Original Articles

Genetic imbalance and human papillomavirus states in vulvar squamous cell carcinomas

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Summary

Purpose of investigation: Vulvar squamous cell carcinoma (VSCC) is a disease of significant clinical importance, which arises in the presence or absence of human papillomavirus (HPV). *Methods*: We used comparative genomic hybridization (CGH) to document non-random chromosomal gains and losses with HPV positive and negative VSCCs. *Results*: Gains of 3q and 12q were significantly more common in HPV-positive cancers compared to HPV-negative cancers where chromosome 8q was more commonly gained in HPV-negative compared to HPV-positive cancer chromosomes and, 4p and 3p were lost in both categories of VSCCs. *Conclusions*: The data indicate that one or more oncogenes important in the development and progression of HPV-induced carcinomas are located on 3q and 12q .

Key words: Vulvar squamous cell carcinomas (VSCC); Comparative genomic hybridization (CGH); Human papillomavirus (HPV).

Introduction

Vulvar squamous cell carcinoma (VSCC) accounts for around 80-90% of female vulvar malignant tumors [1]. However the etiology has remained elusive up to now. As demonstrated by laboratory and clinical epidemiological studies, HPV infection correlates closely with the occurrence and development of VSCC [2]. However, the reality of a high infection rate of HPV and a low incidence of VSCC suggests that the occurrence and development of VSCC is a multi-factor and multi-step process with the participation of multiple genes [3]. Therefore employing advanced molecular genetic technology to search for the abnormal chromosome alterations of VSCC, further discovering the relevant oncogenes and tumor suppressor genes, and studying the alterations of these genes during the occurrence and development of VSCC will be important.

Comparative genomic hybridization (CGH) was employed in the present study to detect the gain and loss of genomic DNA copy numbers, alteration characteristics and patterns in both HPV-positive and HPV-negative VSCC, to search for and localize the genes related to vulvar carcinogenesis, and to deepen our mechanistic understanding of vulvar carcinogenesis.

Materials and Methods

Materials

The 21 patients with VSCC used in this study were treated surgically between November 1994 and October 2004 at Shengjing Hospital of China Medical University and Liaoning Provincial Tumor Hospital. Pathologic review was performed and the histologic type confirmed as VSCC. All the samples were frozen with liquid nitrogen and stored at -70°C.

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PCR

The primers were synthesized by TaKaRa Biotechnology (Dalian) Co., Ltd. A pair of HPV consensus primers (forward, 5'–CGT CCM ARR GGA WAC TAG TC-3'; backward, 5'–GCM CAG GGW CAT AAY AAT GG-3'. (M= A+C,R= A+G, W=A+T,Y=C+T). HPV16 primers (forward, 5'–ACT ATC CAG CGA CCA AGA–3'; backward 5'– CCA ATG CCA TGT AGA CGA–3'). HPV18 (forward 5'–TGT TCG TGA CCG TCG TGG-3'; backward, 5'–ATG GAG GAG TTC ATG GAG TGG A-3'). Cycle conditions: 94°C primary denaturation 3 min → 94°C denaturation 45 sec → 56°C annealing 1 min→ 72°C extension 1 min, 35 cycles and finally 72°C extension for 7 min. Controls with known HPV carrying samples or without the DNA template were included in each amplification to avoid false-positive and false-negative results. DNA quality and adequacy was evaluated by amplification of the ß-globin gene.

CGH

CGH was carried out according to the methods described previously [6], with a slight modification. Briefly, tumor and reference DNA were labeled with SpectrumGreen-dUTP and SpectrumRed-dUTP, respectively, by nick translation using a commercial kit (Vysis, Downers Grove, IL). The sizes of the probes were optimized to a range of 600-2000 base pairs. The labeled tumor and normal probes, 300 ng each, were mixed with 10 μg human Cot–I DNA (Vysis, Downers Grove, IL) ethanol precipitated twice, and 2 μlTE (pH 8.0) was added. After virtual dissolution, 8 μl hybridization solution was then added. The mixture was denatured in a 75°C water bath for 5 min and prehybridized at 37°C for 30 min. The slides of metaphase spreads prepared from karyotypical normal blood cells were heated at 60°C for 2 h, incubated in 0.1 μg/μl RNAase inhibitor solution at 37°C for 1 h, shaker-washed with 2×SSC, dehydrated with serial ethanol (70%, 85% and 100%) 1 min each and let air-dry. After reacting in the denaturing buffer solution at 73°C for 5 min, dehydrating with serial ethanol (70%, 85% and 100%) and air-drying, the hybridization mixture solution was added to the regions of normal chromosome on a carrier slide. After placing well the cover slide, the sample was incubated in a dark moist box at 37°C for 48 h. After hybridization, the chromosome sam-

ple was rinsed for 2 min in a 74°C wash solution 1 (0.4× SSC/0.3% detergent nonidet P-40, NP-40), rinsed for 30 sec in a room temperature wash solution 2 (2× SSC/0.1%NP-40) and air-dried in a dark place. After restaining with 6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI), the film was blocked with 50% glycerin containing antifade agents. The fluorescent chromosome images of every case of vulvar squamous cell carcinoma were taken by a fluorescent microscope and their analytic graphs obtained by the analysis software QCGH (German Laica Inc.). The green/red fluorescence ratio for each chromosome was calculated. For analysis of each sample, there were at least five high-quality genotypes. The results were summarized to obtain the full profile of either gain or loss in copy numbers of the whole chromosome set for this sample. Green/red ratio ≤ 0.8 was loss while ≥ 1.2 gain [6].

CGH controls

DNA from an immortalized breast cancer cell line MPE600 with previously known aberrations (losses in 1 pter, 9p, 11q14-q25,16q, and gain at 1q) was used as a positive control. For negative controls, DNA samples from the same normal individual or from two different normal individuals were hybridized against each other. A CGH profile from positive control experiments was noted with gains or losses as published, and no gain or loss was detected in the negative controls.

Statistical analysis

Fisher's exact test was used to compare proportions from two independent samples, while the two-sample independent-groups *t*-test was used to compare the difference between means. All tests were two-sided and results with a *p*-value of less than 0.05 were considered statistically significant.

Results

HPV analysis showed that there were 11 HPV-positive tumors and ten HPV-negative tumors. All HPV-positive tumors contained subtype 16. Table 1 represents the overall gains and losses in the 21 VSCCs, mapped to specific chromosome locations. The mean number of overall changes seen in the 21 VSCCs was 4.9 per case (range 1-7). This comprised a mean of 2.6 (range 1-4) losses per case and a mean of 2.3 (range 1-4) gains per case. There were more CGH changes in the HPV-positive group compared to the HPV-negative group (mean 5.2 *vs* 4.5, respectively) but there was no statistic significance ($p > 0.05$).

Table 2 shows the five most common CGH changes in the VSCCs and Figure 1 shows a representative CGH profile on an individual case. Chromosomal location 3q (9 out of 21, 43%), 8q (8 out of 21, 38%) and 12q (7 out of 21, 33%) were most frequently affected by genetic gain, while 4p (11 out of 21, 52%) and 3p (9 out of 21, 43%) were the most frequently lost. There was a statistically significant difference at the p = 0.05 level, in 3q and 12q gain when analyzed by HPV states, with 3q and 12q gain being more common in HPV-positive VSCCs (8 out of 11 *vs* 1 out of 10; 7 out of 11 *vs* 0 out of 10, $p < 0.01$, respectively), and 8q gain more common in HPV-negative VSCCs (7 out of 10 *vs* 1 out of 11, $p < 0.01$). There was no statistically significant difference by HPV status for 4p and 3p loss.

Discussion

As found by the present study, the gain at 3q and 12q is notely higher in the HPV-positive group than in the HPV-negative group. Up to the present, there are few studies of VSCCs employing the technology of CGH. Some scholars have reported that there was an aberration at chromosome 3q of VSCCs. All these aberrations belong to the high-frequency gain [4-8]. Allen *et al.* [5]

Table 1. — *Aberration status of genomic DNA in tissues of vulvar squamous cell carcinomas.*

Case No.	HPV	Amplification locus	Loss locus
1	positive	1p13-24, 3q24-27, 12q12-15	5p, 4p13-ter, 8p22-23, 18q21-22
2	negative	2p16-24, 3q24-27, xq	3p22-24, 9p21-22, 12q13-21
3	positive	3, 8q13-23, 12q13-15	2p14-22, 5q31-35, 13q
4	positive	1p21-24, 3q13, 26	4p13-ter, 6q24-31, 11q22-25
5	positive	3q24-27, 14q21-24, 9p21-22	3p24, 15q14-24
6	negative	5p, 8q21-23, 17q11-22	4p13-ter, 11q14-25, 20q
7	positive	3q24-27, 12q13-15	3p24 13q14-22
8	positive	3q24-27, 12q12-21, xq13-24	7q33-36, 10q26-ter
9	positive	5p14-ter, 12q13-15	2p21-24, 3p24, 4p13-ter, 22q
10	positive	9p21-22, 12q13-15	1q21-41, 4p13-ter, 16q22-24
11	negative	14q12-24, xq12-13	3p24, 15q24-27, 21q
12	negative	1p21,8q21-23, 20p	4p13-ter, 15q24-26, 21q
13	positive	9p21-22	3p21-24, 10p13-ter
14	negative	8q12-24, 5p13-14.2, 19q	3p24, 16q21-24
15	negative	8q12-24	4p13-ter, 9p21-24
16	negative	7p13-21	3p24, xp
17	positive	3q, 7q11-23, 12q13-15	3?4p13-ter, 5q, 17q12-24
18	negative	8q12-24, 17q12-24	4p13-ter, 7p13-21
19	negative	8q12-23, 13q12-21	17q23, 26
20	negative	8q21-23, 10p	4p13-ter, 19q12-13
21	positive	3, 9p21-22	4p13-ter, 11q23-25

studied 18 cases of VSCC samples and found out that the gain at 3q in the HPV-positive group (5/10) was markedly higher than the HPV-negative group (0/8). Huang *et al.* [8] studied eight cases of VSCC samples and two cell lines of VSCCs and obtained similar results. It is thought that the gain at 3q is correlated with the occurrence and development of HPV-positive VSCCs. Some other materials also reported a link between HPV infection and the high-frequency gain at 3q of such reproductive canal squamous cell carcinomas as cervical cancer and anal canal cancer [9,10]. The results of the present experiment further illustrate that the high-frequency gain at chromosome 3q plays an important role in the occurrence and development of HPV-positive VSCCs. Data of the present experiment and previous reports [4-8] showed that there was a locus in chromosome 3q permitting HPV genomic integration. It is possible that insertion of exogenous DNA leads to amplification of one or multiple oncogenes at locus 3q. These oncogenes play important roles in the occurrence and development of HPV-infected reproductive canal carcinomas. Furthermore, the present study also revealed the occurrence of a higher-frequency gain at chromosome 12 q. Previously only Yen *et al.* [11] reported such a finding with esophageal cancer. Maybe to key undiscovered oncogenes closely related to the occurrence and development of HPV-positive VSCCs are located within this region. It is believed that further and deeper-level researches will provide important clues to the occurring and developing mechanisms of HPV-positive VSCCs.

The present study also showed that the gain frequency of 70% at chromosome 8q of HPV-negative VSCCs was higher than the HPV-positive group of 9%, and such difference was statistically significant. This is in accordance with the results reported by Allen *et al.* [5]. Misa *et al.* [7] studied ten cases of VSCC cell lines and their results also supported this viewpoint. Kirchhoff et al. [12] reported similar findings in cervical cancer and suggested that the gain at chromosome 8q is related to the occurrence and development of HPV-negative VSCCs. Some oncogenes closely related to the occurrence and development of HPV-negative VSCCs may be located at this locus. This becomes the starting point to further explore the etiology and causative mechanism of HPV-negative VSCCs.

Table 2. — *Common CGH changes in the VSCCs by HPV status.*

Groups	Gain (%) 3q	8q	12q	Loss (%) 3p	4p
HPV-positive	8 /11 (73)	1/11 (9)	7/11 (64)	5/11 (46)	6/11 (55)
HPV-negative	1/10 (10*)	7/10 (70*)	0/10 (0*)	4/10 (40)	5/10 (50)
Total tumors	9/21 (43)	8/21 (38)	7/21 (33)	9/21 (43)	11/21 (52)
P	< 0.01	< 0.01	< 0.01	> 0.05	> 0.05

* HPV-positive vs HPV-negative

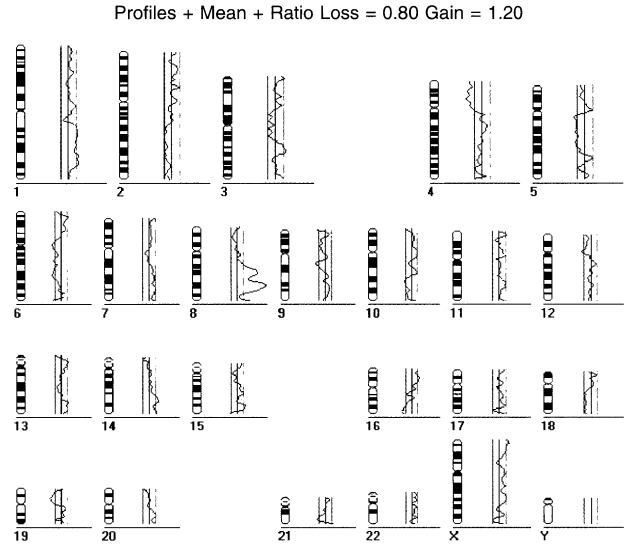

Figure 1. — Computer software analytic graphs of chromosome in case no. 15. Note: gain at right and loss at left.

The losses at chromosomes 4p and 3p were relatively common in the present study. According to Flowers *et al.* and Allen *et al.* [2, 5], the methods of loss of heterozygosity (LOH) and CGH were used, respectively, to study the loss of chromosome 3p in VSCCs. This alteration was found not to be related to HPV infection. Rosenthal *et al.* [13] employed the method of LOH to test 42 cases of VSCCs. It was found that the loss at chromosome 3p was higher in the negative group than in the positive group. In conjunction with the present data, it further suggests the possibility of the loss of tumor suppressor genes closely related to vulvar carcinogenesis at chromosome 3p. Nonetheless whether the functions of tumor suppressor genes are independent of HPV needs to be further studied with a larger sample size. It was also revealed by the present study that there was a gain and loss of whole arms at chromosome 3 of VSCCs. We surmised that there were the high-frequency breakage of centromeres at chromosome 3 and formation of isochromosomes during the occurrence of VSCCs. Within the following mitosis, the imbalance can be passed on to the offspring and form many instances of gain and loss of the isochromosome. This is in accordance with the report on cervical cancer by Heselmeyer [12, 14]. It is thought that this kind of alteration can probably promote the progression of cervical cancer. Jin Y. *et al.* [15] discovered that there was a higher breakage frequency of centromeres at chromosome 8 in squamous cell carcinomas of the head and neck. As a result, an isochromosome was formed and the whole arms of daughter cells were gained and lost. Formation of isochromosomes and gain and loss of whole arms of chromosomes in such other tumors as liver and breast carcinomas have also been confirmed [16-18]. It can be illustrated that the occurrence and progression of

VSCCs may share some common patterns with other tumors, which can be used for reference in future studies. Jee *et al.* [6] reported the loss of chromosome 4p in VSCCs but detected no relationship with HPV. It is of significance that synthesizing the previous data revealed the existence of loss at chromosomes 3p and 4p of VSCCs quite similar to the alterations in cervical cancer [8, 9]. This suggests that there are some similar pathological modes and identical genetic background between VSCCs and cervical cancer. It also further shows that the tumor suppressor genes closely related to the occurrence and development of VSCCs and cervical cancer are located at chromosomes 3p and 4p.

Oncogenes, tumor suppressor genes and other relevant genes closely related to the occurrence and development of VSCCs are located at the above abnormal chromosome loci. For example, the MDM2 (murine double minute 2) gene at chromosome 12q13-14 is one kind of oncogene found to have expressions of varying degrees in a variety of human tumors correlated with prognosis [19, 20]. The method of RT-PCR was previously used to analyze 31 cases of VSCC specimens and determined the gene expression rate of MDM2 at 22%. The present study found that there was a 33% gain at 12q. It showed that the MDM2 gene participated in the occurrence and development of VSCCs. This further suggests that the MDM2 gene can be one candidate oncogene in the occurrence and development of VSCCs.

Some important tumor-related genes are localized at the candidate abnormal chromosome loci. The newly cloned tumor suppressor gene FHIT is at 3p14.2. The cancer-inhibiting mechanism of this gene has remained somewhat unclear up to now. It is probably related to cellular apoptosis and p53-mediated cycle inhibition [21]. The FHIT gene and its abnormal transcription product have been found in many primary malignant tumors derived from epithelium [22]. Muller *et al.* [23] studied cervical cancer and found that there was some relationship between LOH of FHIT and gene integration of HPV. The recently discovered PIK3CA gene is also located at 3q26.3 and encodes for the catalytic subunit of PI3 kinase. As one kind of oncogene, PIK3CA participates in the signal transduction pathway of PI3-kinase/AKT and has the function of promoting cellular growth and inhibiting cellular apoptosis [24-27]. As reported recently in foreign studies [28, 29], amplification of the PIK3CA gene is closely related to HPV infection in human cervical cancer. When PIK3CA-amplified tumor cells are treated with PI3-kinase inhibiting factors, the cellular growth weakens and cellular apoptosis increases [30]. However relevant studies on these and VSCCs have not been reported. Further studies should be carried out to understand the functions of these genes. The experiences and results from other tumor researches are combined so as to decipher the relationship between them and the occurrence and development of VSCCs as well as to provide a quick and effective pathway to further accurately localize, screen and clone new related genes of VSCCs.

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