Copy Number Gains at 8q24 and 20q11-q13 in Gastric Cancer Are More Common in Intestinal-Type than Diffuse-Type

Dong-Hao Jin¹, Seong-Eun Park¹, Jeeyun Lee², Kyung-Mi Kim³, Sung Kim⁴, Duk-Hwan Kim¹*, Joobae Park¹*

¹ Department of Molecular Cell Biology, Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Suwon, 135–710, Korea, ² Department of Internal Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, 135–710, Korea, ³ Department of Pathology, Samsung Medical Center, Sungkyunkwan University School of Medicine, 135–710, Seoul, Korea, ⁴ Department of Surgery, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, 135–710, Korea

Abstract

The present study was aimed at discovering DNA copy number alterations (CNAs) involved in the carcinogenesis of stomach and at understanding their clinicopathological significances in the Korean population. DNA copy numbers were analyzed using Agilent 244K or 400K array comparative genomic hybridization (aCGH) in fresh-frozen tumor and matched normal tissues from 40 gastric cancer patients. Some of the detected CNA regions were validated using multiplex ligation-dependent probe amplification (MLPA) in six of the 40 patients and customized Agilent 60K aCGH in an independent set of 48 gastric cancers. The mRNA levels of genes at common CNA regions were analyzed using quantitative real-time PCR. Copy number gains were more common than losses across the entire genome in tumor tissues compared to matched normal tissues. The mean number of alterations per case was 64 for gains and 40 for losses, and the median aberration length was 44016 bp for gains and 4732 bp for losses. Copy number gains were frequently detected at 7p22.1 (20%), 8q24.21 (27%–30%), 8q24.3 (22%–48%), 13q34 (20%–31%), and 20q11-q13 (25%–30%), and losses at 3p14.2 (43%), 4q35.2 (27%), 6q26 (23%), and 17p13.3 (20%–23%). CNAs at 7p22.1, 13q34, and 17p13.3 have not been reported in other populations. Most of the copy number losses were associated with down-regulation of mRNA levels, but the correlation between copy number gains and mRNA expression levels varied in a gene-dependent manner. In addition, copy number gains tended to occur more commonly in intestinal-type cancers than in diffuse-type cancers. In conclusion, the present study suggests that copy number gains at 8q24 and 20q11-q13 and losses at 3p14.2 may be common events in gastric cancer but CNAs at 7p22.1, 13q34, and 17p13.3 may be Korean-specific.
Introduction

Gastric cancer is the third leading cause of cancer deaths worldwide. Despite significant advances in the diagnosis and treatment of gastric cancer, five-year survival rates of gastric cancer patients remain below 30% in most countries [1]. In addition, approximately half of the patients who undergo curative surgical resection still develop loco-regional or distant metastases in spite of the multi-modality therapeutic approach and die from the disease [2,3]. Although most gastric cancers display similar clinical features, there is considerable heterogeneity in its histopathology and associated molecular changes [4]. Accordingly, it is important to identify molecular biomarkers involved in the carcinogenesis of gastric cancer for early detection and targeted therapy of the disease.

DNA copy number alteration (CNA) defined as DNA segments 1 kb or larger in size, is an important type of genetic alteration observed in cancer cells [5]. CNAs can influence gene expression, phenotypic variation and adaptation by disrupting proximal or distant DNA regulatory regions or by altering gene dosage levels [6,7]. In addition, the distribution of copy number is significantly different in distinct ancestral populations, which may result in different susceptibility to diseases across ancestral groups [8]. Recently, several groups have analyzed alterations of DNA copy number in gastric cancer using array comparative genomic hybridization (aCGH) and have identified novel genes important in the pathogenesis of gastric cancer [9–14]. For example, Tsukamoto et al. [10] investigated CNAs in 30 cases of gastric cancer by using BAC or PAC clones, and identified the most frequent regions of DNA copy number gains as 20q13, 20q11, 8q24, and 20p12, and those of losses as 4q34-qter, 5q12, 18q21, and 3p14. Fan et al. [11] detected CNAs in 64 gastric cancer tissues and 8 gastric cancer cell lines by using BAC clones, and observed that 20q12-20q13 and 9p21 were the most frequently amplified and deleted regions, respectively. In addition, Cheng et al. [12] studied CNAs in 27 gastric cancers by aCGH-244K and identified 8p11-q24, 20q11-q13, and 7q21-q22 as the most gained regions and 4q34, 6p25, 18q12, and 18q22 as the most lost regions. In these previous studies, various microarrays (BAC or PAC clone, oligo) were applied to investigate CNAs in gastric cancer, and the reported CNA regions were different for the various study populations.

To identify CNAs important in the pathogenesis of gastric cancer in the Korean population, we first performed a genome-wide analysis of DNA copy number using aCGH-244K or aCGH-400K in 40 gastric cancers and then validated the detected CNAs using a customized aCGH-60K in another set of 48 gastric cancers. The effects of CNAs on gene expression were analyzed in some of the genes with CNAs.

Results

Discovery of CNAs involved in the carcinogenesis of stomach

To discover CNAs involved in the carcinogenesis of the stomach, tumor and matched normal tissues from 40 gastric cancer patients were analyzed using array comparative genomic hybridization (aCGH); 30 cases by aCGH-400K and 10 cases by aCGH-244K. CNAs were detected across the entire genome, and copy number gains were more common than copy number losses (Fig 1A). The number of CNAs was vastly different amongst individuals. The mean number of CNAs per case was 64 for gains and 40 for losses (Fig 1B), and the median length of the CNA region was 44016 bp for gains and 4732 bp for losses (Fig 1C). The common CNAs were detected using a context-corrected algorithm with a p-value threshold of 0.05 and overlap threshold of 0.9 (Fig 1D). Copy number gains were commonly detected on chromosomal regions 7p22.1, 8q24, 8q24.3, 13q34, and 20q11-q13, and copy number losses were frequently observed on 3p14.2, 6q26, 7q36.3, 13q34, and 18q23. The losses were largely detected at the ends of chromosomes.
and the size was relatively small. The CNAs were less common on chromosomes 2 and 15. The common aberration lengths with a low p-value mainly fell within 1 kb-10 kb (Fig 1E). Common aberrations around MYC gene at 8q24.21 are shown in Fig 1F. The aCGH-244K and aCGH-400K data can be downloaded from the NCBI's Gene Expression Omnibus portal (www.ncbi.nlm.nih.gov/geo) (accession number: GSE69318 and GSE69266, respectively).

Validation of the aCGHs by MLPA analysis

Some genomic imbalances detected by the aCGH were validated using PCR-based multiplex ligation-dependent probe amplification (MLPA). Six of the 40 tissue samples analyzed using aCGH were available for MLPA. Copy number alterations of MYC (8q24.21), FHIT (3p14.2), WDR60 (7q36.3), COL4A2 (13q34), NFATC1 (18q23), and NCOA3 (20q12) were analyzed in six matched tumor and normal tissue pairs (268–1, 271–1, 272–2, 301–1, 685–1 and 685–2). MYC was amplified in 271-1T and 301-1T (Fig 2A), NCOA3 was gained in 301-1T and 685-2T (Fig 2B), and FHIT was deleted in 271-1T, 301-1T, and 685-1T (Fig 2A). These results were highly consistent with those detected by aCGH. However, the copy number losses of genes such as WDR60 (Fig 2C), NFATC1 (Fig 2D), and COL4A2 (Fig 2E), which were found to be lost in aCGH, were not detected in the MLPA. The length of the deletion region in the WDR60 gene detected by aCGH was 2907 bp, and COL4A2 and NFATC1 were 1903 bp and 2596 bp, respectively. Based on these observations, it is likely that the aberrations spanning small regions observed by aCGH may be false.

Additional validation of the CNA regions by aCGH-60K

To validate and narrow the CNA regions of recurrent (>20%) copy number gains or losses observed by aCGH in the 40 samples, we further analyzed their aberrations in tumor and...
matched normal tissues from another 48 gastric cancer patients using a customized aCGH-60K. The CNA regions at 8q24, 20q11-q13, 3p14.2, and 18q23 showed coincident alterations of copy number in the aCGH-60K, but CNAs at other regions, such as 20p13-20p12 and 20q21.2, did not show the same patterns as in the 244K and 400K, thereby suggesting heterogeneous results among aCGH platforms. To find minimal common regions (MCRs) of copy number alterations among the three platforms, we overlapped the CNA regions in a total of 88 samples. The MCRs of recurrent (>20%) copy number gains were detected on multiple chromosomal regions including 7p22.1 (20%), 7q22.1 (31%~44%), 8q24.21 (27%~30%), 8q24.3 (22%~48%), 13q34 (20%~31%), and 20q11~q13 (25%~30%) (Table 1 and Table A in S1 File). The MCRs of recurrent (>20%) copy number losses were also found in seven chromosomal regions, including 3p14.2 (43%) harboring FHIT (Table 1 and Table B in S1 File).

**Gene-dependent association between CNAs and mRNA levels**

To investigate the effect of CNAs on gene expression, we measured mRNA levels of multiple genes (MYC, SCRIB, PUF60, BOP1, SNTA1, E2F1, CD40, EYA2, NCOA3, FHIT, CRK, and SMAD2) at the common aberration regions in 48 tumor and matched normal tissues and analyzed the association with the CNAs. The effect of CNAs on gene expression was analyzed by comparing the mRNA fold change (FC) in cancers with and without CNAs. The correlation between copy number alterations and corresponding gene expression was different according to copy number gains or losses. The majority of genes with copy number losses showed downregulation of mRNA: the mRNA level was downregulated in the FHIT (Table 2 and Fig 3A), CRK, and SMAD2 genes (Table 2). However, we found the correlation between copy number gains and upregulation of mRNA levels was gene-specific: the mRNA levels in genes such as MYC, PUF60, BOP1 (Fig 3B), and E2F1 were positively associated with copy number gains. However, no association was found between mRNA levels and copy number gains of genes.

---

**Fig 2. Multiplex Ligation-Dependent Probe Amplification (MLPA).** (A) Upper left panel: representative image of capillary electrophoresis signals of MLPA analyzed by GeneMaker 2.0.0. Blue squares represent internal controls. Copy number alterations of MYC and FHIT were analyzed in 6 samples. (B-E) DNA copy number of NCOA3 (B), WDR60 (C), NFATC1 (D), and COL4A2 (E) were also analyzed using MLPA. Peak ratios within 1.25 – 0.75 was regarded as an indication of a normal DNA copy number, below 0.75 as an indication of a deletion, and above 1.25 as an indication of amplification. The T and N represent tumor and matched normal tissues, respectively.

doi:10.1371/journal.pone.0137657.g002
| Chromosomal regions | Frequency (N = 88) | Gene list | Gains |
|---------------------|-------------------|-----------|
| 1p36.33             | 26%               | SKI       |
| 1q42.13             | 28%               | DUSP5P    |
| 2p25.3              | 35%               | KIAA1106, MYT1L |
| 5p15.33             | 27%               | CCDC127   |
| 6p25.3              | 28%               | EXOC2     |
| 6q21                | 43%               | SCML4     |
| 6q25.3              | 32%               | C6orf35, SLC22A1 |
| 7p22.1              | 20%               | FBXL18, ACTB, ACTG1, BC044606, DL492006, FBXL18, FSCN1, RNF216 |
| 7q22.1              | 31%               | DP601342, PMS2L13, SPDYE3 |
| 7q22.1              | 44%               | SPDYE2, SPDYE6 |
| 7q36.3              | 31%               | PTPRN2    |
| 8p23.1              | 28%               | AK307331, FLJ00326 |
| 8q24.13             | 25%               | TIB11, BX648371 |
| 8q24.21             | 27%               | DQ515896, DQ515899, LOC727677, POUSF1B, POUSF1P1 |
| 8q24.21             | 30%               | BC042052, MYC |
| 8q24.3              | 48%               | AX748239, TRAPPC9 |
| 8q24.3              | 23%               | CHRAC1, EIF2C2, MAPK15, SCRIB, ZNF707, PUF60, BOP1, RECQL4, SLC39A4, VPS28 |
| 8q24.3              | 22%               | KIAA1688 |
| 9q22.31             | 30%               | PHF2      |
| 10p15.3             | 52%               | ADARB2, NCRNA00168 |
| 13q34               | 20%               | QRTTP1    |
| 13q34               | 31%               | FLJ44054  |
| 13q34               | 22%               | RASA3     |
| 16p13.3             | 24%               | HMNF1876, LA16c-360B4.1, LMF1 |
| 18q21.1             | 34%               | BC040860  |
| 19q13.12            | 22%               | BC045185  |
| 20q11.21–q11.22     | 25%               | ID1, NCRNA00028, PSIMCT-1, REM1, BCL2L1, SNTA1, E2F1 etc. |
| 20q13.12            | 27%               | WFD3, CTSA, UBE2C etc.  |
| 20q13.12            | 28%               | CD40, CDH22, ETA2, NCOA3 etc. |
| 20q13.31            | 30%               | RAE1, RBM38, ZBP1 etc.  |
| 20q13.33            | 27%               | CDH4      |
| 20q13.33            | 28%               | ZBTB46, TPDS2L2 etc.  |
|                      |                   |           |
| Losses              |                   |           |
| 3p14.2              | 43%               | FHIT      |
| 4q22.1              | 30%               | FAM190A, KIAA1680 |
| 4q35.2              | 27%               | BC034307, BC038717, FAT1, MTNR1A, ZFP42 etc. |
| 6q26                | 23%               | PARK2, Parkin |
| 17p13.3             | 22%               | NXN       |
| 17p13.3             | 20%               | ABR, NXN, TIMM22 |
| 17p13.3             | 22%               | TUSC5     |
| 17p13.3             | 23%               | CRK, YWHAE |
| 18q21.1             | 20%               | SMAD3     |
| 18q23               | 20%               | SALL3     |

*a* MCRs (Minimal Common Regions) were identified through the analysis of three arrays (aCGH-244K, aCGH-400K, aCGH-60K). MCR was defined as a 100 percent overlapping common region that was observed in all three arrays.

*b* When CNAs occurred within whole DNA sequences of a gene, the gene is depicted as bold.

doi:10.1371/journal.pone.0137657.t001
such as SCRIB, BCL2L1, SNTA1, CD40, EYA2 and NCOA3 (Table 2) suggesting that the relationship between copy number gains and expression may be gene-specific.

Association of CNAs with clinicopathological characteristics

The association between copy number alterations and clinicopathological variables was analyzed in 88 gastric cancer patients. Fifteen genes with recurrent (>20%) copy number alterations were selected for the analysis. Copy number losses of CRK (P = 0.07), SMAD2 (P = 0.09), FHIT (P = 0.68), and NFATC1 (P = 0.11) genes did not vary significantly between diffuse-type cancers and intestinal-type cancers (Fig 3C). However, copy number gains tended to occur at a high prevalence in intestinal-type cancers than in diffuse-type cancers (Fig 3D and 3E, Table C in S1 File). For SCRIB (P = 0.36), PUF60 (P = 0.07), MAPK15 (P = 0.08), E2F1 (P = 0.14), SNTA1 (P = 0.15), BCL2L1 (P = 0.15), NCOA3 (P = 0.22), and EYA2 (P = 0.06), copy number gains occurred at a high prevalence in intestinal-type cancers than in diffuse-type cancers, but the difference was not statistically significant. Copy number gains of MYC (P = 0.03), BOP1 (P = 0.03), and CD40 (P = 0.01) were found at a significantly high prevalence in intestinal-type cancers compared to diffuse-type cancers. To detect age-related CNAs, we analyzed correlation between patient’s age and copy number change using Pearson’s correlation coefficients but found no correlation was found between copy number change of 15 genes and patient’s age (Fig 4A). Hierarchical clustering analysis was performed in order to group patients with similar CNAs. Most of the patients with copy number gains at 8q24 also had copy number gains at 20q11.21 or 20q13.12 (Fig 4B). Data were further divided into 4 clusters according to the presence of copy number gains at 8q24 and 20q11.21 (or 20q13.12). Copy number gains at 8q24 was significantly associated with copy number gains at 20q11.21 or 20q13.12 (P = 0.005, Fisher’s exact test; Table D in S1 File). These observations suggest that the two regions, 8q24 and 20q11.21 (or 20q13.12), may be similarly susceptible to copy number gains in gastric cancer.

Table 2. The association of the expression of selected genes with copy number alterations.

| Chromosomal regions | Gene | Total Number (Without CNA + with CNA) | mRNA FC | P-value* | mRNA FC without CNA | P-value* | mRNA FC with CNA | P-value* |
|---------------------|------|--------------------------------------|---------|----------|---------------------|----------|------------------|----------|
| Gains               |      |                                      |         |          |                     |          |                  |          |
| 8q24.21             | MYC  | 26 (20 + 6)                          | Up      | 0.003    | -                   | 0.050    | Up               | 0.010    |
| 8q24.3              | SCRIB| 26 (22 + 4)                          | Down    | <0.001   | Down               | 0.014    | -                | 0.503    |
| 8q24.3              | PUF60| 26 (22 + 4)                          | Up      | 0.018    | -                   | 0.111    | Up               | 0.038    |
| 8q24.3              | BOI  | 26 (22 + 4)                          | Up      | <0.001   | Up                  | <0.001   | Up               | 0.024    |
| 20q11.21            | SNTA1| 26 (21 + 5)                          | Down    | <0.001   | Down               | <0.001   | -                | 0.202    |
| 20q11.21            | E2F1 | 24 (20 + 4)                          | Up      | <0.001   | Up                  | <0.001   | Up               | 0.030    |
| 20q13.12            | CD40 | 26 (21 + 5)                          | Down    | 0.002    | Down               | 0.010    | -                | 0.147    |
| 20q13.12            | CYA2 | 26 (21 + 5)                          | Down    | <0.001   | Down               | <0.001   | -                | 0.058    |
| 20q13.12            | NCOA3| 26 (21 + 5)                          | Down    | <0.001   | Down               | 0.002    | -                | 0.177    |
| Losses              |      |                                      |         |          |                     |          |                  |          |
| 3p14.2              | FHIT | 25 (9 + 16)                          | Down    | <0.001   | Down               | 0.003    | Down             | <0.001   |
| 17p13.3             | CRK  | 26 (13 + 13)                         | Down    | <0.001   | Down               | <0.001   | Down             | <0.001   |
| 18q21.1             | SMAD2| 26 (16 + 10)                         | Down    | <0.001   | Down               | <0.001   | Down             | <0.001   |

* The significance of mRNA fold change (FC) of individual genes was statistically analyzed by one-sample t-test.

doi:10.1371/journal.pone.0137657.t002
Discussion

The change of gene dosage by CNA is being increasingly recognized as an important component of tumorigenesis. To discover novel CNAs involved in the pathogenesis of gastric cancer, we performed a genome-wide analysis of CNAs in tumor and matched normal tissues from 88 gastric cancer patients and identified recurrent (> 20%) copy number gains at multiple chromosomal regions including 7p22.1, 8q24.21, 8q24.3, 13q34, 20q11~q13 and a recurrent losses at 3p14.2, 4q35.2, 6q26, and 17p13.3. The CNAs at 7p22.1, 13q34, and 17p13.3 have not been reported in other populations. The 7p22.1 regions identified in the present study contain the FBXL18, ACTB, ACTG1, and RNF216 genes. Although CNAs at 7p22.1 have not been reported in gastric cancer, several studies reported their impact on the development of ovarian clear cell adenocarcinoma [15] and endometriosis [16]. In this study, copy number gains of MYC (8q24.21), FHIT (3p14.2), and NCOA3 (20q12) were validated using MLPA, but copy number losses (WDR60, COL4A2, NFATC1) of around 2000bp were not validated by MLPA.

We failed to perform extensive computational estimation of false positive rates of array-based calling. Instead, we have compared the prevalence of copy number losses between aCGH-244k & -400K and the aCGH-60K with highly dense probes according to the sizes of copy number losses: 1kb-5kb, 5kb-10kb, 10kb-50kb, 50kb-100kb, and 100k-. Statistically significant differences were found only in the copy number losses of small size (1kb-5kb) (Table E in S1 File). In addition, the significant differences were found in chromosomal locus-specific manner: no differences were found in chromosomes 3, 6, 16, 17, and 20 (data not shown). Therefore, it is possible that copy number losses of small size detected in aCGH-244K and aCGH-400K may be false in some loci.

doi:10.1371/journal.pone.0137657.g003
We furthermore analyzed minimal common regions of recurrent (≥10%) amplification or deletion in 88 gastric cancers (Table F in S1 File) and compared them with the large gastric cancer TCGA (The Cancer Genome Atlas) study (14) and three previous studies (Table G in)

![Correlations between age with CNAs](image)

**Fig 4. Correlations of CNAs levels with patient’s age at diagnosis and other CNAs in different chromosomal regions.** (A) Correlations of CNAs levels with patient’s age were analyzed using Pearson’s correlation coefficient. Multiple tests were corrected using the Bonferroni correction. The Bonferroni-corrected P-values were calculated by multiplying the observed (uncorrected) P-values by the number of tested genes. Violet colors indicate Bonferroni-adjusted P-value < 0.05. (B) Unsupervised hierarchical clustering analysis of 15 genes in regions with recurrent (>20%) CNAs was performed to investigate correlations among CNAs levels of 11 genes showing copy number gains on 8q24.21, 8q24.3, 20q11.21, and 20q13.12, and 4 genes showing copy number losses. The numbers on the right side of the figure indicate patient identification number. The color scales indicate log2 intensity ratios of CNAs at individual gene. Value “zero (= log(2/2))” indicate a copy number of 2. Green and red colors represent copy number gain and loss, respectively.

doi:10.1371/journal.pone.0137657.g004

We furthermore analyzed minimal common regions of recurrent (≥10%) amplification or deletion in 88 gastric cancers (Table F in S1 File) and compared them with the large gastric cancer TCGA (The Cancer Genome Atlas) study (14) and three previous studies (Table G in...
The TCGA study was comprised of 295 primary gastric adenocarcinomas and identified 30 focal amplifications and 45 focal deletions. Amplification (≥ 5 copies) at 8q24.21 (MYC), 17q12 (ERBB2 etc.), 20q11.1-q13.33 (EYA2, NCOA3 etc.), and deletion (0 copies) at 3p14.2 (FHIT) were observed in our data as well as the data from the TCGA and others’ studies (Table G in S1 File). However, CNAs at 7p22.1, 13q34, and 17p13.3 have not been reported in the TCGA study and other populations. The number of the regions of the CNAs identified in the TCGA study was larger than the present study, which might result from the different subgroups of sample members. The TCGA study consists of larger intestinal-type (66.4%) compared to diffuse-type cancers (23.4%).

Among the genes located on 8q24.21, MYC is known to promote the growth and proliferation of normal gastric cells, and knockdown of MYC restrains the growth and proliferation of gastric cancer cells [17]. MYC encodes a transcriptional factor that regulates a variety of genes related to proliferation, differentiation, and apoptosis [18]. MYC is amplified and over-expressed in gastric cancer [19], and its expression increases progressively as the cancer develops [20]. MYC amplification is associated with the aggressive behavior of gastric cancer cells [21,22]. In this study, copy number gains of MYC were found at a high prevalence in the intestinal-type cancers as compared to the diffuse-type cancers, supporting the observation that MYC protein expression is more frequently observed in intestinal-type tumors than in diffuse-type tumors [23]. We have analyzed the effect of MYC CNAs on overall survival within each type. Patients with copy number gains of MYC had poor overall survival compared to those without, but the difference was not statistically significant in diffuse type and intestinal type cancers (S1 Fig). The copy number gains of the POU5F1B (POU domain class 5 transcription factor 1B) pseudogene on 8q24.21 were found in 27% of the samples analyzed. POU5F1B is known to be associated with mRNA abundance and an aggressive phenotype in gastric cancer [24].

The 8q24.3 and 20q11-q13 regions contain hundreds of genes (Table A in S1 File), but many are unlikely involved in oncogenesis. Among the genes located in these regions, we analyzed the mRNA levels of SCRIB, PUF60, and BOP1 at 8q24.3 and SNTA1, E2F1, CD40, EYA2, and NCOA3 at 20q11-q13 (Table 2). In the present study, copy number gains of SCRIB, SNTA1, CD40, EYA2, and NCOA3 were not associated with a fold change in mRNA levels. However, the PUF60, BOP1, and E2F1 genes were found to be significantly over-expressed in tumor tissues with copy number gains. PUF60 was over-expressed in cancers with CNAs (P = 0.038), but its expression was not significantly different between tumor tissues and matched normal tissues in samples without CNAs (P = 0.111). PUF60 (poly-U binding splicing factor 60kDa), a FUSE-binding protein-interacting repressor (FIR), plays a role in nuclear processes such as pre-mRNA splicing and transcriptional regulation. In addition, PUF60 suppresses MYC transcription at the P2 promoter through the core-TFIIB basal transcription factor [25]. Recently, Gumireddy et al. [26] reported that PUF60 is required for the regulator function of translational regulatory IncRNA (treRNA), which is involved in tumor invasion and metastasis. Copy number gains of PUF60 show a strong positive correlation with expression in gastric cancer [27] and in ovarian cancer [28]. These observations suggest that copy number gains of PUF60 may be a major mechanism underlying the over-expression of the gene in gastric cancer.

In contrast to PUF60, the BOP1 and E2F1 were found to be over-expressed in tumor tissues with copy number gains as well as in those without. Copy number gains of BOP1 and E2F1 in this study occurred in 23% and 25% of samples studied, respectively. Increased mRNA fold change of BOP1 was significant in tumor tissues with copy number gains (P = 0.024) as well as in those without (P < 0.001). BOP1 (block of proliferation 1) is a component of the PeBoW (Pes1, Bop1, and WDR12) complex, which is required for maturation of 28S and 5.8S
ribosomal RNAs and formation of the 60S ribosome [29]. BOP1 plays an oncogenic role in hepatocellular carcinoma by inducing epithelial-mesenchymal transition (EMT) and promoting actin cytoskeleton remodeling [30]. The BOP1 gene is known to be over-expressed in rectal cancer with 8q gain [31], and dosage increase of the BOP1 gene is associated with an increase of BOP1 mRNA in colorectal cancer [32]. The E2F1 was also over-expressed in tumor tissues with copy number gains \( (P < 0.001) \) and in those without \( (P = 0.03) \). E2F1 plays a crucial role in the control of the cell cycle and its activity is regulated through binding to retinoblastoma protein in a cell-cycle-dependent manner. Over-expression of E2F1 is associated with the development of a variety of tumors, and the increased copy number of E2F1 is known to be associated with over-expression of the gene in melanoma [33] and cervical cancer [34]. Based on these observations, it is likely that the overall impact of copy number gains on gene expression in gastric cancer varies in a gene-dependent manner.

Although copy number gains at 13q34 were not reported in gastric cancer, the gains were found in 20–30% of samples studied. Copy number gains at 13q34 are known to be associated with the progression of cervical intraepithelial neoplasia to squamous cell carcinoma [35] and with small bowel adenocarcinoma [36]. Copy number gains at 17q12 are frequent in gastric cancer. In the present study, several genes, including ERBB2, GRB7, STARD3, PPP1R1B, RARA, and C17orf37, were amplified in 15–20% of the 88 cases, consistent with other studies [37,38]. We did not evaluate the correlation of copy number and expression levels of the genes, but several groups have reported that the genes are important in the development of gastric cancer. Among them, ERBB2 (HER2) is frequently amplified and over-expressed in gastric cancers [39–41], and amplification of HER2 was strongly associated with poor survival, particularly in the intestinal type of gastric cancer [42]. Immunoreactivity of ERBB2 also occurs at a higher prevalence rate in intestinal type than in the diffuse subtypes [43]. Furthermore, the PPP1R1B-STARD3 fusion transcript in human gastric cancer increases colony formation through the activation of phosphatidylinosil-3-kinase and AKT signaling [44]. Frequent amplification of GRB7 and positive changes in expression were also reported in gastric cancer [41,43].

The most frequent losses in this study were detected on 3p14.2 (39% in diffuse-types and 37% of intestinal types), where FHIT is located. FHIT is a well-known tumor suppressor gene [45], and is often involved in the loss of heterozygosity (LOH) and deletions in human tumors [46]. Primary gastric carcinomas represent a rearrangement of the FHIT gene and 20 of 30 (67%) samples exhibited an absence of FHIT protein expression [47]. Loss of FHIT protein expression correlates with disease progression and poor differentiation in gastric cancer [48]. In the present study, we observed that FHIT expression was reduced in gastric cancers with or without its CNA, suggesting that gene dosage as well as other mechanisms regulate FHIT expression in gastric cancer. A somatic missense mutation (exon 6, codon 61, ACG \( \rightarrow \) ATG) of FHIT has also been identified in gastric cancers [49]. Furthermore, a high frequency of promoter hypermethylation of FHIT (62%) is observed in gastric cancers [50]. Therefore, integrating copy number data with additional genomic data is essential to comprehensively understanding the genetic control of gene expression [51].

Copy number losses of several genes in this study were not significantly different between diffuse-type cancers and intestinal-type cancers. However, the prevalence of copy number gains was different between both types in certain genes, suggesting that environmental factors may be more influential in copy number gains than losses. In addition, patients with copy number gains on 8q24.21 and 8q24.3 tended to have gains on 20q11-q13, suggesting both regions may be equally susceptible to copy number variation. This study was severely limited due to the small number of samples and the lack of survival data. Further study in a large cohort is required to understand the functional significance of CNAs discovered in this...
study. In addition, mRNA measurements were not performed at a genome level. We analyzed relationship between mRNA levels of some genes known to be important in the pathogenesis of human cancer and the CNAs. A significant correlation was found between the expression levels of \textit{MYC}, \textit{PUF60}, \textit{BOP1}, and \textit{E2F1} genes and their CNAs (Table 2). A statistically significant correlation between CNAs of \textit{MYC}, \textit{PUF60}, and \textit{E2F1} genes and their expression levels was also found by Fan et al. (11). However, further study is required to clearly understand the effect of CNAs on gene expression. In conclusion, the present study suggests that DNA copy number gains at 8q24.21, 8q24.3, 20q11-20q13 and losses at 3p14.2 may be common events in gastric cancer. However, CNAs at 7p22.1, 13q34, and 17p13.3 may be Korean-specific. In addition, copy number gains may be more frequent in intestinal-type than diffuse-type gastric cancer.

Materials and Methods

Study population and DNA extraction

A total of 88 patients, 35 women and 53 men, who had undergone curative surgical resection for gastric cancer between November 2004 and October 2010 at the Department of Surgery in the Samsung Medical Center, Seoul, Korea, participated in this study. Surgically removed tumor tissues were collected after obtaining written informed consent from all of the patients. This study was approved by the Samsung Medical Center (SMC) Institutional Review Board (IRB). The tumors were snap-frozen in liquid nitrogen and stored at \(-80\degree\)C until needed. Prior to DNA extraction from the fresh frozen tissues, the sections were placed on slides and stained with H&E to evaluate the admixture of tumorous and non-tumorous tissues. Tumor and non-tumor areas were microdissected carefully under a microscope. The microdissected tissues were digested with proteinase K, and the genomic DNA was isolated according to the instructions of the manufacturer (DNeasy Tissue kit, Qiagen, Valencia, CA). The sample consisted of 43 diffuse-type cancers, 41 intestinal-type, and 4 mixed-type cancers.

CNA analysis using aCGH

The aCGH was performed according to the manufacturer’s recommendations. After DNA hybridization and washing, slides were scanned immediately using an Agilent microarray scanner, and raw data were extracted using Feature Extraction Software at the default CGH parameter settings (Agilent Technologies). Putative CNA intervals in each sample were identified using Agilent Genomic Workbench v7.0.4.0 software. Cy5/Cy3 ratios were converted into log2-transformed values. Centralization and fuzzy zero corrections were applied to the microarray. The Aberration Detection Method 2 (ADM-2) algorithm at threshold 6.0 was used to identify the CNAs in individual samples and to determine aberration frequencies in gastric cancer samples (Fig 5). The following filters were employed: minimum number of probes in region \(> = 3\), minimum absolute average log ratio of region \(> = 0.25\). Common aberrations were detected by using the context-corrected algorithm at p-value \(< 0.05\) and an overlap threshold of 0.9. The CNAR (Copy Number Alteration Region) was defined as the union of more than 90 percent overlapping aberrant segments across multiple samples. The UCSC genome assembly hg19 was used as the human reference genome sequence. For each platform (244K, 400K, and 60K), the within array global Lowess normalization method was applied to correct for local spatial bias and continuous spatial gradients. After the within array normalization, a quantile between array normalization was applied to compare the aberration results across arrays. These normalizations were carried out using the limma package in R. The MCR (Minimal Common Region) was defined as a 100 percent overlapping common region between samples in the CNAR. There are several MCRs in the CNAR according to the possible overlapping frequency. The
MCR of amplification and deletion was analyzed. Amplification and deletion was defined when the normalized log2 ratio was ≥ 0.8 and ≤ −0.8, respectively. All statistical methods and visualization of individual aberrant regions were conducted using R statistical language v.3.0.2 (www.r-project.org).

Fig 5. A schematic diagram for identifying a minimal common region. The Aberration Detection Method 2 (ADM-2) algorithm with a sensitivity threshold of 6.0 was used to identify the CNAs in gastric cancer and to determine the frequencies of CNAs in each sample. The MCR (Minimal Common Region) of copy number gains or losses was identified through the analysis of CNAs in the three kinds of aCGHs (244K, 400K, and 60K).

doi:10.1371/journal.pone.0137657.g005

MCR of amplification and deletion was analyzed. Amplification and deletion was defined when the normalized log2 ratio was ≥ 0.8 and ≤ −0.8, respectively. All statistical methods and visualization of individual aberrant regions were conducted using R statistical language v.3.0.2 (www.r-project.org).

Multiplex Ligation-Dependent Probe Amplification (MLPA) Analysis

MLPA analysis was performed using the SALSA MLPA kit P200 (MRC-Holland, Amsterdam, Netherlands) according to the manufacturer’s instructions [52]. The P200 kit contains 14 internal control probes to assess DNA denaturation and DNA quantity, and also for the X and Y chromosome. DNA samples were diluted with TE to 5 μl and were heated at 98°C for 5 min in PCR tubes in a thermocycler with a heated lid. After the addition of 1.5 μl MLPA buffer and 1.5 μl probe mix, samples were further heated for 1 min at 95°C and then incubated for 16 h at 60°C. The probe sequences for detected genes are listed in Table H in S1 File. Ligation of annealed oligonucleotides was performed by diluting the samples to 40 μl with a dilution buffer containing 1 U Ligase-65 enzyme, and incubating 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. While at 60°C, 10 μl of a buffered solution containing the PCR primers, dNTPs and SALSA polymerase (MRC-Holland, Amsterdam, Netherlands) were added. PCR was carried out for 35 cycles (30 s at 95°C, 30 s at 60°C and 1 min at 72°C). The MLPA PCR reactions were separated using the capillary electrophoresis system, ABI-Prism 3130 (Applied Biosystems, Foster City, CA), and the data was analyzed using a GeneMarker 2.0.0 (SoftGenetics, State College, PA). Data was population-normalized, and probe ratios below 0.75 were regarded as an indication of deletion, while probe ratios above 1.25 were regarded as an indication of amplification.
Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated using PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA), and RT-PCR was carried out using SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Real-time PCR was carried with SYBR green dye (Qiagen, Valencia, CA) under the following conditions: an initial denaturation step of 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 30 s. The PCR primers (Table I in S1 File) were designed using Primer Express 3 (Applied Biosystems, Foster City, CA), and the specificity of primer sets was checked with BLAST. The target mRNA amount in each sample was normalized to an internal control of RPLP0, and fold change was calculated by comparing the tumor with its matched normal.

Statistical analysis

Statistical significance of log2 ratio of mRNA fold change was analyzed by a one-sample t-test. Associations between CNAs of individual genes and Lauren’s classification were tested by the Pearson’s chi-square test (or Fisher’s exact test). Correlations between two continuous variables were analyzed using Spearman’s (or Pearson’s) correlation coefficients. The agglomerative hierarchical clustering algorithm was used for detecting clusters in copy number alterations. The effect of CNAs on overall survival was analyzed by Kaplan-Meier survival curves, and the significance of differences in survival between the two groups was evaluated by the log-rank test. All statistical analyses were two-sided, with a 5% type I error rate.

Supporting Information

S1 Fig. The effect of MYC CNA on overall survival. The Kaplan-Meier approach was used to estimate survival curves according to the CNA of MYC. The effect of CNA of MYC on overall survival was analyzed using log-rank test in 88 gastric cancers (A), 43 diffuse type cancers (B), and 41 intestinal type cancers (C). The CNAs of MYC tended to reduce the overall survival rate in diffuse and intestinal types, but the difference was not statistically significant.

S1 File. Titles of supporting tables. Table A. Minimal common regions of recurrent (>20%) copy number gains. Table B. Minimal common regions of recurrent (>20%) copy number losses. Table C. Copy number alterations according to Lauren’s classification. Table D. Prevalence of copy number gains at 8q24 and 20q11.21 (or 20113.12). Table E. Comparison of copy number losses between aCGH-244K/-400K and aCGH-60K. Table F. Minimal common regions of recurrent (>10%) amplifications or deletions. Table G. Comparison of recurrent amplifications or deletions among studies. Table H. MLPA probe sequences. Table I. qPCR primer sequences.

Acknowledgments

The authors wish to thank Eunkyung Kim and Jin-Hee Lee for data collection and management, and Hoon Suh for sample collection. This work was supported by grants from the National R&D Program for Cancer Control, Ministry for Health and Welfare (#1120270) and from the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare (HI14C1979), Republic of Korea.
Author Contributions
Conceived and designed the experiments: DHJ DHK JP. Performed the experiments: DHK SEP. Analyzed the data: DHJ DHK. Contributed reagents/materials/analysis tools: JL KMK SK. Wrote the paper: DHK DHK JP.

References
1. Brenner H, Rothenbacher D, Arndt V. Epidemiology of stomach cancer. Methods Mol Biol. 2009; 472: 467–477. doi:10.1007/978-1-60327-492-0_23 PMID: 19107449
2. Macdonald JS, Smalley SR, Benedetti J, Hundahl SA, Estes NC, Stemmermann GN, et al. Chemoradiotherapy after surgery compared with surgery alone for adenocarcinoma of the stomach or gastroesophageal junction. N Engl J Med. 2001; 345: 725–730. PMID: 11547741
3. Lee J, Lim do H, Kim S, Park SH, Park JO, Park YS, et al. Phase III trial comparing capecitabine plus cisplatin versus capecitabine plus cisplatin with concurrent capecitabine radiotherapy in completely resected gastric cancer with D2 lymph node dissection: the ARTIST trial. J Clin Oncol. 2012; 30: 268–273. doi: 10.1200/JCO.2011.39.1953 PMID: 22184384
4. Boussioutas A, Taupin D. Towards a molecular approach to gastric cancer management. Intern Med J. 2001; 31: 296–303. PMID: 11512601
5. Shlien A, Malkin D. Copy number variations and cancer susceptibility. Curr Opin Oncol. 2010; 22: 55–63. doi: 10.1097/CCO.0b013e328333dca4 PMID: 19952747
6. Park CH, Rha SY, Jeung HC, Kang SH, Ki DH, Lee WS, et al. Identification of novel gastric cancer-associated CNVs by integrated analysis of microarray. J Surg Oncol. 2010; 102: 454–461. doi: 10.1002/jso.21585 PMID: 20872948
7. Kleinjans DA, van Heyningen V. Long-range control of gene expression: emerging mechanisms and disruption in disease. Am J Hum Genet. 2005; 76: 8–32. PMID: 15549674
8. White SJ, Vissers LE, Geurts van Kessel A, de Menezes RX, Kalay E, Lehesjoki AE, et al. Variation of CNV distribution in five different ethnic populations. Cytogenet Genome Res. 2007; 118: 19–30. PMID: 17901696
9. Koizumi Y, Tanaka Si, Mou R, Koganei H, Kokawa A, Kitamura R, et al. Changes in DNA copy number in primary gastric carcinomas by comparative genomic hybridization. Clin Cancer Res. 1997; 3:1067–1076. PMID: 9815785
10. Tsukamoto Y, Uchida T, Kaman S, Noguchi T, Nguyen LT, Tanigawara M, et al. Genome-wide analysis of DNA copy number alterations and gene expression in gastric cancer. J Pathol. 2008; 216: 471–482. doi: 10.1002/path.2424 PMID: 18798223
11. Fan B, Dachrut S, Coral H, Yuen ST, Chu KM, Law S, et al. Integration of DNA copy number alterations and transcriptional expression analysis in human gastric cancer. PLoS One. 2012; 7: e29824. doi: 10.1371/journal.pone.0029824 PMID: 22593939
12. Cheng L, Yang S, Yang Y, Zhang W, Xiao H, Gao H, et al. Global gene expression and functional network analysis of gastric cancer identify extended pathway maps and GPRC5A as a potential biomarker. Cancer Lett. 2012; 326: 105–113. doi: 10.1016/j.canlet.2012.07.031 PMID: 22867948
13. Cheng L, Wang P, Yang S, Yang Y, Zhang Q, Zhang W, et al. Identification of genes with a correlation between copy number and expression in gastric cancer. BMC Genomics. 2012; 5:14. doi: 10.1186/1755-8794-5-14 PMID: 22559327
14. Bass Bass AJ, Thorsson V, Shmulevich I, Reynolds SM, Miller M, Bernard B, et al. Comprehensive molecular characterization of gastric adenocarcinoma. Nature. 2014; 513: 202–209. doi: 10.1038/ nature13480 PMID: 25079317
15. Sung CO, Choi CH, Ko YH, Ju H, Choi YL, Kim N, et al. Integrative analysis of copy number alteration and expression profiling in ovarian clear cell adenocarcinoma. Cancer Genet. 2013; 206: 145–153. doi: 10.1016/j.cancergen.2013.04.002 PMID: 23726144
16. Gogusev J, Bouquet de Joliniere J, Telvi L, Doussau M, du Manoir S, Stojkoski A, et al. Detection of DNA copy number changes in human endometriosis by comparative genomic hybridization. Hum Genet. 1999; 105: 444–451. PMID: 10598611
17. Zhang L, Hou Y, Ashtorab H, Gao L, Xu Y, Wu K, et al. The impact of C-MYC gene expression on gastric cancer cell. Mol Cell Biochem. 2010; 344: 125–135. doi: 10.1007/s11010-010-0536-0 PMID: 20737197
18. Dang CV, Resar LM, Emison E, Kim S, Li Q, Prescott JE, et al. Function of the c-Myc oncogenic transcription factor. Exp Cell Res. 1999; 253: 63–77. PMID: 10579912
19. Calcagno DQ, Leal MF, Seabra AD, Khayat AS, Chen ES, Demachki S, et al. Interrelationship between chromosome 8 aneuploidy, C-MYC amplification and increased expression in individuals from northern Brazil with gastric adenocarcinoma. World J Gastroenterol. 2006; 12: 6207–6211. PMID: 17036397

20. Xu AG, Li SG, Liu JH, Gan AH. Function of apoptosis and expression of the proteins Bcl-2, p53 and C-myc in the development of gastric cancer. World J Gastroenterol. 2001; 7: 403–406. PMID: 11819799

21. Calcagno DQ, Freitas VM, Leal MF, de Souza CR, Montenegro R, et al. MYC, FBXW7 and TP53 copy number variation and expression in gastric cancer. BMC Gastroenterol. 2013; 13: 141. doi: 10.1186/1471-230X-13-141 PMID: 24053468

22. Sonoda A, Mukaiho K, Nakayama T, Diem VT, Hattori T, Andoh A, et al. Genetic lineages of undifferentiated-type gastric carcinomas analysed by unsupervised clustering of genomic DNA microarray data. BMC Med Genomics. 2013; 6: 25. doi: 10.1186/1755-8794-6-25 PMID: 23866769

23. de Souza CR, Leal MF, Calcagno DQ, Costa Sozinho EK, Borges Bdo N, Montenegro RC, et al. MYC deregulation in gastric cancer and its clinicopathological implications. PLoS One. 2013; 8: e64420. doi: 10.1371/journal.pone.0064420 PMID: 23717612

24. Hayashi H, Arao T, Togashi Y, Kato H, Fujita Y, De Velasco MA, et al. The OCT4 pseudogene POUSF1B is amplified and promotes an aggressive phenotype in gastric cancer. Oncogene. 2015; 34: 199–208. doi: 10.1038/onc.2013.547 PMID: 24362523

25. Matsushita K, Tomonaga T, Shimada H, Shiyoa H, Higashi M, Matsumura H, et al. An essential role of alternative splicing of c-myc suppressor FUSE-binding protein-interacting repressor in carcinogenesis. Cancer Res. 2006; 66: 1409–1417. PMID: 16452196

26. Gumireddy K, Li A, Yan J, Setoyama T, Johannes GJ, Orom UA, et al. Identification of a long non-coding RNA-associated RNP complex regulating metastasis at the translational step. EMBO J. 2013; 32: 2672–2684. doi: 10.1038/emboj.2013.188 PMID: 23974796

27. Cheng L, Wang P, Yang S, Yang Y, Zhang Q, Zhang W, et al. Identification of genes with a correlation between copy number and expression in gastric cancer. BMC Med Genomics. 2012; 5: 14. doi: 10.1186/1755-8794-5-14 PMID: 22559327

28. Ramakrishna M, Williams LH, Boyle SE, Bearfoot JL, Sridhar A, Speed TP, et al. Identification of candidate growth-promoting genes in ovarian cancer through integrated copy number and expression analysis. PLoS One. 2010; 5: e9983. doi: 10.1371/journal.pone.0009983 PMID: 20386695

29. Rohmoser M, Holzel M, Grimm T, Malamoussi A, Harasim T, Orban M, et al. Interdependence of Pes1, Bop1, and WDR12 controls nucleolar localization and assembly of the PeBoW complex required for maturation of the 60S ribosomal subunit. Mol Cell Biol. 2007; 27: 3682–3694. PMID: 17353269

30. Chung KY, Cheng IK, Ching AK, Chu JH, Lai PB, Wong N. Block of proliferation 1 (BOP1) plays an oncogenic role in hepatocellular carcinoma by promoting epithelial-to-mesenchymal transition. Hepatology. 2011; 54: 307–318. doi: 10.1002/hep.24372 PMID: 21520196

31. Lips EH, van Eijk R, de Graaf EJ, Oosting J, de Miranda NF, Karsten T, et al. Integrating chromosomal aberrations and gene expression profiles to dissect rectal tumorigenesis. BMC Cancer. 2008; 8: 314. doi: 10.1186/1471-2407-8-314 PMID: 18959792

32. Killian A, Sarafan-Vasseur N, Sesboue R, Le Pessot F, Blanchard F, Lamy A, et al. Contribution of the BOP1 gene, located on 8q24, to colorectal tumorigenesis. Genes Chromosomes Cancer. 2006; 45: 874–881. PMID: 16804918

33. Nelson MA, Reynolds SH, Rao UN, Goulet AC, Feng Y, Beas A, et al. Increased gene copy number of the transcription factor EZF1 in malignant melanoma. Cancer Biol Ther. 2006; 5: 407–412. PMID: 16481740

34. Scotto L, Narayan G, Nandula SV, Arias-Pulido H, Subramaniam S, Schneider A, et al. Identification of copy number gain and overexpressed genes on chromosome arm 20q by an integrative genomic approach in cervical cancer: potential role in progression. Genes Chromosomes Cancer. 2008; 47: 755–765. doi: 10.1002/gcc.20577 PMID: 18506748

35. Oh EK, Kim YW, Kim IW, Liu HB, Lee KH, Chun HJ, et al. Differential DNA copy number aberrations in the progression of cervical lesions to invasive cervical carcinoma. Int J Oncol. 2012; 41: 2038–2046. doi: 10.3892/ijo.2012.1644 PMID: 23023522

36. Diosdado B, Buffart TE, Watkins R, Carvalho B, Ylstra B, Tijssen M, et al. High-resolution array comparative genomic hybridization in sporadic and celiac disease-related small bowel adenocarcinomas. Clin Cancer Res. 2010; 16: 1391–1401. doi: 10.1158/1078-0432.CCR-09-1773 PMID: 20179237

37. Varis A, Wolf M, Monni O, Vakkari ML, Kokkola A, Moskaluk C, et al. Targets of gene amplification and overexpression at 7q in gastric cancer. Cancer Res. 2002; 62: 2625–2629. PMID: 11900659

38. Kokkola A, Monni O, Puolakkainen P, Nordling S, Haapiainen R, Kivilaakso E, et al. Presence of high-level DNA copy number gains in gastric carcinoma and severely dysplastic adenomas but not in moderately dysplastic adenomas. Cancer Genet Cytogenet. 1998; 107: 32–36. PMID: 9809031
39. Yano T, Doi T, Ohtsu A, Boku N, Hashizume K, Nakanishi M, et al. Comparison of HER2 gene amplification assessed by fluorescence in situ hybridization and HER2 protein expression assessed by immunohistochemistry in gastric cancer. Oncol Rep. 2006; 15: 65–71. PMID: 16328035
40. Yan B, Yau EX, Bte Omar SS, Ong CW, Pang B, Yeoh KG, et al. A study of HER2 gene amplification and protein expression in gastric cancer. J Clin Pathol. 2010; 63: 839–842. doi: 10.1136/jcp.2010.076570 PMID: 20696687
41. Rossi E, Klersy C, Manca R, Zuffardi O, Solcia E. Correlation between genomic alterations assessed by array comparative genomic hybridization, prognostically informative histologic subtype, and patient survival in gastric cancer. Hum Pathol. 2011; 42: 1937–1945. doi: 10.1016/j.humpath.2011.02.016 PMID: 21676433
42. Jorgensen JT, Hersom M. HER2 as a Prognostic Marker in Gastric Cancer—A Systematic Analysis of Data from the Literature. J Cancer. 2012; 3: 137–144. doi: 10.7150/jca.4090 PMID: 22481979
43. Myllykangas S, Junnila S, Kokkola A, Autio R, Scheinin I, Kiviluoto T, et al. Integrated gene copy number and expression microarray analysis of gastric cancer highlights potential target genes. Int J Cancer. 2008; 123: 817–825. doi: 10.1002/ijc.23574 PMID: 18506690
44. Yun SM, Yoon K, Lee S, Kim E, Kong SH, Choe J, et al. PPP1R1B-STARD3 chimeric fusion transcript in human gastric cancer promotes tumorigenesis through activation of PI3K/AKT signaling. Oncogene. 2014; 33: 5341–5347. doi: 10.1038/onc.2013.472 PMID: 24276243
45. Siprashvili Z, Sozzi G, Barnes LD, McCue P, Robinson AK, Eryomin V, et al. Replacement of Fhit in cancer cells suppresses tumorigenicity. Proc Natl Acad Sci U S A. 1997; 94: 13771–13776. PMID: 9391102
46. Ohta M, Inoue H, Cotticelli MG, Kastury K, Baffa R, Palazzo J, et al. The FHIT gene, spanning the chromosome 3p14.2 fragile site and renal carcinoma-associated t(3;8) breakpoint, is abnormal in digestive tract cancers. Cell. 1996; 84: 587–597. PMID: 8598045
47. Baffa R, Veronese ML, Santoro R, Mandes B, Palazzo JP, Rugge M, et al. Loss of FHIT expression in gastric carcinoma. Cancer Res. 1998; 58: 4708–4714. PMID: 9788626
48. Rocco A, Schandl L, Chen J, Wang H, Tulassembly Z, McNamara D, et al. Loss of FHIT protein expression correlates with disease progression and poor differentiation in gastric cancer. J Cancer Res Clin Oncol. 2003; 129: 84–88. PMID: 12669232
49. Gemma A, Hagiwara K, Ke Y, Burke LM, Khan MA, Nagashima M, et al. FHIT mutations in human primary gastric cancer. Cancer Res. 1997; 57: 1435–1437. PMID: 9108441
50. Roa JC, Anabalon L, Roa I, Tapia O, Melo A, Villaseca M, et al. Promoter methylation profile in gastric cancer. Rev Med Chil. 2005; 133: 874–880. PMID: 16163424
51. Taylor BS, Barretina J, Socci ND, Decarolis P, Ladanyi M, Meyerson M, et al. Functional copy-number alterations in cancer. PLoS One. 2008; 3: e3179. doi: 10.1371/journal.pone.0003179 PMID: 18784837
52. Schouten JP, McElgunn CJ, Waaijer R, Zwijsenburg D, Diepvens F, Pals G. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res. 2002; 30: e57. PMID: 12060695