AMY2A: A possible tumor-suppressor gene of 1p21.1 loss in gastric carcinoma

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Abstract. Homozygous deletions (HDs) are major genomic forces contributing to the development of many solid tumors. To identify critical tumor-suppressor loci involved in the pathogenesis of gastric carcinoma (GC), a high-resolution microarray-CGH was performed in a series of 27 GC patients. On a genome-wide profile, five distinct HD ($\log_2$ ratio <-1) loci, including 1p21.1, 2q21.1, 10q24.32, 13q34 and 15q11.2 were identified. These regions contained representative tumor-related genes, such as the *FGF8* and *NPM3* genes at 10q24.32, and the *LAMP1* gene at 13q34, which have been reported in connection with various tumors. The most frequent HD encompassed chromosome band 1p21.1 in 5 of 27 GC cases (18.5%). A hemizygous deletion (-0.5< $\log_2$ ratio ≤-1) or a single copy loss ($\log_2$ ratio <-0.25) from the 1p21.1 region was noted in 51.9% (14/27) and 88.9% (24/27) of GCs, respectively. A 30 Kb HD of the 1p21.1 chromosomal region was shown to contain a potential candidate tumor-suppressor gene (TSG) of *AMY2A*. Quantitative real time PCR analysis further confirmed complete loss of expression of the *AMY2A* gene located at the 1p21.1 region. We demonstrated that *AMY2A*, a possible TSG, is frequently silenced in GC deletion 1p21.1. The identified gene could provide a basis for further functional validation and may lead to the identification of novel candidates for tumorigenesis and targeted therapies in GC.

Introduction

Gastric carcinoma (GC) is the second most common cause of cancer-related deaths in the world (1). GC is characterized by a complex pattern of cytogenetic and molecular genetic changes, and chromosomal aberrations are a major genomic force contributing to the development in GC.

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Bi-allelic loss, also called homozygous deletion (HD), is one of several known mechanisms leading to gene inactivation. In cancer genomes, such HDs can cause the inactivation of genes with tumor-suppressor activity and thus contribute to cancer development and progression (2). It has been proposed that inactivation of tumor-suppressor genes (TSGs) plays an important role in the development of many human solid tumors (3). Therefore, detection of specific gene deletions in tumor cells can lead to the identification of genes putatively involved in growth control and tumorigenesis.

Previous analyses of the GC genome with low-resolution chromosomal or BAC array-CGH have demonstrated several typical regions with genomic deletions. The most common HDs in GC have been repeatedly detected on 2q, 3p, 9p, 16q, 17p and 20q (4-11). Many significant genes that map to these regions have previously been described to be deleted in GC (e.g., *FHIT*, *CDKN2A/p16*, *RB1* and *WWOX*) (8-11) and are known to promote the carcinogenesis of GC, but little is known about the specific underlying genes that affect tumorigenesis in GC.

Array CGH is recognized as a successful tool for the analysis of chromosome copy-number alterations that may prove suitable for individualized diagnostic, prognostic and therapeutic decision-making. The significantly improved resolution of the array-based CGH technique permits highly accurate mapping of DNA copy number changes throughout the genome (2-5). Therefore, array-CGH is a promising starting point for the identification of novel candidate genes affected by genomic imbalances contributing to deregulation of the expression levels of TSGs.

Although many low-resolution chromosomal or array-CGH-based copy number analyses of GC primary tumors have been reported, including our own, and have successfully identified a series of copy-number changes (4-13), few possible target genes for these alterations have been determined, suggesting that various critical genes for the pathogenesis of GC remain to be identified.

In this study we therefore employed a high-resolution array-CGH to identify critical candidate TSGs that could be important mediators in the formation or progression of GC.

Materials and methods

Study materials. Two early gastric carcinomas (EGC) and 25 advanced gastric carcinomas (AGC) were selected for array-

CGH analysis from the Department of General Surgery at Chungnam National University Hospital in Taejeon, Korea. The histopathologic classification was based on the World Health Organization. Tumor staging was in accordance with the International Union Against Cancer (UICC) recommendations (14). The demographic and pathologic data, including age, gender, lymph node metastasis, and the tumor stage were obtained by a review of the medical records.

Tissue preparation. DNA was extracted from fresh frozen tissues of AGC and micro-dissected cells from EGC. Hematoxylin and eosin-stained sections for each sample were graded and micro-dissected under the guidance of a gastric pathologist. Micro-dissected samples containing at least 70% cancer cells were digested from the frozen tissue or micro-dissected cells with a lysis buffer, adding 10 mg/ml of proteinase K, incubating the samples overnight at 55˚C, removing the RNA with RNAse, precipitating the unnecessary proteins, and aliquoting the DNA from the supernatants followed by washing and suspending the DNA. The genomic DNA was extracted using a Genomic DNA purification kit (Promega, Madison, WI, USA).

Array-CGH. Array-CGH was performed using the MacArray™ Karyo 1.4 K BAC-chip (Macrogen, Seoul, Korea) (15-19). The information on each individual clone was obtained from the UCSC Genome Bioinformatics database [http://genome.ucsc.edu; Build 36, Version Mar. 2006 (hg18)]. The locus specificities of the chosen clones were confirmed by removing multiple loci-binding clones individually under standard fluorescence *in situ* hybridization (FISH) (20). These clones were prepared using the conventional alkaline lysis method to obtain BAC DNA. The arrays were manufactured using an OmniGrid arrayer (GeneMachine, San Carlos, CA, USA) using a 24-pin format. BAC clones were represented on an array as triplicated spots and each array was pre-scanned using a GenePix 4200A scanner (Axon Instruments, Foster City, CA, USA) for proper spot morphology. The array-CGH experiment, imaging and data analysis were carried out as described earlier (19). Briefly, 500 ng of tumor and reference DNA were labeled with Cy5 and Cy3, respectively, using a Bioprime labeling kit (Invitrogen, Carlsbad, CA, USA). Array hybridization was carried out for 48 h at 37˚C. After hybridization, array slides were scanned on a GenePix 4200A two-color fluorescent scanner (Axon Instruments, Union City, CA, USA); quantification was performed using GenePix software (Axon Instruments).

We applied Lowess normalization, a form of smoothing adjustment that removes intensity-dependent variations in dye bias. Spot quality criteria were set as foreground-to-background >3.0 and standard deviation of triplicates <0.2. The breakpoint detection and status assignment of genomic regions was performed by GLAD software (21). The average $\log_2$ Cy3/Cy5 signal ratios of the triplicate BAC clones were calculated for each sample, and ± 0.25 ($\log_2$ ratio) was used as a threshold for defining the copy number increases (gains) and decreases (losses). Homozygous deletion of clones was defined when the intensity ratios were <-1 in the $\log_2$ scale (22-24). This threshold value was defined empirically as a value 3-fold that of the standard deviation calculated from 30 normal males to normal females in hybridization experiments. The software MAC viewer (v1.6.6), CGH-Explorer 2.55 and Avadis 3.3 Prophetic were used for graphical illustration and image analysis of array-CGH data. A multiple testing correction [Benjamini-Hochberg false discovery rate (FDR)] was applied to correct for the high number of false positive calls. The R 2.2.1 package of the Bioconductor Project (http://www.bioconductor.org) was used for detection of the frequency of gain or loss and statistical analysis.

Table I. Demographic data for patients with 27 gastric carcinomas.

Characteristic	Number (%)
Gender	
Male	21 (77.8)
Female	6 (22.2)
Age (M ± SD)	
≤65	11 (40.7)
>65	16 (59.3)
Borrmann's type	
AGC[a]	25 (92.6)
EGC[b]	2 (7.4)
WHO differentiation	
WD[c]	3 (11.1)
MD[d]	10 (37.0)
PD[e]	14 (51.9)
pTNM classification[f]	
Tumor status	
pT1	10 (37.0)
pT2	9 (33.3)
pT3	5 (18.5)
pT4	3 (11.1)
Lymph node status	
Nx	3 (11.1)
N0	7 (25.9)
N1	6 (22.2)
N2	8 (29.6)
N3	3 (11.1)
Metastatic status	
Mx	2 (7.4)
M0	22 (81.5)
M1	3 (11.1)
Tumor stage	
I	8 (29.6)
II	9 (33.3)
III	7 (25.9)
IV	3 (11.1)

[a]AGC, advanced gastric cancer; [b]EGC, early gastric cancer; [c]WD, well differentiated; [d]MD, moderately differentiated; [e]PD, poorly differentiated, [f]pTNM, tumor-node-metastasis.

Table II. Overview of 5 homozygous deletion regions detected by array-CGH in 27 gastric carcinomas.

Cytogenetics locations	Bac start of the HD[a]	Bac end of the HD[a]	Size (kb) of the HD[a]	Possible target genes	% of loss in GCs[b]	% of HD in GCs[c]
1p21.1	103,961,840	103,992,000	30,160	*AMY2A*	88.9% (24/27)	18.5% (5/27)
2q21.1	130,422,046	130,519,616	97,570		96.3% (26/27)	3.7% (1/27)
10q24.32	103,451,002	103,545,553	94,551	*FGF8*, *NPM3*, *MGEA5*	22.2% (6/27)	7.4% (2/27)
13q34	113,034,986	113,108,890	73,904	*LAMP1*, *GRTP1*	11.1% (3/27)	3.7% (1/27)
15q11.2	22,856,147	22,956,737	100,590		33.3% (9/27)	3.7% (1/27)

[a]Genomic positions were retrieved from the UCSC Genome browser web page [http://genome.cse.ucsc.edu; Build 36, Version Mar. 2006 (hg18)]. [b]Alterations were defined by $\log_2$ ratio thresholds of -0.25 for copy number loss. Using this threshold, we generated a frequency table. [c]Alterations were defined by $\log_2$ ratio thresholds of -1 for copy number homozygous deletion.

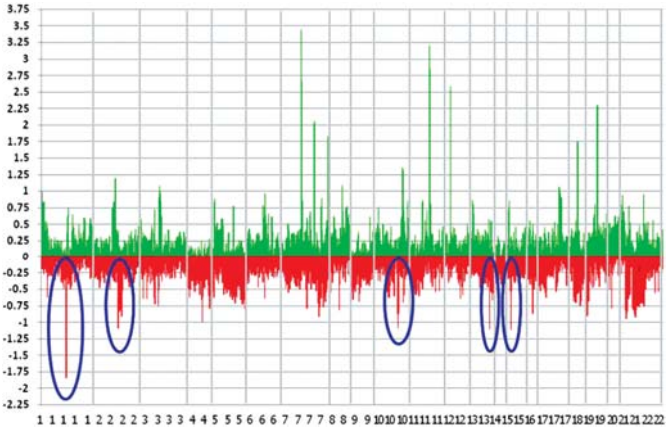

Figure 1. Frequency of chromosomal copy number changes in the 27 GC cases. Five homozygous deletion (HD) regions, including 1p11.2, 2q21.1, 10q24.32, 13q34 and 15q11.2, are highlighted in blue. Log_2 ratios for all clones were plotted based on the chromosome position, with the vertical dotted bars representing the separation of chromosomes. The clones were ordered from the 1p telomere on the left to the 22q telomere on the right. Gains and losses are shown as green and red color bars, respectively.

Quantitative real-time PCR analysis (Q-RT-PCR). Q-RT-PCR analysis was conducted to validate the array CGH results using the ABI PRISM 7900HT sequence detection system and TaqMan Gene Expression assays according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). The 2-primer/TaqMan probe combinations were designed based on sequences from the NCBI public database, and as follows: sense for *AMY2A*, GTGGAAGTTA CTTCAACCCTGGAA; and anti-sense for *AMY2A*, ATATT TACCATCATTGAAATCCCATCCA. Amplifications were performed using the Universal Master Mix (Applied Biosystems) and cycling conditions of 15 sec of denaturing time (95˚C) and 1 min of annealing/amplification time (60˚C) for 40 cycles after an initial activation step of 10 min at 95˚C. All samples were amplified in triplicate and data were analyzed using Sequence Detector software (Applied Biosystems). We quantified sample DNAs using standard curves generated using three reference DNAs. All data analysis used ArrayAssist® (Stratagene, La Jolla, CA, USA) and R (version 2.7.2). The correlation between the BAC chip and Q-RT-PCR data was performed by Pearson correlation analysis ($P<0.05$). Normalized normal human pooled genomic DNAs (Promega) were used as reference DNAs.

Results

Homozygous deleted chromosomal regions in GCs by genome-wide array-CGH. High-density genomic arrays were employed to characterize common regions throughout the whole genome deletion in a series of 27 GC patients. The clinicopathologic data for the 27 GC cases are summarized in Table I. Since the most common genetic aberrations had already been identified in GCs, we focused on more remarkable patterns of chromosomal alterations, such as HDs, which are likely to be landmarks of TSGs in GC.

On a genome-wide profile, five distinct lost genomic loci were identified as candidate regions for HD ($\log_2$ ratio <-1) in 6 of the 27 GC cases [22% (6/27)]. Only well-defined HDs, excluding alterations that spanned several mega-bases or whole chromosome arms, were included. The 5 HD loci of the 27 GC cases are shown in Table II.

The most frequently deleted HDs of the cases were observed on 1p21.2 [18.5% (5/27)] followed by 10q24.32 [7.1% (2/27)], 2q21.1, 13q34, and 15q11.2 [3.7% (1/27)]. Several such alterations corresponded to known cancer-related genes, including the *FGF8* and *NPM3* genes at 10q24.32, and the *LAMP1* gene at 13q34. In addition, we identified possible candidate TSGs, namely the *AMY2A* (1p21.2), *MGEA5* (10q24.32) and *GRTP1* genes at 13q34 that previously had not been assumed to play a pathogenic role in GCs. The frequency of chromosomal copy number changes with 5 HD loci in the 27 GCs are shown in Fig. 1.

Homozygous deletion and high frequency loss at 1p21.1 region in GCs. Genome-wide array-CGH analysis showed that 25 of 27 cases (92.6%) of GCs involved copy number losses ($\log_2$ ratio <-0.25) on the short arm of chromosome 1. The minimal common region identified by the array-CGH was located between BAC 22_M23 and BAC 22_P12.

A more detailed analysis of chromosome 1p identified 3 distinct regions of alteration across the chromosome. One

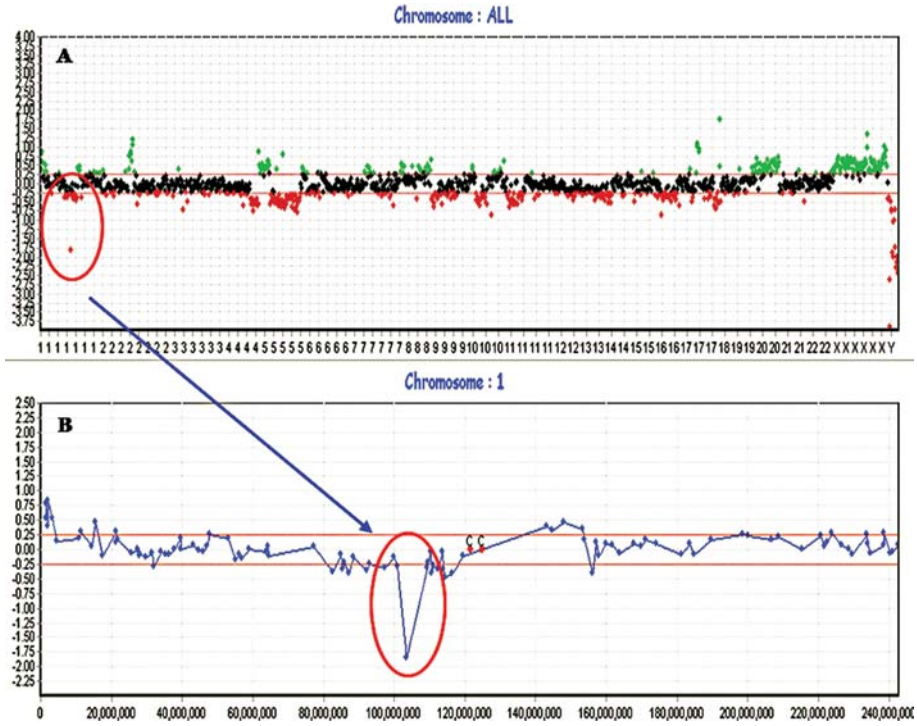

Figure 2. (A) Results of array-based CGH analysis from a patient sample (tumor 27). Normalized $\log_2$ signal intensity ratios of the 1,440 clones were plotted on the basis of chromosome position. Each dot represents the signal ratio for an individual BAC clone (dots with gains in green and losses in red). A $\log_2$ ratio >0.25 represents a genomic copy number gain, and a $\log_2$ ratio <-0.25 represents a genomic copy number loss. Clones are in the order from chromosome 1p to 22q. (B) Genomic profiles of chromosome 1, including HDs at 1p21.1 from a tumor (T27). Vertical lines indicate the lowest locus of chromosome 1 in the BAC clone containing the *AMY2A* gene. Log_2 ratio was <-1 in this BAC clone, suggesting that HD occurs at the *AMY2A* gene locus.

region spanning approximately 82-77 kbp on 1p32.3-p31.1 containing candidate TSG, namely Disabled I (*DAB1*) was detected in 29.6% (8/27) of the GC cases.

The second candidate locus spanned 100 kbp, mapping at 1p22.1, and harbored putative TSG of growth factor independent-1 (*GFI1*). The single copy number loss at 1p22.1 was noted in 22.2% (6/27) of the cases. Five of 6 cases at the 1p22.1 losses were found in high-stage tumors (III and IV; data not shown).

The third interval spans, 78-30 kbp on 1p21.3-p21.1, comprised the possible TSGs of the α amylase (*AMY2A*) and dihydropyrimidine dehydrogenase (*DPYD*) genes [92.6% (25/27)]. More specifically, HDs ($\log_2$ ratio <-1) or hemizygous deletions (-0.5< $\log_2$ ratio ≤-1) at the 1p21.1 region were detected in 18.5% (5/27) and 51.9% (14/27), respectively. Representative genome profiles of HDs at 1p21.1 are shown in Fig. 2. Whole genome profiles from a GC case (tumor 27) (A) are shown in the upper portion and individual chromosome profiles with HDs at the 1p21.1 region (B) are presented in more detail below (T27). Fig. 3A shows a weighted frequency (%) diagram for chromosome 1 with HDs at 1p21.1 from all of the 27 GC cases and an example of an individual profile with cases 5, 19, 24, 26 and 27 showing HDs from the same region in 27 GCs is presented in Fig. 3B.

Quantitative real-time PCR analysis. To delineate the consequences of HDs, we subsequently performed Q-RT-PCR analysis of the *AMY2A* gene at the 1p21.1 region, which had been detected as an HD ($\log_2$ ratio <-1) by analysis on the array-CGH. Twenty-two tumors with 1p21.1 loss and three patients without this loss were analyzed. The value of array-CGH was depicted by linear ratios and N-values were delineated in Q-RT-PCR. Although the absolute values of the selected gene were different between the two analyses, statistically significant correlation was observed between the two data sets (P<0.05). The correlation coefficient between the gene expression levels in Q-RT-PCR and array-CGH analysis for the gene (*AMY2A*) was 0.459 (P=0.021). Twenty-two patients with 1p21.1 loss showed complete loss of expression of *AMY2A* compared with three normal cases (Fig. 4A). Fig. 4B represents the scatter plot analysis of all data points for the *AMY2A* gene by array CGH and Q-RT-PCR analysis in GC cases.

Discussion

Homozygous deletions (HDs) provide an important resource for identifying the location of candidate TSGs (25). The importance of such deletions has been demonstrated in previous studies, but little is known about the specific underlying genes that could be important mediators in tumor initiation or progression.

Array-CGH has been recognized as a successful tool for evaluation of the whole genome. One of the advantages of array-CGH is the ability to convincingly detect HDs, an alteration that strongly suggests the presence of a TSG in the deleted regions (26). Using a whole genomic profile, we identified five HD ($\log_2$ ratio <-1) loci, including 1p21.1, 2q21.1, 10q24.32, 13q34 and 15q11.2, with detection of at least one HD in 27 GC patients.

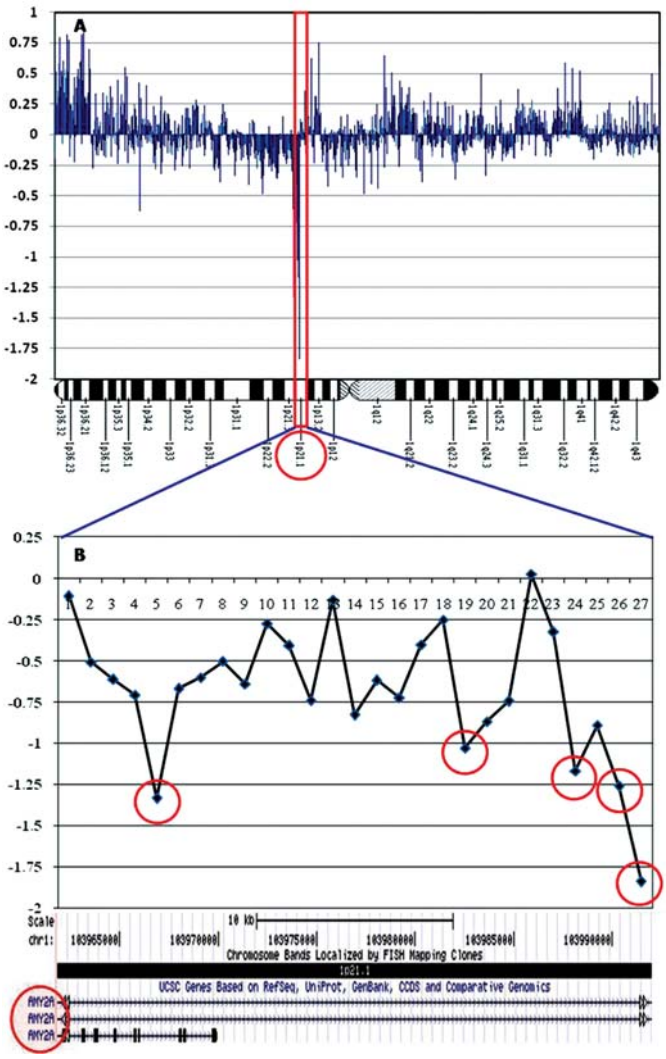

Figure 3. (A) Weighted frequency (%) diagram for chromosome 1 with HDs at 1p21.1 from all of the 27 GCs. The HDs at 1p21.1 are highlighted in red. In the intensity ratio profiles, the x-axis represents the map position of the corresponding clone and the intensity ratios were assigned to the y-axis. (B) Individual profiles with cases 5, 19, 24, 26 and 27 showing HDs at 1p21.1 region in 27 GCs (circled in red). The schematic presentation of cytogenetic bands, as well as a map position, is shown below the plot. HDs are clearly seen at chromosome 1p21.1 (*AMY2A*).

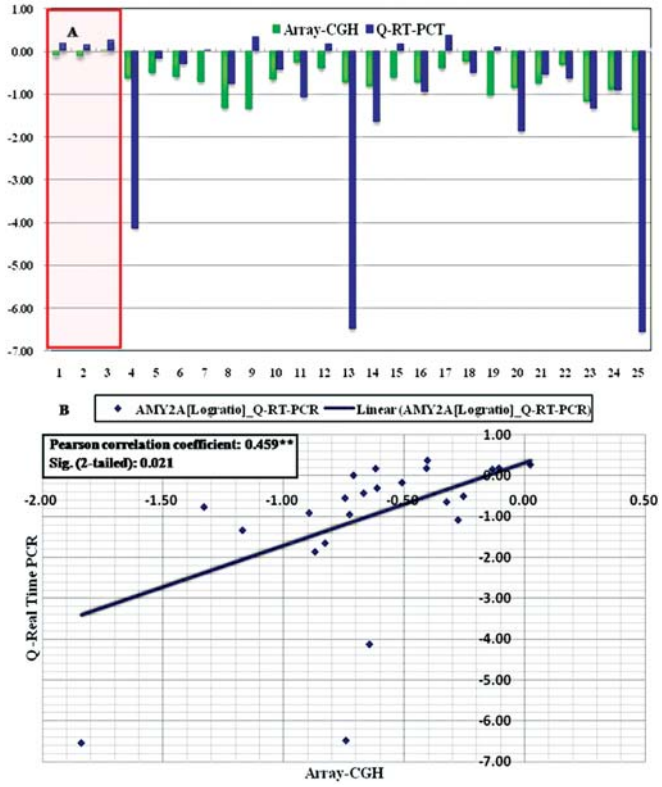

Figure 4. (A) The results of quantitative real-time PCR (blue bar) were compared with those of the array CGH (green bar) for the *AMY2A* candidate gene. The horizontal axis indicates sample number and the vertical axis indicates $\log_2$ ratio of copy number changes. Three patients without loss at 1p21.1 are shaded in red. (B) The scatter plot analysis of all data points of the gene *AMY2A* by Array CGH (*X axis*) and Q-RT-PCR (*Y axis*) analysis in GC patients. Each dark square is the $\log_2$ ratio value of the clone containing *AMY2A* gene for one case. The correlation coefficients (r) and P-value between the two data sets are given in the upper left corner of the figure.

HD of 2q21.1 region was observed in 27 GC cases, albeit infrequently [1/27 (3.7 %)]. However, this region was lost in the majority of GC cases [26/27 (96.3%)] without homozygous loss, suggesting that the 2q21.1 region contains multiple GC suppressor genes and/or genomic features which are fragile during gastric carcinogenesis. Further studies are needed to validate this hypothesis.

Homozygous deletion was also detected at 10q24.32 [7.1% (2/28)], containing several putative cancer-related genes (*FGF8*, *NPM3* and *MGEA5*). HD at this region has not been described in GC thus far, but is commonly found in other cancers (27-30). Bashyam *et al* (27) showed an HD at 10q24 in pancreatic cancer and Narayan *et al* (28) demonstrated a hemizygous deletion at 10q24 in cervical cancer. Loss of heterozygosity (LOH) or microsatellite instability (MSI) was also reported from the same region. Hui *et al* (29) showed frequent loss at 10q24.32-26.2 regions in medulloblastomas and the high frequency (74%) of LOH at 10q24 in small cell lung cancer has also been described (30). Taken together, these and our own results suggest that inactivation of TSGs within this region may play a critical role in development or progression of a variety of neoplasms, including GC.

A 74-kbp HD at the 13q34 region in GC is described here for the first time and contains several possible candidate TSGs, such as, *LAMP1* and *GRTP1*, which have not been previously reported as deletions in GCs. However, loss at another region of 13q is commonly identified in GCs (31,32). Wozniak *et al* (31) demonstrated that genomic losses often affected 13q with two minimal overlapping regions at 13q14.11-q14.2 and 13q32.3-q33.1 in gastrointestinal stromal tumors and the high frequency of LOH (83%) at the 13q region has also been reported in GC cases (32). These results suggest that this region could be the site of a candidate TSG in GC.

The HDs at 15q11.2 (7.1%, 2/28) do not encompass any known TSGs. However, a high frequency of single-copy loss or hemizygous deletion from the same region was detected in 33.3% (9/27) and 7.1% (2/28), respectively, suggesting that this might be affected in tumorigenesis of GC.

Previous low-resolution cytogenetic or LOH analyses have implicated the short arm of chromosome 1 as having a major

role in the initiation and/or progression of GC (31,33-35), suggesting the presence of multiple TSGs. Derre *et al* (33) documented that consistent DNA losses on the short arm of chromosome 1 is the most striking feature of malignant gastrointestinal stromal tumors and Wozniak *et al* (31) reported that the deletion of 1p (1p36.32-1p35.2, 1p34.1 and 1p22.1-1p21.3) was the second most frequent chromosomal imbalance (59%), with predominance in gastrointestinal stromal tumors.

In the present study, the loss of the short arm of chromosome 1 detected in 25 of 27 (92.6%) GC cases, revealing the following 3 minimal overlapping regions: 1p32.2-p31.1 (82-77 kbp), 100 kbp (mapping at 1p22.1) and 1p21.3-p21.1 (78-30 kbp). Among these regions of imbalance, loss at 1p21.1 seems particularly interesting, because a single-copy number loss ($\log_2$ ratio <-0.25) was noted in 88.9% (24/27) of the cases. More specifically, HDs at 1p21.1 were identified in 5 of 27 (18.5%) cases. This region has a 30 kbp gene-specific copy number loss comprising the one potential candidate TSG of the *AMY2A* gene, coding for salivary and pancreatic amylases. The estimated extent of the HD was 30 kbp; the *AMY2A* gene is the only gene within that region, and it must represent the actual and sole target for HDs in GC. Although, the *AMY2A* gene has not been previously documented as being deleted in GC, lack of the *AMY2A* gene has been described in connection with human cancers. Fibach *et al* (36) described that leukemia represents a deficiency of pancreatic enzymes leading to a state of arrested development in leukocytes and lacking mature leukocytes, to compensate, the body continues producing more pro-leukocytes, as is found in leukemia. Furthermore, the lack of fetal amylase has been suggested as the cause of cancer eclampsia and use of amylase along with trypsin was proposed for cancer treatment (37). These findings support our hypothesis that *AMY2A* gene may have potential for a tumor suppressor of GC. Therefore, an HD of the *AMY2A* gene seems to be involved in carcinogenesis of human cancers, including that of GC. Q-RT-PCR analysis further confirmed complete loss of expression of the *AMY2A* gene located at 1p21.1 region. Further functional and biological studies are expected to validate and to evaluate the role of the *AMY2A* gene as a novel candidate TSGs in GC in larger series and on multiple samples.

In the study presented herein, we successfully identified the *AMY2A* gene as having the greatest potential as a 1p21.1 HD target in GC. Frequent detection of reduced copy number of the *AMY2A* gene implicates it as suppressors of GC tumorigenesis and might be a good candidate as a therapeutic target.

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