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Loss of *CDC4/FBXW7* in gastric carinoma

A.N. Milne [a,*], R. Leguit [a], W.E. Corver [b], F.H.M. Morsink [a], M. Polak [c], W.W. de Leng [a], R. Carvalho [d] and G.J.A. Offerhaus [a]

[a] *Department of Pathology, University Medical Centre, Utrecht, The Netherlands*
[b] *Department of Pathology, Leiden University Medical Centre, Leiden, The Netherlands*
[c] *Department of Pathology, Academic Medical Centre, Amsterdam, The Netherlands*
[d] *ZF-Screens BV, Leiden, The Netherlands*

Abstract. *Background*: *CDC4/FBXW7,* encoding a ubiquitin ligase, maps to 4q32 and has been implicated as a tumor suppressor gene and therapeutic target in many tumor types. Mutations in colonic adenomas, and the frequent losses on 4q described in gastric cancer prompt speculation about the role of *CDC4/FBXW7* in gastric carcinogenesis.

Methods: We assessed the role of *CDC4/FBXW7* in gastric cancer, through loss of heterozygosity (LOH) and multiplex ligation-dependent probe amplification (MLPA) on 47 flow-sorted gastric carcinomas including early-onset gastric cancers (EOGC) and xenografted conventional gastric carcinomas. Ploidy analysis was carried out on 39 EOGCs and immunohistochemistry of CDC4/FBXW7 and its substrates c-myc, c-jun, Notch and cyclin E was performed on 204 gastric carcinomas using tissue microarrays (TMAs). Sequence analysis of *CDC4/FBXW7* was carried out on gastric carcinoma cell lines and xenografts.

Results: Loss of heterozygosity of *CDC4/FBXW7* occurred in 32% of EOGCs, and correlated with loss of expression in 26%. Loss of expression was frequent in both EOGC and conventional gastric cancers. No *CDC4/FBXW7* mutations were found and loss of *CDC4/FBXW7* did not correlate with ploidy status. There was a significant correlation between loss of CDC4/FBXW7 expression and upregulation of c-myc.

Conclusion: Loss of *CDC4/FBXW7* appears to play a role in both EOGC and conventional gastric carcinogenesis, and c-myc overexpression is likely to be an important oncogenic consequence of *CDC4/FBXW7* loss.

Keywords: Gastric cancer, hcdc4, FBXW7, early-onset gastric cancer, MLPA

1. Introduction

Gastric cancer is the fourth most common malignancy in the world and ranks second in terms of cancer-related death [35]. Several classification systems have been proposed, but the most commonly used are those of the World Health Organization (WHO) and of Laurén who describes the diffuse and intestinal histological types [23]. Gastric cancer is thought to result from a combination of environmental factors and the accumulation of generalized and specific genetic alterations, and consequently affects mainly older patients often after a long period of atrophic gastritis. The commonest cause of gastritis is infection by *Helicobacter Pylori*, which is the single most common cause of gastric cancer [12,36].

Research to date has not revealed a specific pathway for gastric cancer, although numerous molecules have been implicated [26,29]. Aberrations of the *CDH1* gene, which encodes E-Cadherin, have been shown to be an important initiator of gastric cancer [13] yet even in hereditary diffuse gastric cancer mutations of this gene explain only 30–40% of cases [34]. Thus the need to uncover other such critical regulators of gastric cancer is undoubted.

The *CDC4/FBXW7* gene, which maps to 4q32, encodes a ubiquitin ligase and has been implicated as a tumor suppressor gene in many tumor types. CDC4/FBXW7 forms part of the ubiquitin-proteasome mediated programmed degradation system whereby the addition of ubiquitin polymers to protein substrates directs them to the proteasome for destruction. Ubiquitin ligases called SCF complexes couple to any one of several "F-box" proteins, such as CDC4/FBXW7, which confer specificity of substrate, and over 70 different F-box proteins have been identified in man [32]. FBXW7 targets several oncoproteins, including cyclin E, c-myc, c-jun, Notch 1 and Notch 4 for degradation

*Corresponding author: Anya N. Milne, Department of Pathology, H04-312, University Medical Center Utrecht, Postbox 85500, 3508 GA, Utrecht, The Netherlands. Tel.: +31 887 555555; Fax: +31 882 544990; E-mail: a.n.a.milne@umcutrecht.nl.

and its tumor suppressor function is thought to be exerted through these substrates. Mutations that disrupt CDC4/FBXW7 are expected to deregulate cyclin E, c-myc, c-jun and Notch signaling thus simultaneously deregulating cell division, cell growth, apoptosis and cell differentiation [52]. The tumor suppressor role of CDC4 is said to be p53 dependent [24]. In addition, mutations in the *CDC4/FBXW7* gene have shown to be associated with DNA aneuploidy in endometrial cancer [16].

The finding of mutations in colonic adenomas, suggest that dysregulation of *CDC4/FBXW7* is a critical step in colon carcinogenesis [37]. This prompts speculation about the role of *CDC4/FBXW7* in the stomach and as to whether it acts as an important tumor suppressor in gastric cancer, as parallels can often been drawn between gastric cancer and cancer occurring in other parts of the gastrointestinal tract. The finding of frequent losses on 4q in gastric cancers using comparative genomic hybridization [18,20,44,45,50] furthers curiosity about the role of *CDC4/FBXW7* in gastric cancer and indeed 6% of gastric tumors have recently been reported to have a somatic mutation of *CDC4/FBXW7* [2,24].

In order to examine the role of *CDC4/FBXW7* we have chosen to primarily investigate early-onset gastric cancers (EOGC; $\leqslant$45 years old) as it is more likely that genetic factors are more important in this group when compared to conventional gastric cancer ($>$45 years old) [9,29]. Indeed, EOGCs are known to have a different molecular and genetic phenotype from that of the older age group [5,6,27–29,39]. In this study we have carried out ploidy analysis and assessed the status of *CDC4/FBXW7* in EOGCs using loss of heterozygosity (LOH) and multiplex ligation-dependent probe amplification (MLPA) on flow-sorted tumor cells. We also carried out sequence analysis on 13 gastric cancer cell lines and xenografted gastric cancers. In addition we assesed the expression of the p53, CDC4/FBXW7, cyclin E, c-myc, c-jun and Notch in 204 gastric cancers including 113 early-onset and 91 conventional gastric cancers.

2. Methods

2.1. Patients/study groups

Ninety one conventional gastric cancers ($>$45 years old), diagnosed between 1993 and 2003, were obtained from the Academic Medical Centre, Amsterdam. One hundred and thirteen cases of gastric carcinoma in patients under 45 years of age (coded with the letter Y), 90% diagnosed between 1994 and 2002 and 10% diagnosed between 1980–1994, with an unknown family history, were obtained from 24 different institutions throughout the Netherlands through the nationwide database system, and from the Department of Pathology at the Jorvi Hospital (Espoo, Finland). This age cut-off was chosen in order to obtain enough cases to achieve meaningful result. The tumors were classified by an experienced gastrointestinal pathologist (GJAO) according to the Laurén classification as intestinal, diffuse or mixed gastric adenocarcinomas (Table 1) and location was deduced from the pathological report. In addition, 8 xenografted gastric carcinomas (coded with the letter X) described by Milne et al. [30] were included. This study was carried out in accordance to the ethical review committee of our institution and in accordance to the Helsinki Declaration of 1975, as revised in 1983.

2.2. Tissue microarray

Tissue microarrays were constructed from formalin-fixed and paraffin-embedded (FFPE) archive specimens. Three core biopsies (0.6 mm cylinders) were taken from histologically representative regions (including heterogeneous areas) of paraffin-embedded gastric tumors and arranged in a new recipient paraffin block (tissue array block) using a custom-built instrument (Beecher Instruments, Silver Spring, MD, USA) as described previously [22,28]. Normal gastric mucosa from each case was also included where available (33%, 69/204). Cores were arranged in 2 or 3 separate subdivisions together with insertion of liver, lymph node and kidney cores to assist analysis. 195 cases

Table 1
Clinicopathological characteristics

Age	Histology			Location			Total
	Intestinal	Diffuse	Mixed	Cardia	Non-cardia	Unknown	
$\leqslant$45 yrs old	24	80	9	9	74	30	113
$>$45 yrs old	49	31	11	49	42	0	91

were informative for all markers. In most cases sections were stained immediately after cutting, but if stored, this was done so by wrapping in aluminium foil and freezing at −20°C to prevent loss of antigenic epitopes. Immunohistochemistry was carried out using full tissue sections for the 8 xenografted gastric cancers.

2.3. Immunohistochemistry

Sections (4 μm) were deparaffinized and endogenous peroxidase activity was blocked by immersion in 0.3% H_2O_2 in methanol for 20 min. Antigen retrieval was carried out by 10 min of boiling in 10 mM Tris/1 mM EDTA (pH 9) except for c-myc and cdc4/Fbw7 where no antigen retrieval was used and for C-jun where a pepsine pre-treatment was performed at a concentration of 0.25% in 0.01 M HCl/pH 2.0 for 15 min at 37°C. Non-specific binding was blocked with 5% normal goat serum in PBS (pH 7.4) for 15 min at room temperature. The sections were incubated for 1 h at room temperature with the following primary antibodies: CDC4/FBXW7 (Zymed, South San Francisco, CA, USA) 1:50, p53 (monoclonal antibody combination of DO-7 and BP53-12 Neomarkers, Union City, CA, USA) 1:2000 dilution, NCL c-jun (Nova Castra) 1:200, Cyclin E Ab-5 (clone CYE05), monoclonal antibody (Neomarkers, Fremont, CA, USA) 1:40 dilution, and c-myc (Santa Cruz) 1:50. Notch rabbit polyclonal antibody (Cell Signaling Technologies, Danvers, MA, USA) was incubated overnight at 4°C, 1:50. The Powervision+poly-HRP detection system (ImmunoVision Technologies, Co., Daly City, CA, USA) was used to visualize antibody binding sites with 3,3′-diaminobenzidine as a chromogen. Sections were counterstained with haematoxylin.

2.4. Immunohistochemical scoring

All immuhistochemical staining was scored independently by 2 of the authors (G.J.A.O and A.N.M). For the TMA, the overall score of the tumor was the highest score found of the 3 cores [28]. In all cases where the staining was not obviously abnormal, it was placed in the normal category. No slides showed increased negative staining around the edges. The following scoring systems were used: P53 – cases scored as positive showed strong nuclear staining in >30% of cells [49] whereas normal mucosa showed scant staining in the proliferative zone only; c-myc and c-jun – Nuclear staining in >5% was scored as positive [14,28], with lack of staining or presence of cytoplasmic staining only, scored as negative (as found in normal gastric mucosa); cyclin E – this was initially graded in 4 categories 0–4%, 5–10%, 11–50% and 51–100% positivity in the nucleus [27]. Thereafter, samples in the 0–10% categories were deemed negative/normal (and comparable with the variation in normal gastric mucosa) and 11–100% in the positive/overexpression category. CDC4/FBXW7 was scored in 4 categories: 0 – completely negative, 1 – reduced (nuclear staining in <20% of cells; clearly reduced compared to normal gastric mucosa), 2 – moderate (21–50%; similar to normal gastric mucosa) and 3 – strong (>50%). Two categories of negative (0) and positive (1, 2 and 3) were ultimately used in the statistical analysis. Notch scoring was negative if 0% of cells showed nuclear staining, all other staining patterns were scored as positive. Normal mucosa showed varying levels of positivity for Notch (no cases of normal gastric mucosa showed absence of staining).

For all antibodies, negative controls for the immunohistochemistry were carried out when optimising the antibody. Here we performed immunohistochemistry using a variety of antibody dilutions and buffers on both normal gastric mucosa and gastric mucosa with cancer to optimise the antibody, ensure its specificity and to attain minimal background.

2.5. Statistical analysis

The SPSS 11.5 software package was used for statistical analysis. A chi-squared test was applied to the groups of gastric cancer to determine whether the differences found between antibodies were statistically significant ($p < 0.05$). A binary logistic regression model was used to adjust for potential confounding factors such as location, histological type, T stage, N stage, ploidy, age of blocks and the hospital from which the block was derived.

2.6. Tissue dissociation and staining

Four 60 μm sections pre-trimmed to exclude non-cancerous epithelial tissue were collected in individual 100 μm-pore gauze bags (Verseidag-Industretextilien GmbH, Kempen, Germany). Sections were deparaffinized, incubated in 0.1 M citrate buffer pH 6.0 for 5 min followed by 60 min at 80°C. After cooling and

washing with phosphate buffered saline (PBS), enzymatic dissociation (0.1% collagenase I-A (Sigma) and 0.1% dispase) (Gibco BRL, Paisly, Scotland) was carried out as described previously [10].

Cell concentrations were determined using a Bürcker haemocytometer and cells were incubated with 100 µl of antibody mixture (anti-keratin 5,6,8,17 clone MNF116 (Dako) 1:50, clones AE1, 3 (Chemicon) 1:100 and V9-2b (anti-vimentin; Department of Pathology, LUMC [48]) 1:5) per million cells and incubated overnight at 4°C. After washing, cells were then incubated for 1 h at 4°C with 100 µl of FITC and RPE labeled secondary antibodies (Southern Biotechnology Associates, Birmingham, AL, USA) diluted 1:100 in PBS/0.5% BSA/0.05% Tween 20 (PBATw). Cells were washed and incubated for 30 min at room temperature with 500 µl DNA staining solution (0.1% DNase free RNase (Sigma), 10 µM propidium iodide (PI) (Calbiochem, San Diego, CA, USA) in PBATw). Samples were stored overnight at 4°C before flow cytometric analysis.

2.7. Flow cytometry and cell sorting

Data from 10,000 single keratin-positive tumor cells were collected using a FACScaliber flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). FITC (FL1) and RPE (FL2) fluorescent signals were collected in the logarithmic mode and PI fluorescent signals were collected in the linear mode (FL3). The FL3-A versus FL3-W pulse processor was used to enrich for single cell events during acquisition and analysis. Data were analyzed using the WinList 6.0 and ModFit 3.1 software packages (Verity Software House Inc., Topsham, ME, USA). For DNA-ploidy analysis, the vimentin-positive, keratin-negative cell fraction was used as an intrinsic DNA diploid reference. DNA-ploidy was defined as follows: DI $<$ 0.95: hypoDNA diploid, 0.95 $<$ DI $<$ 1.05: DNA diploid, 1.05 $<$ DI $<$ 1.95 DNA aneuploid, 1.95 $<$ DI $<$ 2.05 tetraploid and DI $>$ 2.05 DNA aneuploid. N-color compensation was used for post-acquisition spectral cross-talk correction according to the manufacturer's instructions. Keratin-positive tumor cells and vimentin-positive normal stromal cells were flow-sorted using a FACSVAntage flow sorter (BD Biosciences, San Jose, CA, USA) using the 488 laser line at 300 mW.

2.8. Cell lines

Cell lines TMK1, NUGC4, HSC39, HS746T, ST42, GP220, MKN45, MKN7, MKN74 and MKN28 were cultured in RPMI 1640 (GIBCO BRL, MD, USA) supplemented with 10% fetal calf serum (FCS, GIBCO BRL, MD, USA) and 2% penicillin–streptomycin (GIBCO BRL, MD, USA). Cell line HM51 was cultured in DMEM (GIBCO BRL, MD, USA) supplemented with 10% FCS and 2% penicillin–streptomycin.

2.9. DNA extraction

DNA from paraffin embedded and fresh frozen xenografts tissue was isolated using the PUREGENE® DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA) according to manufacturer's instructions. DNA isolation from cell lines was performed using Proteinase K treatment, followed by phenol/chloroform extractions and precipitation using ammonium acetate and ethanol. DNA from flow-sorted cells were pelleted and treated with proteinase K before use. DNA concentrations were measured using a NanoDrop spectrophotometer (Isogen Life Science, IJsselstein, The Netherlands).

2.10. LOH analysis

LOH analysis was carried out using the polymorphic microsatellite repeat markers D4S2934 and D4S2998 which lie 723 and 7723 bp from the telomeric and centromeric side of the *CDC4/FBXW7* gene. The sequences of the primers and their corresponding location were selected through the Genomic Data Base (http://www.gdb.org). PCR amplification was performed using 35 cycles in a 20 µl reaction volume containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1.5 unit of Platinum® Taq DNA polymerase in buffer supplied by the manufacturer, and 0.5 mM of each primer, one of which was labeled with a fluorescent marker with an annealing temperature of 58°C. A minimum of 10 ng of DNA was used for each PCR reaction to prevent induced losses. LOH analysis was carried out using an automated ABI 3100 sequencer with a Genescan™ 350ROX size standard (Applied Biosystems) and the manufacturer's Genescan® 2.1 software. The ratio of peak heights of the two alleles for tumor sample (keratin-positive cells) and normal (DNA diploid, vimentin-positive celles) was calculated and subsequently the normal ratio was divided by the tumor

ratio. A value below 0.6 or above 1.6 was interpreted as evidence of LOH whereas values between these figures were considered retention of heterozygosity. All losses were confirmed by at least one repeat reaction.

2.11. Mutation analysis

All 11 exons of *CDC4/FBXW7* were amplified using primers described previously [3] and shown in Table 2. At least 10 ng of DNA and 10 pmol of primer was used for each reaction and an annealing temperature of 57°C for all exons except exon 8, where an annealing temperature of 55°C was used. 35 cycles were carried out using 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 mM forward and reverse primers, at an annealing temperature of 55°C and 4 units of Platinum® Taq DNA polymerase (Invitrogen) in a buffer supplied by the manufacturer, with an end volume of 50 μl in a Peltier Thermal Cycler (DYAD™) (Biozyme, Blaenavon, South Wales, UK).

Following purification of PCR products with the QIA quick® PCR purification kit (Qiagen, Leusden, The Netherlands), the sequences were analyzed using the ready reaction Big Dye™ Terminator Cycle Sequence kit (Applied Biosystems, Foster City, CA, USA) and an ABI 3100 automated sequencer (Applied Biosystems).

Table 2
CDC4/FBXW7 primers

CDC4/FBXW7	Primers
Exon 1C	FWD atctgatccctctgctgctg
	REV ctgcagccatctctgatgatac
Exon 2	FWD tcatcacacactgttcttctgg
	REV tgcttgtgtcctacttcaaaatg
Exon 3	FWD tgtgatctctgggaagaaagg
	REV gaatcaacatgtggccttttg
Exon 4	FWD gccttcttcaatgccttgtg
	REV caatttacagccctctttaccac
Exon 5	FWD aaggcaagagcagagacagag
	REV attcggctcatctgaatgtg
Exon 6	FWD ccaatttcctcacatcttcacc
	REV tcaagcacaattagttgtcagattag
Exon 7	FWD cgtcagccaggaaaaataatg
	REV ggggaaaaattgagccaag
Exon 8	FWD catgttttatatcatatgtttgctttc
	REV acaaaacgctatggctttcc
Exon 9	FWD atgcagcattctaggcttcc
	REV ttcttcatgccaattttaacg
Exon 10	FWD tcattgccactttatcttagtacctc
	REV aagtcagggtgaaaatattcataaac
Exon 11	FWD tttcttacatccatgctgatcc
	REV gaagggcagggagtatatcg

2.12. Multiplex ligation-dependent probe amplification (MLPA)

MLPA was carried out by MRC-Holland, Amsterdam, The Netherlands using *CDC4/FBXW7* probes designed for this study as described by Schouten et al. [38]. These included 6 probes targeted to each of the possible exon 1 variants (exons 1A–C) as well as exons 2, 6 and 11 of the CDC4/FBXW7 gene together with 15 control probes. DNA samples were diluted with TE to 5 μl and were heated at 98°C for 5 min in 200 μl tubes in a thermocycler with a heated lid (Biometra Uno II). After addition of 1.5 μl salt solution (1.5 M KCl, 300 mM Tris–HCl pH 8.5, 1 mM EDTA) mixed with 1.5 μl probe mix (1–4 fmol of each synthetic probe oligonucleotide and each M13-derived oligonucleotide in TE), samples were heated for 1 min at 95°C and then incubated for 16 h at 60°C. Ligation of annealed oligonucleotides was performed by diluting the samples to 40 μl with dilution buffer (2.6 mM $MgCl_2$, 5 mM Tris–HCl pH 8.5, 0.013% non-ionic detergents, 0.2 mM NAD) containing 1 U Ligase-65 enzyme, and incubation for 15 min at 54°C. The ligase enzyme was inactivated by heating at 98°C for 5 min and ligation products were amplified by PCR. For most experiments, 10 μl of the ligation reaction was added to 30 μl PCR buffer. While at 60°C, 10 μl of a buffered solution containing the PCR primers (10 pmol), dNTPs (2.5 nmol) and 2.5 U *Taq* polymerase (Promega) or SALSA polymerase (MRC-Holland) were added. Alternatively, the 10 μl solution containing PCR primers, dNTPs and polymerase was added to the complete MLPA reactions while at 60°C. PCR was for 33 cycles (30 s at 95°C, 30 s at 60°C and 1 min at 72°C). The sequence of the labelled primer is 5′-GGGTTCCCTAAGGGTTGGA-3′ and that of the unlabelled primer is 5′-GTGCCAGCAAGATCCAATCT AGA-3′. The exact gene and sequence recognised by each probe used in this article can be found at www.mrc-holland.com.

Amplification products were detected and quantified by capillary electrophoresis using the ABI 3100 automated sequencer (Applied Biosystems) using a ROX-labeled internal size standard (ROX-500 Genescan, Applied Biosystems, Warrington, UK). The data was analyzed using the MLPAnalyzer (MRC-Holland) according to manufacturer's instructions as previously described [8] using the single iteration analysis mode. Reference DNA from normal non-cancerous lymph nodes and non-neoplastic gastric mucosa from three different individuals was used. All MLPA reactions were repeated at least twice, or repeated a third time so that the presence of loss/gain/retention was always

confirmed in a second sample. A ratio of less than 0.60 was considered a deletion and a ratio higher than 1.35 considered a gain.

3. Results

3.1. Status of the CDC4/FBXW7 locus (4q32)

LOH analysis at the *CDC4/FBXW7* locus was carried out on 39 early-onset gastric cancers, after tumor tissue dissociation using flow cytometry whereby the tumor cells were separated from the normal cells using vimentin and keratin staining and selective flow-sorting as can be seen in Fig. 1. In addition LOH was also carried out on 8 paraffin-embedded xenografted gastric cancers [30]. Results of this analysis can be seen in Table 3. Of note, 34% of cases (16/47) showed LOH of at least one marker, with 13/40 (32.5%) showing loss at D4S2998 and 13/44 (29.5%) showing loss at D4S2934. Interestingly, in case Y132, LOH of the

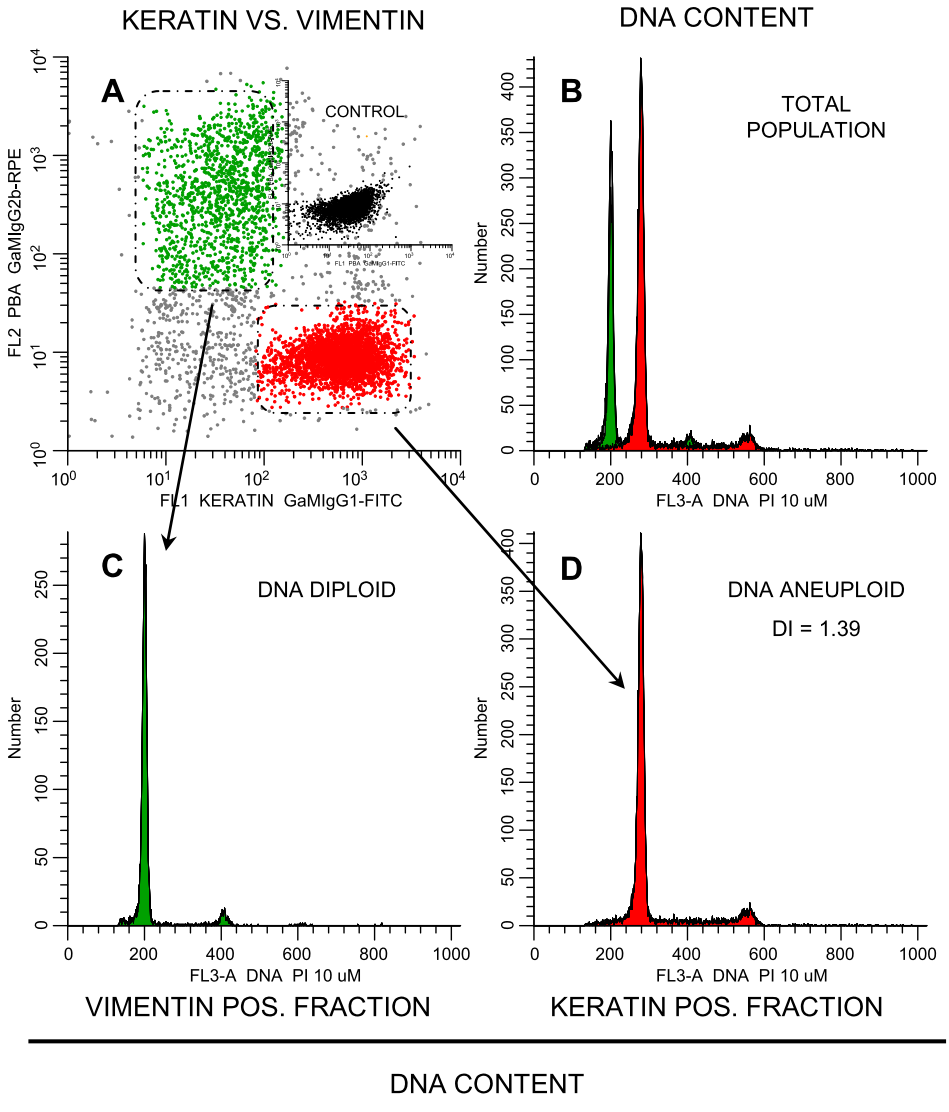

Fig. 1. Multiparameter DNA flowcytometry of a FFPE gastric cancer sample. Cells were isolated from a FFPE gastric cancer sample (Y5), stained as described and analyzed on a FACSCalibur flow cytometer. (A) Keratin versus vimentin dot plot. Inlay: negative control only stained for DNA. (B) DNA histogram of the total cell suspension showing a bimodal DNA histogram. (C) Gating on the vimentin-positive cell fraction (green) yields a unimodal DNA histogram representing stromal cells. (D) Gating on the keratin-positive cell fraction also reveals unimodal DNA histogram, with a DI of 1.39, representing gastric carcinoma cells.

Table 3
Results of LOH, MLPA and IHC on cdc4/FBXW7

Case	D4S2998	D4S2934	MLPA	IHC	DNA ploidy
Y5	LOH	LOH	Not performed	Moderate	1.39
Y11	ROH	NI	Not performed	Not assessable	2.23
Y67	ROH	ROH	Retention	Moderate	1.15
Y102	ROH	ROH	Not performed	Weak	DNA diploid
Y103	LOH	LOH	Not performed	Strong	1.49
Y106	ROH	ROH	Not performed	Moderate	0.9
Y107	LOH	LOH	Not performed	Strong	1.33
Y110	ROH	ROH	Retention	Negative	1.61
Y111	LOH	LOH	Loss exons 6–11	Strong	1.5
Y112	NI	ROH	Retention	Weak	DNA diploid + 1.57
Y113	Non amplifiable	LOH	Not performed	Negative	1.4 + 1.65
Y117	LOH	ROH	Not performed	Moderate	1.49
Y118	ROH	ROH	Not performed	Weak	1.08
Y121	ROH	ROH	Not performed	Moderate	1.81
Y126	NI	ROH	Retention	Moderate	1.33
Y130	ROH	ROH	Retention	Strong	1.53
Y132*	LOH	ROH	Not performed	–	DNA diploid
Y132	LOH	ROH	Loss exons 6–11	Weak	1.52
Y133	ROH	ROH	Retention	Moderate	DNA diploid
Y135	ROH	ROH	Retention	Moderate	DNA diploid
Y137	Non amplifiable	ROH	Retention	Moderate	Tetraploid
Y138	ROH	ROH	Not performed	Strong	1.18
Y139	ROH	Non amplifiable	Not performed	Moderate	DNA diploid
Y140	ROH	LOH	Retention	Negative	1.75
Y143	ROH	ROH	Not performed	Moderate	DNA diploid
Y150	ROH	ROH	Not performed	Moderate	1.73
Y152	LOH	NI	Not performed	Moderate	0.81
Y156	ROH	ROH	Retention	Moderate	DNA diploid
Y158	ROH	ROH	Not performed	Moderate	1.84
Y159	LOH	LOH	Not performed	Strong	1.15
Y160	LOH	LOH	Gain exon 1B	Strong	2.23
Y161*	LOH	LOH	Not performed	–	0.78
Y161	LOH	LOH	Not performed	Negative	1.75
Y166	ROH	ROH	Retention	Weak	DNA diploid + 1.71
Y168	LOH	LOH	Gain exon 2	Strong	DNA diploid
Y169	ROH	ROH	Retention	Weak	1.6
Y177	ROH	ROH	Retention	Negative	1.29 and 1.57
Y180	ROH	ROH	Not performed	Moderate	DNA diploid
Y182	ROH	ROH	Not performed	Weak	DNA diploid
Y185	ROH	ROH	Retention	Moderate	DNA diploid
8.11	NI	LOH	Retention	Moderate	1.2
X1	ROH	ROH	Not performed	Moderate	Not performed
X2	ROH	ROH	Not performed	Moderate	Not performed
X3	LOH	LOH	Not performed	Negative	Not performed
X4	NI	ROH	Not performed	Moderate	Not performed
X5	NI	ROH	Not performed	Moderate	Not performed

Table 3
(Continued)

Case	D4S2998	D4S2934	MLPA	IHC	DNA ploidy
X6	LOH	LOH	Not performed	Negative	Not performed
X7	ROH	ROH	Not performed	Moderate	Not performed
X8	ROH	ROH	Not performed	Moderate	Not performed
Total	13/40 (32.5%)	13/44 (29.5%)			

Notes: LOH – loss of heterozygosity, ROH – retention of heterozygosity, NI – non-informative, IHC – immunohistochemistry.
* Both keratin positive clones were subject to LOH analysis in these cases.

Table 4
Results of CDC4/FBXW7 TMA immunohistochemistry

Type of staining	Negative	Weak/Reduced	Moderate	Strong
N (nearest percent)	34/195 (17%)	35/195 (17%)	79/195 (41%)	47/195 (24%)

CDC4/FBXW7 locus was observed in both the DNA diploid keratin-positive cell fraction and hyperploid keratin-positive cell fraction, suggesting that this is an early event. This was also the case with the hypoploid and hyperploid keratin-positive cell fraction of Y161.

Just under half of all cases (19/47, 40%, according to availability of tumor DNA) were subject to MLPA analysis of the *CDC4/FBXW7* gene, as shown in Table 3. Interestingly case Y132 MLPA revealed a partial deletion of the *CDC4/FBXW7* gene on the side where microsatellite loss was also found and this corresponded to weak/reduced expression of *CDC4/FBXW7*. In case Y111 however, despite seeing loss of both microsatellite markers, only a partial deletion of the coding region exons 6–11 was seen with MLPA. Curiously in case Y140, we found loss of D4S2934 on LOH and an intact *CDC4/FBXW7* coding region, yet protein expression was absent, suggesting that the nearby loss on the exon 1 side disrupts the promoter region or affects the binding of molecules important in the transcription of *CDC4/FBXW7*. Interestingly in two cases (Y160 and Y168) an amplification of the *CDC4/FBXW7* gene was found with MLPA, and LOH showed a loss of both markers. This highlights the limitations of LOH analysis, which is often assumed to correspond to loss of an allele, whereas it actually measures a change in ratio only, i.e. it measures a relative loss and does not necessarily represent a real loss of the region in question, and amplifications have also been found in other genes in gastric cancer where LOH was observed [4].

3.2. Expression of CDC4/FBXW7 in gastric carcinoma

Normal fundic gastric mucosa showed moderate nuclear expression of CDC4/FBXW7 on immunohistochemistry, which was occasionally more pronounced in the proliferative zone. Weak cytoplasmic staining seen in some cells was deemed non-specific. Lack of CDC4/FBXW7 expression was observed in 5/16 (31%) of the cases with LOH as seen in Table 3, and reduced CDC4/FBXW7 expression was seen in an additional case (Y132), where partial loss of coding sequence was found by MLPA.

Expression of the CDC4/FBXW7 protein was subsequently examined in 204 gastric carcinomas (of which 195 were assessable) including both EOGCs and conventional gastric cancer using tissue microarrays as summarized in Table 4 and as seen in Fig. 2. Negative staining was seen in 17% of gastric cancers, and reduced staining was seen in 35% of cases. There was no difference in the expression of CDC4/FBXW7 between EOGCs and conventional gastric cancers using a χ^2 test when assessed on the basis of negative versus positive categories. CDC4/FBXW7 expression did not correlate with histological type or location of the tumor, T-stage or the presence of lymph node metastases.

3.3. The relationship of Cdc4/FBXW7 expression with its substrates

CDC4/FBXW7 expression and expression of its substrates c-jun, c-myc, cyclin E and Notch was assessed immunohistochemically on 204 gastric cancers using tissue microarrays (Fig. 2). A summary of the results can be seen in Table 5. Of note, the results presented for c-jun, c-myc and cyclin E are as published previously [27,28]. A significant correlation was found between CDC4/FBXW7 expression and the substrate c-myc ($p = 0.041$). CDC4/FBXW7 expression did not correlate with cyclin E ($p = 0.422$), c-jun ($p = 0.87$)

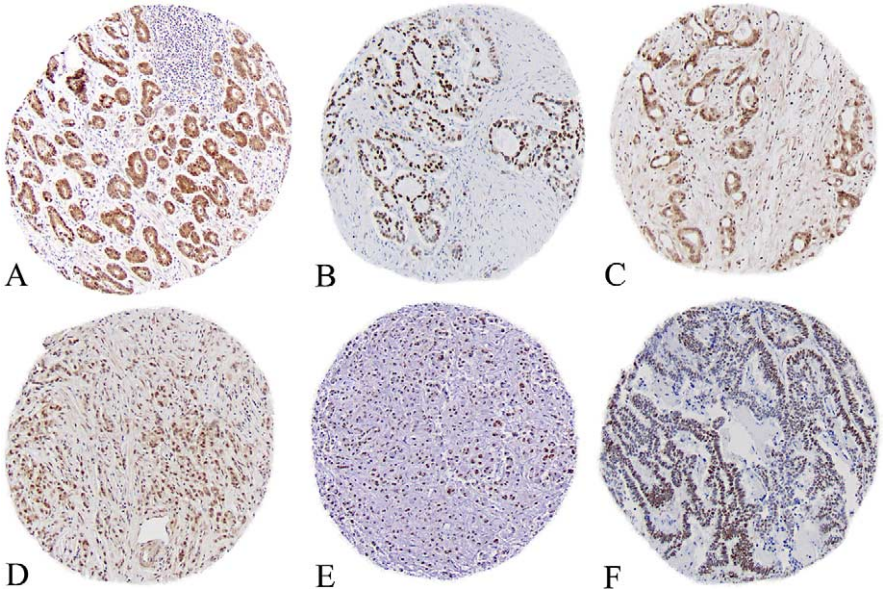

Fig. 2. Immunohistochemistry for cdc4 (A), cyclin E (B), c-myc (C), c-jun (D), Notch (E) and p53 (F) (×100) on tissue microarray cores.

Table 5
TMA immunohistochemistry together with statistical correlation with CDC4/FBXW7

Antibody	Total abnormal staining*	Significant correlation with CDC4/FBXW7 (χ^2)
CDC4/FBXW7	17% negative	–
Notch#	25% negative	None
cyclin E	36% positive	None
c-myc	43% positive	$p = 0.024$
c-jun	70% positive	None
p53	42% positive	$p = 0.057$

Notes: *to the nearest percent; #significant difference between notch staining in EOGC and conventional GC ($p = 0.004$, χ^2-test).

or Notch ($p = 0.174$) and correlation with p53 staining was of borderline significance ($p = 0.057$). In addition, loss of Notch staining occurred more frequently in conventional gastric cancers (35%) than in EOGCs (18%) and this difference was statistically significant using a χ^2 test ($p = 0.004$), as well as on using a binary logistic regression model to correct for histology and location ($p = 0.034$).

3.4. Mutation analysis

Mutation analysis of all 11 exons of the *CDC4/FBXW7* gene was carried out on DNA from 11 gastric cancer cell lines as well as on DNA from fresh frozen xenografted tissue of the 2 cases with LOH. No mutations of the *CDC4/FBXW7* gene were found. In this study we chose to carry out mutation analysis on available fresh/frozen tissue only (cell lines and fresh/frozen xenografted tissue) due to the high percentage of induced mutations/false positives which can be found in paraffin-embedded material [42,53].

3.5. Ploidy status

In the process of obtaining pure EOGC tumor cell populations using vimentin and keratin staining and selective FACS sorting (see Fig. 1) ploidy analysis was carried out, the results of which can be seen in Table 3. Overall, 36% of diffuse cancers (8/22) were DNA diploid whereas 8% of intestinal cancers (1/13) and 25% of mixed cancers (1/4) were DNA diploid. All other cases were DNA aneuploid (or had a DNA aneuploid component). In 6 cases, bimodal distributions were found after gating on the keratin-positive fraction. In all other cases only unimodal distributions were found in the keratin-positive fraction. Interestingly, in Y161, we found both a hypoDNA diploid and hyperdiplod peak, both of which showed loss of both microsatellite markers. In other cases (Y113, Y177), two DNA aneuploid peaks were visualized and in 3 cases (Y112, Y132, Y166) both a DNA diploid and DNA aneuploid subclone of the tumor was observed. Of note, CDC4/FBXW7 expression, LOH of *CDC4/FBXW7* or cyclin E expression did not correlate with ploidy status using Spearmans and Pearson corre-

lation tests. In addition, ploidy did not correlate with histological type.

4. Discussion

CDC4/FBXW7 is believed to be an important tumor suppressor gene in many cancers, as well as a potential therapeutic target [3], with mutations found in ovarian, breast, pancreatic, colorectal, endometrial tumors [3,7, 16,31,37,40,43] and recently in gastric tumors [2,24]. In fact, loss of chromosome region 4q32 has been reported in 31% of all neoplasms, including 67% of lung cancers, 63% of head and neck cancers, 41% of testicular cancers, and 27% of breast cancers [21] thus raising the possibility that *CDC4/FBXW7* may also be involved in the genesis of many other tumor types. In this study we investigated the role of *CDC4/FBXW7* in gastric cancer with particular attention to early-onset gastric cancer. We found LOH in microsatellite markers next to the *CDC4/FBXW7* gene in 34% of cases and in addition, loss of CDC4/FBXW7 expression was seen in $5/16$ (31%) of cases harboring LOH. Not all cases with LOH showed loss of CDC4/FBXW7 expression, but it is known that *CDC4/FBXW7* can lose its tumor suppressor function when haploinsufficient [25] meaning that loss of function of only one allele and a reduction of protein expression or reduced function rather than complete loss is enough to promote carcinogenesis, thus there may be functional significance of loss of the *CDC4/FBXW7* locus despite presence of the protein immunohistochemically. Interestingly 15% of cases showed negative or weak expression in the absence of LOH, suggesting that the loss of CDC4/FBXW7 expression in a subset of gastric cancers may occur through mechanisms other than allelic deletion, such as genetic or epigenetic events in nearby promoters or in molecular regulators of *CDC4/FBXW7*.

Cyclin E, p53, c-jun, c-myc and Notch are reported substrates of CDC4/FBXW7 *in vitro* [52,54], however only the substrate c-myc showed a significant correlation with CDC4/FBXW7 levels in gastric cancer in this study. Expression levels of c-myc are increased in many malignant tumors, and many c-myc mutations affect the stability of the encoded protein [1]. C-myc accumulates in mouse Fbxw7$^{-/-}$ cells [54] as well as in lymphomas from Fbxw7$^{+/-}$ mice [25] and a nucleolar isoform of the FBXW7 ubiquitin ligase is reported to regulate c-myc and cell size [51]. C-myc is known to play a role as an oncogene in gastric cancer [15] and our findings suggests that c-myc overexpression is likely to be an important oncogenic consequence of CDC4/FBXW7 loss in gastric cancers. We postulate that less functional cdc4 would imply decreased targeting of c-myc for ubiquitination and subsequent breakdown and this would lead to an accumulation of the functional c-myc protein. However, the exact mechanism by which this occurs is yet to be elucidated and requires further investigation.

Deregulation of cyclin E is considered to be a major factor in tumorigenesis with increased levels of cyclin E being associated with various malignancies, and it is claimed that constitutive expression of cyclin E leads to genomic instability [41]. However, recent studies do not support this notion and expression of cyclin E is not always increased in cancer cells in which *CDC4/FBXW7* is mutated [11,25,33]. Furthermore, expression of cyclin E is unaffected in *Fbxw*7$^{-/-}$ embryos [25,46,47]. It appears likely that cdc4/Fbw7 contributes to cyclin E proteolysis in a context-dependent manner and the mechanisms for the degradation of cyclin E appear complex, occurring though different pathways depending on whether the molecule is phosphorylated or not [33]. This may explain why no correlation was found between cyclin E and CDC4/FBXW7 in this study, as we did not examine phosphorylated and unphosphorylated forms of cylcin E separately. In addition, how CDC4/FBXW7 interacts with low molecular weight isoforms of cyclin E, which are known to occur more frequently in EOGC [27], is unknown.

Our lack of correlation between CDC4/FBXW7 and cyclin E, Notch or c-jun highlights the complexity of the relationship between CDC4/FBXW7 and its substrates [11,25,33]. CDC4/FBXW7 has been described to be p53 dependent [25] and it has been reported that CDC4/FBXW7 is a transcriptional target of p53 [19]. The relationship of p53 with CDC4/FBXW7 in this study had a correlation of borderline significance, despite recent evidence showing a significant relationship between p53 mutations and CDC4/FBXW7 expression [55]. Interestingly, aside from its relationship with CDC4/FBXW7 expression, we found that loss of Notch expression occurred less frequently in EOGC than conventional gastric cancer. These findings add further support to the idea that EOGC have a different molecular phenotype from conventional gastric cancers [5,6,27–29].

DNA ploidy did not correlate with histological type in this study and these findings are this is undoubtedly accounted for by increased accuracy of ploidy analysis using the recently developed technique by Corver

et al. [10]. This technique involves double staining and dissociation of normal and tumor cells which eliminates the contamination of normal lymphocytes and stromal cells within diffuse tumors. In the past there have been mixed reports of comparative genomic hybridization studies/genetic studies with respect to karyotypes/genomes in diffuse cancer. In our current study only 36% of diffuse tumors were found to be DNA diploid and the accuracy of these results is strongly by the fact that contamination with normal cells in our double staining and flow-sorted tumor dissociation technique has been dramatically reduced compared to prior tumor dissection techniques, especially for the diffuse type. Furthermore, as LOH of the *CDC4* locus was also seen in DNA diploid cases, the actual number of tumors which are technically DNA aneuploid is probably greater than stated in this study.

Although *CDC4/FBXW7* was proposed as a chromosomal instability (CIN) gene, particularly in colorectal carcinoma [37], more recent reports on primary tumors do not support this theory, and similar to our results, they find no association with *CDC4/FBXW7* aberrations and ploidy [17]. Our findings also emphasize that the loss we observed at the *CDC4/FBXW7* locus does not necessarily reflect a more generalized chromosomal instability, but rather is more likely to be a loss critical in gastric carcinogenesis. However, its possible role in chromosomal instability is not entirely ruled out, as indeed diploid tumors can still posses numerous "balanced" chromosomal abberations.

In conclusion, loss of heterozygosity of *CDC4/FBXW7* occurs commonly in early-onset gastric cancer, and is correlated with loss of expression in 31% of cases. Loss of expression was seen frequently in both early-onset gastric cancers and conventional gastric cancers on tissue microarray analysis, suggesting that *CDC4/FBXW7* may play an important role, not only in early-onset gastric cancer but in gastric carcinogenesis in general. In addition, the correlation found between loss of cdc4/FBXW7 expression and upregulation of c-myc suggests that c-myc overexpression is likely to be an important oncogenic consequence of *CDC4/FBXW7* loss in gastric cancers.

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