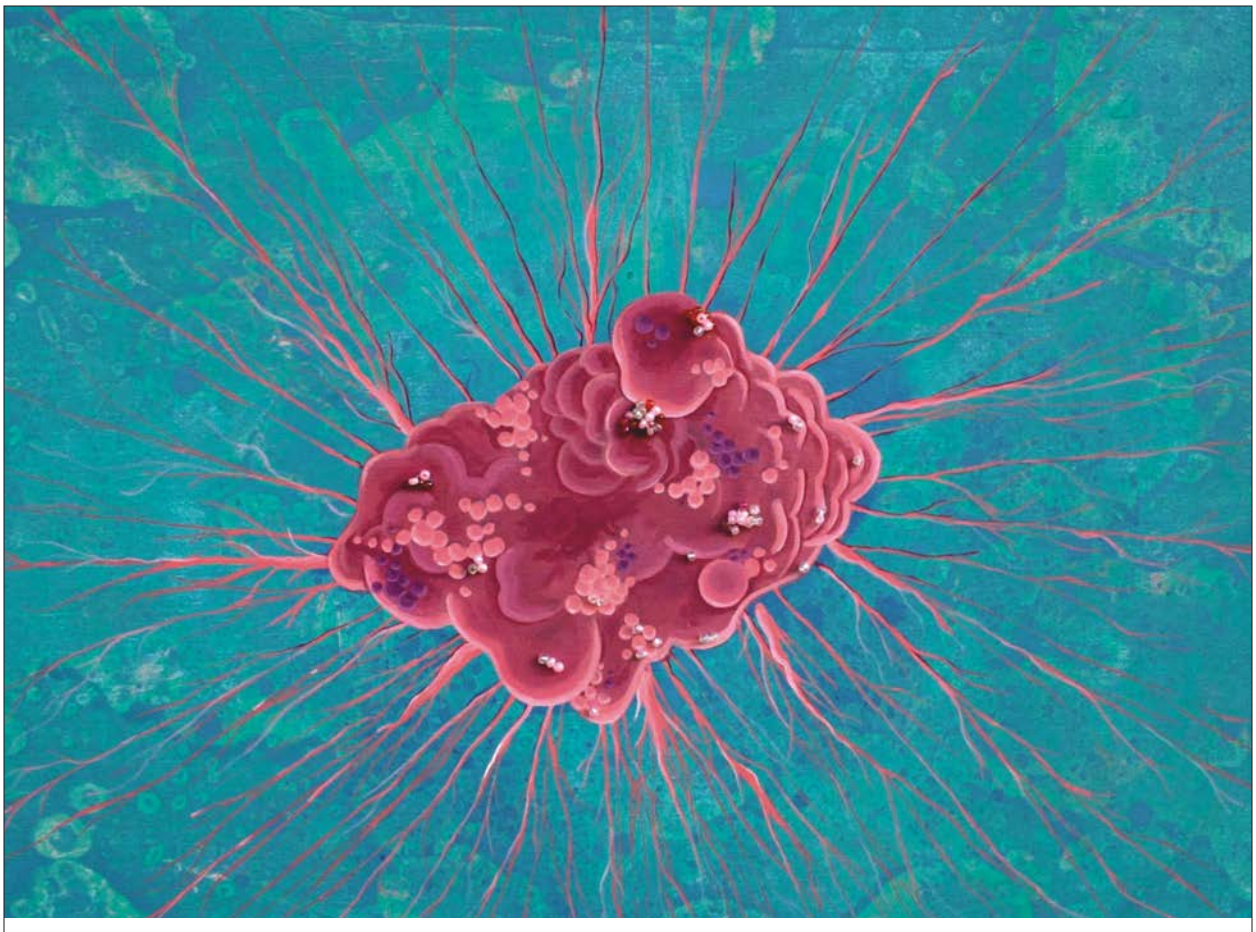

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Research Article
Frequent Loss of Chromosome 4q, Homozygous *FSTL5* Deletion at the 4q32.2 in Gastric Adenocarcinoma

Jiun Kang

Department of Biomedical Laboratory Science, Korea Nazarene University, 331-718 Cheonan, Republic of Korea

Abstract

Background and Objective: Chromosomal alterations are a predominant genetic force that contributes to the development of gastric adenocarcinoma (GC). This study was performed to identify critical genetic landmarks that may be important mediators in the formation or progression of GC. **Materials and Methods:** The whole genome-wide copy numbers were screened in 25 patients with GC using array comparative genomic hybridization (CGH) consisting of 4,030 bacterial artificial chromosome clones. Categorical analyses were applied to analyze whether chromosomal changes were related to clinico-pathological characteristics. **Results:** The most notable finding was the high frequency of copy number losses and hemizygous deletions on the long arm of chromosome 4, which was detected in 96.0 and 24.0% of the cases, respectively. More strikingly, three homozygous deletions in the 4q27-q34.2 regions were detected in 12.0% of GCs. Among the homozygous deleted regions, it was identified a potential tumor suppressor gene of *FSTL5* at the 4q32.2 region (14.3%), which has not been previously implicated to play a pathogenic role in GC. Furthermore, it was identified possible target genes that have not been previously described in GC, such as the losses of *LPHN3* on 4q13.1, *MGC35043* on 4q21.21, *DKFZP434G072*, *RG9MTD2* and *MTP* on 4q23, *Tenr* and *IL2* on 4q32.2, *FSTL5* on 4q32.2 and *FAT* on 4q35.2. **Conclusion:** This study confirmed and expanded upon a previous finding that 4q genetic alterations accumulate during the multistage pathogenesis of GC. The newly identified candidate genes at the 4q chromosomal sites could provide important clues with regard to the genetic mechanisms of initiation and progression as well as provide novel targets for therapeutic intervention in GC.

Key words: Gastric adenocarcinoma, copy number loss, homozygous deletion, prognostic markers, cancer genetics

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Corresponding Author: Jiun Kang, Department of Biomedical Laboratory Science, Korea Nazarene University, 456 Ssangyong-dong, Seobuk-gu, Cheonan city, 330-718 Chungnam, South Korea Tel: 82-41-570-4128 Fax: 82-41-570-4258

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Gastric carcinoma (GC) remains a major public health issues, as it is the fifth most common malignancy and the third leading cause of cancer death in both sexes worldwide[1]. Despite its recent decline, gastric cancer is the fourth most common cancer and the second leading cause of cancer-related death worldwide[2,3].

Gastric tumorigenesis is a heterogeneous process that occurs after a series of clonal molecular genetic alterations, including genomic gains and losses, particularly the deletion of tumor-suppressor genes (TSGs) and the amplification of oncogenes[4]. Unveiling the abnormalities of specific genes may offer novel insights into the mechanisms of local growth or the metastatic potential cases of GC and allow for the stratification of patients into different risk categories or treatment with novel options for targeted therapy[5].

Genomic instability with frequent DNA copy number variations is one of the key hallmarks of gastric carcinogenesis[6]. Tumor progression seems to depend on the successive acquisition of chromosomal aberrations, leading to gains or losses of parts of the genome[7]. To improve the prognosis of GC cases, the identification of suitable markers was required to select patients with a poor prognosis who may benefit from adjuvant therapy subsequent to surgery.

Previous studies suggested that GC progression depends on the successive acquisition of chromosomal aberrations leading to gains or losses of part of the tumor cell genome[5,7]. However, there was no clear agreement on the genetic changes underlying gastric carcinogenesis and identification of the predictive markers was crucial.

In the present study, genomic array comparative genomic hybridization (CGH) was performed to investigate DNA copy number alterations and new candidate target genes that may be indicative and specific for GC cases.

MATERIALS AND METHODS

Preparation of patient samples: A total of 25 GC tumor samples were obtained from patients treated at the Department of General Surgery of Chungnam National University Hospital in Daejeon, South Korea. All specimens used in this study had been submitted for pathologic and cytogenetic evaluation at institute over a 9 years period (April, 2004-March, 2013). None of these patients had received pre-operative chemotherapy or radiation. The stage of disease was based on the tumor-node-metastasis (TNM) classification using the UICC (Union Internationale Contre Le Cancer) staging system. The original diagnostic material of all GC patients was reviewed to verify the previous histopathological diagnosis and staging according to the World Health Organization classification system[8]. This study has been reviewed and approved by the Institutional Review Board of the Chungnam National University Hospital.

Array-CGH analysis: Microarray-CGH was performed the MacArray™ Karyo 1.4K BAC-chips (Macrogen, Inc., Seoul)[9-11] according to the manufacturer's instructions and as described in previous studies[12,13]. Briefly, all clones were two-end sequenced using an ABI Prism 3700® DNA analyzer (Applied Biosystems, Foster City, CA, USA) and their sequences were blasted [using basic local alignment search tool (BLAST)]. The labeled probe and human Cot-I DNA were mixed and dissolved in hybridization solution. Preparation of DNA targets, labeling, hybridization, washing, staining and scanning was conducted according to the manufacturer's instructions (Macrogen, Seoul, Korea, 2013)[14-16].

Statistical analysis: To adjust for effects due to the variation between the red and green dyes, Lowess normalization was applied. Breakpoint detection and status assignment of the genomic regions were performed using GLAD software. The median of the signal ratio of each triplicate spot was defined as a gain or a loss when it was >0.25 or <-0.25, respectively. High-level amplification of clones was defined when their intensity ratios were >1.0 in log2 scale and vice versa for homozygous deletion. The Benjamini-Hochberg false discovery rate (FDR) was applied for multiple testing correction for the high number of false-positive calls. One-way ANOVA, probability was used in the comparisons of the differences in the mean number of chromosomal alterations (gain or loss) between TNM stages.

RESULTS

Whole genome array analysis in GC cases: Samples from 25 patients with GC were analyzed using microarray-CGH to identify DNA copy number alterations and new candidate target genes associated with GCs. All the profiled patients (100.0%) showed multiple segmental alterations, including single copy and high-level losses. In the first step of the analysis, the decision was made to focus on chromosome 4q, the most frequently lost (log_2 ratio<-0.25) (96.0%, 24/25) and the hemizygously deleted (-0.5>log2 ratio>-1) (24.0%, 6/25) regions in GCs. Specifically, three loci of homozygous deletions (HDs) (log2 ratio<-1) were found in 12.0% (3/25) of the cases,

Table 1: Most frequent lost regions of overlap detected by microarray comparative genomic hybridization in 25 gastric adenocarcinomas, together with candidate genes

Chromosomal band	Clone name	Genes contained in clones	Cases with copy number losses (%)*
4q27	D4S427		24(96.0)
4q32.2	D4S1598	*FSTL5*	12(48.0)
4q28.2	D4S852		11(44.0)
4q35.2	4q terminal		10(40.0)
4q21.21	FGF5	*MGC35043*	8(32.0)
4q27	IL2	*Tenr, IL2*	8(32.0)
4q28.1	SGC34174		8(32.0)
4q13.1	WI-21794	*LPHN3*	7(28.0)
4q23	MTP	*DKFZP434G072, RG9MTD2, MTP*	7(28.0)
4q34.1	SHGC4-1602		7(28.0)
4q35.2	SHGC-141214	*FAT*	5(20.0)

Alterations were defined by log2 ratio thresholds of -0.25 for copy number loss

centered at 102.4, 106.1 and 105.9 Mb. The minimal common region identified by the array-CGH was located between BAC165_H02 and BAC84_P22. A list of the delineations of the 4q chromosomal region and possible target genes of GCs is presented in Table 1.

Chromosomal alterations on the long arm of chromosome 4 in GC cases: A more detailed analysis of chromosome 4q identified three distinct regions of alteration across the chromosome.

The first interval spanned 58.9-59.1 Mb and was mapped to then 4q12-q13.3, containing 12 target clones and was identified as having copy number losses in 28.0% (7/25) of cases. According to the information archived by the human genome database (http://genome.ucsc.edu/), it was flanked by the BAC clones between BAC165_H02and BAC1_C16(2.0 Mb segment).

The second locus spanned 81.8-82.1 Mb and was mapped to the 4q21.21-q28.2 regions. Notably, a high-frequency of copy number losses (-0.25>log2 ratio) and hemizygous deletions (-0.5>log2 ratio>-1) in these regions were detected in 96.0% (24/25) and 28.0% (7/25) of the cases, respectively. The most frequently lost clone was BAC247_H12 at the 4q27 region, which is located in the tenascin-R (*Tenr*) and interleukin 2 (*IL2*) genes. Specifically, one HD locus in the 4q27 was noted in 8% (2/25) of cases. Interestingly, the copy number losses in 4q23 and 4q27 were significantly correlated with advanced clinical stages. The incidence of copy number loss at these regions was directly related to the stage of disease progression and exhibited a tendency to increase with the progression of the tumor stage. Representative genome profiles of HDs at the 4q27 region are presented in Fig. 1. Whole genome profiles are shown in the upper portion (Fig. 1a) and an individual profile of chromosome 4, including HDs at the 4q27 region, is presented in more detail below (Fig. 1b).

The third locus spanned 74.7-112.2 Mb, mapped to the 4q31.1-q35.2 and demonstrated a high frequency of copy number losses in 13 of 25 cases (52.0%). This regions displayed a varying degree of copy number losses, predominantly from 4q32.2, 4q35.2, 4q34.1 and 4q35.2. Specifically, two HD loci in the 4q32.2-q34.3 region were noted in 8% (2/25) of cases. One locus at 4q32.2 contained homozygous clones covering a region of ~106.1 kb and comprised the transcription factor follistatin-like 5 (*FSTL5*) gene. Another locus spanning ~89.6 kb on 4q34.2 was without an associated gene. The median span of the HDs was 7.9 Mb (range, 105.9-106.1 kb) and all HDs were located between BAC152_C21 and BAC186_J15. A representative weighted frequency (%) diagram with HDs in the 4q27-q34.2 regions for all 25 GC cases is displayed in Fig. 2.

DISCUSSION

In this array profile, the most notable finding was the high frequency of copy number losses and hemizygous deletions in the long arm of chromosome 4, which occurred in 96.0% and 24.0% of cases, respectively. More strikingly, three HDs in the 4q27-q34.2 regions were detected in 12.0% of Gcs. Genomic changes on chromosome 4q have long been considered one of the major drivers of cancer progression and are suspected to include critical TSGs in GCs[14-18]. Fan *et al.*[17] reported that recurrent copy number losses of chromosome 4q is one of the most prevalent genomic alterations in GC and a high frequency of copy number losses on 4q in diffuse type of GC was also documented[18]. Similarly, Kimura *et al.*[19] summarized that frequent copy number losses at 4q was associated with venous invasion in GC cases and a high frequency of copy number losses of 4q (40%) was also reported most frequently in GCs[20]. Additionally, Xu *et al.*[21] indicated that loss of heterozygosity (LOH) patterns were clearly detected in GC cases and recurrent allelic losses (>50%) were also identified on 4q in gastric cardia adenocarcinoma[22].

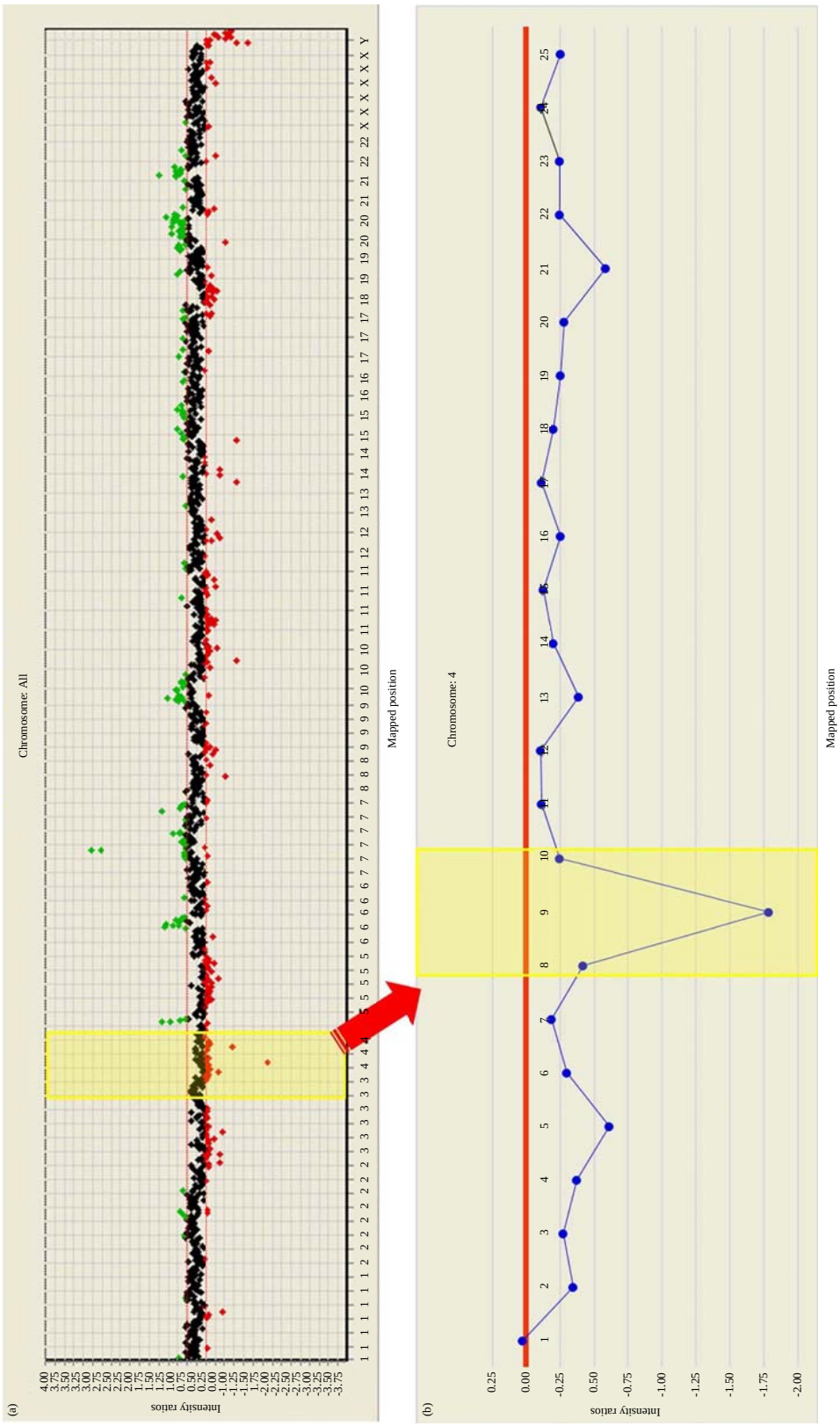

Fig. 1: Examples of microarray-comparative genomic hybridization results from a patient sample (Tumor 9), (a) A log2 ratio >0.25 represents a genomic copy number gain and a log2 ratio <-0.25 represents a genomic copy number loss. Clones are ordered from chromosome 1p to 22q. The homozygous deletions (HDs) at 4q32.2 are highlighted in yellow and (b) Genomic profiles of chromosome 4 from a patient sample (Tumor 9). The vertical lines indicate a ratio of < -1 in this bacterial artificial chromosome (BAC) clone, suggesting HD regions at 4q32.2. The HDs at 4q32.2 are highlighted in yellow

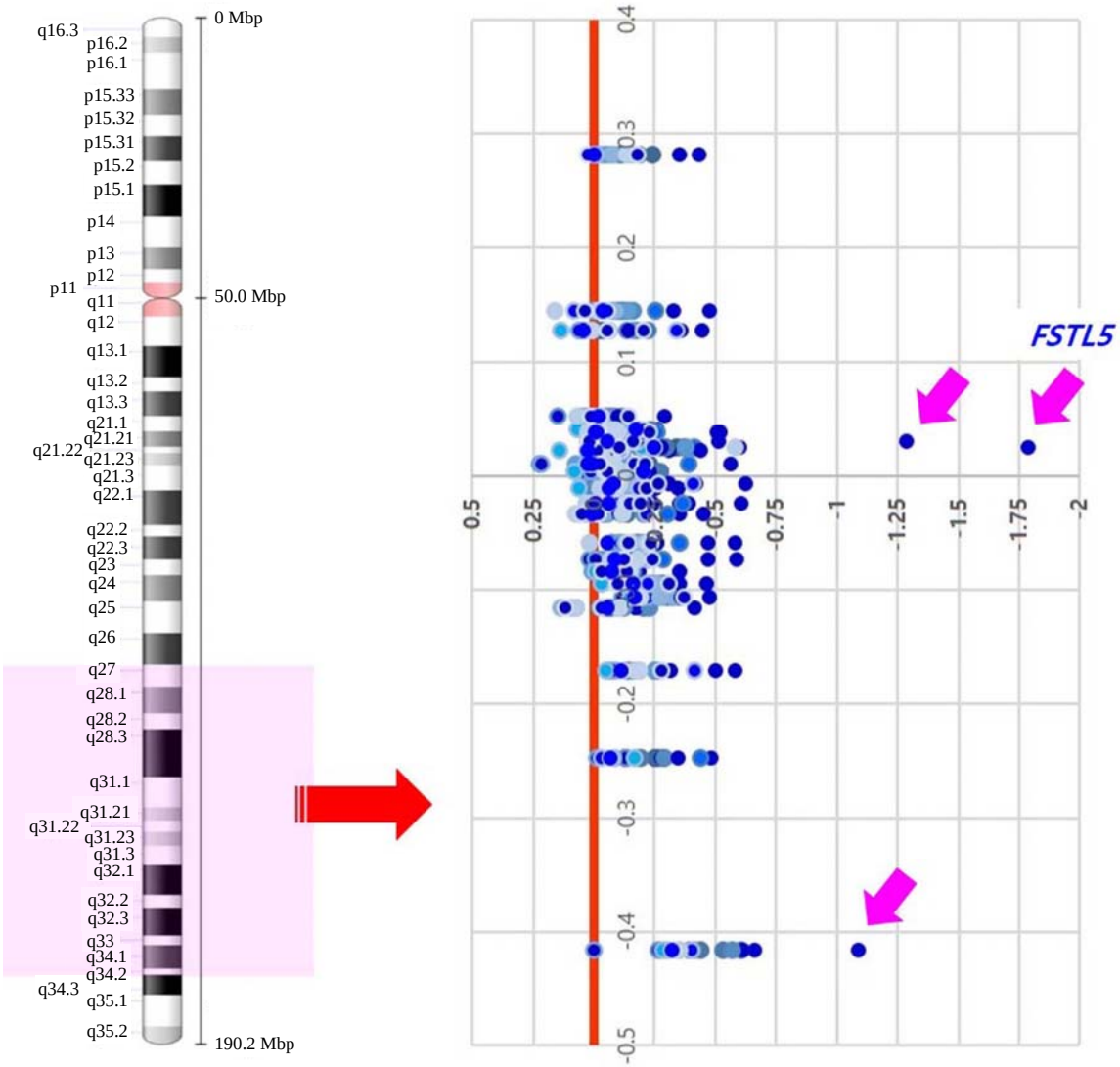

Fig. 2: Weighted frequency (%) diagram of the 4q27-q34.2 region in GCs. In the intensity ratio profiles, cytobands in the ideogram are shown on the left. Log_2 ratio was <-1 in this BAC clone, suggesting that homozygous deletion occurred at the *FSTL5* gene loci

Allelic losses on 4q have also been documented in other carcinomas, with most studies uncovering a complex pattern that cannot be reduced to a single minimally deleted region. Luebke *et al.*[23] reported that deletions on chromosome 4q showed prognostic significance for overall survival and tumor recurrence in operable ductal pancreatic adenocarcinoma and recurrent copy number losses at 4q in hepatocellular carcinoma (HCC) was also identified by array-CGH analysis[24]. Moreover, Rumpel *et al.*[25], suggested that in tumors that exhibit chromosome 4q deletion in esophageal adenocarcinomas, over half showed total or near total LOH. However, the remaining neoplasms exhibited localized deletion or a patchy distribution of chromosomal loss. Additionally, Chen *et al.*[26], indicated high frequency of LOH at 4q seem to play an important role in oral cancer biology and survival in oral squamous cell carcinoma. These results and the findings of the present study suggested that copy number losses on chromosome 4q are an important genetic event in the pathogenesis of many cancers including GC.

Although several candidate genes at 4q regions have been suggested, undiscovered TSGs might reside here. In this study, it was identified possible target genes that have not been previously described to play a pathogenic role in GCs, such as the loss of *LPHN3* on 4q13.1, *MGC35043* on 4q21.21,

DKFZP434G072, *RG9MTD2* and *MTP* on 4q23, *Tenr* and *IL2* on 4q32.2, *FSTL5* on 4q32.2 and *FAT* on 4q35.2 (≥20% of patients). Interestingly, the losses of *DKFZP434G072*, *RG9MTD2* and *MTP* at 4q23 and *Tenr* and *IL2* on 4q27 were significantly correlated with the TNM tumor stage. The incidence of copy number losses at these regions was directly related to the disease progression and exhibited a tendency to increase with the progression of the tumor stage. These findings suggested that GC has a complex pattern of chromosomal alterations that can arise from general chromosomal instability related to the advanced stage of gastric carcinogenesis and that the disease stage could be estimated by an analysis of the genomic alterations on certain chromosomal regions prior to the initiation of treatment[27]. Although this result supports previous findings that 4q loss is one of the major drivers of GC progression, it also suggested the concept of a genomic stage and others routes of progression of GC. The possible candidate genes identified in this study might contribute to the progression of GC. Additional genome-wide studies with a larger number of patients are warranted to confirm the results of the present study and to improve the understanding of GC.

The HDs are major genomic forces that contribute to the development of many solid tumors and provide an important resource for the identification of the location of candidate TSGs[28]. In cancer genomes, HDs can cause the inactivation of genes with tumor suppressor activity and thus contribute to cancer development and progression[29]. In this study, the most noteworthy observation consisted of three HDs from the 4q region that occurred in 28.6% of cases. In the homozygous deleted region, the 4q32.2 locus comprised the transcription factor follistatin like 5 (*FSTL5*) gene. The *FSTL5*, a member of the follistatin family of genes, encodes a secretory glycoprotein[30]. Although the involvement of the *FSTL5* gene in the pathogenesis of GC has not been previously described, it was regarded as a candidate TSG that plays a role in the development and progression of other cancers. Zhang *et al*.[31] reported that *FSTL5* plays a suppressive role in HCC and suggested that the down-regulated of *FSTL5* could increase the growth and survival of HCC cells through the activation of Wnt/β-catenin signaling. In this article, the authors also described that the extracellular matrix protein *FSTL5* plays a suppressive role in HCC through the inhibition of Wnt/β-catenin signaling, but was down-regulated in HCC. Furthermore, in the study of Remke *et al*.[32], as *FSTL5* expression denoted a dismal prognosis both within and between medulloblastoma subgroups and the authors suggested that *FSTL5* is a marker of poor prognosis in non-wingless/non-sonic hedgehog medulloblastoma. Collectively, the results of the present study and the findings of other studies present evidence that *FSTL5* is a new candidate target gene, regulated by the 4q32.2 risk region, which could be defined as an independent target region for chromosome 4q alterations in various tumors, including GCs. Further functional and biological studies, in larger series and on multiple samples, are required to validate and evaluate the role of the *FSTL5* gene as a novel candidate oncogene in GC.

CONCLUSION

The present study confirms and expands upon previous observations that 4q genetic alterations were significantly implicated in GCs. These results warrant future studies to identify the putative TSGs on 4q to gain a better understanding of the molecular pathogenesis of GC. The genomic analysis also allowed the proposition of novel candidate genes that may be associated with the pathogenesis of GC. The newly identified target genes might contribute to GC pathogenesis as well as provide novel targets for therapeutic intervention in GC that require functional validation.

SIGNIFICANCE STATEMENTS

This study reported a detailed deletion mapping for 4q in GCs and identified a homozygous deletion regions at 4q27-q34.2 which is likely to contain important tumor suppressor genes related to the GC case. Furthermore, high-resolution analysis allowed us to propose new target genes that could be potential candidates for tumorigenesis of GC. These results indicated that 4q is the most frequent target of homozygous deletions in GC, suggesting that the arm contains multiple gastric tumor suppressor genes and/or genomic features fragile during gastric carcinogenesis. The newly identified candidate target genes are thought to be a potential candidate for GC tumorigenesis and may be highly attractive candidate marker for the GC cases.

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