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**Establishment of a murine model of bone invasion  
by oral squamous cell carcinoma**

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**Key words:** animal model, bone invasion, cytokine, SCC cell line, C3H

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**Running title:** murine model of bone invasion

**Abstract:**

**Purpose:** This study examines the establishment an animal model of bone invasion by oral squamous cell carcinoma to clarify the mechanisms of osteoclast-mediated bone invasion.

**Materials and Methods:** C3H/HeN mice were inoculated with SCCVII cells into the masseter region. At the end of week 3, all surviving mice were sacrificed and analyzed by three-dimensional imaging using micro-computed tomography, histopathological observation using Hematoxylin-Eosin staining and Tartrate-Resistant Acid Phosphatase staining, and confirmation of mRNA expression of the osteoclast-related cytokines IL-6, TNF- $\alpha$ , and PTHrP.

**Results:** SCCVII cells rapidly multiplied in the masseter muscle of the mice. Bone invasion was evident only in the SCCVII transplanted group on micro-computed tomography. The histopathologic findings obtained with H-E and TRAP staining indicated that the tumor cells in the mandible of all animals of the SCCVII transplanted group exhibited funicular invasion and presented a serrated pattern of bone resorption. The mRNA expression of IL-6, PTHrP, and TNF- $\alpha$  increased as the control decreased.

**Conclusion:** SCCVII cells were highly invasive into mandibular bone in C3H/HeN mice. This model was similar to the invasion of human oral cancer into maxillary and mandibular bone. Our mandibular invasion

model may provide a powerful new modality for the diagnosis and treatment of oral cancer with bone invasion.

## Introduction

Oral squamous cell carcinoma (OSCC) is known to have a potent activity of local invasion. Its invasion into the adjoining maxilla and mandible, in particular, is a major clinical problem.<sup>1,2</sup> While gingival and oral floor carcinoma are frequently associated with mandibulectomy the extent of bone invasion can only be assessed by diagnostic imaging. It has been pointed out that resection of the maxilla and mandible occasionally amounts to oversurgery. In recent years, it has been reported that osteoclast associated with bone invasion and metastasis to the bone by cancer cells is mediated by osteoclasts rather than being directly caused by cancer cells (i.e., osteoclastic bone resorption).<sup>3</sup> We have carried out immunohistological analysis using specimens obtained from oral squamous cell carcinoma patients following mandibulectomy.<sup>4</sup> The results of these analytical studies have demonstrated in particular, that the presence of the osteoclast activation factors interleukin-6 (IL-6), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and parathyroid hormone-related protein (PTHrP) have a strong involvement in this process.<sup>5</sup> In this study, we have prepared a murine model of mandibular invasion by cancer cells and investigated the presence of the osteoclast activation factors IL-6, TNF- $\alpha$ , and PTHrP in order to shed light on the mechanism of bone invasion by OSCC.

## Materials and Methods

**Cell culture.** The SCCVII used in this study is an established cell strain derived from OSCC on the oral floor in mice.<sup>6</sup> It has been reported both as a model for distant metastasis and as a model for salivary gland tumor transplanted to the submandibular gland.<sup>7</sup>

The basal medium was Dulbecco Modified Eagle Medium (DMEM) with a 10% addition of fetal bovine serum (FBS). This SCCVII cell strain was cultured in DMEM+10%FBS and used for experiments. This was allowed to stand in a 100mm dish for incubation at 37°C in the presence of 5%CO<sub>2</sub>. The cell concentration was approximately 1.3 x 10<sup>5</sup>/ml. The cells were collected by centrifugation, and the cell concentration was adjusted to 1.0 x 10<sup>5</sup>/ml with DMEM.

**Animal model.** The experimental animals consisted of twelve male C3H/HeN mice weighing approximately 20g (Sankyo Laboratories, Tokyo, Japan). They were divided into two groups: the control group and the SCCVII transplanted group, consisting of three and nine animals, respectively. All of the mice were maintained in the ordinary manner and treated in accordance with the Guidelines for Animal Experiments of the Tokyo Dental College. The animals in the SCCVII transplant group were maintained normally for three weeks after injection, into the left masseter region, of 50µL SCCVII cells adjusted to 2 x 10<sup>7</sup>/mL in a serum-free

medium in such a manner that no lesions were caused to the periosteum. The mice of the control group received 50 $\mu$ L of physiological saline solution alone, likewise injected into the left masseter region. Both groups were maintained under normal conditions in the same manner for three weeks.

Treatment was given for 3 weeks. On days 1, 7, and 14, the mice were weighed and the longest diameter of the mass in the buccal region was measured in three directions. At the end of week 3, all surviving mice were sacrificed. The tumors were resected and preserved at -70°C, and the heads of the mice were fixed in 10% formalin (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

The specimens were analyzed by (1) three-dimensional imaging using micro-computed tomography (micro-CT), (2) histopathological observation using Hematoxylin-Eosin (H-E) staining and Tartrate-Resistant Acid Phosphatase (TRAP) staining, and (3) confirmation of mRNA expression of the osteoclast-related cytokines IL-6, TNF- $\alpha$ , and PTHrP.

#### **Micro-computed tomography (CT) and 3-dimensional reconstruction.**

The head and neck of the mice was imaged with a micro-CT (ultra-micro focus X ray CT unit KMS-755, Kashimura, Osaka, Japan), at a minimum tomographic width of 20 - 30 $\mu$ m. The imaging conditions were: lamp

voltage 45.0kV, lamp current 100.0 $\mu$ A, multiplying factor 2.0. After preparing two-dimensional slice data from the resulting images by the back projection method, a steric image was constructed with 3-dimensional reconstruction software (TRI/3D Bon; R2.1.16-S Ratoc, Tokyo, Japan).

**Hematoxylin-eosin (H-E) staining and tartrate-resistant acid phosphatase (TRAP) staining.** After subsequent decalcification with 10% formalin formate, the frontal sections of the occipital region were embedded in paraffin by the routine method to prepare thin slices for microscopy using a H-E double-stain.

After decalcification with a 10% aqueous solution of EDTA, paraffin-embedded specimens were prepared and TRAP staining was performed using a TRAP Kit (Hokudo Co., Sapporo, Japan) and Mayer's hematoxylin (Mutoo Pure Chemicals Ltd., Tokyo, Japan).

**Preparation of mRNA and semiquantative RT-PCR.** Specimens were taken from the SCCVII cultured cells as well as from the injected portions of the mice in the control group and the SCCVII transplanted group for RNA isolation using the AGPC method. After heating for five minutes, 1 $\mu$ g of the RNA sample was mixed with the reverse transcriptase solution to synthesize cDNA. The reaction took place at 30°C for 60 minutes. IL-6, TNF- $\alpha$ , and PTHrP were then amplified by the PCR method and the

DNA product was confirmed by electrophoresis with 1.5% agarose gel, stained with ethidium-bromide, and photographed.

**Statistical analysis.** To assess the statistical significance of body weights and tumor size, student's *t* test was performed. Differences of means among the percentage of mRNA of 3 cytokines in the two groups were compared by  $\chi^2$  test. A value of  $p < 0.05$  was considered statistically significant (SPSS 11.0 for windows 2001 software, USA).

## Results

During the 3 weeks of treatment, 12 mice survived. In our bone invasion models, tumors usually grew for about 3-4 weeks and died after that (Fig 1). Body weights changed in each group during the initial three weeks. Compared with the control, the body weights of SCCVII transplanted group tended to decrease (Fig 2). The differences in tumor size were significant between the two groups. The tumors transplanted into the SCCVII transplanted group of mice grew rapidly. Mean tumor diameter was  $2263.1\text{mm}^3$  in the SCCVII transplanted group after 3 weeks (Fig 1,2).

**Three-dimensional image analysis using micro-CT.** Micro-CT showed bone invasion only in the SCCVII transplanted group. Anatomical observation of the maxilla and mandible using the micro-CT revealed an inclination of the teeth and bone resorption in the mandibular angle, on the

surface of the mandible and in the zygomatic arch, being different from the ordinary facial bones. The mandibles were obviously asymmetric, and the zygomas were bowed (Fig 3).

**Appearance of resorption in the mandibular bone.** The histopathologic findings obtained with H&E staining indicated that the tumor cells in the mandible of all animals of the SCCVII transplanted group exhibited funicular invasion and presented a serrated pattern of bone resorption (Fig 4). The TRAP stain findings showed evidence of accumulated osteoclasts in considerable numbers around the mandible (Fig 5).

**Expression of the osteoclast-related cytokine mRNA.** It has been confirmed that SCCVII cells express IL-6, TNF- $\alpha$ , and PTHrP at the mRNA level. Similarly, tumors of the SCCVII transplanted mice showed mRNA expression of IL-6, TNF- $\alpha$ , and PTHrP. In contrast, the control group showed no evidence of expression (Fig 6). This makes it clear that the newly established murine model of invasive OSCC closely reflects the clinical features of human invasive OSCC and is thus useful in shedding light on the mechanism of bone invasion. Given that IL-6, TNF- $\alpha$ , and PTHrP are involved in the same manner as in humans it can be surmised that bone resorption is mediated by the cytokines produced by the tumor cells.

## Discussion

Animal models are essential for studies of the mechanism of bone invasion by OSCC. SCCVII is a malignant tumor cell line derived from the mouse and grows without being affected by the immune system.<sup>6,7</sup> This cell line was first used to develop an animal model of oral cancer of the oral floor.<sup>6</sup> Tumor growth, cervical lymph node metastasis, and pulmonary metastasis have been confirmed. In the present study, SCCVII cells were injected into the masseter region of mice. It has been confirmed that the transplantation of SCCVII cells into the masseter region of C3H/HeN mice resulted in significant tumor proliferation after three weeks.<sup>3</sup> This tumor is characterized by its bone invasion potential, and marked bone resorption was detected in the mandible and the facial bones. The histopathologic findings showed evidence of funicular invasion of the tumor cells into the mandible with a serrated bone resorption pattern in all specimens of the SCCVII transplanted group. Moreover, it was possible to extract osteoclasts not only in the HE but also in the TRAP stained specimens. This was clear evidence of the fact that bone resorption was osteoclast-mediated. The present experimental setup can thus be evaluated as being extremely useful as a bone invasion model that closely reflects the clinical features.

In human invasive OSCC the tumor cells are known to show a strong expression of IL-6, TNF- $\alpha$ , and PTHrP.<sup>2,3,8</sup> In our animal models, it was possible to confirm the expression of these cytokines from the SCCVII cultured cells at the mRNA level. Roughly the same results (i.e., enhanced expression of all cytokines at the mRNA level) were also obtained with neoplastic tumor tissues that had been transplanted. This substantiates the fact that the SCCVII cell strains are also subject to the influence of the osteoclast-related cytokines.

Much of the significant progress made in the determination of the bone invasion mechanism is due to the recent spectacular advances in the basic study of bone metabolism.<sup>8,9</sup> The isolation and identification of the PTHrP, the causal factor of hypercalcemia associated with cancer, in particular, was a milestone in the development of research on bone metastasis and bone invasion by cancer cells.<sup>9</sup> Okamoto et al. produced evidence in their in-vitro study that IL-6 is involved in the bone invasion of OSCC.<sup>2</sup> Recently, it has been clarified that the differentiation and functions of osteoclasts are strictly controlled by the RANKL expressed on the cell membranes of the osteoblasts or marrow interstitial cells.<sup>10</sup> We, for our part, have carried out immunohistochemical analyses on various osteoclast related cytokines, and have been able to demonstrate the possibility that IL-6, TNF- $\alpha$ , and PTHrP may be involved at least in bone

invasion by human gingival cancer.<sup>3,5</sup> In much the same manner it has also been possible, in our present study with the SCCVII cell strain, to substantiate the production of these cytokines and their involvement in bone invasion and in causing the formation of osteoclasts in large numbers. The use of the animal model may provide a means of shedding light on the mechanism by which OSCC with the potential for cytokine production causes bone invasion.

There is no doubting the fact that the clarification of the cytokine network is strategically most important in establishing a method for the diagnosis and treatment of bone invasion by gingival cancer. The preoperative testing of biopsy samples to determine the extent to which cytokines are present is likely to become, in the foreseeable future, a therapy planning approach that is consistent with the principles of evidence-based medicine.

In any event, however, these studies make use of animal models related to bone invasion, but the bone invasion model involving the cell transplantation of OSCC has been established only at our Department. It is our understanding that this model reflects the clinical features most closely and is thus most effective and useful in elucidating the invasion mechanism and in clarifying the treatment method. In this context we would like to emphasize that the determination of the mechanism of bone

invasion in the oral cancer domain in fuller detail is bound to substantially benefit the further progress of dental medicine.<sup>11,12</sup>

In the future, it will be necessary to investigate the expression of cytokines and calcium in the serum in fuller detail at the protein level. It is also felt essential for progress studying research and in the interest of developing new methods for cancer therapy targeting the osteoclast activating factors to introduce antisense oligonucleotides of the respective cytokines in this cell strain to inhibit the expression of these cytokines and investigate their involvement in bone invasion and suppress bone invasion through the administration of their antibodies and bone resorption inhibitors.

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FIGURE 1. 0.2ml of SCCVII ( $1.0 \times 10^5/\text{ml}$ ) in DMEM was injected into the left masseter region. Tumor enlarges in 3 weeks.

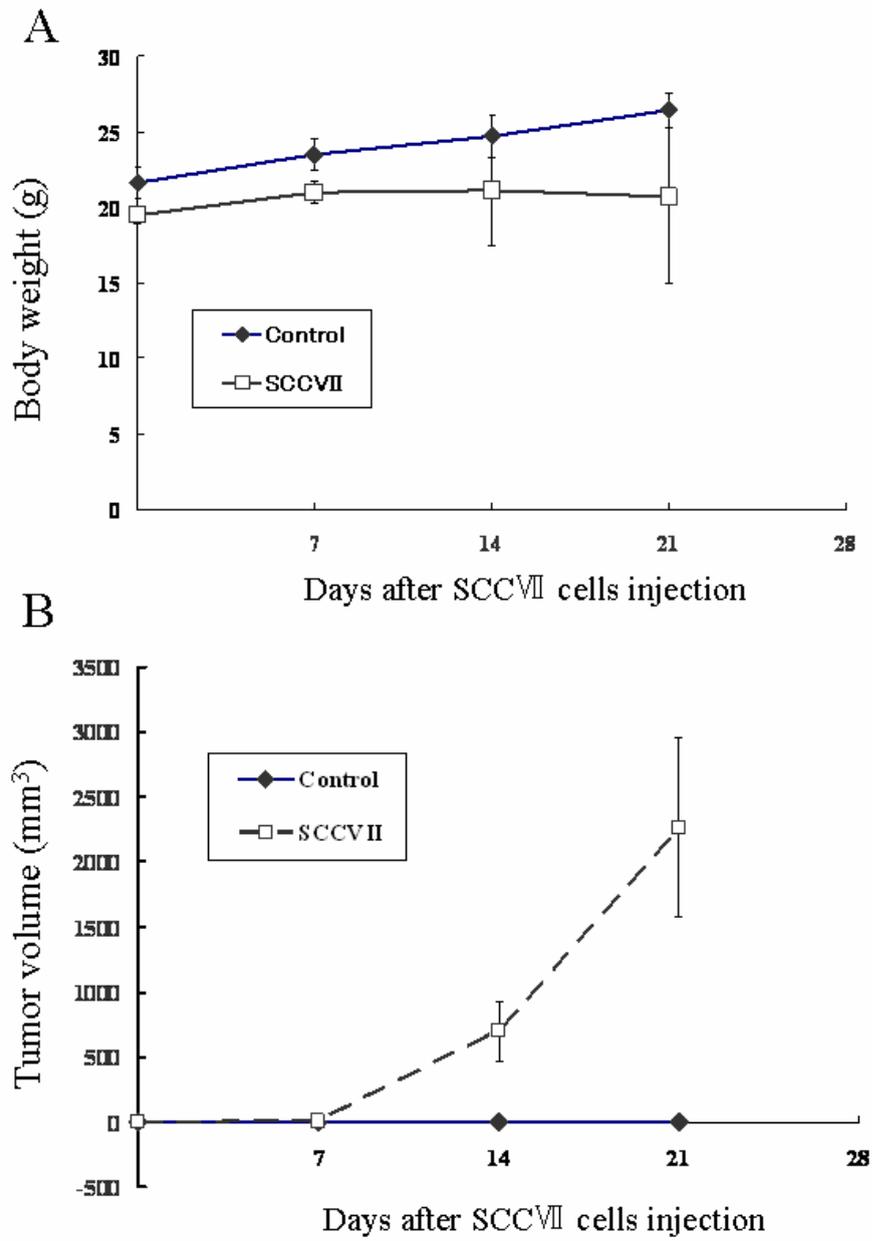


FIGURE 2. Body weights (A) and tumor size (B) of animals control and SCCVII transplanted group. Bars:SD.  $p < 0.01$  by Student's *t* test versus Control group.

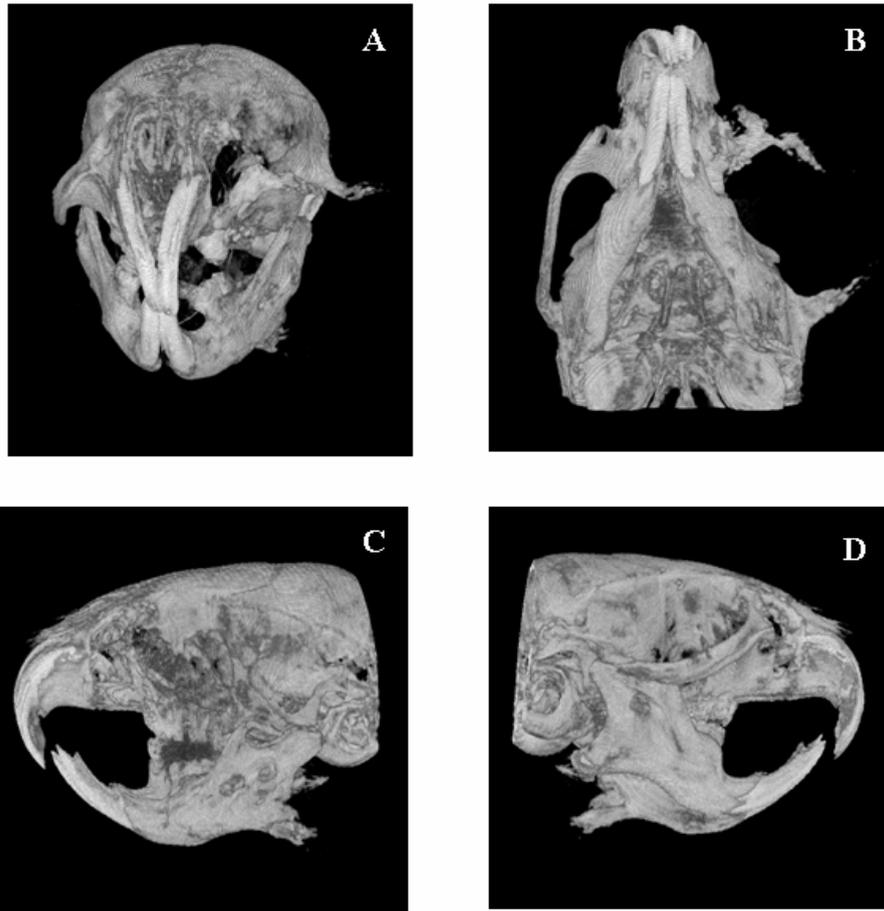


FIGURE 3. 3-D reconstruction of the skull. SCCVII transplanted group show looking from the front (A), from the bottom (B), form the left side (C), and from the right (normal) side (D). The mandible and zygoma were severely destroyed.

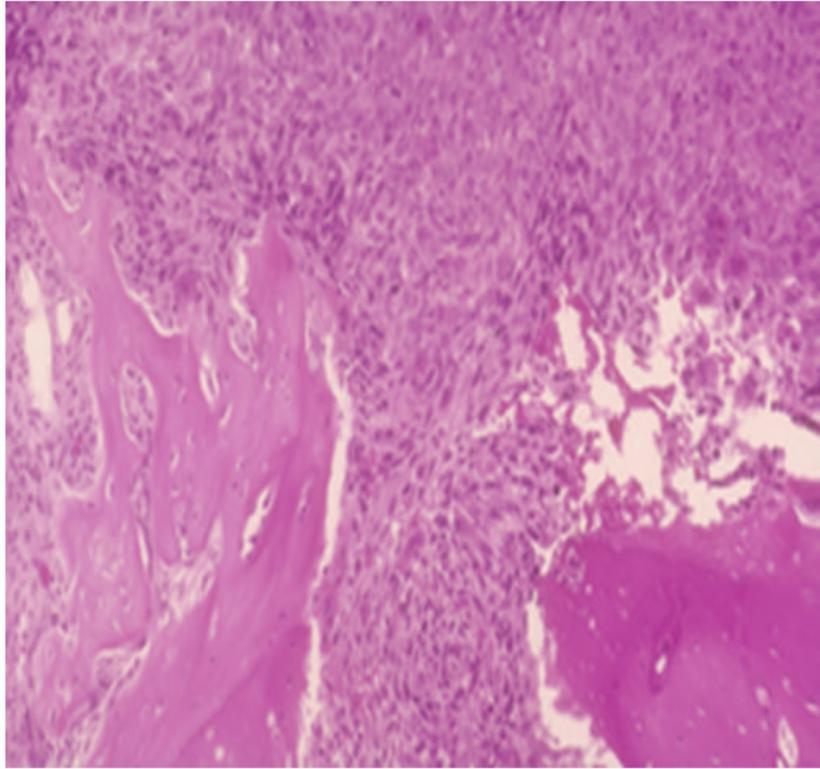


FIGURE 4. Photomicrograph showing exhibition funicular invasion and presented a serrated pattern of bone resorption in the SCC<sup>VII</sup> transplanted group (hematoxylin and eosin stain original magnification x200).

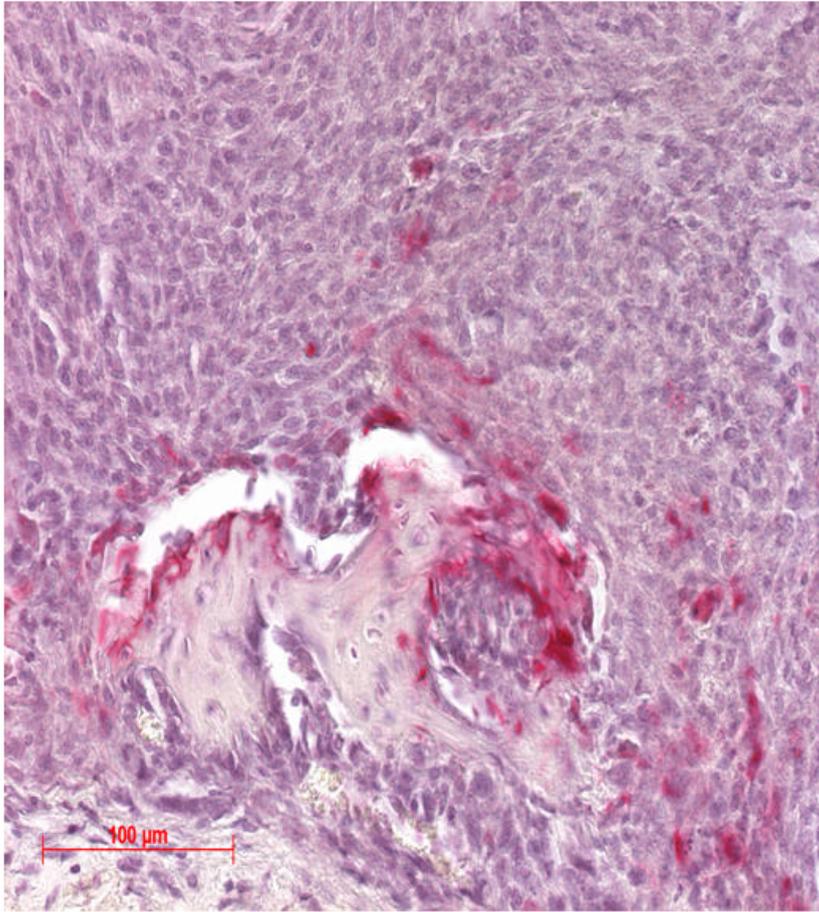


FIGURE 5. Photomicrograph showing the osteoclast violet and evidence of accumulated osteoclasts in considerable numbers around the bone in the SCCVII transplanted group (TRAP stain original magnification x400).

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**IL-6 and TNF- $\alpha$**

**Denaturing** : 94°C , 1 min

**Annealing** : 51°C , 1 min

**Extention** : 72°C , 1 min

**Cycle** : 35cycles

**PTHrP**

**Denaturing** : 94°C , 1 min

**Annealing** : 53°C , 1 min

**Extention** : 72°C , 1 min

**Cycle** : 35cycles

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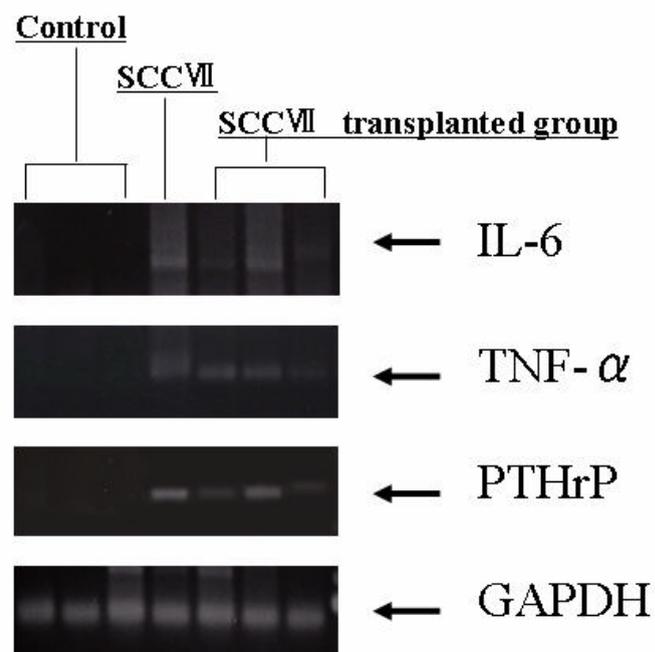


FIGURE 6. SCCVII cells and the SCCVII transplanted group showed mRNA Expression of IL-6, TNF- $\alpha$ , and PTHrP. The control showed no evidence of expression. The PCR system of IL-6, TNF- $\alpha$ , and PTHrP are shown in upper table.

**Table 1. The primer sequences of IL-6, TNF- $\alpha$ , and PTHrP**

| <b>Cytokine</b>                |                  | <b>Nucleotides</b>             |
|--------------------------------|------------------|--------------------------------|
| <b>IL-6</b>                    | <b>sense</b>     | <b>ACTGATGCTGGTGACAACCACG</b>  |
|                                | <b>antisense</b> | <b>GTTTTCTGCAAGTGCATCATCG</b>  |
| <b>TNF-<math>\alpha</math></b> | <b>sense</b>     | <b>GAAAGCATGATCCGCGACGTGG</b>  |
|                                | <b>antisense</b> | <b>GTAGACCTGCCCGGACTCCGCAA</b> |
| <b>PTHrP</b>                   | <b>sense</b>     | <b>GCGACGATTCTTCCTTCACC</b>    |
|                                | <b>antisense</b> | <b>AGAGTCTAACCAGGCAGAGC</b>    |
| <b>GAPDH</b>                   | <b>sense</b>     | <b>GGATGACCTTGCCACAGCCT</b>    |
|                                | <b>antisense</b> | <b>CATCTCTGCCCCCTCTGCTGA</b>   |

**Table 2. Percentages of mRNA of 3 cytokines in control and SCCVII transplanted group (%)**

|                                | IL-6        | TNF- $\alpha$ | PTHrP       |
|--------------------------------|-------------|---------------|-------------|
| <b>Control group</b>           | <b>0</b>    | <b>0</b>      | <b>0</b>    |
| <b>SCCVII translated group</b> | <b>56.3</b> | <b>68.8</b>   | <b>93.8</b> |

*p*<0.05 versus Control group by  $\chi^2$  test of cross tabulation

*p*<0.01 versus Control group by  $\chi^2$  test of cross tabulation