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Original Article

Early discovery of disseminated tumor cells during carcinogenesis in a 4NQO-induced mouse model of oral squamous cell carcinoma

Yong Cao[1], Qiang Shi[1], Baosheng Wei[1], Yun Mu[1], Jing Li[2], Fengqiang Chen[1], Dahai Yu[2]

[1]College of Stomatology, Guangxi Medical University, Nanning, Guangxi, P. R. China; [2]Department of Stomatology, The First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi, P. R. China

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Abstract: Objectives: Heterogeneous cells appear in multiple organs during the same time period as the primary lesion of some tumors is clinically detected. These heterogeneous cells are also known as disseminated tumor cells (DTCs). However, the characteristics of DTCs that disseminate during oral carcinogenesis remain unclear. Materials and methods: A mouse 4NQO model of lymph node metastasis in oral squamous cell carcinoma was established. Tissue samples of the tongue, bone marrow and submandibular lymph node were collected. Five stages (stage 0~stage IV) of carcinogenesis in each experimental animal were classified by two pathologists. After immunohistochemical staining of cytokeratin, the DTCs were isolated from bone marrow samples (stage II) by the laser capture microdissection (LCM) technique during oral carcinogenesis. Genomic amplification of bone marrow DTCs was performed, and homozygous deletion of the RB1CC1 gene was analyzed. After confirming the presence of disseminated tumor cells in stage II bone marrow samples, a comprehensive study among various stages of lymph node tissue was conducted using the same method. Results: DTCs that spread from the primary tumor were discovered in stage II bone marrow samples and in stage I, stage II and stage III submandibular lymph node samples through immunohistochemical staining. These spreading cells had different levels of homozygous exon deletion in the RB1CC1 and TP53 genes. Conclusion: Early spreading of epithelial cells may occur during the carcinogenesis of oral cancer. DTCs of oral carcinoma may show different chromosome aberrations from matched primary tumor cells.

Keywords: Disseminated tumor cells (DTCs), oral squamous cell carcinoma (OSCC), whole-genome amplification (WGA), homozygous deletion, metastasis

Introduction

Invasion and metastasis are the leading causes of death due to oral cancer. Tumor metastasis is a complex and multi-step process that requires cancer cells to lose adhesion, invade blood or lymphatic vessels, survive in the circulatory system, colonize metastatic organs and eventually form metastases after adapting to the local microenvironment [1]. Traditionally, metastasis occurs during the final stage of cancer. In the advanced stages of tumor evolution, some primary tumor cells accumulate sufficient gene mutations, acquire stronger invasive and survival capabilities, and migrate to adjacent tissue or distant organs through the vascular system. These cells form new tumor clones in the colonized organs, eventually leading to macrometastasis [2-4]. However, this notion has been recently challenged. Many studies have found that the spreading of heterogeneous cells begins in the early stages of tumor progression. These cells are also known as disseminated tumor cells (DTCs) [5]. A study of breast cancer DTCs showed that tumor cells can spread after obtaining a small amount of genetic variation and continue to acquire genomic aberrations in the new microenvironment [6]. Eyles and colleagues confirmed the presence of tumor cells in the early stages of tumorigenesis using an RET. AAD mouse melanoma model [7]. DTCs may exhibit latent metastasis in the shape of dormant cells, which may transform into malignant cells under certain conditions due to the local microenvironment [8]. Insights into the dynamic process of head

and neck carcinoma metastasis are warranted before auxiliary treatment plans are developed after removal of the primary tumor.

To date, few studies have focused on the spreading of tumor cells in oral cancer, and we speculate that oral cancer may demonstrate the early spreading phenomenon of primary tumor cells during tumorigenesis. To this end, in this study, we intended to preliminarily observe and confirm the early spreading of DTCs into bone marrow and lymph node tissue during carcinogenesis in a 4NQO-induced mouse model of oral squamous cell carcinoma to further understand the mechanisms of metastasis and dormancy of DTCs.

Presently, the most commonly used tumor metastasis model is the xenograft animal model, which is advantageous for its short tumor formation time and high metastasis rate [9]. However, this animal model cannot simulate the spontaneous occurrence of cancer, and the use of advanced tumor cell lines is unsuitable for exploring the early events of tumor metastasis. Our pre-constructed 4-nitroquinoline-1-oxide (4NQO) oral carcinoma animal model is a chemically induced carcinogenesis mouse model with a high occurrence rate of submandibular lymph node metastasis [10]. This model is useful for studying the mechanisms of tumor formation, progression and metastasis. Pan-cytokeratin (pan-CK) is a commonly used marker for detecting occult metastasis in epitheliogenic malignant tumors and can be used to identify disseminated tumor cells [11], and thus, pan-CK was selected to detect disseminated epithelial cells in bone marrow and lymph nodes in this study. Oncogene or tumor suppressor gene mutations represent one factor that differentiates tumor cells and somatic cells. The TP53 mutation is commonly found in the epithelial tissue of head and neck squamous cell carcinoma [12], and the RB1CC1 mutation is an early cancerous marker [5]. Genetic mutation analysis of immunohistochemical positive-stained cells can help to further identify whether these cells have undergone malignant transformation in bone marrow or lymph node tissue.

The current study sought to search for DTCs in bone marrow and submandibular lymph node tissues during the carcinogenesis of oral carcinoma from our pre-constructed 4NQO oral carcinoma animal model and to further verify the results by evaluating the homozygous deletion of the RB1CC1 gene and the TP53 exome of these cells.

Materials and methods

Tissue sample collection

Twenty-four BLAB/c mice were administered drinking water containing 200 mg/l 4NQO for 20 weeks to induce oral carcinoma according to the previous method [10]. Four mice were sacrificed bi-weekly. Tissue samples (tongue, submandibular lymph nodes and bone marrow) were harvested from week 21 to 36. Four normally raised mice that served as controls were sacrificed at the 40th week. The tongue tissue was immersed in 4% formalin solution. The submandibular lymph nodes were placed in cryostat tubes and stored in liquid nitrogen. The bone marrow was washed out from the femur with 1 ml of PBS solution and then placed in heparinized tubes. The animal experimental protocol was approved by the Animal Ethics Committee of Guangxi Medical University, China.

Pathological staging

The tongue lesions were stained by HE (hematoxylin & eosin), and lymph node metastasis was confirmed by the appearance of clumped tumor cells according to HE and pan-CK staining. The pathological stage of each animal was then classified by two experienced pathologists. Oral mucosa dysplasia was classified as normal mucosa (stage 0), moderate dysplasia (stage I), or severe dysplasia (stage II) according to the grading and diagnostic criteria of the World Health Organization (WHO) in 2005, and oral squamous cell carcinoma (OSCC, stage III) was classified according to the American Joint Committee on Cancer (AJCC) Cancer Staging Manual (Fifth Edition). OSCC combined with lymph node metastasis was classified as metastatic oral squamous cell carcinoma (stage IV). Bone marrow samples from stage 0, stage II and stage III animals were used for preliminary experiments, followed by a more comprehensive study of lymph node samples from stage 0~IV animals.

Sample processing

Mononuclear cells from the bone marrow were isolated by a Ficoll gradient centrifugation

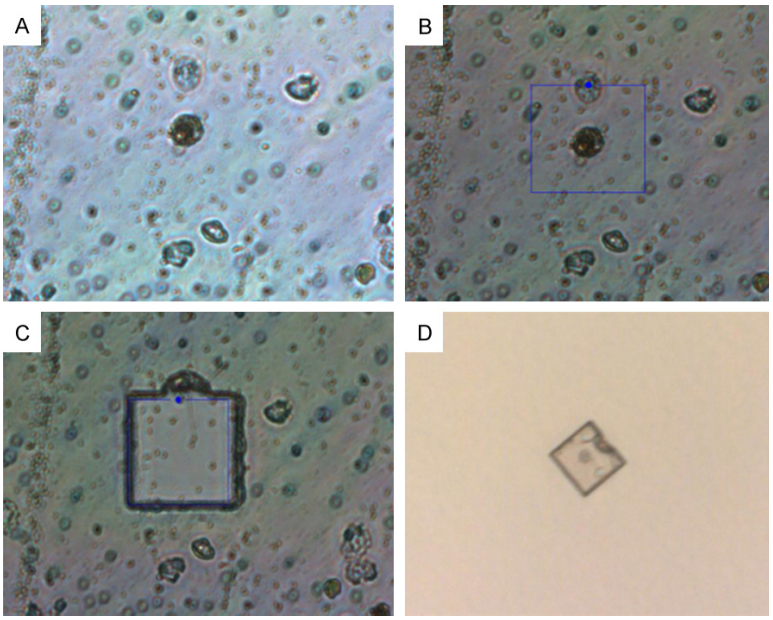

Figure 1. Microdissection procedure. A. Recognition of CK-positive cells. B. Adjustment of the cutting area. C. Removal of DTCs from the slide. D. Single DTCs left on the cut chip.

method [13]. The mononuclear cell suspension obtained by the above method was centrifuged and then washed twice with D-Hank's solution. The monocyte sediment was resuspended in 150 μl of PBS after centrifugation. The stage II monocyte suspension was coated evenly on (polyethylene PEN) membrane slides (ZEISS, Germany) dedicated for microdissection (50 μl for each smear, 3 smears for each sample, 36 smears in total), while the stage 0 and stage III monocyte suspensions were coated evenly on positively charged glass slides (50 μl for each smear, 3 smears for each sample, 24 smears in total).

Serial sections (5-8 μm) were cut from the frozen submandibular lymph node blocks using a cryostat microtome (SLEE, Germany). Using four lymph nodes for each pathological stage, serial sections of twenty lymph nodes from stages 0~IV were cut. One section was placed on a positively charged glass slide every 15 sections, and the adjacent section was placed on a PEN membrane slide dedicated for microdissection (3 positively charged glass slides and 3 PEN slides were collected for each lymph node).

Immunohistochemistry

All the monocyte smears and positively charged glass slides of lymph nodes were subjected to immunohistochemical staining. The procedure was performed as previously described [14]. Briefly, the slides were treated with 3% hydrogen peroxide to block endogenous peroxidase activity and were boiled for 30 min in 0.01 M sodium citrate buffer to retrieve the antigen. Rabbit anti-mouse polyclonal antibody (AE1/AE3, 1:100 dilution, Boster, Wuhan, China) was used as the primary antibody. Biotin-labeled secondary antibody was added after complete removal of the primary antibody. Specific antigen antibody reactions were visualized using a DAB staining kit (ZSGB-BIO, Beijing, China). All sections were counterstained with hematoxylin after immunohistochemical staining.

Laser capture microdissection (LCM)

Pan-CK-positive monocyte smears underwent laser capture microdissection (LCM) to extract single DTCs using the method described in a previous study [15], (3 DTCs were isolated from each CK-positive smear), while the lymph node PEN slide adjacent to the CK-positive slide was pretreated with HE stain before the same procedure (3 DTCs were isolated from each positive lymph node sample). The procedures are shown in **Figure 1**. For lymph node DTC extraction, the dissection area was located in the same region of the CK-positive cells according to the adjacent CK-positive positively charged glass slide. The collected DTCs were placed in a centrifuge tube filled with DEPC water and stored at -20°C after brief centrifugation at 14000 g.

Whole-genome amplification (WGA) and purification of DTCs

Whole-genome amplification of single DTCs was performed using the Single Cell Whole Genome Amplification Kit (Sigma, USA) and the multiplex displacement amplification technique [16]. The PCR purification kit (Qiagen, Germany) was used to clean up the amplified DNA, and the DNA concentrations were determined using a NanoDrop spectrophotometer (Nanodrop Technologies, USA). The amplification product

Table 1. Primer sequences of RB1CC1 exons 2, 6, and 7 and TP53 exons 5-8

Gene	Primer sequence	Length (bp)	Annealing temperature (°C)
GAPDH	F: AGCGAGACCCCACTAACATC R: TCCCCACTGCCTACATACCA	419	57.3
RB1CC1 (Exon 2)	F: CATTTCTTTTTACCCCCCAC R: GGAATCTCTTACTTCAGTCTGACG	315	53.1
RB1CC1 (Exon 6)	F: GTTTTTGGCAGTCTCTTATAGTGG R: GTATTCGTGGCTGTTACCTGAG	211	55.4
RB1CC1 (Exon 7)	F: CAACAGGCATTACAACCACTAC R: CACAAACTCATTCACAGCAGAC	245	54.2
TP53 (Exon 5)	F: TACTCTCCTCCCCTCAATAAGC R: ACGACCTCCGTCATGTGCT	143	55.5
TP53 (Exon 6)	F: GGCTCCTCCCCAGCATCT R: CTCGGGTGGCTCATAAGGT	109	53.5
TP53 (Exon 7)	F: CGGCTCTGAGTATACCACCAT R: GGAGTCTTCCAGTGTGATGATG	106	52.5
TP53 (Exon 8)	F: CTTTGAGGTTCGTGTTTGTGC R: CTCCCTGGGGGCAGTTCA	102	53.5

Table 2. Immunohistochemical results of pan-CK in bone marrow and lymph node samples

Stage	Bone marrow			Lymph node		
	n	+	-	n	+	-
0	4 (12)	0 (0)	4 (12)	4 (12)	0 (0)	4 (12)
I	0	0	0	4 (12)	1 (1)	3 (11)
II	12 (36)	5 (12)	7 (24)	4 (12)	1 (1)	3 (11)
III	4 (12)	4 (12)	0 (0)	4 (12)	2 (2)	2 (10)
IV	0	0	0	4 (12)	4 (9)	0 (3)

Note: The numbers in parentheses show the corresponding number of smears (slides).

containing the GAPDH gene was considered a successful whole-genome amplification product, and the product could be used for subsequent chromosomal mutation analysis.

DNA extraction from tongue tissue

Stage 0~IV animal tongue tissue (4 samples of each pathological stage) was made into a cell suspension with a homogenizer. DNA extraction was performed using the Qiagen QLAamp DNA Mini Kit (Qiagen, Germany) following the manufacturer's protocol.

Homozygous deletion analysis for RB1CC1 and TP53

Homozygous deletion analysis of the DNA products from DTCs and tongue tissue was performed as described previously [17]. All the primer sequences were designed by Takara Biotech (Dalian, China) according to Gene Bank. Exons 2, 6, and 7 of RB1CC1 were amplified in stage II bone marrow-derived DTCs and stage 0, II, and III tongue tissue, while exons 5-8 of TP53 were amplified in lymph node-derived DTCs and stage 0~IV tongue tissue. Detailed information on the primers is shown in **Table 1**. The PCR reaction bands were separated by 2.0% agarose gel electrophoresis and were visualized under a UV illuminator. For homozygous deletion specimens, the PCR array was repeated to exclude false-positive results.

Results

Tissue samples and pathological staging

Twenty-eight animal tissue samples (tongue, submandibular lymph node, and bone marrow) were collected at the end of the fortieth week, including four cases of normal mucosa (stage 0), four cases of moderate dysplasia (stage I), twelve cases of severe dysplasia (stage II), four cases of squamous cell carcinoma (stage III) and four cases of metastatic oral squamous cell carcinoma (stage IV). Representative results are published in our previous article [10].

Immunohistochemical staining results

Four stage 0 bone marrow samples were judged as pan-CK negative. Five of the 12 stage II bone marrow samples were judged as pan-CK positive, of which CK-positive cells were discovered on 12 smears. All 4 stage III bone marrow samples were judged as pan-CK positive, of which CK-positive cells were discovered on all smears. Four stage 0 lymph node samples were judged as pan-CK negative. One of the 4 cases of stage I and stage II lymph node samples was judged as pan-CK positive, of which CK-positive cells were discovered on 1 slide respectively.

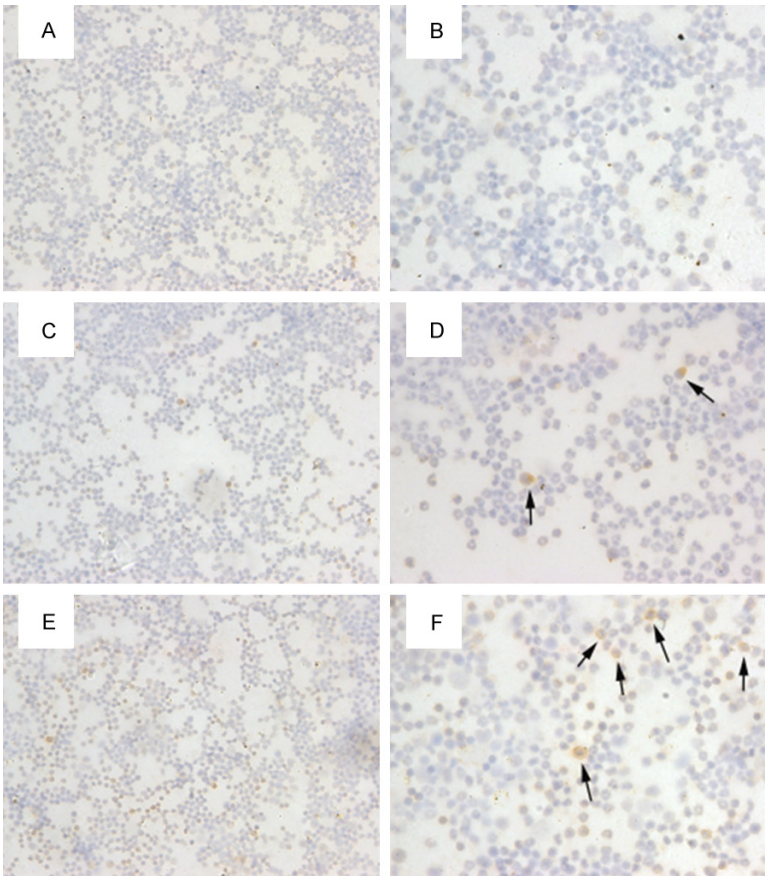

Figure 2. Micrograph of immunohistochemical staining of monocyte smears. (A) The stage 0 animal monocyte smear shows no positive-stained cells; original magnification, 200×. (B) An enlarged view of (A); original magnification, 400×. (C) The stage II animal monocyte smear shows scattered positive-stained cells; original magnification, 200×. (D) An enlarged view of (C). The black arrows show a single positive cell; original magnification, 400×. (E) The stage III animal monocyte smear shows clustered positive-stained cells; original magnification, 200×. (F) An enlarged view of (E). The black arrows show clustered positive cells; original magnification, 400×.

Two of the 4 stage III lymph node samples were judged as pan-CK positive, of which CK-positive cells were discovered on 1 slide respectively. All 4 stage IV lymph node samples were judged as pan-CK positive, of which CK-positive cells were discovered on 9 slides. Detailed information is shown in **Table 2**, and representative pictures are shown in **Figures 2** and **3**.

Microdissection results

Thirty-six DTCs were isolated from 12 stage II CK-positive smears, and twenty-four DTCs were isolated from CK-positive lymph nodes by microdissection technology, including three from stage I lymph nodes, three from stage II lymph nodes, six from stage III lymph nodes, and twelve from stage IV lymph nodes.

WGA results

The genomic DNA of three DTCs (8.3%) was successfully amplified from 36 stage II bone marrow-derived DTCs. The genomic DNA of fifteen DTCs (62.5%) was successfully amplified from 24 submandibular lymph node-derived DTCs, including two genome samples from stage I lymph nodes, two from stage II lymph nodes, three from stage III lymph nodes, and eight from stage IV lymph nodes.

PCR results

Homozygous deletion of RB-1CC1 exon 2 occurred in stage II animal bone marrow DTCs (2/3) and OSCC tongue tissue (2/4). There was no homozygous deletion of RB1CC1 exons 6 and 7 in severe dysplasia tongue tissue, OSCC tongue tissue or stage II animal bone-marrow DTCs. The electrophoresis results are shown in **Figure 4**.

There was no homozygous deletion of TP53 exon 5 in the tongue tissues of normal or precancerous lesions, although homozygous deletion of TP53 exon 5 was found in tongue tissues of OSCC (1/4) and metastatic OSCC (1/4). Homozygous deletion of TP53 exon 5 occurred in DTCs derived from stage I (1/2), stage II (1/2), stage III (1/3) and stage IV (2/8) animal lymph nodes. Homozygous deletion of TP53 exon 6 occurred in only metastatic OSCC tongue tissue (1/4). Homozygous deletion of TP53 exon 7 was not found in tongue tissue or DTCs. Homozygous deletion of TP53 exon 8 occurred in metastatic OSCC tongue tissue (1/4) and DTCs derived from stage II (1/2) and stage IV (1/8) animal lymph nodes. Detailed information is shown in **Table 3**, and representative electrophoretic bands are shown in **Figure 5**.

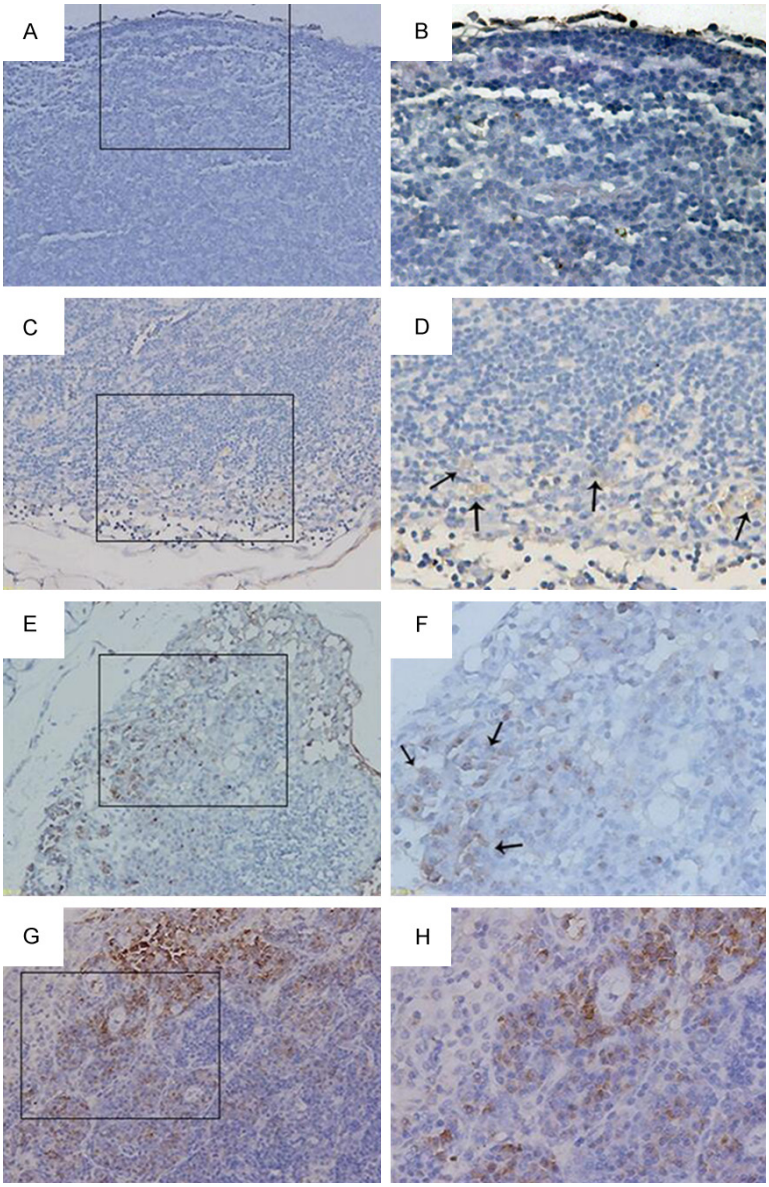

Figure 3. Micrograph of the immunohistochemical staining of submandibular lymph nodes. A. The stage 0 animal lymph node slide shows no positive-stained cells; original magnification, 200×. B. An enlarged view of the box on the left; original magnification, 400×. C. The stage I~II animal lymph node slide shows scattered positive-stained cells; original magnification, 200×. D. An enlarged view of the box on the left; the black arrows show single positive cells; original magnification, 400×. E. The stage III animal lymph node slide shows clustered positive-stained cells; original magnification, 200×. F. An enlarged view of the box on the left; the black arrows show clustered positive cells; original magnification 400×. G. The stage IV animal lymph node shows a wide range of positive-stained cells; original magnification, 200×. H. An enlarged view of the box on the left; original magnification, 400×.

Discussion

Xenograft models and chemo-inducing models are commonly used animal models in the study of oral cancer metastasis [9]. The xenograft model is advantageous because of its fast tumor formation time and controllable tumor size. Nevertheless, the aim of this study was to investigate the spreading of DTCs in different stages of oral carcinogenesis. Therefore, a chemically induced carcinogenesis model was used to simulate the whole process of tumorigenesis. Thus far, chemically induced oral cancer models have not yet achieved satisfactory lymph node metastasis [18, 19]. According to our previous study, submandibular lymph node metastasis was successfully obtained after 32 weeks of administration by increasing the 4NQO dose in drinking water and prolonging the administration time. The rate of lymph node metastasis was 100% after 32 weeks in the current study, which was much higher than the rate of a xenograft model reported by Zhao et al [20].

Disseminated tumor cells are single or small clusters of tumor cells that migrate from primary tumors and colonize in regional lymph nodes, peripheral blood, or bone marrow to initiate early micrometastasis [21]. Cytokeratin is an epithelial cell-specific marker [22] that is not expressed in mesenchymal tissue. Immunohistochemical staining of cytokeratin in lymph node and bone marrow tissues can discriminate epithelial derived disseminated cells and is a commonly used method to identify oral cancer-disseminated tumor cells [23, 24]. Pan-CK (AE1/AE3) is a mixture of CK monoclonal antibodies that recognize CK1-CK8, CKI0, CKI4, CKI6, and CKI9. The current study used pan-CK antibody to identify DTCs in tissue samples because of its high specificity and sensitivity. In this study,

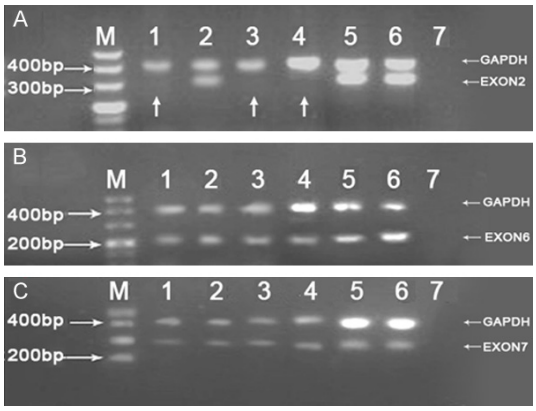

Figure 4. The results of agarose gel electrophoresis of RB1CC1 gene exons 2, 6, and 7. A: Exon 2, B: Exon 6, C: Exon 7. M: DNA marker. 1-3: DTCs, 4: OSCC tongue tissue, 5: severe dysplasia tongue tissue, 6: normal tongue tissue, 7: blank control.

CK-positive cells were found in stage I~II lymph nodes and in stage II bone marrow tissues but not in stage 0 tissues, indicating that epithelial cells may spread from the primary lesion during the early stage of tumorigenesis. DTCs were primarily discovered after the primary tumor diagnosis [25]. However, DTCs were found during the period of oral precancerous lesions in the current study, furthering our understanding of tumor metastasis. Due to the limitations of medical ethics, it is difficult to obtain lymph node and bone marrow samples from patients with dysplasia of the oral mucosa for disseminated cell detection. Therefore, it is difficult to determine whether heterogeneous cells disseminate from the primary site of human oral precancerosis. The epithelial-mesenchymal transition (EMT) [26] may reduce the expression of cytokeratin in tumor cells during invasion and metastasis [27]. In this study, we used CK to mark DTCs, which may result in false-negative results, resulting in a portion of the disseminated cells being undetected. We intend to use EMT epithelial markers such as N-cadherin or FSP1 [28] to assist in the detection of DTCs in a future study.

Because of the high false-positive rate of immunohistochemical detection [29], we sought to further confirm the characteristics of DTCs identified by pan-CK staining by evaluating the chromosome aberration of these cells. In this study, direct and indirect LCM techniques were used to isolate bone marrow and lymph node DTCs, respectively. Direct separation of CK-positive cells from monocyte smears can eliminate the contamination of mesenchymal cells, resulting in high-purity DTC genetic material. However, in this study, only 8.6% of the cell genome obtained by this method was successfully amplified by WGA. This may be due to the degradation of cellular nucleic acids during the antigen retrieval process of immunohistochemical staining [30, 31]. To decrease the degradation of cellular nucleic acids during immunohistochemistry, microdissection of lymph node DTCs was performed in the same region in which DTCs appeared on HE-staining slides, adjacent to the immunohistochemical-positive slide. In this way, the percent of the cell genome successfully amplified by WGA was improved to 62.5%. However, this method may result in low-purity DTC genetic material because of mesenchymal cell contamination. In future studies, improving the isolation technique of single DTCs is expected to increase the success rate of WGA.

Mutations or deletions of tumor suppressor genes are hallmarks of tumor cells [32]. The characteristics of DTCs isolated from bone marrow or lymph nodes can be further determined by detecting the genetic deletion of these cells. TP53 is located in the chromosome 17p13 region, and its mutation is a key factor in the occurrence of head and neck squamous cell carcinoma [33]. The most commonly mutated loci in TP53 are exons 5-8 [34]. RB1CC1 is a tumor suppressor gene located in the chromosome 8q11 region, and the loss of its heterozygosity was found in bone marrow DTCs of breast cancer patients [6]. Overexpression of the RB1CC1 protein in human and mouse oral cancer tissues was also found in our previous study [35]. The current study detected the homozygous deletion of exons 5-8 of TP53 and exons 2, 6, and 7 of RB1CC1 due to limited DNA resources. We revealed that the homozygous deletion of RB1CC1 and TP53 occurred in bone marrow- and lymph node-derived DTCs, respectively, during the precancerous phase of oral squamous cell carcinoma and in OSCC tongue tissue, further verifying that the pan-CK-positive cells discovered in the lymph nodes and bone marrow had disseminated from oral premalignant lesions in an early stage. Interestingly, we found no deletion of TP53 or RB1CC1 exons in dysplastic tongue tissue, and homozygous deletion of TP53 exon 6 existed in metastatic OSCC tongue tissue but not in lymph node DTCs. This may be due to the presence of dis-

Table 3. Homozygous deletion of exons 5-8 in TP53 of the primary lesion and DTCs

Pathological stage	Cell origin	N	Exon 5		Exon 6		Exon 7		Exon 8	
			Normal	Loss	Normal	Loss	Normal	Loss	Normal	Loss
Stage I	Tongue tissue	4	4	0	4	0	4	0	4	0
	DTCs	2	1	1	2	0	2	0	2	0
Stage II	Tongue tissue	4	4	0	4	0	4	0	4	0
	DTCs	2	1	1	2	0	2	0	1	1
Stage III	Tongue tissue	4	3	1	4	0	4	0	4	0
	DTCs	3	2	1	3	0	3	0	3	0
Stage IV	Tongue tissue	4	3	1	3	1	4	0	3	1
	DTCs	8	6	2	8	0	8	0	7	1

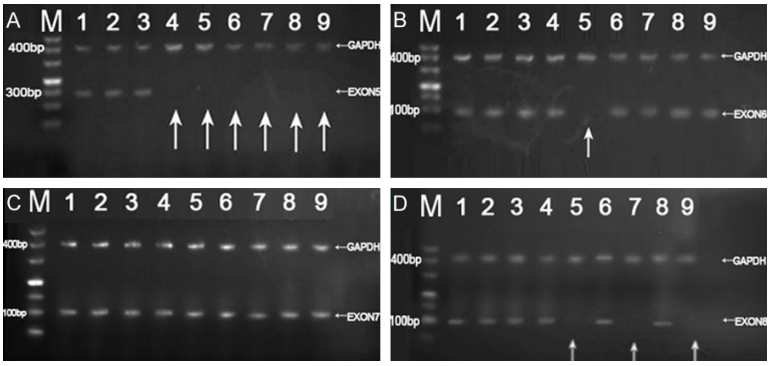

Figure 5. The results of agarose gel electrophoresis of TP53 gene exons 5-8. A: Exon 5, B: Exon 6, C: Exon 7, D: Exon 8. M: DNA marker. 1: normal tongue tissue, 2: moderate dysplasia tongue tissue, 3: severe dysplasia tongue tissue, 4: OSCC tongue tissue, 5: metastatic OSCC tongue tissue, 6: stage I DTCs, 7: stage II DTCs, 8: stage III DTCs, 9: stage IV DTCs.

proportionate chromosomal mutations among disseminated cells and primary lesion cells, suggesting that tumor cells disseminated during an early stage [6]. Moreover, the homozygous deletion of TP53 exon 8 occurred in metastatic OSCC tongue tissue and matched lymph node DTCs, but no deletion was found in OSCC tongue tissue or in matched lymph node DTCs, suggesting that the loss of TP53 exon 8 may rouse dormant DTCs, resulting macrometastasis.

Disseminated tumor cells of oral cancer have gained increasing interest in recent research [36]. In our study, we found disseminated tumor cells in the bone marrow and lymph nodes during the oral precancerous stage in a 4NQO-induced mouse model of oral squamous cell carcinoma by immunohistochemical staining. We further confirmed this finding by gene mutation analysis to preliminarily verify our speculation of early spreading during oral tumorigenesis. However, due to the insufficient number of animal tissue samples and the low success rate of whole-genome amplification techniques, the obtained results were not able to be statistically analyzed. In further studies, a larger number of animal tissue samples and an improved whole-genome amplification technique combined with chromosome sequencing of the disseminated cells at different stages of oral tumorigenesis will better reveal the mechanism of metastasis of head and neck carcinoma.

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Disclosure of conflict of interest

None.

Address correspondence to: Dahai Yu, Department of Stomatology, The First Affiliated Hospital of Guangxi Medical University, 6 Shuangyong Road, Nanning 530021, Guangxi, P. R. China. Tel: +86 18877181688; Fax: +86 0771 5315946; E-mail: yudahai813@aliyun.com

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