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Identification of novel candidate target genes, including *EPHB3*, *MASPI* and *SST* at 3q26.2–q29 in squamous cell carcinoma of the lung

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Abstract

Background: The underlying genetic alterations for squamous cell carcinoma (SCC) and adenocarcinoma (AC) carcinogenesis are largely unknown.

Methods: High-resolution array-CGH was performed to identify the differences in the patterns of genomic imbalances between SCC and AC of non-small cell lung cancer (NSCLC).

Results: On a genome-wide profile, SCCs showed higher frequency of gains than ACs ($p = 0.067$). More specifically, statistically significant differences were observed across the histologic subtypes for gains at 2q14.2, 3q26.2–q29, 12p13.2–p13.33, and 19p13.3, as well as losses at 3p26.2–p26.3, 16p13.11, and 17p11.2 in SCC, and gains at 7q22.1 and losses at 15q22.2–q25.2 occurred in AC ($P < 0.05$). The most striking difference between SCC and AC was gains at the 3q26.2–q29, occurring in 86% (19/22) of SCCs, but in only 21% (3/14) of ACs. Many significant genes at the 3q26.2–q29 regions previously linked to a specific histology, such as *EVII*, *MDS1*, *PIK3CA* and *TP73L*, were observed in SCC ($P < 0.05$). In addition, we identified the following possible target genes (> 30% of patients) at 3q26.2–q29: *LOC389174* (3q26.2), *KCNMB3* (3q26.32), *EPHB3* (3q27.1), *MASPI* and *SST* (3q27.3), *LPP* and *FGF12* (3q28), and *OPA1*, *KIAA022*, *LOC220729*, *LOC440996*, *LOC440997*, and *LOC440998* (3q29), all of which were significantly targeted in SCC ($P < 0.05$). Among these same genes, high-level amplifications were detected for the gene, *EPHB3*, at 3q27.1, and *MASPI* and *SST*, at 3q27.3 (18, 18, and 14%, respectively). Quantitative real time PCR demonstrated array CGH detected potential candidate genes that were over expressed in SCCs.

Conclusion: Using whole-genome array CGH, we have successfully identified significant differences and unique information of chromosomal signatures prevalent between the SCC and AC subtypes of NSCLC. The newly identified candidate target genes may prove to be highly attractive candidate molecular markers for the classification of NSCLC histologic subtypes, and could potentially contribute to the pathogenesis of the squamous cell carcinoma of the lung.

Background

Lung cancer is responsible for the highest cancer-related morbidity and mortality worldwide [1]. Non-small cell lung cancer (NSCLC) comprises approximately 80% of all lung cancers; squamous cell carcinoma (SCC) and adenocarcinoma (AC) are the two most common subtypes of NSCLC [2]. Cumulative information suggests that the SCC and AC subtypes' progress through different carcinogenic pathways [2-4], but the genetic aberrations promoting such differences, especially for the molecular difference between two subtypes, remain unclear.

The most prevalent known chromosomal changes in NSCLC include gains/amplifications at 3q, 5p, 7p, and 8q, and losses at 3p, 8p, 9p, 13q, and 17p [5-7]. Many significant genes that map to these regions had previously been associated with specific histologies [2-5]. Gains of 3q, 7p, 12p, and 20q, as well as losses of 2q, 3p, 16p, and 17p, are more frequently detected in SCC, whereas gains of 1q and 6p as well as losses of 9q and 10p are more prevalent in AC [7-10]. One of the most prevalent and significant differences between SCC and AC, a gain at the chromosome 3q location, has been recognized in several molecular cytogenetic studies [3-5]. Emerging data suggests that regions of amplification of 3q have a profound effect on tumor development and house candidate biomarkers of disease progression, response to therapy, and prognosis of SCC [11]. These findings suggest that genes located at these chromosomal regions progress through differing pathogenic pathways, but the genetic aberrations promoting such differences are largely unknown.

Array CGH has been recognized as a successful and valuable tool for evaluation of the whole genome, as well as significant genetic information at the single gene level, and has enabled us to classify different neoplasm's based on characteristic genetic patterns [12]. It has been used extensively to study various human solid tumors including NSCLC [13-15]. Although, recurrent genetic alterations in NSCLC have been studied extensively, to our knowledge, only a few studies have been performed to date to correlate the molecular difference between histologic subtypes of NSCLC using high-resolution microarray CGH. Therefore, further investigations are needed to gain additional insight into the clinical significance of recurrent chromosomal alterations between the two subtypes of NSCLC.

In this study, therefore, we performed high-resolution array-CGH to compare the different patterns of genetics alterations, and to identify potential candidate genes that may be associated with phenotypic properties that differentiate early stage SCC from AC.

Methods

Tumor Samples and DNA Extraction

Twenty two SCCs and 14 ACs of the lung patients undergoing surgery as a primary treatment, without previous radiation or chemotherapy, were analyzed. This study has been reviewed and approved by the Institutional Review Board of the Chungnam National University Hospital. All cases were reviewed by pathologists to verify the original histopathological diagnosis, depth of tumor, invasion, tumor differentiation and lymph node metastasis. The written informed consent was obtained from each patient according to institutional regulations. The demographic and pathological data, including age, gender and the tumor stage were obtained by a review of the medical records. All of the patients were classified according to the WHO histologic typing of lung carcinomas and the UICC TNM (tumor-node-metastasis) staging system. Some of these samples were previously profiled for copy number variations [15].

Tumor preparations were performed as described previously [7]. DNA isolation was performed following the manufacturer's instructions (Promega, Madison, WI, USA), with some modifications as described before [15,16]: commercial genomic DNA was used as a reference (Human Genomic DNA: Female; Promega Corporation, Madison, WI; Cat. No. G1521).

Contraction of BAC clones mediated array CGH microarray

The characteristics of the MacArray™ Karyo4000 chips (MacroGen, Inc., Seoul) [17-20] were used in this study consist of 4,046 human BACs, which were applied in duplicate and a resolution of 1 Mbp <http://www.macrogen.co.kr>. BAC clones were selected from the proprietary BAC library of MacroGen, Inc. All clones were two-end sequenced using an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, CA), and their sequences were blasted (using BLAST; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and mapped according to their positions, as described in the University of California, Santa Cruz (UCSC) human genome database <http://www.genome.ucsc.edu> (Build 36, Version Mar. 2006 (hg18)). Locus specificities of chosen clones were confirmed by removing multiple loci-binding clones individually under standard fluorescence *in situ* hybridization (FISH) [21]. 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) using a 24-pin format. Each BAC clone was represented on an array as triplicate spots and each array was pre-scanned using a GenePix 4200A scanner (Axon Instruments, Foster City, CA) for proper spot morphology.

Array CGH experiment

Array CGH was performed as described previously [15]. Briefly, arrays were pre-hybridized with salmon sperm DNA to block repetitive sequences in the BACs. 500 ng of normal male DNA (reference) and digested tumor DNA (test) were labeled with Cy5-dCTP and Cy3-dCTP, respectively, by randomly primed labeling (Array CGH Genomic Labeling System; Invitrogen, CA, USA). The labeled probe and human Cot-1 DNA were mixed and dissolved in hybridization solution. Hybridizations were performed in a sealed chamber 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).

Analyzed BAC clones

After scanning, the fluorescent intensities of the red and green channels were saved as two TIFF image files and the background was subtracted from these. \log_2 -transformed fluorescence ratios were calculated from background-subtracted median intensity values, and these ratios were used for normalization using intensity normalization methods. 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 \log_2 transformed. Spot quality criteria were set as foreground to background greater than 3.0 and standard deviation of triplicates less than 0.2. The breakpoint detection and status assignment of genomic regions is performed by the GLAD software was used [22]. The total number of 3,776 BAC clones was analyzed excluding the 31 missing values and sex chromosomes (238) since female tumor DNA was hybridized with male control DNA to serve as an internal control. A low-level copy number gain was defined as a \log_2 ratio > 0.25 and a copy number loss was defined as a \log_2 ratio < -0.25 . High-level amplification of clones was defined when their intensity ratios were higher than 0.8 in \log_2 scale and vice versa for homozygous deletion [23-25]. This threshold value was defined empirically as a value 3-fold that of the standard deviation calculated from 30 normal male to normal females in hybridization experiments. MacroGen's 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.

Statistical analysis for array CGH

For group comparison, the differences in \log_2 ratios, as well as the Fisher exact test were used to determine whether there was any significant gain or loss of genomic content within particular cytobands with cancer type. The Fisher exact test utilized two categories normal and abnormal (loss and gain), with the null hypothesis that the relative proportions of each of the two imbalance categories

would be expected to be the same in the groups. The counts of abnormal versus normal were summarized by subtype of NSCLC (SCC and AC) for each BAC, providing 2×2 tables for analysis. A multiple testing correction (Benjamini-Hochberg false discovery rate (FDR)) 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. MacroGen's 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.

Quantitative Real-time PCR analysis

Real-time quantitative PCR analysis was performed using the ABI PRISM 7900HT Sequence Detection system and TaqMan Gene Expression assays according to the manufacturer's instructions (Applied Biosystems, Foster city, CA). In brief, samples (2.5% of the reverse transcription reaction) were amplified using the Universal Master Mix (Applied Biosystems) and cycling conditions of 15 s 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 assayed in triplicates. To enable detection of possible contaminating genomic DNA, we analyzed non-reversed transcribed total RNA from all tumors in parallel with the cDNAs. Normalized normal human pooled genomic DNAs (Promega, Madison, WI, USA) were used as reference DNAs. All data analysis was used ArrayAssist® (Stratagene, La Jolla, USA) and R (Ver.2.7.2). Correlation between BAC chip and Q RT-PCR data was performed by Pearson correlation analysis ($P < 0.05$).

Results

Array CGH analysis in SCCs and ACs of the lung

One-megabase through put whole genome array-CGH was performed to establish distinct differences in chromosomal copy number changes between the SCC and AC histologic subtypes of NSCLC. Clinicopathological data for the 22 SCCs and 14 ACs patients are summarized in Table 1. All of the NSCLC patients (100%) had copy number aberrations and each patient evidenced numerous copy number changes. On average, 173 clones were gained (range, 14–579), and 136 clones were lost (range, 5–537) per patient. Although the difference in the copy number gain, between the two histologic subtypes was not statistically significant, we found higher frequency of gains in SCC compared to AC (203 vs. 125, respectively, $P = 0.067$). To visualize both common and specific altered chromosomal regions in each subtype of NSCLC, signal intensity ratios for each spotted BAC clones were calculated and displayed as \log_2 plots (Figures 1 and 2). Most of the chromosomes in this profile showed multiple seg-

Table 1: Summary of clinico pathological data of the samples

No	Gender	Age	Histology	TNM classification	
				Lymph node status	Tumor stage
1	M	61	AC	Absent	IB
2	F	50	AC	Present	IIIA
3	F	47	AC	Absent	IB
4	M	66	AC	Absent	IA
5	M	65	AC	Present	IIIA
6	F	61	AC	Absent	IA
7	F	56	AC	Present	IIB
8	M	72	AC	Absent	IA
9	M	61	AC	Present	IIB
10	F	70	AC	Present	IIIA
11	M	60	AC	Present	IIIA
12	M	70	AC	Present	IIB
13	M	75	AC	Present	IIIA
14	F	69	AC	Absent	IA
15	M	67	SCC	Present	IIA
16	M	51	SCC	Present	IIIA
17	M	59	SCC	Absent	IB
18	M	69	SCC	Absent	IB
19	M	62	SCC	Absent	IA
20	F	72	SCC	Absent	IB
21	M	60	SCC	Present	IIIA
22	M	64	SCC	Absent	IB
23	M	65	SCC	Absent	IB
24	M	65	SCC	Present	IIB
25	M	64	SCC	Present	IIB
26	M	65	SCC	Present	IIB
27	M	64	SCC	Absent	IB
28	M	64	SCC	Absent	IB
29	M	66	SCC	Present	IIIA
30	M	74	SCC	Absent	IB
31	F	72	SCC	Absent	IA
32	M	74	SCC	Absent	IB
33	M	69	SCC	Absent	IB
34	M	55	SCC	Present	IIIA
35	M	90	SCC	Present	IIB
36	F	69	SCC	Absent	IA

mental alterations, including single copy as well as high level gains and losses.

Distinct genomic signatures between SCC and AC histologic subtypes

Across the whole genome, we identified specific genomic alterations between the two subtypes. Gains at 2q, 3q, 12p, and 19p, as well as losses at 3p, 4p, 16p, and 17p were found specific to SCC, whereas gains at 6p and 7q and losses at 4q and 15q were more prevalent in AC. A summary of preferentially gained and lost genomic changes for patients with SCC and AC are listed in Table 2 (> 30% of patients). More specifically, statistically significant differences were observed across the histologic subtypes. We identified significant chromosomal regions

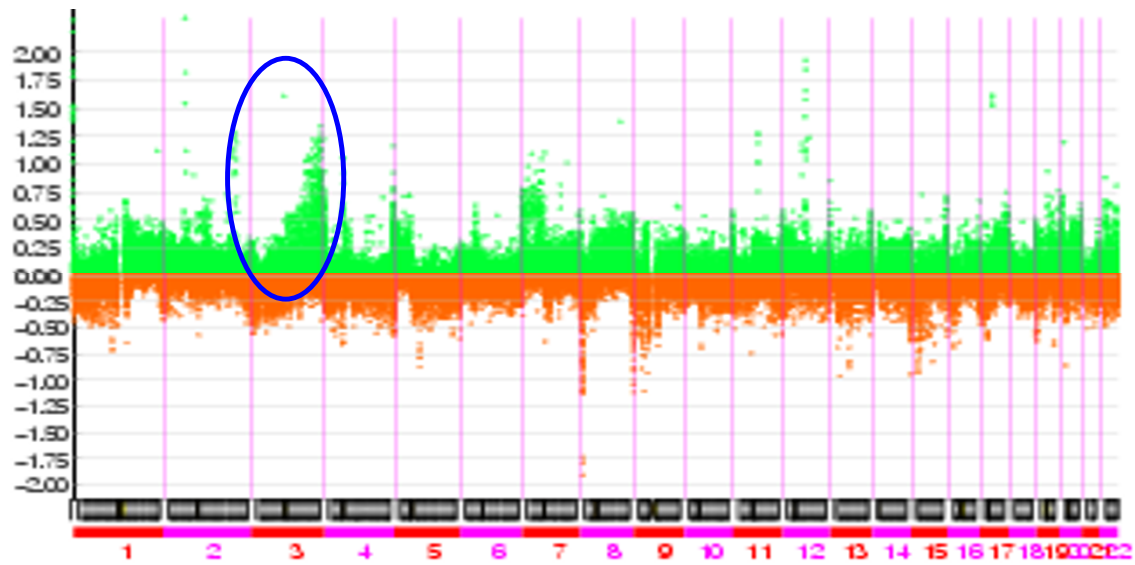
between the two subtypes for gains of 2q14.2, 3q26.2–q29, 12p13.2–p13.33, and 19p13.3, as well as losses of 3p26.2–p26.3, 16p13.11, and 17p11 in SCC ($P < 0.05$), and a gain of 7q22.1 and losses of 15q22.2–q25.2 in AC ($P < 0.05$). The statistically significant genomic regions preferentially gained and lost by histologic subtypes and the potential target genes are summarized in Table 3 and Figure 3 (see Additional file 1).

Significant differences were not reached for gains at 3q29 (*LMLN*, *LOC348840*, *LOC442100*, and *BDH*; $P = 0.085$) or losses at 3p25.1 (*ZFYVE20* and *LOC344875*; $P = 0.084$), 3p26.1 (*GRM7*; $P = 0.084$), 3p26.3 (*CHL1*; $P = 0.084$) and 4p16.1 (*WFS* and *PPP2R2C*; $P = 0.084$) in the SCC series, and gains at 6p25.3 (*LOC44118*; $P = 0.065$), 7q21.11 (*CD36*; $P = 0.069$), 7q32.1 (*SND1*; $P = 0.069$), 7q34 (*EPHB6*, *TRPV6*, *TRPV5*, *C7orf34* and *KEL*; $P = 0.069$) or losses at 4q13.3 (*ADAMTS3*; $P = 0.069$) in the AC group; however, all had borderline significances (data not shown).

Significant copy number differences of target genes at 3q26.2–q29 between SCC and AC

In this array profile, the most striking difference between SCC and AC was gains at 3q26.2–q29, with 19 of 22 patients showing gains in at least part of these chromosomal regions (86%) in SCCs, but only 21% (3/14) were observed in ACs. Several putative cancer-related genes at the 3q26.2–q29 regions were previously linked to specific histologic subtypes; specifically, gains at *EVI1* and *MDS1* (3q26.2), *PIK3CA* (3q26.32), and *TP73L* (3q28) were significantly observed in SCCs ($P < 0.05$). Additionally, we identified possible candidate target genes in these chromosomal regions, that are not yet known for their involvement in the pathogenesis of SCC (> 30% of patients): namely, *LOC389174* (3q26.2), *KCNMB3* (3q26.32), *EPHB3* (3q27.1), *MASP1* and *SST* (3q27.3), *LPP* and *FGF12* (3q28), and *OPA1*, *KIAA022*, *LOC220729*, *LOC440996*, *LOC440997* and *LOC440998* (3q29), all of which were significantly targeted in SCC ($P < 0.05$). A representative high level amplification observed recurrently at 3q27 (*MCF2L2* and *B3GNT5*) in AC (A), and an example of an individual profile of high level amplifications around the 3q26.1–q28 regions in SCC (B) is presented in Figure 4.

Among these significantly associated genes at 3q26–q29 in SCC, high-level amplifications were detected for the genes, *EVI1* and *MDS1* at 3q26.2 (23 and 5%, respectively), and *EPHB3*, *MASP1*, and *SST* at 3q27.1–q27.3 (18, 18, and 9%, respectively). For this analysis, we defined a high level amplification as log2 signal intensity ratio reaching +0.8.



(A) SCC



(B) AC

Figure 1

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 NSCLC are shown. Vertical lines indicate chromosomal boundaries. (A) Frequencies of genes increased in copy number or decreased in copy number in SCC of the lung. (B) Frequency of gene copy number abnormalities in the AC. Gains and losses are shown as green and red color bars, respectively, which indicate the overall chromosomal copy number aberrations detected.



Figure 2
Signal intensity ratios for each spotted BAC clones of SCCs and ACs displayed as log₂ plots. A total of 4,046 BAC clones were ordered (x-axis) according to the map positions and the chromosomal order from 1pter to 22qter.

As a gain of *EVI1* and *MDS1* at 3q26.2 has been described previously in SCC, we sought to determine whether there exist any correlations between *EVI1* or *MDS1* and the newly amplified genes of *EPHB3*, *MASP1*, or *SST*. *Interestingly*, co-amplifications were demonstrated for *EVI1* and *EPHB3* in 18% and for *EVI1* and *SST* in 14%. All of the amplified genes, including significantly associated targets in SCC at the 3q26.2–q29 regions, are summarized in Table 4. Figure 5A shows a weighted frequency (%) diagram for chromosome 3 with high-level amplifications, and Figure 5B represents more detailed profiles at the 3q26.2–q29 regions with the significantly associated genes in SCC. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession

number GSE 16597 <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE16597>.

Real Time quantitative PCR analysis

To validate the consequences of gene amplification by array CGH, we performed subsequently real-time quantitative PCR analyses for three potential oncogenes (*EPHB3*, *MASP1*, or *SST*) at 3q27.1–q27.3. Primers for the three genes (*EPHB3*, *MASP1*, or *SST*) are presented in Table 5.

Although the absolute values of selected genes were different between two analyses, significant correlations were observed between two data sets ($P < 0.05$). The value of array CGH was depicted by linear-ratios and *N*-value was delineated in real time PCR. Correlation coefficients

Table 2: Summary of histology-enriched chromosomal alterations by array CGH analysis

Chromosomal alterations	Histology	
	Squamous cell carcinomas	Adenocarcinomas
Gained regions	1p36.33-1q44 2q14.2 3q13.12-3q29 5p12-5p15.33 7p12.2-p22.3 9q34.3 10q26.3 11p13.3-11q15.5 12p11.1-12q24.33 13q21.1-13q34 15q22.2-q26.3 16p13.3-16q24.3 17q23.2-17q25.3 19p13.3-19q12 20q13.33	1p36.33-1q44 5p13.2-5p15.33 6p25.3 7q22.1-7q36.3 16p13.3 17q21.2-17q25.3 20q13.33
Lost regions	1p21.1-1p36.33 2q37.1 3p12.2-3p26.3 4p12-p16.1 5q11.2-5q35.1 8p21.2-8p23.3 9p12-p13.1 10q11.22-10q26.3 13q12.11-13q14.3 14q32.33 16p12.3-16p13.11 17p11.2-17p13.3 18q12.3-18q23 20p11.23 21q11.2 22q11.1	1q21.1 4q13.3 5q13.2 8p12-8p23.3 13q12.11-13q21.1 14q32.33 15q11.2-15q25.3 18q21.2 19q13.2-19q13.31 20p11.23 21q11.2 22q11.1

between gene expression levels in real time PCR and array CGH analysis for three genes (*EPHB3*, *MASP1*, or *SST*) were 0.694, 0.723 and 0.752, respectively. Figure 6A shows a comparison of mean relative expression levels for *EPHB3*, *MASP1*, or *SST* genes between array CGH and real time PCR results in NSCLCs and three cases with amplification at 3q27.1-q31.3 are shown in Figure 6C. Moreover, real time PCR analysis demonstrated higher expression levels for *EPHB3*, *MASP1*, and *SST* genes in SCCs compare to ACs. This corresponds are shown in Figure 6B.

Discussion

Non-small cell lung cancer is the most frequently occurring type of lung cancer, with SCC and AC being the two main subtypes [12]. Since the most common genetic aberrations in NSCLC had already been identified in our previous study [15], we paid attention to explore candidate genes that may be associated with phenotypic properties

that differentiate early stages SCC from AC. A whole genomic strategy allowed us to define candidate regions that may contain specific cancer-related genes involved in early stages of SCC and AC.

Gains of 2q, 3q, 12p, and 19p, as well as losses of 3p, 4p, 16p, and 17p, were found to be specific to SCC, whereas gains of 6p and 7q and losses of 4q and 15q were more prevalent in AC. More specifically, statistically significant differences were observed between the two subtypes for gains at 2q14.2, 3q26.2-q29, 12p13.2-p13.33, and 19p13.3, as well as losses at 3p26.2-p26.3, 16p13.11 and 17p11.2 in SCCs, whereas gains at 7q22.1 and losses at 15q22.2-q25.2 were observed in ACs ($P < 0.05$). These findings support the notion that the two histologic subtypes progress through different pathogenic pathways and further indicate that SCC and AC can be readily distinguished from each other at the chromosomal level.

Table 3: BAC clones with significant frequency differences between SCC and AC subtypes of NSCLC

BAC clone	Regions	Gene contained in clones	% of gain in SCCs [‡]	% of gain in AC [†]	p-value*
Gains[†]					
BAC13_I04	2q14.2		55	7	0.046
BAC215_M07	3q26.2	<i>EVII</i>	77	29	0.007
BAC28_H10	3q26.2	<i>MDS1</i>	45	7	0.034
BAC127_C12	3q26.32	<i>PIK3CA, KCNMBS</i>	50	14	0.050
BAC78_P09	3q26.2	<i>LOC389174</i>	45	7	0.034
BAC97_L20	3q27.1	<i>EPHB3</i>	32	0	0.039
BAC187_G13	3q27.3	<i>MASPI</i>	45	7	0.034
BAC195_F06	3q27.3	<i>SST</i>	32	0	0.039
BAC62_G09	3q28		64	7	0.002
BAC29_O18	3q28	<i>TP73L</i>	45	7	0.034
BAC74_E06	3q28		50	7	0.015
BAC143_F11	3q28	<i>FGF12</i>	45	7	0.034
BAC144_N20	3q28	<i>LPP</i>	48	0	0.032
BAC95_M19	3q29	<i>OPA1</i>	55	7	0.046
BAC96_C05	3q29	<i>KIAA0226, LOC220729, LOC440996, LOC440997, LOC440998</i>	45	7	0.034
BAC42_D19	7q22.1	<i>TRRAP</i>	0	29	0.023
BAC141_P01	7q22.1	<i>ZKSCAN1, ZNF38, ZNF3</i>	0	29	0.023
BAC118_C23	12p13.2	<i>CDKN1B, DKFZP434F0318</i>	50	0	0.003
BAC26_P09	12p13.32	<i>TSPAN9, LOC399986, LOC441627</i>	59	7	0.001
BAC159_B18	12p13.33	<i>LOC440123, SLC6A12, SLC6A13</i>	45	0	0.002
BAC179_J19	12p13.33	<i>SLC6A12, SLC6A13</i>	32	0	0.003
BAC18_B14	19p13.3	<i>SCHC2, C19orf19, MADCAM1, C19orf20, CDC34, GZMM, BSG</i>	41	0	0.008
Losses[‡]					
BAC83_J18	3p26.2	<i>LOC440943</i>	36	0	0.018
BAC229_D19	3p26.3	<i>CCR2, CCRL2, APOD</i>	45	0	0.005
BAC57_A15	15q22.2	<i>RORA</i>	5	36	0.033
BAC175_C05	15q25.2	<i>DNM1DN11-6, LOC388160, LOC440300, LOC388152, LOC161527, LOC440301, LOC159170</i>	5	36	0.033
BAC169_L10	16p13.11		55	7	0.046
BAC130_G23	17p11.2		64	36	0.039

Abbreviations: †AC, Adenocarcinoma; ‡SCC, Squamous cell carcinoma; * P values from Fisher's exact test on categorized log2 ratio values.

†Alterations were defined by log2 ratio thresholds of 0.25 for copy number gain. Using this threshold, we generated a frequency table. ‡Alterations were defined by log2 ratio thresholds of -0.25 for copy number loss. Using this threshold, we generated a frequency table.

Although, these findings are similar to the findings reported in previous studies [26-29], four chromosomal regions for gains at 2q14.2 or 19p13.3 in SCC, and gains at 7q22.1 or losses at 15q22.2-q25.2 in AC, have not been described as focal regions of lung cancer.

We found 55% of copy number gains at the 2q14 region in SCCs, but only 7% of gains were detected in ACs ($P = 0.046$). Although, little is known about gains at chromosome 2q14, the genomic region containing this region may harbor potential oncogenes involved in the tumorigenesis of SCC. Future studies will be needed to verify the significances of this outcome.

A gain at 19p13.3 was observed in 41% of SCCs, whereas no gains in ACs ($P = 0.008$), which harbored the genes *SHC2, C19orf19, MADCAM1, C19orf20, CDC34, GZMM,* and *BSG*. Emerging data [30] have described that the

C19orf19 gene product is *EGFR*-associated and phosphorylated at 5 tyrosines in response to *EGFR* activation and, therefore, represents a new component of the *EGFR* signaling network. The over expression of the *EGFR* gene in SCC has been well recognized in several molecular cytogenetic studies [8,31,32]. Although no significant correlation was found regarding the association between *EGFR* and *C19orf19* in this study (data not shown), further investigations will enable us to determine the functional associations of these two genes, and whether these genes or additional genes at 19p13.3 contribute to the genome differentiating SCC from AC.

The present study revealed that gain of 7q22.1 is more specific for lung AC than for SCC ($P = 0.023$). This region detected as an 89-kbp gene-specific copy number gain, entered at *TRRAP, ZKSCAN1, ZNF38* and *ZNF3*. Loukopoulos et al. [33] recently showed that the frequent ampli-

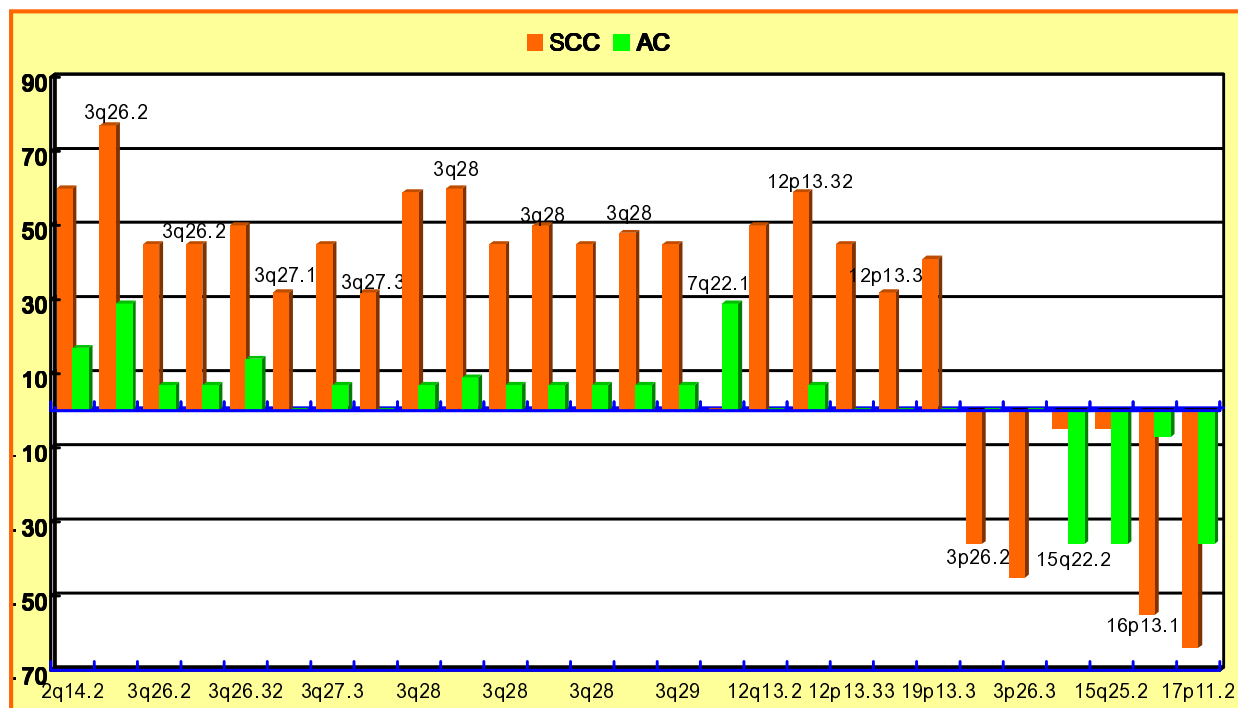


Figure 3
The statistically significant genomic regions preferentially gained and lost between SCC and AC of NSCLC patients. Significant genomic regions between SCC and AC of NSCLC represent in the x-axis and the percentage of gains (upper panel) and losses (lower panel) expressed regions in each chromosomal regions is illustrated to the y-axis (red bars represent the SCC and green bars represent ACs).

fication of the *TRRAP* gene in AC from the pancreas, and another study using array-CGH reported high level amplifications at 7q21–q22 in gastric ACs [34]. Taken together, these results and our own results suggest that this region might be affected in tumorigenesis of AC.

Chromosome 15q was detected in 36% of copy number losses with 15q22.2–q25.2 in the AC group, whereas only 5% of loss were detected in SCC ($P = 0.033$). These regions have not been described as a common change in AC of the lung thus far, but are commonly found in ACs from follicular and clear cell AC. Roque *et al.* [35] demonstrated that 15q loss was significantly associated with follicular adenocarcinomas, and Okada *et al.* [36] reported that the loss of heterozygosity (LOH) at 15q was detected in at least 50% of clear cell ACs, indicating that these candidate regions may contain specific cancer related genes involved in AC. Future work will validate of these findings.

In this array survey, the most salient discriminators between SCC and AC were gains at 3q26.2–q29, occurring in 86% of SCCs, whereas only 21% were observed in ACs. Furthermore, high-level amplifications in these regions were more prevalent in SCCs than ACs (6/22 = 27% vs. 1/14 = 7%, respectively). Our data also pointed out that 4 of 6 (67%) high level amplifications at 3q26.2–q29 regions were detected in stage (I+IIA) SCCs without lymph node metastatic lesions. It is very interesting to note that the high level amplifications at 3q26.2–q29 were more prevalent in stage (I+IIA) SCCs than more advanced stages. Heselmeyer *et al.* [37,38] reported that gain of chromosome 3q could be found in early dysplastic lesions as well as in invasive cervical cancer, but at a reduced frequency in advanced stages of disease. Furthermore, Yen *et al.* [39] described that the high-level amplifications on 3q25.3–qter were all found in stage IB tumors in esophageal squamous cell carcinoma. By combining results of this study with other reports, it is very likely that amplification of genes located on 3q may occur in early stages in the cancer. One possible explanation is that the alterations of

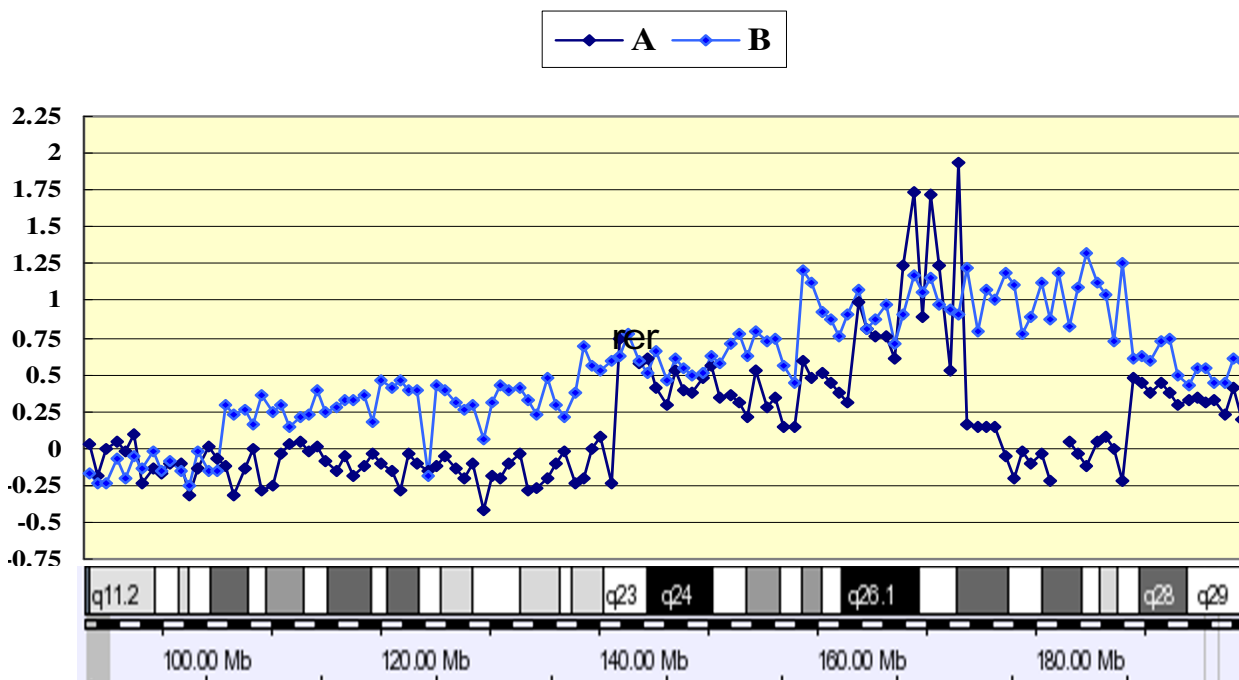
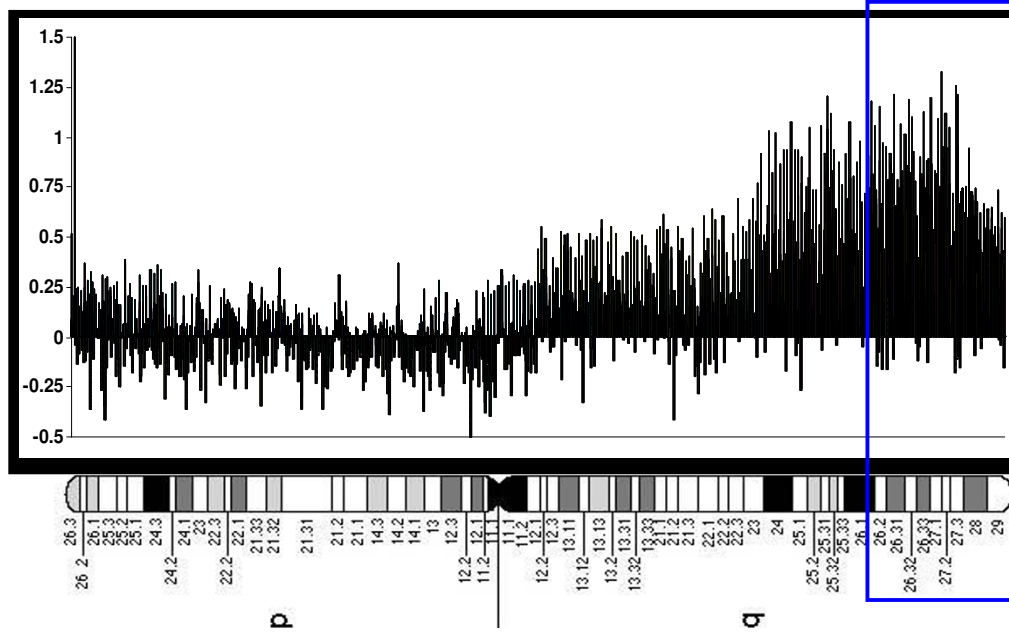


Figure 4
Individual profiles of high copy number changes at 3q. A. High-level amplifications on 3q27 for AC from patient 12, B. 3q26.2–q28 for SCC from patient 27. In the intensity ratio profiles, the x-axis represents the map position of corresponding clone and the intensity ratios were assigned to the y-axis. The schematic presentation of cytogenetic bands as well as a map position is shown below the plot.

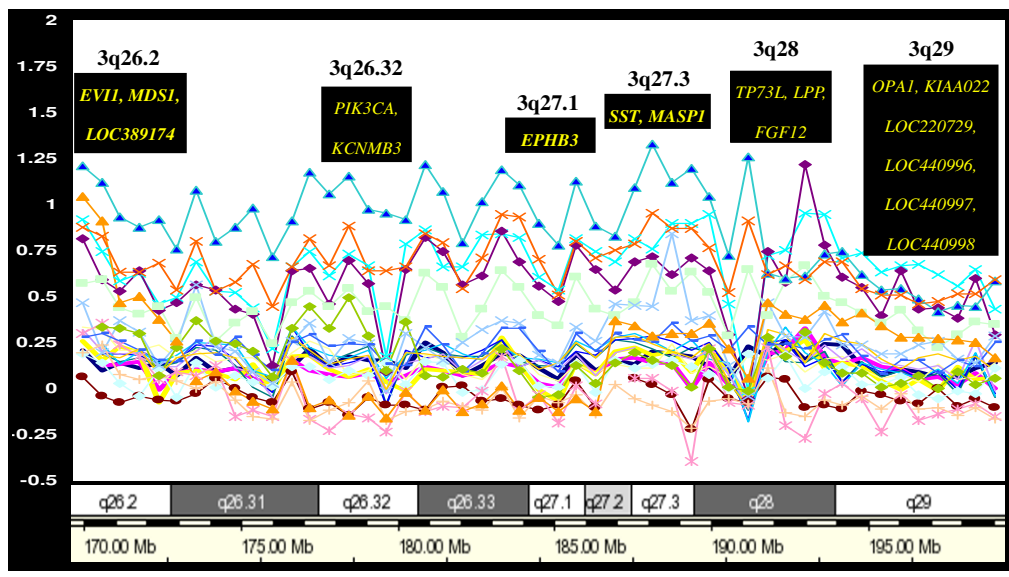
Table 4: High-level amplifications at 3q26.2–q29 by array CGH analysis in NSCLC, together with candidate genes

BAC clone	Chromosome location	Gene contained in clones	% of case of amplifications
SCC[‡]			
BAC215_M07	3q26.2	<i>EVII</i>	23 (5/22)
BAC28_H10	3q26.2	<i>MDSI</i>	5 (1/22)
BAC130_O18	3q26.31	<i>NAALADL2,</i>	9 (2/22)
BAC1_N14	3q26.32		9 (2/22)
BAC231_III	3q26.33	<i>LOC401102,</i>	14 (5/22)
	3q27.1	<i>EPHB3,</i>	18 (4/22)
BAC187_G13	3q27.3	<i>MASPI</i>	18 (4/22)
BAC179_E01	3q27.3	<i>BCL6, LOC389185</i>	14 (3/22)
BAC195_F06	3q27.3	<i>SST</i>	14 (3/22)
BAC74_E06	3q28	<i>CLDN1,</i>	9 (2/22)
BAC144_N20	3q28	<i>LPP</i>	9 (2/22)
AC[†]			
BAC176_G15	3q27.1	<i>MCF2L2, B3GNT5</i>	7 (1/14)

Abbreviations: Abbreviations: [‡]SCC, Squamous cell carcinoma; [†]AC, Adenocarcinoma.



(A)



(B)

Figure 5
(A) Weighted frequency (%) diagram for chromosome 3 with high-level amplifications. 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)** More detailed profile on 3q26.2–q29 with the significantly associated genes in the SCC. The schematic presentation of cytogenetic bands as well as a map position is shown below the plot.

Table 5: Primers used for Real-time quantitative PCR analysis

Gene name	Chromosome location	Primer forward	Primer reverse
EPHB3	3q27.1	GGGTCAGAACCTTCCCTAAAGTG	GTAAAGATACCCCAATAAGTACTGCCT
MASPI	3q27.3	CCTTGCAAGATGAGGGAGTTCAG	GCCCCTATACTCTTCCTTCTATGT
SST	3q27.3	TGACCGACTGCGTTATCATG	TCCCGAAGCTCTTGAGAAAATATC

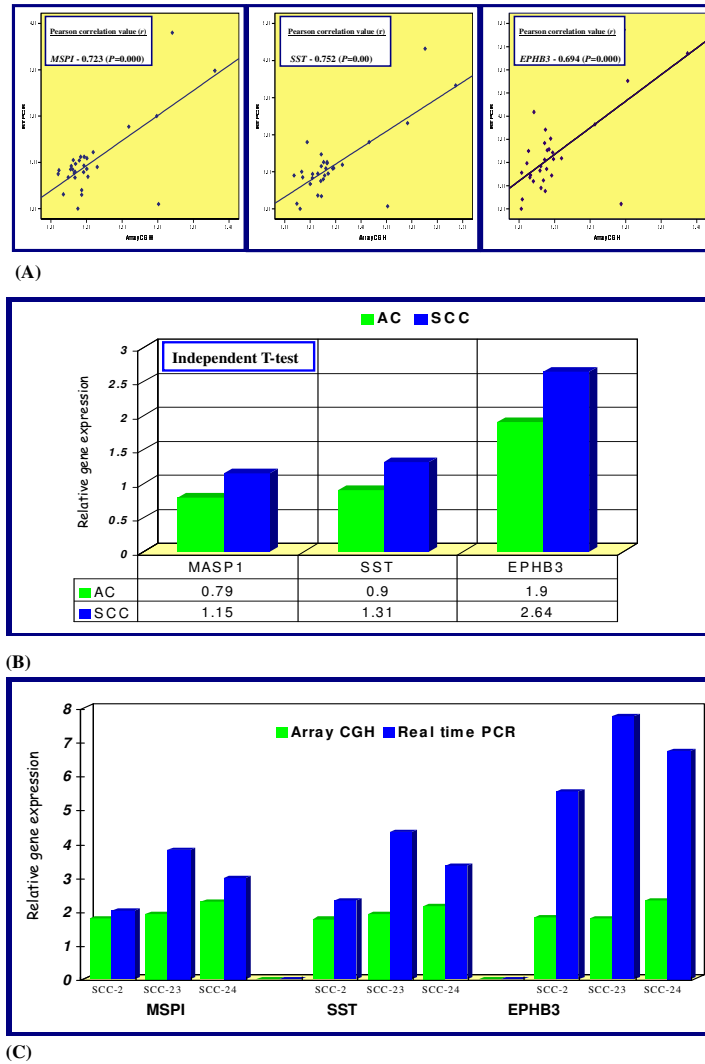


Figure 6

(A) The scatter plot analysis of all data points of three genes (*EPHB3*, *MASPI* and *SST*) by Array CGH (X axis) and Q-RT-PCR (Y axis) analysis in NSCLC patients. Each dark square, the log₂ ratio value of the clone containing one of these genes for one case. The correlation coefficients (r) and P-value are given in the upper left corner of the figure. (B) Comparison of real time PCR analysis of *EPHB3*, *MASPI*, or *SST* between SCCs and ACs. Note that the mean expression levels for selected genes were higher in SCCs compared to ACs. (C) Gene expression levels in Q RT PCR of three genes in three cases seen with amplification at 3q27.1–q27.3 by Array CGH. Each samples are depicted (x-axis) and the fold difference of array CGH was depicted by linear-ratios, and N-value was delineated in real time PCR (y-axis). The heights of the bars represent mean expression level of the indicated genes in each SCC cases.

genes are no longer necessary for maintenance of cancer cells survival [40]. Further studies are needed to confirm this hypothesis.

Previous analyses of the NSCLC genome with low-resolution chromosomal or BAC array-CGH have consistently demonstrated genome differentiation between SCC and AC in the telomeric subregion, 3q26-qter [40-43]. Several interesting cancer-related genes located in these genomic alteration regions have previously been identified with specific histologies, such as *EVI1* (ecotropic viral integration site 1) and *MDS1* (myelodysplastic syndrome 1) at 3q26.2, *PIK3CA* (phosphoinositide-3-kinase, catalytic, alpha polypeptide) at 3q26.32, and *TP73L* (tumor protein p73-like) at 3q28 region [40,41]. In this array survey, we sought to determine whether there exist additional candidate genes at 3q26-qter regions that drive genome differentiation of SCC from AC subtypes of NSCLC, and we were capable of detecting the following possible target genes previously not assumed to play a pathogenic role in SCC; *LOC389174* (3q26.2), *KCNMB3* (3q26.32), *EPHB3* (3q27.1), *MASP1* and *SST* (3q27.3), *LPP* and *FGF12* (3q28), and *OPA1*, *KIAA022*, *LOC220729*, *LOC440996*, *LOC440997*, and *LOC440998* (3q29), all of which were significantly targeted in SCC ($P < 0.05$). These genes have not been described in squamous cell carcinoma of the lung thus far, but are commonly found in other cancers or cancer cell lines [40-44]. Lukashova-v Zangen *et al.* [43] described that the overexpression of *EPHB3* gene in ependymomas with high proliferation indices was associated with a poor outcome, and Kuraya *et al.* [45] demonstrated the high expression of *MASP1* gene in glioma cell line. More strikingly, among these possible target genes within 3q26-q29 regions, *EPHB3* (3q27.1), and *MASP1* and *SST* (3q27.3) showed high-level amplifications, in more than three patients each in SCC, implicating that these genes may be major potential targets for characterization of NSCLC histologic subtypes. Real time PCR analysis demonstrated over expression level for the three genes (*EPHB3*, *MASP1* and *SST*) in SCCs compare to ACs. These results were in agreement with array-CGH results.

Conclusion

The high-resolution analysis allowed us to propose novel candidate target genes that may be associated with phenotypic properties that differentiate early stages of SCC from AC. The newly identified candidate genes could be useful biomarkers for the early detection and characterization of NSCLC histological subtypes as well as novel targets for therapeutic interventions of early stages of squamous cell carcinoma of the lung.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JUK performed the experiments, data analysis, and wrote the manuscript. SHK contributed to the design of the study and critically reviewed the manuscript. KCK was involved in reviewing the manuscript and supervision of the study. JWP participated in data analysis and its interpretation. JMK provided the material obtained from Korea and participated in case selection and pathologic review. All authors have read and approved the manuscript.

Additional material

Additional file 1

The physical position of each BAC. The data provided represent the physical position of each BAC start and stop points.

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