

***ORAOVI* is a probable target within the 11q13.3 amplicon in lymph node metastases from gastric adenocarcinoma**

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Received July 29, 2011; Accepted September 21, 2011

DOI: 10.3892/ijmm.2011.811

Abstract. The lymph node metastatic (LNM) spread of tumor cells is a frequent event in the initial process of cancer dissemination and is a powerful independent prognostic indicator in gastric adenocarcinoma (GAC). High density genomic arrays were conducted to identify molecular markers associated with lymph node metastasis in GAC. In the genome-wide profile, large copy number gains involving chromosomes 1p, 3q, 8q, 9q, 11q, 16p, 19p, and 20q (\log_2 ratio >0.25) ($>40\%$ of patients) were more prevalent than copy number losses. The most notable finding was copy number gains at the long arm of chromosome 11, which occurred in 75.0% of lymphatic metastasis GAC cases, and the delineated minimal common region was 11q24.2-q12.1. More specifically, 2 amplified ($>1 \log_2$ ratio) loci on the 11q13.3 region were detected in 12.5% of the cases. The first locus, covers a region of ~ 7.7 Mbp, and comprises the representative oncogene of cyclin D1 (*CCND1*). This finding occurred in 12.5% of the cases. Additionally, an oral cancer overexpressed 1 (*ORAOVI*) gene was identified as a probable target within the 11q13 amplicon, which previously was not assumed to play a pathogenic role in GACs (12.5%). A second locus spanning 7.8 Mbp on 11q13.3 without associated genes also showed high-level amplifications in 12.5% of the GACs. This study indicates that the long arm of chromosome 11 harbors protooncogenes that are associated with lymphatic metastasis formation and the *ORAOVI* gene at the 11q13.3 region could be a potential target and serve as an indicator for the presence of occult metastases in GAC.

Introduction

Gastric adenocarcinoma (GAC) is the second most common cause of cancer-related deaths in the world (1). Although the

overall incidence of GAC has declined, the incidence remains high in Asian countries and there is a compelling need to explore novel therapeutic targets for its management (2).

Lymph node metastasis (LNM) is a frequent event in the initial process of GAC dissemination and is the major determinant of GAC-related mortality (3). In most solid tumors, including GAC, the spread of cancer cells through the lymphatics to the regional lymph nodes (LN) is considered as an important prognostic indicator for patient outcome (4-8). Therefore, a better understanding of molecules promoting LNM may open the way to a host of innovative therapeutic strategies for GAC and could provide additional information concerning the presence of occult metastases of GACs (2). However, little is known about the specific underlying genes that affect LN metastasis and the genomic markers that predict aggressive clinical behavior of GAC still remain to be identified.

In the present study, we conducted a whole genome analysis to investigate invasion and metastasis related to DNA copy number alterations and new candidate genes that may be indicative and specific for the metastatic phenotype for GACs.

Materials and methods

Preparation of patient samples. A total of 16 tumor tissues were obtained from patients treated in the Department of General Surgery at Chungnam National University Hospital in Taejeon, South Korea. Original diagnostic material of all patients was reviewed to verify the original histopathological diagnosis and staging according to the WHO classification system. The stage of disease was based on the tumor-node-metastasis (TNM) classification using the Union for International Cancer Control (UICC) staging system. None of these patients had received preoperative chemotherapy or radiation. This study was reviewed and approved by the Institutional Review Board of the Chungnam National University Hospital. Written informed consent was obtained from each patient according to the institutional regulations.

Array-CGH experiment. DNA isolation was performed following the manufacturer's instructions (Promega, Madison, WI, USA), with some modifications as previously described (9,10). Array-CGH was performed using the MacArray™ Karyo 1.4 K BAC-chip (Macrogen, Seoul, Korea) (11-13) according to the manufacturer's instructions and as described in

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Key words: gastric adenocarcinoma, array CGH, copy number gain, high-level amplification

Table I. Overview of the DNA copy number changes in the 16 GAC cases.

No.	TNM classification ^a		Chromosomal alterations on 11q	Start ^b (Mbp)	End (Mbp)	Size (Mbp)
	Lymph node stage	Tumor stage				
1	N1	IIB	-	-	-	-
2	N1	IV	-	-	-	-
3	N1	IIIB	11q13.3-q25	69.0	133.3	64.3
4	N1	IIB	-	-	-	-
5	N1	IIIB	-	-	-	-
6	N2	IIB	11q12.2-q13.5 11q23.3	60.7	118.8	58.1
7	N2	IIIA	11q13.1-q13.3	64.3	69.1	4.8
8	N2	IIB	-	-	-	-
9	N2	IV	11q12.2-q13.1	60.7	64.5	3.8
10	N2	IIB	11q13.1	63.9	64.5	6.0
11	N2	IIB	11q12.2-q13.1	60.7	63.9	3.2
12	N2	IIB	11q23.3	118.7	118.8	0.1
13	N2	IIIB	11q12.2-q15.5 11q23.3	60.7	118.8	58.1
14	N3	IIB	11q13.3-q15.5	69.0	70.0	1.0
15	N3	IIIA	11q12.2-q13.3 11q23.3	69.0	118.8	49.8
16	N3	IV	11q12.2-q15.5	60.7	65.0	4.3

^apTNM, tumor-node-metastasis. ^bStart and end positions are based on the UCSC March 2006 assembly.

our previous studies (14,15). 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 BLAST; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Mapping of large insert clones was performed according to the genomic location in the UCSC Genome Bioinformatics database [<http://genome.ucsc.edu>; Build 36, version March 2006 (hg18)].

Hybridizations were carried out using a standard direct method as previously described (14,15). Briefly, 500 ng of normal male DNA (reference) and digested tumor DNA (test) were labeled with Cy5-dCTP and Cy3-dCTP, respectively, by random primed labeling (Array-CGH Genomic Labeling System; Invitrogen, Carlsbad, CA, USA). Hybridizations were performed in a sealed chamber for 48 h at 37°C. After hybridization, slides were washed according to the manufacturer's instructions and immediately scanned on a GenePix 4200A two-color fluorescence scanner (Axon Instruments, Union City, CA, USA). The acquired images were analyzed using GenePix Pro 4.1 imaging software (Axon Instruments).

Data analysis. To adjust for effects due to variation between the red and green dyes, Lowess normalization was applied. Then, the ratio of the red/green channel of each clone was calculated and log₂ transformed. The spot quality criteria were set as foreground-to-background >3.0 and standard deviation of triplicates <0.2. Breakpoint detection and status assignment of the genomic regions were performed using the GLAD soft-

ware (16). The median of the signal ratio (test signal/reference signal) 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 threshold value was determined empirically as a value 3-fold that of the standard deviations calculated from 30 normal males and normal females in hybridization experiments. The R 2.2.1 package of the Bioconductor Project (<http://www.bioconductor.org>) was used for the detection of the frequency of gain or loss and for statistical analysis. The Benjamini-Hochberg false discovery rate (FDR) was applied for multiple testing correction for the high number of false-positive calls. 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 the array-CGH data.

Results

Whole genome array analysis in lymph node-involved GAC cases. A genome-wide platform was performed to investigate invasion and metastasis-related DNA copy number alterations and new candidate genes in GAC. All of the patients (100.0%) in this profile showed multiple segmental alterations including single copy as well as high level gains and losses. Notably, large copy number gains involving chromosomes 1p, 3q, 8q, 9q, 11q, 16p, 19p and 20q (>40% of patients) were more prevalent than copy number losses. A detailed overview of

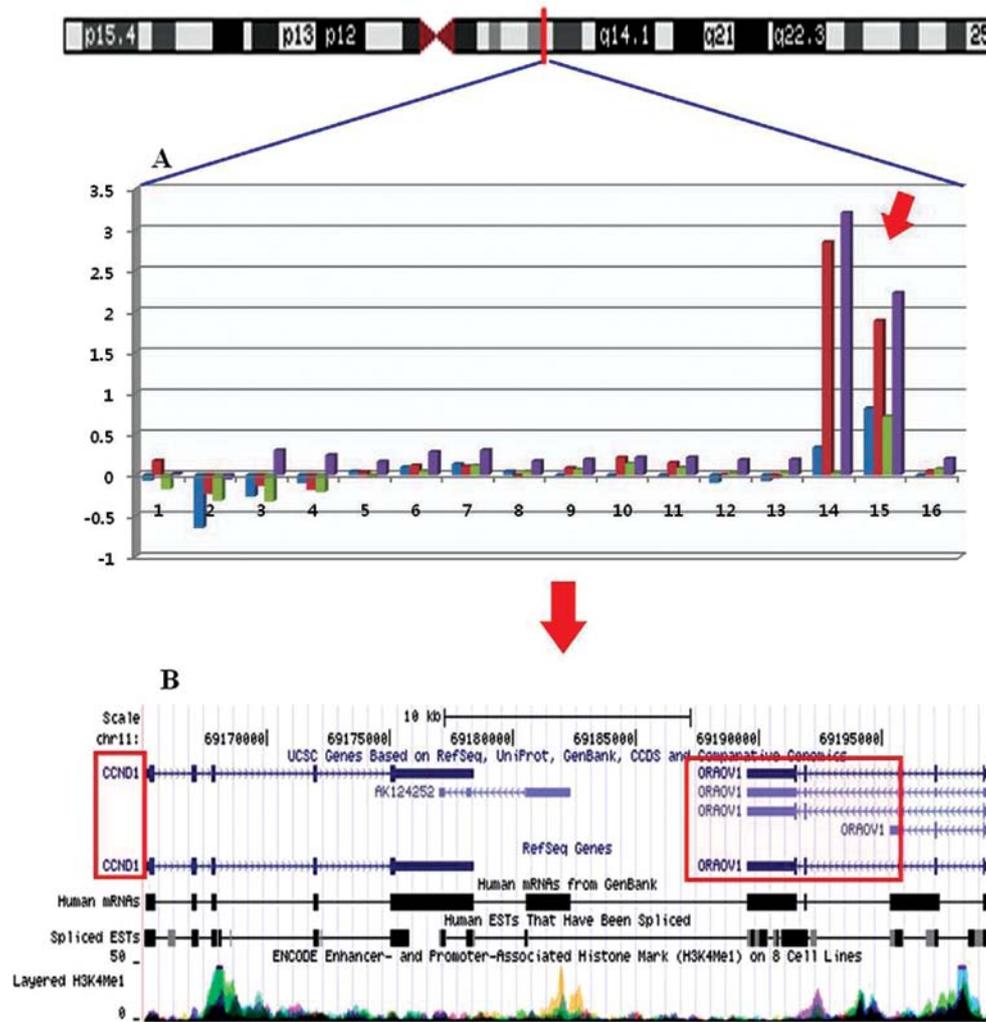


Figure 1. (A) Example of an individual profile at the 11q13.3 region in the 16 gastric adenocarcinoma cases. High-level amplifications are clearly seen in cases 14 and 15. (B) The schematic presentation of cytogenetic bands, as well as a map position from the UCSC genome browser (Mar 2006 freeze), is shown below the plot. The candidate target genes (*CCND1* and *ORAOV1*) at the 11q13.3 region are boxed in red.

clinicopathological data of 16 GACs is shown in Table I. To visualize both common and specific altered chromosomal regions of GAC, signal intensity ratios for each spotted BAC clone were calculated and displayed as log₂ plots (Figs. 1-3).

Delineation of the 11q amplicon in gastric adenocarcinomas.

Genome-wide array-CGH analysis showed that 12 of 16 (75.0%) cases were involved in the copy number gains (log₂ ratio >0.25) on the long arm of chromosome 11. The minimal common region identified by the array-CGH was located between BAC50_K14 and BAC250_C10. A more detailed analysis of chromosome 11q identified 3 distinct regions of alteration across the chromosome.

One region spanning ~99.9-100.0 Mbp on 11q24.2-q22.1 regions, containing 15 target clones, was identified as having copy number gains in 37.5% (6/16) of the cases. The most frequently gained clone was BAC141_J03 at the 11q23.3 region (31.3%, 5/16), which is located in the *CBL*, *MCAM*, *RNF26*, *CIQTNF5*, *MFRP* and *USP2* genes. Notably, copy number gains of these genes occurred at higher frequencies in stage IV GACs as compared to stage II-III tumors (data not presented).

The second candidate loci spanned 63.9-91.7 Mbp, and mapping at the 11q14.3-q13.1 regions determined a high frequency of gains in 10 of 16 cases (62.5%). More specifically, 2 amplified (>1 log₂ ratio) loci on the 11q13.3 region were detected in GACs. One locus contained contiguous amplified clones covering a region of ~7.7 Mbp and comprised the representative oncogene of cyclin D1 (*CCND1*) in 12.5% (2/16) of the cases. In addition, one candidate target gene for oral cancer overexpressed 1 (*ORAOV1*) was identified in 12.5% (2/16) at the 11q13.3 region, which was previously not assumed to play a pathogenic role in GACs. The other locus spanning ~7.8 Mbp on 11q13.3 without associated genes also showed high-level amplifications in 2 of 16 cases (12.5%). The median span of the copy number amplifications was 9.3 Mbp (range, 68.3-69.3 Mbp), and all copy number amplifications were located between BAC241_H14 and BAC220_G02. Examples of an individual profile showing high-level amplifications at 11q13.3 region are presented in Fig. 1A and the schematic presentation of cytogenetic bands, as well as map positions are shown in Fig. 1B. High-level amplifications are clearly seen in patients 14 and 15. Representative genome profiles of high-level amplifications at the 11q13.3 region are

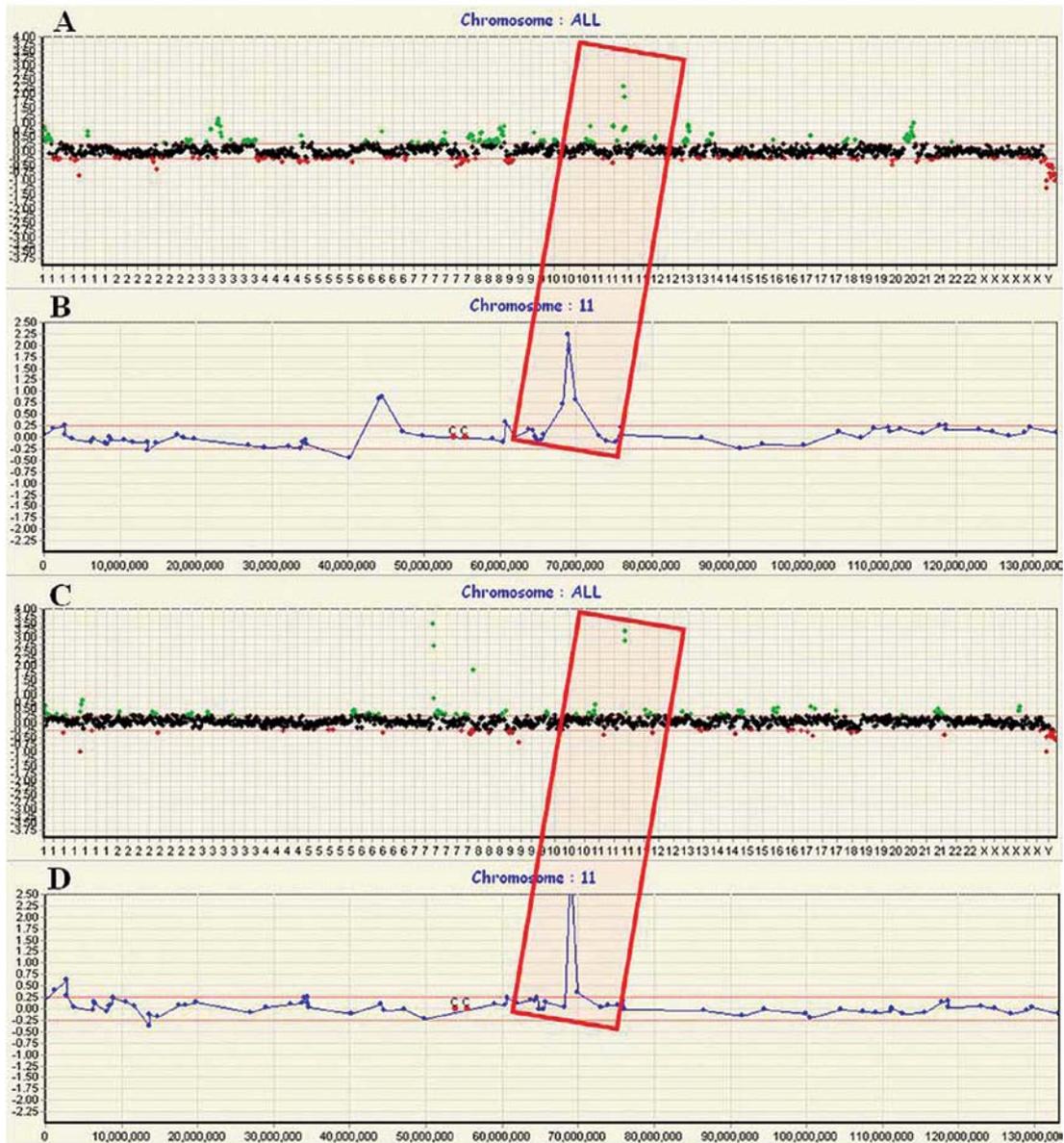


Figure 2. Examples of microarray CGH results from patients 14 and 15. Whole genome profiles (A and C) are shown in the upper portion, and individual chromosome profiles (B and D) are shown in more detail below the profile of the entire genome. Vertical lines indicate the highest locus of chromosome 11 in the BAC clone containing the *CCND1* and *ORAOVI* genes. The X-axis represents chromosome number (1-22) and the Y-axis represents the genome-wide frequencies of gains (>0.25 of intensity ratio) and losses (<0.25 of intensity ratio) for each clone of GAC. The copy number amplified regions at the 11q13.3 region are highlighted in red.

presented in Fig. 2. Whole genome profiles (Fig. 2A and C) are shown in the upper portion and individual chromosome profiles, and copy number amplifications at the 11q13.3 region (Fig. 2B and D) are presented in more detail below.

The third interval spans 58.9-59.1 Mbp on 11q12.3-q12.1, and this region contains 14 possible target genes according to the information archived by human genome databases (<http://genome.ucsc.edu/>). Notably, high-frequencies of copy number gains (>0.25 \log_2 ratio) and high-level gains (>0.5 \log_2 ratio) from the 11q12.3-q12.1 region were detected in 37.5% (6/16) and 12.5% (2/16) of the cases, respectively. A list of the delineation of 11q chromosomal regions and possible target genes in the GACs is shown in Table II. Fig. 3A represents the frequency of the copy number changes on chromosome 11, and a weighted frequency (%) diagram with high-level ampli-

fications at the 11q13.3 region from all of the 16 GAC cases is shown in Fig. 3B.

Discussion

Lymph node metastasis (LNM) status is considered as an important prognostic indicator for gastric carcinoma (GC) and research on the molecules causing LNM is a promising venue to improve outcomes (3). In most solid tumors, including GC, the spread of cancer cells through the lymphatics to a regional LN is a frequent event in the initial process of cancer dissemination and is considered as an important prognostic indicator for patient outcome (4-7). Therefore, identification of molecular markers associated with a higher tendency of LNM formation should be useful during the initial tumor staging and

Table II. Chromosomal recurrent minimal regions of genetic alterations on the long arm of chromosome 11 in 16 GACs.

Regions	Gene contained in clones	% gains ^a	% amplifications ^b
11q24.2-q22.1	<i>PKNOX2, FEZ1, SCN3B, CBL, MCAM, RNF26, C1QTNF5, MFRP, USP2, MFRP, USP2, LOC94105, THY1, ATP5L, MGC13053, MLL, FLJ11783, TTC12, ANKK1, DRD2, DIBD1, C11orf1, CRYAB, PPP2R1B, DIBD1, NPAT, ATM, OR2AL1P</i>	37.5 (6/16)	
11q14.3-q13.1	<i>FLJ22104, GARP, LOC390226, C11orf30, UVRAG, POLD3, CHRDL2, KIAA0280, PLEKHB1, CPT1A, CCND1, ORAOV1, PPF1A1, CTTN, SHANK2, PACS1, KLC2, RAB1B, MGC50896, SF1, MAP4K2, MEN1, HSMDPKIN, EHD1, KIAA0404, PPP2R5B, TIGD3, LOC283130, FKSG44, LOC387781, ESRRRA, HSPC152, PRDX5, FLJ37970, RPS6KA4, MGC11102, BANF1, CST6, CATSPER1, GAL3ST3, SF3B2, PACS1, NAALADL1, LOC256676, CDCA5, ZFPL1, LOC399904, C11orf2, TM7SF2, ZNHIT2, FAU, MRPL49, HRD1</i>	62.5 (10/16)	12.5 (2/16)
11q12.3-q12.1	<i>MGC5395, EEF1G, RBM21, FLJ20847, PGA5, HSPA5BP1, SLC15A3, CD6, OR4D6, OR4D10, OR4D11, OR4D9, LOC390200, OSBP</i>	37.5 (6/16)	

^aAlterations were defined by \log_2 ratio thresholds of 0.25 for copy number gain. ^bAlterations were defined by \log_2 ratio thresholds of 1 \log_2 ratio for high-level amplification. Genomic positions were retrieved from the UCSC genome browser web page [<http://genome.cse.ucsc.edu>; Build 36, version March 2006 (hg18)].

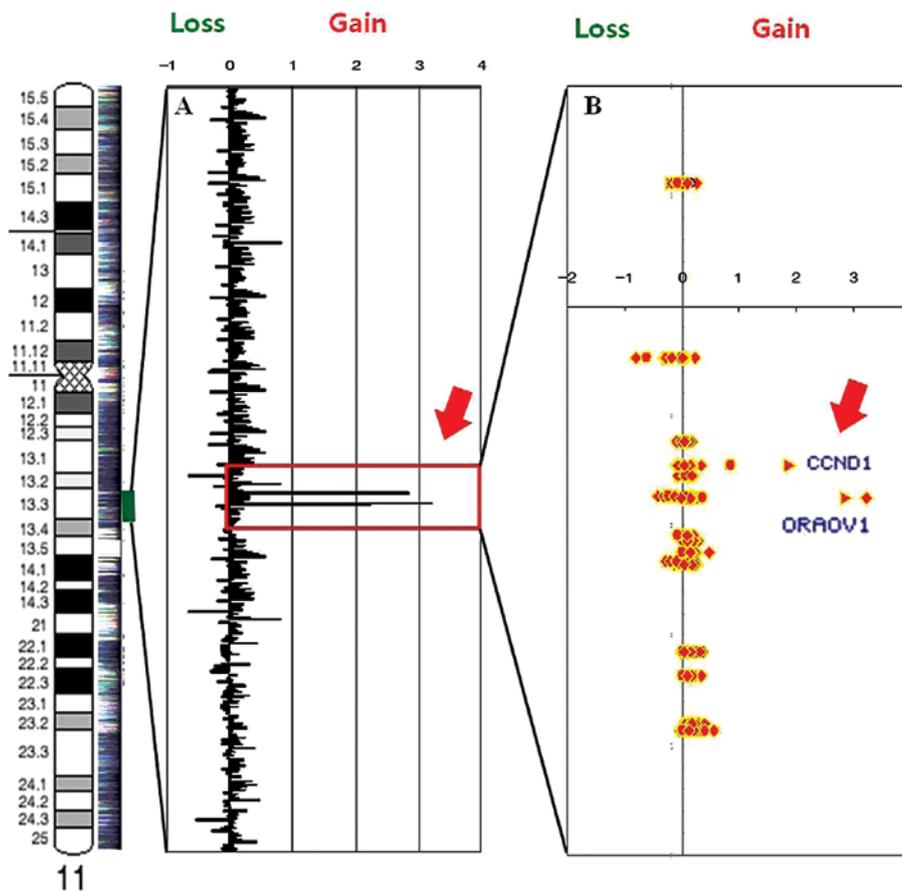


Figure 3. (A) Frequency (%) diagram for chromosome 11 generated from combined scores of 16 GAC cases. The \log_2 ratio was >1 in this BAC clone, suggesting copy number amplifications at the 11q13.3 region, and is highlighted in red. Cytobands in the ideogram are shown at the left. (B) Weighted frequency (%) diagram of the 11q13.3 region from the GAC cases. In the intensity ratio profiles, the Y-axis represents the map position of the corresponding clone, and the intensity ratios are assigned to the X-axis.

may provide additional information concerning the presence of occult metastases of GACs (2).

In the present study, we performed a genome wide array-CGH to assess molecular markers associated with LNM formation in GACs. On array based profiles, copy number changes on chromosomal arms with gains on 1p, 3q, 8q, 9q, 11q, 16p, 19p and 20q (\log_2 ratio >0.25) and losses on 14q and 18q21 (\log_2 ratio <-0.25) ($>40\%$ of patients) were investigated as potential metastatic targets for GACs. These results support the previous findings that GAC has a complex pattern of chromosomal alterations that can be due to general chromosomal instability related to the advanced stages of gastric carcinogenesis (17-19).

In these array profiles, the most notable finding was the high frequency of copy number gains at the long arm of chromosome 11, which occurred in 75.0% (11/16) of the LN involved GACs, and the delineated minimal common region was mapped to 11q24.3-q12.2. Chromosomal band 11q has long been implicated as one of the most frequently amplified regions, and its rearrangements are regarded to be independent prognostic factors for GACs (14-19). According to Zhu *et al* (17) chromosomal gains at 11q is one of the most frequent events in GC cases, and Weiss *et al* (18) also documented the copy number gains of chromosome 11q as a potential target for malignant GAC. These findings implied that activation of oncogenes within this region may play a critical role in the development or progression of GACs.

More strikingly, high-level amplifications at the 11q13.3 region were detected in 12.5% (2/16) of the cases. The corresponding chromosomal region harbors the putative oncogene of cyclin D1 (*CCND1*), which has been shown to contribute to a more aggressive tumor progression of GCs (20,21). Bizari *et al* (20) described the genetic amplification and mRNA expression of the *CCND1* gene in LNM GC cases. Moreover, overexpression of *CCND1* in clear cell GCs has also been documented (21). These results suggest that the *CCND1* gene may play a critical role in the development or progression of GC cases.

Additionally, one potential candidate oncogene of oral cancer overexpressed 1 (*ORAOVI*) from the 11q13.3 region was identified in 12.5% (2/16) of the cases, as a potential metastatic target within the 11q13 amplicon. Although, involvement of the *ORAOVI* gene in the pathogenesis of GAC has not been previously described, it is regarded as a candidate oncogene with a role in the development and progression of various human cancers. Previous studies suggested that the high-level expression of *ORAOVI* is tightly correlated with prognosis-related clinicopathological parameters and clinical grades in different cancer types (22,23). According to Xavier *et al* (22), *ORAOVI* exhibited increased gene expression levels and higher copy numbers in the 11q13 amplified chromosomal region in oral squamous cell carcinoma, and Jiang *et al* (23) also proposed the *ORAOVI* gene as a crucial protooncogene and a novel candidate therapeutic target for cervical cancer. These findings support our hypothesis that *ORAOVI* may have biological impacts on inducing tumor cells to spread from the primary site to local lymph nodes and be a probable target within the 11q13 amplicon. Further investigations are needed to validate and clarify the vital functions of *ORAOVI* as a novel target for GACs in larger studies using multiple samples.

In this study, we extended previous findings showing that the long arm of chromosome 11 harbors protooncogenes that

are associated with lymphatic metastasis formation (14-18). Furthermore, we described here for the first time high-level amplifications of the *ORAOVI* gene as a potential metastatic target for the underlying amplification on 11q13.3 in GACs. This noble *ORAOVI* gene could be a useful target for therapy and may serve as an indicator for occult metastasis status for GACs.

Acknowledgements

This study was financially supported by the research fund of Korea Nazarene University in 2011.

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