ORIGINAL ARTICLE

Chromosomal Gain of 3q and Loss of 11q Often Associated with Nodal Metastasis in Early Stage Cervical Squamous Cell Carcinoma

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Background/Purpose: Cervical cancer remains a health problem among women worldwide. Delineation of genetic changes is critical to understanding the molecular basis of tumor progression, as well as for identifying genetic markers for early identification of patients at high risk for a poor outcome.
Methods: To provide comparative genomic hybridization data for cervical squamous cell carcinoma in Taiwan, and to gain further insight into genetic markers associated with lymph node metastasis of this disease, we performed comparative genomic hybridization analysis of 30 consecutive cases of cervical squamous cell carcinoma (24 stage IB and 6 stage IIB).
Results: The results disclosed that higher staged tumors or those with lymph node metastasis had more chromosomal imbalances. The commonly recurrent chromosomal imbalances were gains of 3q (46.7%), 1q (36.7%) and 8q (20.0%) and losses of 11q (36.7%), 3p (33.3%), 6q (23.3%), and 2q (20.0%). The frequencies of these chromosomal imbalances in stage IB and IIB tumors did not differ significantly. However, when compared with tumors without lymph node metastasis, the loss of 11q14-q22 (5/9 *vs.* 3/21, $p=0.019$) and gains of 3q11-q22 and 3q26-qter (6/9 *vs.* 5/21, $p=0.026$) were significantly more prevalent in tumors with lymph node metastasis.
Conclusion: The results suggest that certain tumor-associated genes residing on 3q and 11q warrant further investigation to elucidate their role in the progression of this disease. [*J Formos Med Assoc* 2007;106(11): 894–902]

Key Words: cervix, chromosomal imbalance, comparative genomic hybridization, squamous cell carcinoma

Cervical cancer remains a key health problem among women worldwide.[1] In recent years, this disease accounted for 6.5% of cancer deaths in Taiwanese women (http://www.doh.gov.tw/statistic/). Though human papilloma viral infection appears to be an important factor in the etiology of cervical cancer, only a small fraction of women harboring oncogenic human papilloma virus in their lower genital tract will have progression to an invasive lesion.[2] It is clear that a human papilloma viral infection alone is insufficient for progression to a malignant phenotype. Mounting evidence indicates that additional genetic aberrations are required for the multistep

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process of tumor initiation and progression to an invasive carcinoma.[3] Delineation of these genetic changes is critical for an understanding of the molecular basis of cervical carcinogenesis, as well as to discover markers that would identify patients at high risk for a poor outcome who would benefit from aggressive adjuvant treatment.

The search for recurrent chromosomal aberrations in cervical cancers has often been hampered by technical difficulties in karyotype analysis.[4] Comparative genomic hybridization (CGH) is a molecular cytogenetic technique that allows comprehensive analysis of chromosomal imbalance in an entire genome with a single test. Notably, it does not require cell culture and metaphase preparation of the test samples.[5] Identification of characteristic chromosomal imbalances in a particular cancer would imply the presence of oncogenes or tumor suppressor genes in these regions of gain or loss, respectively. In previous CGH studies of cervical cancer, many recurrent chromosomal imbalances were reported, including gains of 1q, 3q, 5p, 8q and X, and losses of 2q, 3p, 4p, 6q, 8p, 11q, 13q and 18q.[6–15] However, the prognostic significance of these recurrent chromosomal imbalances[7,9,13] as well as the recurrent loss of heterozygosity identified in different allelotype studies[16,17] often vary greatly and need to be further elucidated.

Chromosomal imbalance pattern of cervical cancer in Taiwanese women was reported in a study of 20 cases of adenocarcinoma.[18] For the more common squamous cell carcinoma (SCC), there has been no publication describing the genome-wide pattern of chromosome imbalance in Taiwan except for two reports of allelotype analysis of the short arms of chromosomes 3 and 5.[19–21] Moreover, due to recent improvements in the Pap smear screening strategy, most Taiwanese women with invasive cervical cancer are now diagnosed at an early stage. Surgery and radiation therapy are relatively effective for controlling cervical cancer at its primary site. However, mortality from this disease is usually caused by its metastasis to lymph nodes and distant organs.[22,23] In an attempt to provide CGH data for cervical SCC in Taiwan, and to gain further insight into the genetic markers that are significantly associated with lymph node metastasis (LNM) of this disease, we performed CGH analysis of 30 consecutive cases of cervical SCC. The commonly recurrent chromosomal imbalances detected in this study were further correlated with the pathologic stage and the LNM status to evaluate their ability to predict which tumors demonstrated high-risk behavior.

Materials and Methods

Tumor specimens

Thirty consecutive cases of cervical SCC treated by radical hysterectomy and lymphadenectomy at Chi Mei Medical Center between January 2003 and September 2005 were collected for CGH analysis. All samples were classified according to the International Federation of Gynecology and Obstetrics (FIGO) criteria. For the histological identification of the pathologic lesions, the criteria set by the World Health Organization and the International Society of Gynecological Pathologists were used. Twenty-four cases were classified as stage IB and six as stage IIB. LNM was present in nine cases (Table).

Under the guide of a hematoxylin-eosin stained section, the paraffinized tissue of a tumor-enriched region containing more than 80% carcinoma cells, about $5 \times 5 \times 2$ mm in volume, was selected. The selected tissue was deparaffinized by treating it twice with xylene at 55°C for 15 minutes each time, followed by washes with absolute ethanol and air-drying. The tissue was then incubated in proteinase K solution (Sigma Co., St Louis, MO, USA; with 0.5 mg/mL in 10 mM Tris, pH 7.8, 5 mM EDTA, and 0.5% SDS) at 55°C overnight or longer, if needed. DNA in suspension was purified using a phenol/chloroform procedure, and resuspended in 1X TE buffer.

CGH

The CGH procedure was modified from that described by Kallioniemi et al[5] and is provided in

Table. Clinicopathologic features and comparative genomic hybridization findings in 30 cases of early stage cervical squamous cell carcinoma

Case	Age	LNM	Gain	Loss
IB tumors				
1	45	ND	20	6p12-p23, 6q11-q26, 7q31-q35
2	54	ND	3q26.1-qter	3p14-p21, 3p23-p25
3	60	ND	Xq13-q21	—
4	70	ND	1q22-q23, 1q31-q43	8p21-p23
5	71	ND	1q22-q24, 1q32, 1q41, 3q (**3q21-q23**, **3q26-qter**), 21q11.2	3p13-p24
6	37	No	—	2q36-q37
7	43	No	1q23, 1q41-q44, 3q23-q24, 3q25, 19q13.3	7p13-p22, Xq25-q25
8	44	No	8q22-q23	—
9	46	No	5p, 13q22-q31	3p14-p24, 4q21-q22, 6p21.1-p22, 6q16-q25
10	46	No	1p13-q22, 3q13.1-q21, 5q32-q33, 6p12, **9p21-pter**	2q36-qter, 3p14-pter, 4p12-p14, 4p16, 4q, 11p12-p15, 11q14-q25
11	51	No	—	2p23-p24, 2p25-pter, 2q37, 6p24, 6q22-q24, 11q23, 16q23
12	56	No	2p16-p21, 5p13-p14, 6p11-p12, 15q11-q13	—
13	56	No	1q12-q23, 1q31-q41, 3q25-qter (**3q26.3-qter**), 11q21-q22, 17q23-q24	10q24-q25, 11q24-q25, 13q12-q13, 20p1.2-p12
14	60	No	1q31	22q12-q13
15	60	No	3q21-q24, 8q12-q21.1, 8q21.3, 8q24.1-q24.3	1p13-p21, 1p31-p32, 3p24, 10q11.2, 10q22, 13q21
16	60	No	3q, 8q (**8q24**), 18q11.2-q12, Xq13-q21	2q36-qter, 6q24-q25, 11p14-pter, 11q21-q24
17	64	No	1q21-q25, 1q31-q32	—
18	67	No	15q23-q24	14q11.2-q13
19	34	Yes	6p11.2-p12	—
20	44	Yes	1p21, 1p22, 1p31, 1q25-q31, 3q21-q26.3, 8q13, 20p11.2, 20q11.2-q13.2, 21q11-q22	7q31-q32, 10q21, 10q24, 11p11.2-p13, 11q22-qter, 12p12, 12q21-q24.1, 13q12, 17p11.2-p13, 17q11.2-q24
21	47	Yes	7p11-p13, Xp22.1-q21	—
22	49	Yes	1q22, 1q24-q31, 2p13-p14, 7q11.2, 8q22-q24.2, 9p22-pter, 13q21-q34	1p34.3, 1p32-p33, 3p21, 6q23-q24, 11p14-pter, 11q14
23	50	Yes	3q13.1-q21, 3q23-q26.1, 9p11-p13	2q37, 7q31, 11q13-qter
24	62	Yes	3q13.1-q27, 5p13-p15.3, Xp11-p21, Xp11-p21	—
IIB tumors				
25	42	No	3q, 6p12, 19q13.1	4p13
26	45	No	6p11.2-p12, 19q13.1	5q23, 17p12-p13
27	63	No	1q11-q41, 2q11.2-q13, 8p11-q24, 20p12-qter	2q37, 6p21.2-p21.3, 6p22, 6q23, 6q24-q25, 9q34, 11q13-q25
28	46	Yes	1p35-p36.1, 3q22-q23, 3q26.1-q29	3p14-p21, 8p12-p21, 10p12-p13, 10q21, 10q22, 10q25-q26, 11q14-q22, 11q21-q22, 15q11.2-q13
29	49	Yes	1q11-q41, 2p12-p23, 3q, 5p13-pter, 21q11.2-q22	3p14-p26, 4p15.1-pter, 9p13-pter, 9q32-q34, 11p13-pter
30	60	Yes	3q13.3-q21, 3q26.1-q26.3	3p14-p21, 6q22-q24, 11q14-q22

*LNM = lymph node metastasis; ND = not done. Chromosomal regions in **bold** exhibited high-level amplification.*

detail elsewhere.[24] Briefly, the metaphase slides of normal females were kept in 95% ethanol at −20°C for at least 48 hours before processing for CGH. DNA from a tumor and genomic DNA from a healthy female donor (reference DNA) were directly labeled with fluorescein-12-dUTP or Texas red-5-dUTP (NEN Life Science, Boston, MA, USA), respectively, using the standard nick-translation procedure. After precipitating the DNA in the presence of Cot1 DNA (Gibco BRL, Gaithersburg, MD, USA), the labeled DNA mixture was hybridized to metaphase spreads on a glass slide for 2–3 days. The slides were washed and then counterstained with 4,6-diamidino-2-phenylindole in an antifading solution.

Image acquisition, processing, and evaluation were performed using a fluorescence microscope (Olympus BX51, Tokyo, Japan) equipped with a Sensys charge-coupled device camera (Kodak KAF 1400 chip; Photometrics, Tucson, AZ, USA), which was controlled using the CytoVision imaging system (Applied Imaging, Santa Clara, CA, USA). Chromosomal imbalances were determined based on the calculation of standard reference intervals using CytoVision High-Resolution CGH software, by which we stringently defined DNA losses or gains as significant whenever the tumor profile and the standard reference interval profile at 99.5% confidence did not overlap.[25] However, short chromosomal segments with a test-to-reference fluorescence ratio >1.5 were construed as showing high-level amplification.

In each CGH experiment, a negative and positive control with a known chromosomal gain or loss was also included. The negative control DNA was isolated from an individual with a normal karyotype. The positive control DNAs were prepared from EBV-transformed lymphoblastoid cell lines with either trisomy 21 (with a size of approximately 50 Mb) or an interstitial deletion of 2q23 (with a size of about 15 Mb).

Statistical analysis

For the analysis of the differences between two comparison groups, we used Fisher's exact two-tailed test, the χ^2 test, or the Mann–Whitney *U* test. Values of $p<0.05$ were considered statistically significant.

Results

In each experiment, the green-to-red fluorescence ratios of the negative control were all within the standard reference interval of each chromosome. The known chromosomal imbalances in the positive controls could also be unequivocally detected. All tumor samples analyzed in this study revealed chromosomal imbalance(s), ranging from 1 to 16 involved chromosome arms, with an average number of 5.1 arms per tumor. A higher average number of chromosomal imbalances were found in stage IIB tumors (6.8, $n=6$) than in stage IB tumors (4.7, $n=24$). Similarly, the average was also higher in tumors with LNM (6.8, $n=9$) than in those without LNM (4.2, $n=21$). However, with respect to gain and loss, the frequencies were very similar in stage IB (2.4 *vs.* 2.3) and stage IIB (3.2 *vs.* 3.6) tumors, as well as in tumors with (3.4 *vs.* 3.4) and without (2.1 *vs.* 2.1) LNM.

All chromosomal imbalance(s) observed in this study are described in detail in the Table, and also summarized in Figure 1. The commonly recurrent chromosomal imbalances were gains of 3q ($n=14$, 46.7%), 1q ($n=11$, 36.7%), and 8q ($n=6$, 20.0%) and losses of 11q ($n=10$, 33.3%), 3p ($n=9$, 30.0%), 6q ($n=7$, 23.3%), and 2q ($n=6$, 20.0%). Comparison of these common chromosomal imbalances between stage IB and IIB tumors, as well as tumors with and without LNM, is depicted in Figure 2. There was no apparent difference in the frequencies in the shortest overlapping region of these chromosomal imbalances between stage IB and IIB tumors. However, compared with tumors without LNM, those with LNM were significantly more prevalent in the loss of 11q14-q22 (5/9 *vs.* 3/21, $p=0.019$) and in gains of 3q11-q22 and 3q26-qter (6/9 *vs.* 5/21, $p=0.026$), as highlighted in the lower panel of Figure 2. High-level amplifications were detected at four chromosomal sites. However, the only recurrent amplification, located on 3q26-qter, was

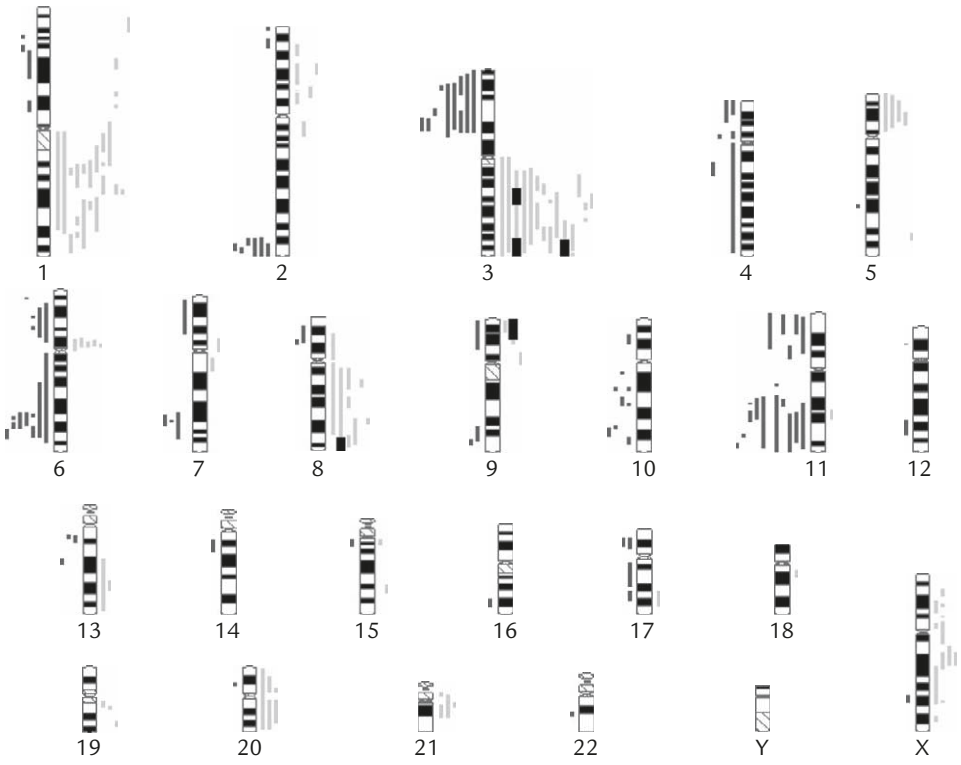

Figure 1. Summary of all chromosomal imbalances identified in 30 cases of early stage cervical squamous cell carcinoma. The bars on the right side and left sides of each chromosome ideogram indicate gains and losses, respectively.

present in two stage IB tumors without LNM. These regions are indicated by the dark boxes in Figures 1 and 2.

Discussion

All samples examined in this study, as well as in most other CGH studies of cervical cancer,[6,8–10,13,14] exhibited chromosomal imbalance(s). Some studies revealed that 10–20% of the stage IB cervical SCC have no detectable chromosomal imbalances.[7,11,12] The trend of the chromosomal imbalance number increasing along with the tumor stage and LNM status are consistently observed in this and other CGH studies of cervical cancer,[6,7] confirming that genetic aberrations often accumulate gradually during tumor progression. The common chromosomal imbalances observed in this study were gains of 3q, 1q and 8q, and losses of 11q, 3p, 6q and 2q, a result consistent with other similar studies.[6,8–14] Notably, as depicted in Figure 2, the results of the present study indicate that the loss of 11q14-q22 and gains of 3q11-q22 and 3q26-qter were significantly more prevalent in tumors with LNM than in those without LNM.

Loss of 11q has been repeatedly identified as a common chromosomal imbalance in cervical SCC in other CGH studies[9,10,12] and in most allelotype screening analyses.[16,17] In other allelotype studies focused primarily on chromosome 11, the frequencies of loss of heterozygosity on bands p12-p15, q12-13, q14-q22, and q23-q25 were estimated to be in the range of 28–33%, 34–40%, 43–62%, and 61–62%, respectively.[26,27] Notably, the results of this study reveal that losses at bands 11q14-q22, but not at bands 11q23-qter, are significantly associated with LNM at an early stage of cervical SCC. A similar finding was also reported in another allelotype study,[16] albeit the only two microsatellite markers selected for screening of 11q all mapped to 11q23.3. Further studies are needed to more precisely define the lost region on 11q, where tumor suppressor genes important for controlling LNM in cervical cancer would most likely be harbored, as may also be true in many other human cancers.

Different study groups have recently focused on 11q13 and 11q23 in the search for the potential

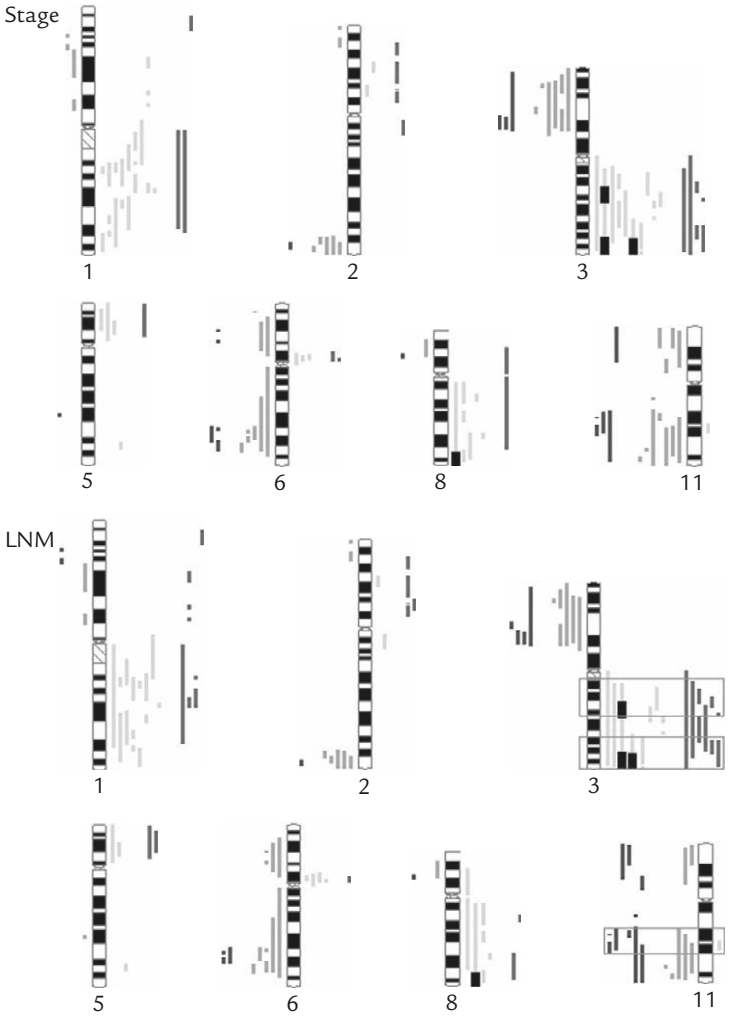

Figure 2. Comparison of the common chromosomal imbalances detected between pathological stage IB ($n = 24$) and IIB ($n = 6$) cervical squamous cell carcinomas (Stage), and between tumors without lymph node metastasis ($n = 21$) and with lymph node metastasis ($n = 9$) (LNM). The bars on the right side and left side of each chromosome ideogram indicate gains and losses, respectively. The light gray bars represent the chromosomal imbalances detected in stage IB tumors and those without LNM. The dark gray bars depict chromosomal imbalances detected in stage IIB tumors and those with LNM. The shortest overlapping regions in 3q11-q22, 3q26-qter and 11q14-q22 (rimmed by boxes) were significantly more prevalent in tumors with LNM than in those without LNM.

tumor suppressor genes. Among the known genes mapping to 11q13, Zainabadi et al excluded *SF3B2*, *BRMS1*, *RIN1* and *RAB1B* as tumor suppressor genes in cervical carcinogenesis, but suggested that *PACS1* may have such potential.[28] Many genes mapped to 11q23, including *PPP2R1B*, U90916 mRNA of unknown function, *ZNF202*, *LOH11CR2A*, *HSC71*, and neurogranin, have also been excluded from consideration as tumor suppressor genes due to lack of evidence supporting their tumorigenic potential.[26] However, a recent study found that the *TSLC1* gene mapping to 11q23 often exhibits promoter hypermethylation in lesions of high-grade cervical intraepithelial neoplasms (CIN; 35%, 7/20) and SCC (58%, 30/52), but not in lesions of low-grade CIN (0/9), supporting its potential role as a tumor suppressor gene.[29] For the known genes mapping to 11q14-q22, no study elucidating their carcinogenic potential has been conducted.

Gain of 3q was the most frequent aberration (46.7%, 14/30) found in this study, comparable to the overall detection rate of 47.8% (141/295) found in many other CGH studies of cervical SCC.[6–14] Furthermore, our data also pointed out that gains of 3q at bands 11-22 and 26-ter were significantly more prevalent in tumors with LNM than in those without LNM ($p = 0.026$). One other study has described a similar finding.[9] Ironically, as depicted in Figure 2, we noticed that three high-level amplifications on 3q were all found in stage IB tumors without LNM. Moreover, it has also been repeatedly demonstrated by others that gain of 3q could be found occasionally in CIN lesions, but were more prevalent in high-grade CIN lesions and invasive SCC.[8,12,30,31] When taken

together, these observations clearly show that gain of 3q is an important early genetic event in cervical carcinogenesis, but it is not a sensitive enough marker to predict which patients are at high risk for a poor outcome.

Gain of 3q is also a common genetic aberration in many other human cancers. Over 100 candidate genes are present in the shortest region of overlap of 3q amplification defined by CGH. Despite functional assessments of some candidate genes, including *RBP1-RBP2* (on 3q21-q22), *CCNL1* (on 3q25.3), *hTERC*, *eIF-5A2*, *SNO* and *EVI1* (on 3q26.2), *PIK3CA* and *SCCRO* (on 3q26.3), and *p63* and *LAMP3* (on 3q27), the precise target(s) of 3q remains ill-defined. Interestingly, a recent study found that high *LAMP3* expression was significantly correlated with the overall survival of patients with stage I/II cervical cancers,[32] implying that amplification of this gene may likely be associated with an enhanced metastatic potential. Once the amplified targeted gene(s) has been identified, the recent advances in triplex-forming oligonucleotides, which are used to site-specifically direct DNA damage in oncogenes, could increase the range of effectiveness of antitumor nucleosides in cancer treatment.[33] A similar concept could also be applied to other common gains, including 1q, 5p and 8q detected in this and other studies of cervical SCC.

Besides loss of 11q and gain of 3q, other common losses detected in this study also merit additional attention. Compared with the common chromosomal imbalances of gain, losses were more often suggested to be associated with disease progression or a poor outcome in other studies of cervical cancer.[7,9] In the present study, other than 11q, the shortest regions of overlap in the common losses were 2q36-qter, 3p14-pter, 6p, 6q23-q25 and 11p. However, there was no apparent difference in their prevalence in stage IB and IIB tumors, or in tumors with and without LNM. Notably, these aberrations were found in some preinvasive lesions in other CGH[8,12] or allelotype[34,35] studies, suggesting that they are unlikely to be rate-limiting in the progression of cervical cancer.

A number of genes mapping to 2q35-q36, including *CFLAR*, *CASP10* and *PPP1R7*, were found to be downregulated in some cervical cancer cell lines. However, this downregulation could be reactivated upon exposure to demethylating agents, suggesting that both genetic and epigenetic changes play a role in 2q alterations.[36] Among the cancer-related genes on 3p, *β-catenin* (mapped to 3p21.1) has been excluded from consideration as a tumor suppressor gene in cervical cancer.[37] Notably, *FHIT* (mapped to 3p14.2) spans the fragile site FRA3B, which has been suggested as an integration hot spot for the human papilloma virus.[38] The most important genes on 6p21 could be the HLA molecules, which are required for the immunologic response against human papilloma viral infection. The loss of HLA expression could allow tumors to evade the immune defense system.[39] Loss of 6q23-q25 was present in seven tumors (23%) in this study, a result comparable with the 23–31% detected by other allelotype analyses.[15,16] However, genes residing in this region have rarely been studied for their potential as tumor suppressor genes. Loss of 11p was significantly associated with a poor prognosis in stage IB cervical SCC without LNM in a CGH study,[7] but this was not supported by another allelotype analysis.[26] A number of known tumor suppressor genes were mapped to 11p, including *WT1*, *WT2*, *CDKN1C* and *TSG 101*. The role of these genes in cervical carcinogenesis is not yet known.

In conclusion, the present study revealed that the common chromosomal imbalances in early stage cervical SCC were gains of 1q, 3q and 8q, and losses of 2q36-qter, 3p, 6q23-q25 and 11q. This chromosomal imbalance pattern is largely consistent with the results of other CGH studies reported for other populations. However, compared to tumors without LNM, those with LNM had significantly more prevalent loss of 11q14-q22 and gains of 3q11-q22 and 3q26-qter. The results suggest that certain tumor-associated genes residing on 3q and 11q warrant further investigation to elucidate their role in the progression of this disease.

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