Characterization of amplification patterns and target genes on the short arm of chromosome 7 in early-stage lung adenocarcinoma

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Abstract. Chromosomal alterations are a predominant genomic force contributing to the development of lung adenocarcinoma (ADC). High density genomic arrays were conducted to identify critical genetic landmarks that may be important mediators in the formation or progression of early-stage ADC. In this study, the most noteworthy and consistent observation was a copy number gain on the short arm of chromosome 7, which was detected in 85.7% (12/14) of cases. Notably, three distinct regions of amplification were identified between the 7p22.3 and q11.2 regions in 28.6% (4/14) of cases; at a size of 4.1 Mbp (7p22.3-p21.1), 2.6 Mbp (7p15.2-p14.1) and 1.5 Mbp (7p12.3-p11.2). Variations of the 7p11.2 locus that encodes EGFR are known to be oncogenic. Furthermore, potential target genes were identified that were previously not assumed to be involved in the pathogenesis of ADC, including *CALM1P2* (7p11.2), *HOXA4*, *HOXA5*, *HOXA6*, *HOXA7*, *HOXA9*, *HOXA10*, *HOXA11* and *HOXA13* (7p15.2) and *LOC442586*, *LOC442589*, *LOC442282*, *FAM20C* and *LOC442651* (7p22.3). The present study determined critical regions on the 7p arm of chromosome 7, which were implicated in ADC. The pattern of rearrangements on the 7p arm may be a consequence of the high density of potential targets and the identified genes at the 7p regions may aid in the development of therapeutic targets for ADC.

Introduction

Lung cancer is the most common form of malignancy and a major determinant of the overall cancer-related mortality worldwide (1). Based on the biology, therapy and prognosis, lung cancers are divided into two predominant classes: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLCs consist of three types, adenocarcinoma (ADC), squamous cell carcinoma (SC) and large cell carcinoma (2).

Lung ADC, an epithelial cancer of glandular origin, is the most prevalent of these lung cancer diagnoses. In addition, due to the recent advances in computed tomographic technology, the number of patients diagnosed with small-sized lung ADC as well as stage I lung ADC has increased. To improve the prognosis of ADC patients, the identification of suitable markers is required to select patients with a poor prognosis who may benefit from adjuvant therapy subsequent to surgery (3,4).

Gene amplification is a predominant genomic force contributing to the development of numerous solid tumors, including ADC, and providing an important resource for identifying the location of candidate oncogenes (5). Previous genome-wide analyses for copy number changes in cancer cells have identified various chromosomal loci that are amplified in lung ADCs (5-7). However, as copy number alterations in lung ADC genomes are complex, target genes often remain unclear in amplified chromosomal segments. In addition, the clinical significance of gene amplification in early-stage lung ADC also remains to be elucidated. Thus, in the present study, the copy number changes of 14 early-stage lung ADCs were determined, with the aim of identifying novel high level alterations and candidate genes that may be important in ADC progression.

Materials and methods

Preparation of patient samples. Fourteen lung ADCs were observed from patients undergoing surgery as a primary treatment, without previous radiation or chemotherapy. The original diagnostic material of all ADC patients was reviewed to verify the previous histopathological diagnosis and staging according to the World Health Organization classification system. The stage of disease was based on the tumor-node-metastasis (TNM) classification using the UICC (Union Internationale Contre Le Cancer) staging system. No patients had received pre-operative chemotherapy or radiation. This study was reviewed and approved by the Institutional

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Review Board of the Chungnam National University Hospital (Daejeon, Korea). Written informed consent was obtained from each patient according to the institutional regulations of the Chungnam National University Hospital. The demographic and pathological data, including age, gender and the tumor stage were obtained by a review of the medical records.

Array-comparative genomic hybridization (CGH) analysis. Microarray-CGH was performed on the MacArray™ Karyo 4000 K BAC-chip (Macrogen, Seoul, Korea) (8-11), consisting of 4,046 human bacterial artificial chromosomes (BACs) applied in duplicate at a resolution of 1 Mbp as described in our previous studies (12,13). Briefly, all clones were two-end sequenced using an ABI Prism 3700® DNA analyzer (Applied Biosystems, Foster City, CA, USA) and their sequences were blasted [using basic local alignment search tool (BLAST); http://blast.ncbi.nlm.nih.gov/Blast.cgi]. Mapping of large insert clones was conducted according to the genomic location in the UCSC Genome Bioinformatics database [http://genome.ucsc.edu; Build 36, version Mar. 2006 (hg18)].

Preparation of DNA targets, labeling, hybridization, washing, staining and scanning was conducted according to the manufacturer's instructions (Macrogen, Seoul, Korea) (8-13). Briefly, arrays were pre-hybridized with salmon sperm DNA to block repetitive sequences in the BACs. A total of 500 ng 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, Carlsbad, CA, USA). The labeled probe and human Cot-I DNA (Invitrogen) were mixed and dissolved in hybridization solution.

Statistical analysis. To adjust for effects due to the variation between the red and green dyes, Lowess normalization was applied. The ratio of the red to green channels of each clone was calculated and $\log_2$ transformed. The spot quality criteria were set as foreground to background >3.0 and the standard deviation of triplicates <0.2. Breakpoint detection and status assignment of the genomic regions were performed using GLAD software (14). 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 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 $\log_2$ scale and vice versa for homozygous deletion. The threshold value was determined empirically as a value 3-fold greater than that of the standard deviations calculated from 30 normal males and females in hybridization experiments. The Benjamini-Hochberg false discovery rate (FDR) was applied for multiple testing correction for the high number of false-positive calls.

Results

Whole genome array analysis of ADC cases. To clarify the critical genetic markers associated with ADC pathogenesis, high-resolution array-CGH was conducted on 14 ADC cases. A broad range of aberrations were detected, such as deletions and/or gains of various sizes. All patients (100.0%) in this genomic profile showed multiple segmental alterations, including single copy as well as high level gains and losses. A detailed overview of the clinicopathological data of the 14 ADCs is shown in Table I. Although entire chromosomal arm changes appeared occasionally, the majority of copy number alterations in ADCs were localized regional changes. Notably, large copy number gains involving chromosomes 5p, 7p, 20q, 1p and 16p (>35% of patients) were more prevalent than copy number losses in the cases. The delineation of the most frequently gained chromosomal regions and possible target genes in the ADCs is listed in Table II.

Table I. A detailed overview of clinicopathological data of the 14 early-stage lung adenocarcinomas.

Case no.	Gender	Age (years)	TNM classification	Tumor stage	Smoking status
1	M	61	T2N0M0	1B	Former smoker
2	F	50	T2N2M0	3A	Current smoker
3	F	47	T2N0M0	1B	Former smoker
4	M	66	T1N0M0	1A	Current smoker
5	M	65	T2N2M0	3A	Current smoker
6	F	61	T1N0M0	1A	Never smoked
7	F	56	T2N1M0	2B	Never smoked
8	M	72	T1N0M0	1A	Former smoker
9	M	61	T2N0M0	2A	Current smoker
10	F	70	T2N2M0	3A	Former smoker
11	M	60	T3N1M0	3A	Current smoker
12	M	70	T3N1M0	3A	Current smoker
13	M	75	T3N1M0	3A	Current smoker
14	F	69	T1N0M0	1A	Never smoked

TNM, tumor-node-metastasis.

Copy number alterations on the short arm of chromosome 7 in ADCs. Array-CGH analysis revealed several copy number changes in the ADC cases. Initially, the analysis focused on the short arm of chromosome 7, the most frequently affected regions in the ADC cases (85.7%, 12/14). More specifically, three distinct regions of amplifications were identified in 28.6% (4/14) of the cases. In addition, three minimal overlapping regions were defined on chromosome 7p, (7p22.3-p21.1, 7p15.2-p14.1) and 7p12.3-p11.2. The minimal common region of chromosome 7p was identified, by array-CGH, to be located between BAC41_H11 and BAC178_O13 (position 92.8-123.6 kb).

The first locus of amplification was located distally on 7p22.3-p21.1 regions (82.4-123.6 kb). According to the information archived by human genome database (http://genome.ucsc.edu/), it is flanked by the BAC clones between BAC130_G18 and BAC113_E07 and contains 63 possible target genes (4.1 Mb segment). These terminal gains were often large and were located to the ETS translocation variant 1 (*ETV1*) gene. Notably, a high-frequency of single copy number gains (>0.25 $\log_2$ ratio) and high-level gains (>0.5 $\log_2$ ratio) from the

Table II. Most frequently gained regions of overlap detected by microarray comparative genomic hybridization in early-stage lung adenocarcinomas and the candidate genes.

BAC clone	Chromosome location	Gene contained in clones	BAC size (bp)	Cases with copy number gains[a] (%)
BAC91_J20	5p15.33	*SLC6A19*, *SLC6A18*, *TERT*	115,211	79
BAC151_L22	5p15.33	*Cep72*, *TPPP*	89,632	71
BAC170_A22	7p22.3	*MGC11257*, *LOC393076*, *GPR146*, *GPR30*, *LOC402518*	84,220	62
BAC1_I06	7p11.2	*CALM1P2*	116,026	57
BAC15_B08	7p11.2	*EGFR*	100,083	57
BAC107_K11	20q13.33	*ARFGAP1*, *KIAA1510*, *CHRNA4*, *KCNQ2*	147,123	54
BAC137_F15	7p14.1	*TRGJP1*, *TRGV11*, *TRGVB*, *TRGV10*, *TRGV9*, *TRGVA*, *TRGV8*, *TRGV7*, *TRGV6*, *TRGV5P*, *TRGV5*, *TRGV4*	80,960	50
BAC147_B17	7p22.3	*MAD1L1*, *LOC402663*, *LOC442696*, *LOC442609*	85,873	50
BAC218_N01	7p15.2	*HOXA4*, *HOXA5*, *HOXA6*, *HOXA7*, *HOXA9*, *HOXA10*, *HOXA11*, *HOXA13*	111,540	43
BAC183_C18	7p14.1	*LOC222103*, *TRGJP2*, *TRGC1*, *TRGJ1*, *TRGJP*, *TRGJP1*, *TRGV11*, *TRGVB*, *TRGV10*, *TRGV9*, *TRGVA*, *TRGV8*, *TRGV7*, *TRGV6*, *TRGV5P*, *TRGV5*	98,051	43
BAC38_N15	1p36.33-1p36.32	*SKI*, *FLJ13941*	85,791	43
BAC62_J14	7p21.1	*HDAC9*	78,272	43
BAC97_B23	7p22.3	*OC442586*, *LOC442589*, *LOC442282*, *FAM20C*, *LOC442651*	94,921	43
BAC239_F18	16p13.3	*LOC441443*, *LOC389753*	72,701	43
BAC142_O10	7p22.3	*MAD1L1*, *LOC442699*, *LOC442592*, *LOC442654*, *LOC442593*, *LOC442594*, *LOC442595*, *LOC442700*	144,899	43
BAC122_H21	7p12.2	*GRB10*	85,692	43
BAC139_D05	7p21.2	*ETV1*	87,963	36
BAC161_H20	7p14.3	*PDE1C*	99,714	36
BAC178_O13	7p11.2	*LOC442681*	92,874	36
BAC65_K10	7p21.1	*LOC442511*, *MEOX2*	90,760	36
BAC46_P02	7p13	*CCM2*, *KIAA0363*, *TBRG4*, *RAMP3*	91,839	36

[a]Alterations were defined by $\log_2$ ratio thresholds of 0.25 for copy number gains. Using this threshold, a frequency table was generated. BAC, bacterial artificial chromosome.

7p22.3-p21.1 region were detected in 71.4% (10/14) and 35.7% (5/14) of the cases, respectively. The most frequently gained clone was BAC170_A22 at the 7p22.3 region (57.1%, 8/14), which is located in the *MGC11257*, *LOC393076*, *GPR146*, *GPR30* and *LOC402518* genes.

The second candidate locus spanned 91.8-118.2 kb in the 7p15.2-p14.1 regions, encompassed 15 target clones and was identified as exhibited copy number gains in 7 of 14 cases (50.0%). ADC cases with 7p14.1-p15.2 gains, displayed a varying degree of copy number increases predominantly from 7p14.1 (50%, 7/14), 7p15.2 (42.9%, 6/14) and 7p14.3 (35.7%, 5/14). It was flanked by the BAC clones between BAC137_F15 and BAC218_N01, and encompassed 57 genes (http://genome.ucsc.edu/). Notably, a 111.5 kb high-level amplification of the 7p15.2 region contained *HOXA4*, *HOXA5*, *HOXA6*, *HOXA7*, *HOXA9*, *HOXA10*, *HOXA11* and *HOXA13* genes in one case.

The third region was flanked by BAC157_N08 and BAC178_O13, and mapped at the 7p11.2-p12.3 regions (76.0-92.9 kb). A high frequency of single copy number gains (>0.25 $\log_2$ ratio) from the 7p11.2-p12.3 regions was observed in 57.1% (8/14) of the cases. In addition, two amplified (>1 $\log_2$ ratio) loci on the 7p11.2 region were identified in 14.3% of the cases. One locus contained contiguous amplified clones covering a region of ~100.1 kb and comprised the oncogenic variant of the epidermal growth factor receptor (EGFR) gene in 2 of 14 ADCs (14.3%), with the highest level of amplification in case 13 (Fig. 2).

Furthermore, a candidate target gene for *CALM1P2* was identified in 14.3% (2/14) of cases at the 7p11.2 region. To the

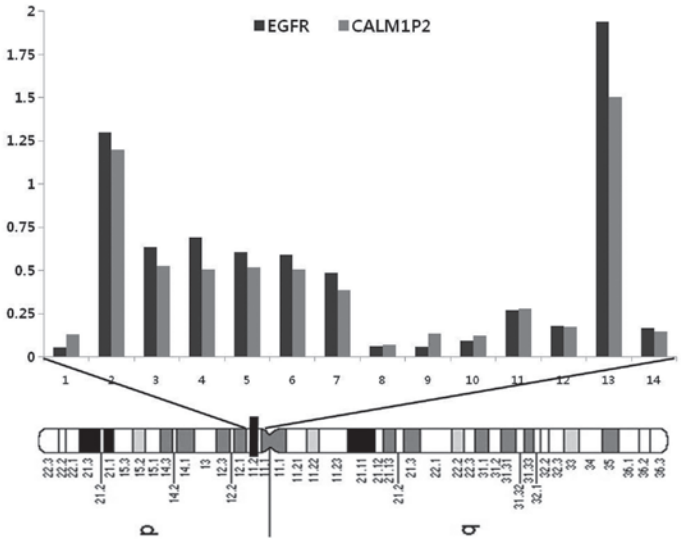

Figure 1. Individual profile at the 7p11.2 region in the 14 ADC cases. High-level amplifications are shown in cases 2 and 13. The schematic presentation of cytogenetic bands, as well as the map position is shown below the plot. ADC, adenocarcinoma.

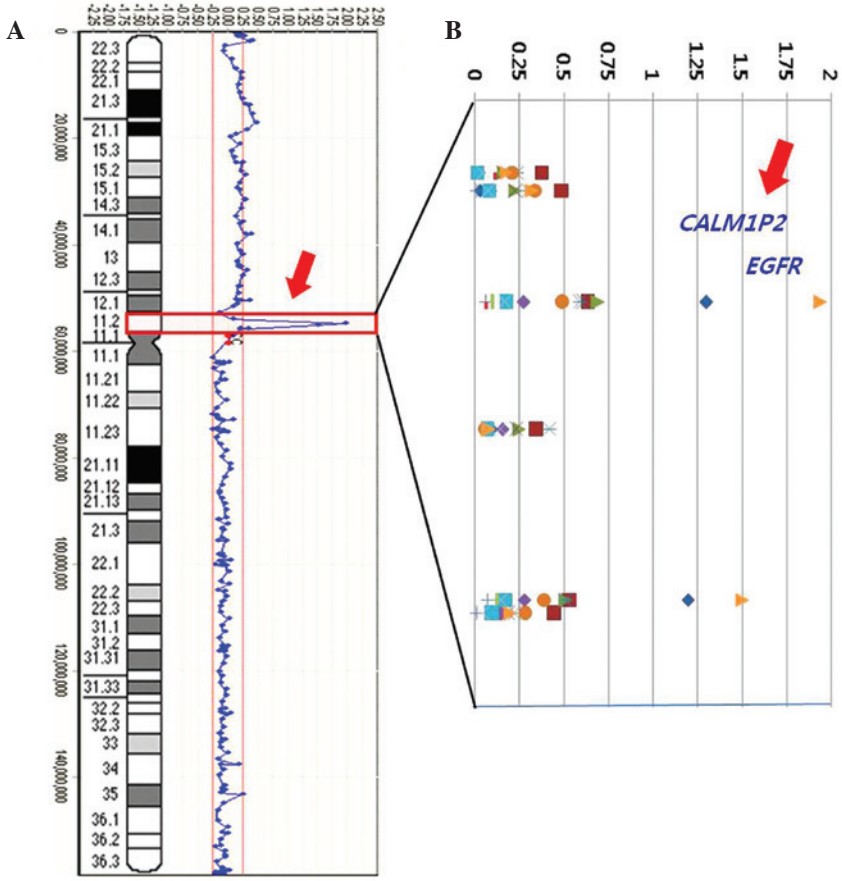

Figure 2. (A) Frequency (%) diagram for chromosome 7 generated from combined scores of the 14 ADC cases. Log_2 ratio was >1 in the BAC clone, suggesting copy number amplifications at the 7p11.2 region (highlighted in red). Cytobands in the ideogram are shown on the left. (B) Weighted frequency (%) diagram of the 7p11.2 region from the ADC 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. ADC, adenocarcinoma; BAC, bacterial artificial chromosome.

best of our knowledge, the pathogenesis of the *CALM1P2* gene has not been previously reported in ADCs. As increased amplification of the *EGFR* gene has been previously observed in ADCs, the present study aimed to determine whether there was a correlation between *EGFR* and the newly identified *CALM1P2* gene. Notably, co-amplifications were demonstrated between *EGFR* and *CALM1P2* genes in 100% (2/2, case 2 and 13; Fig. 1). An example of an individual profile at the 7p11.2

region in the 14 ADC cases is presented in Fig. 1. High-level amplifications are clearly observed in cases 2 and 13. Fig. 2A represents the frequency of the copy number changes on chromosome 7 and a weighted frequency (%) diagram with high-level amplifications at the 7p11.2 region from the 14 ADC cases is shown in Fig. 2B.

Discussion

Array-CGH is a successful and valuable tool for the analysis of chromosome copy-number alterations in human cancer and may be suitable for individualized diagnostic, prognostic and therapeutic decision-making (12). In this study, genome wide array-CGH was conducted to comprehensively characterize genome copy number aberrations associated with early-stage ADC.

The most noteworthy observation in this study was the high frequency of copy number gains at chromosome 7p, in 85.7% (12/14) of the cases. The short arm of chromosome 7 is implicated as being involved in the initiation and/or progression of ADC and has been suggested to include critical cancer related genes in ADC (16-18). Job *et al* (15) demonstrated that the high frequency of copy number gains on chromosome 7p contained *CARD11*, *ETV1* and *IKZF1* genes in 78-92% of the ADCs observed. Single nucleotide polymorphism array analysis determined the high-level amplifications on chromosome 7p (>10%) in small-sized ADCs and lung ADC cell lines (3). Previously, frequent copy number gains on chromosome 7p in ADC cases in non-smokers has also been observed (16). In conclusion, chromosome 7p appears to harbor multiple tumor-related genes that may be implicated in ADC pathogenesis.

Three distinct amplified (>1 $\log_2$ ratio) loci from the 7p22.3-11.2 region were identified in 28.6% of the cases. The first loci located on 7p22.3 contained *FAM20C*, *LOC442586*, *LOC442589*, *LOC442282* and *LOC442651* genes. The *FAM20* family of secreted proteins consists of three members (*FAM20A*, *FAM20B* and *FAM20C*) which have recently been linked to developmental disorders (17).

The second amplification region of 7p15.2 contained *HOXA4*, *HOXA5*, *HOXA6*, *HOXA7*, *HOXA9*, *HOXA10*, *HOXA11* and *HOXA13* genes. Homeobox (HOX) genes encode homeodomain-containing transcription factors critical for development, differentiation and homeostasis. Their dysregulation has been implicated in various types of cancer, including lung adenocarcinoma. Abe *et al* (18) determined that the expression levels of *HOXA5* and *A10* in adenocarcinoma (and *HOXA1*, *A5*, *A10* and *C6* in squamous cell carcinoma of the lung) were significantly higher than those in the non-cancerous tissues. It was suggested that the disordered patterns of *HOX* gene expression were involved in the development of non-small cell lung cancer and in the histological changes (such as adenocarcinoma and squamous cell carcinoma) of the lung. A previous study by Marra *et al* (19) observed the involvement of the *HOX B13* gene in several tumors of the urogenital system. In non-muscle invasive bladder transitional cancer, nuclear *HOX B13* expression showed significant correlation with higher Gleason grade, clinical stage of the tumor and a poor survival outcome, thus determining its potential prognostic value.

HOX genes have also been demonstrated to be a hallmark of numerous hematological malignancies (20). The dysregulation of *HOX* genes is correlated with a number of hematological malignancies, including acute myeloid leukemia (AML) and acute lymphoid leukemia, where they have been shown to support the immortalization of leukemic cells as chimeric partners in fusion genes and when overexpressed in their wild-type form (21). Furthermore, overexpression of individual Hox proteins expanded various bone marrow populations *in vitro*, leading to myeloproliferation and in certain cases inhibition of differentiation and AML *in vivo* (22). A high concentration of *HOXA9* gene product in leukemic blasts has been shown to be an adverse prognostic parameter and *HOXA9* expression was associated with a certain state of myeloid differentiation (23). These results suggest that *HOX* genes represent important prognostic and predictive markers for solid tumors and may be rational targets for therapeutic approaches for the poor prognosis leukemia subset. Additional studies are required to further investigate the mechanism and clinical significance of these results.

The third amplification region was located distally in the 7p22.3 chromosomal region and this locus contained the oncogenic variant of the epidermal growth factor receptor gene (*EGFR*) in 7.1% of the cases. The involvement of the *EGFR* gene as the driver of the 7p11.2 amplicon is well established in ADC cases (24-27).

Liu *et al* (24) determined that *EGFR* gene mutation rates were significantly greater in patients with adenocarcinoma (35.5 versus 9.9% non-adenocarcinoma) and there was a correlation between *EGFR* gene mutation and gene amplification, particularly in early-stage adenocarcinoma. Moreover, Reinmuth *et al* (25) demonstrated that *EGFR* gene mutations are frequently observed in ADC with bronchioloalveolar differentiation and may be linked to chromosomal imbalances. In a study by Sholl *et al* (26), *EGFR* amplification demonstrated a unique association with exon 19 deletion mutations and represented distinct clinicopathological features associated with a significantly worsened prognosis in ADC patients. Furthermore, Yoshizawa *et al* (27) observed that *EGFR* mutations were significantly associated with adenocarcinoma *in situ*, minimally invasive adenocarcinoma and lepidic- and papillary-predominant adenocarcinoma, suggesting that *EGFR* mutations may aid in the prediction of patient prognosis and selection of those who require adjuvant chemotherapy. These results and the results of the present study suggested that the *EGFR* mutation may be an early event in the pathogenesis of lung ADC and may facilitate aggressive behavior of the tumor.

In addition, a potential oncogenic variant of *CALM1P2* was identified in 14.3% (2/14) of cases from the 7p11.2 region. To the best of our knowledge, the involvement of the *CALM1P2* gene in the pathogenesis of ADC has not been previously described; however, genetic mutations of *CALM* genes are observed in other types of cancer (28,29). Toutenhoofd *et al* (28) concluded that the *CALM* gene family is differentially active at the transcriptional level in teratoma cells and that the 5' untranslated regions are required to recover full promoter activation. Furthermore, Stanislaus *et al* (29) suggested that the CALM1 and PLCG2 signaling pathways are the two potential targets for gene knockdown in doxorubicin- and paclitaxel-based

chemotherapy of cervical cancer. As a gain of amplification of the *EGFR* gene has been described previously in ADCs, the present study aimed to determine whether there was a correlation between the *EGFR* gene and the newly identified amplified *CALM1P2* gene. Notably, co-amplification was demonstrated between the *EGFR* and *CALM1P2* genes in 100% (2/2) of cases.

The present study established critical regions on the 7p chromosome implicated in ADC. The present results warrant future studies to identify the putative oncogenes at 7p to gain a better understanding of the molecular pathogenesis of early-stage lung ADC. The genomic analysis allowed the proposition of novel candidate genes that may be associated with the pathogenesis of early-stage lung adenocarcinoma. The newly identified target genes may contribute to ADC pathogenesis as well as provide novel targets for therapeutic intervention in early-stage ADC pending functional validation.

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