	AGC	Missense	Exon 11	2077G > A	E693K

* AGC, advanced gastric carcinoma; EGC, early gastric carcinoma.

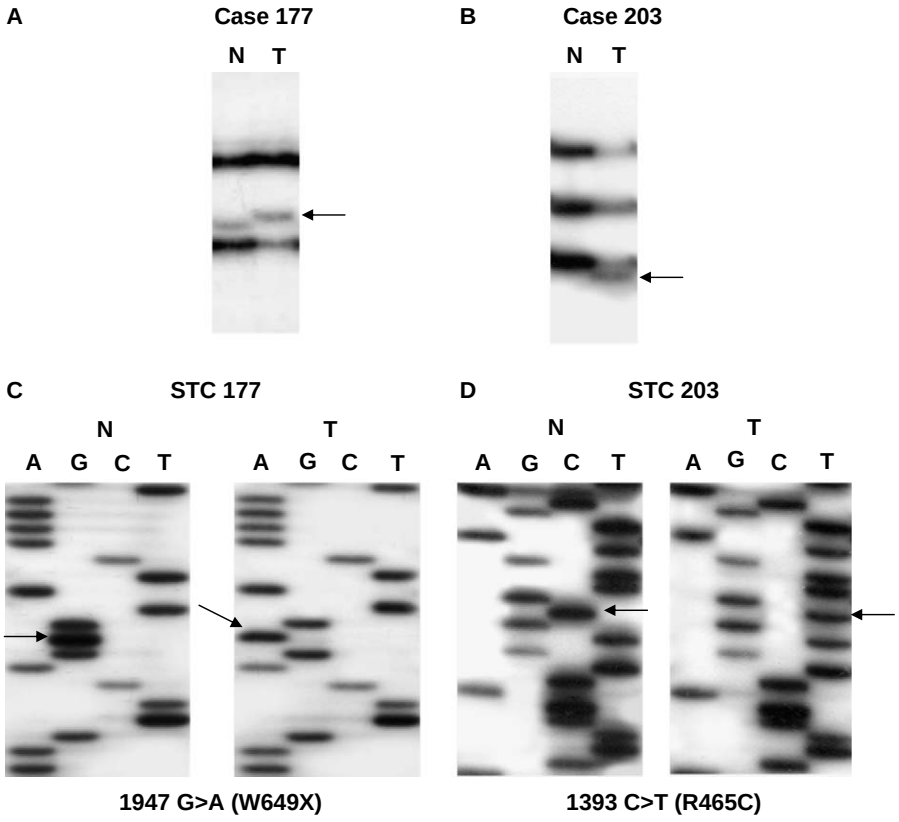

Fig. 1 – PCR-SSCP of *hCDC4* gene in the gastric carcinomas. (A and B) Representative data of SSCP and (C and D) DNA sequencing analysis of *hCDC4* DNA from tumours (lane T) and normal tissues (lane N). (A and B) Single strand conformation polymorphisms (SSCPs) of DNA from two gastric adenocarcinomas (T) show wild-type bands and additional aberrant bands (*arrows*) compared with SSCP from corresponding normal cells (N). (C and D) Sequencing analyses from the aberrant bands in (A and B). Arrows indicate nucleotide changes in tumour tissue compared with normal tissue. Numbering of cDNA of *hCDC4* was carried out in respect to the ATG start codon (GenBank).

there was no significant correlation with *hCDC4* mutations (Fisher's exact test, $P > 0.05$). As a positive control, the known *hCDC4* R505L mutation could also be detected in the SK-OV3 ovarian cancer cell line,[6] demonstrating that the PCR-SSCP methods could detect a common *hCDC4* mutation (data not shown).

4. Discussion

Previous observations that the *hCDC4* gene is mutated in colorectal, endometrial and ovarian cancers led us to analyse whether the *hCDC4* gene is mutated in other human cancers.[7,9–12] It was found that gastric carcinomas harboured *hCDC4* mutation in 3.7% of the tumour samples. The data suggested that *hCDC4* mutation may occur widely in human cancers. Furthermore, it was observed that *hCDC4* mutations could occur in early gastric carcinoma as well as in advanced gastric carcinoma. All gastric cancers presumably begin as early gastric carcinoma, which develop over time into advanced lesions.[16] However, the mechanism of this process is still poorly understood. The presence of the *hCDC4* mutation in early gastric carcinomas suggested that *hCDC4* mutation could be one of the mechanisms involved in the early event of gastric carcinogenesis. Also, both c-Myc and cyclin-E

expression are observed in both in early and advanced gastric cancers.[17,18] Similarly, *hCDC4* mutations occurred in both colorectal carcinomas and their precursor lesions,[7] which, together with our data, support a role for the mutated hCDC4 in the early steps of pathogenesis of common gastrointestinal cancers.

Among the six types of *hCDC4* mutations detected (Table 2), the frameshift mutation 1996delG and the nonsense mutation W649X would change the amino acids, produce a premature termination of hCDC4 protein synthesis, and hence resemble typical loss-of-function mutations. Of note, all of the 6 amino-acid residues affected by the *hCDC4* mutations are conserved among the species of human, mouse, cow and dog (NCBI database). Despite the wide distribution of *hCDC4* mutations, approximately 50% of mutations have been detected in exons 7 and 8.[7,9–12] In this study two *hCDC4* mutations were detected in exon 8, and one (R465C) of the two was a hot-spot mutation in colorectal tumours.[7] The R465C mutation was proven to disrupt the binding of hCDC4 with cyclin-E.[7] Additionally, one of the features of *hCDC4* mutation is that they commonly affected the Arg amino acid residue.[7,9–12] In agreement with such observations, one-third of the mutations detected in the present study would affect the Arg residue (Table 2).

A central aim of cancer research has been to identify the mutated genes that are causally implicated in tumourigenesis. Mutations in cancer could be categorised as either functional alterations affecting key genes underlying the neoplastic process or non-functional 'passenger' changes.[19] Unfortunately, however, the prevalence of passenger mutations in the cancer genomes is not known. In general, high incidence and functional derangements related to the characteristics of cancers may favour that the mutated gene is a cancer-related functional gene, but not a passenger gene.[19] Modest incidences (3.7%) and the intrinsic tumour suppressor function of hCDC4 suggest that the *hCDC4* mutations detected in this study might be functional alterations, but not passenger alterations.

Classically, inactivation of both alleles of a tumour suppressor gene was required to promote tumour progression. However, for some genes, haplo-insufficiency, which is loss of only one allele, may contribute to carcinogenesis.[20] The haplo-insufficiency effects may either be directly attributable to the reduction in gene dosage or may act in concert with other oncogenic or haplo-insufficient events.[20] Many tumour suppressor genes, including $p27^{Kip1}$, *p*53 and *LKB1*, could be categorised into the haplo-insufficient genes.[21–23] Experimentally, $hCDC4^{+/-}$ mice had increased production of tumours compared with wild-type mice, indicating that hCDC4 may be a haplo-insufficient tumour suppressor gene.[8] In colorectal tumours, 22 of 26 *hCDC4* (77%) were mutated heterozygously.[7] Similarly, all of the six mutations found in the current study were heterozygously mutated *hCDC4* mutations (Fig. 1), strongly suggesting the possibility that *hCDC4* also act as a haplo-insufficient tumour suppressor gene in gastric carcinomas.

It is of interest to compare our findings with those reported previously.[7,9–12] The incidence of *hCDC4* mutations in the endometrial carcinomas ranged from 3% to 16%,[9–11] while that in colorectal tumours was 13%.[7] In the ovarian carcinomas, the mutation rate was below 2%.[12] Our data showing *hCDC4* mutations in 3.7% of gastric carcinomas indicated that the incidence of *hCDC4* mutation varied depending on the type of tumour. The $hCDC4^{+/-}$ mice had high frequencies of tumour formations in different tissues,[8] raising the possibility that *hCDC4* gene mutation might be involved in the pathogenesis of other human cancers. Clearly, therefore, studies are now needed to look for *hCDC4* mutations in other common human cancers.

Conflict of interest statement

None declared.

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