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I am submitting herewith a dissertation written by Necip Fazil Erdem entitled “Characterization and comparative analysis of gene expression profiles of three different human oral squamous carcinoma cell lines with different invasion and metastatic capacities”. I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine

David A. Gerard, PhD

Major Professor

We have read this dissertation and
recommend its acceptance:

Eric R. Carlson, DMD, MD

Jack E. Gotcher, DMD, PhD

Darryl L. Millis, DVM, MS

Accepted for the Council:

Carolyn Hodges
Dean of the Graduate School

(Original signatures are on file with official student records)

**Characterization and comparative analysis of gene expression profiles
of three different human oral squamous carcinoma cell lines with
different invasion and metastatic capacities**

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Abstract

Matrix metalloproteinases (MMP's) and cathepsins are thought to play key roles in oral squamous cell carcinoma (OSCC) invasion and metastasis. Three OSCC human cell lines, BHY, HN and HSC-3, have been studied based on their reported ability to invade adjacent bone or metastasize to lymph nodes and/or distant organs. The working hypothesis of this study is that OSCC that demonstrate different actions have different proteolytic enzyme and gene expression profiles.

Immunocytochemistry and flow cytometry were used to determine the expression levels of certain proteolytic enzymes. Complementary DNA (cDNA) microarray technique was used to determine the gene expression profiles of each of the three OSCC cell lines.

The immunocytochemistry and flow cytometry results showed that the BHY cell line expresses MMP- 9 and extracellular matrix metalloproteinase inducer (EMMPRIN). The BHY cell line expressed 139 upregulated genes and 117 downregulated genes. The HN cell line expressed 214 upregulated and 262 downregulated genes. Finally, the HSC-3 cell line expressed 128 upregulated and 117 downregulated genes. There were 13 genes that were upregulated and 83 genes that were downregulated in all three OSCC cell lines. Eight pathways were upregulated and 3 pathways were downregulated in the BHY cell line. In the HN cell line 10 pathways were upregulated and 9 were downregulated. Finally, in the HSC-3 cell line 3 pathways were upregulated and 6 pathways were downregulated.

MMP-1, MMP-9 and EMMPRIN appear to play a role in the invasion of OSCC to bone. KIAA, was upregulated in our study in the HN cell line. Upregulation of KIAA may be involved in the late stage of OSSC. PAM gene was the only downregulated gene in the HN cell line. The TM4SF10 gene was downregulated in all three OSCC cell lines. The COL1A2 and COL4A1 genes were upregulated in the HSC-3 cell line and they may cause the lymph node metastasis of OSCC. Based on this study OSCC involves multiple molecular genetic events that take place in many chromosomes and genes. Upregulation of the Jak-STAT signaling pathway may be involved in bone invasion of OSCC. The MAPK signaling pathway was upregulated in all three OSCC cell lines.

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CHAPTER 1

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common cancer of the oral cavity accounting for ninety percent of all diagnosed oral malignancies. OSCC accounts for approximately 6 % of all human malignancies (6, 96). There is little published information on the capability of some OSCC to invade adjacent bone while other OSCCs do not. Additionally, some OSCCs exhibit metastasis to cervical lymph nodes and/or distant organs while others do not readily metastasize.

In the United States, OSCC is the sixth most common cancer in males (6, 57) and the twelfth most common cancer in females. Approximately 21,000 new cases are diagnosed annually in the United States, and 6,000 of these patients die of this disease each year. Some risk factors for developing OSCC include tobacco use, alcohol consumption, human papilloma virus and syphilis. Other intrinsic factors may include general malnutrition such as vitamin A deficiency and iron deficiency anemia. The treatment of OSCC is radical surgical excision and/or radiation therapy, depending on the clinical stage of the disease. A variety of chemotherapeutic agents are used as adjunctive therapies, but none of them has significantly improved survival rates. As with most tumors, the survival rate decreases with evidence of bone invasion and cervical metastases (6). OSCC may invade tissue locally (1, 2, 3), and often to adjacent bone (1, 4, 7). Also, OSCC often metastasizes to cervical lymph nodes (1, 2, 3) and distant organs (5). In addition, if the tumor invades nearby bone the survivability is significantly lower (6). Resection of OSCC with bone invasion dramatically affects functions such as

swallowing and speaking, and patient esthetics. Thus, reconstruction of the area is a necessity. Tumor usually invades bone through osteolysis, by greatly increasing the number of active osteoclasts at the invasion site (7). This strongly suggests that tumor cells involved with bone invasion promote differentiation and activation of osteoclasts (7). Proteolytic enzymes such as various matrix metalloproteinases (MMP's) (1, 2, 3, 8, 13) and cathepsins (1, 4) are thought to play key roles in OSCC invasion and metastasis, and have been shown to be expressed by OSCC cells (4, 8, 9, 10).

Some studies have focused on the effect MMP-1 (1, 10, 18), MMP-2 (1, 9, 10, 17), MMP-3 (2, 17, 18) and MMP-9 (1, 9, 10, 19) along with cathepsin B, H, L, and D (1, 4) on the ability of OSCC to invade bone. Production of MMP-1 and MMP-9 appear to play a role in the invasion of OSCC to bone (8, 10). These two metalloproteinases are necessary for the complete degradation of the collagen matrix of bone, and have been shown to be expressed by OSCC (10). MMP-2 does not appear to be related to OSCC invasion, but is highly expressed in some OSCCs (9). Over expression of MMP-3 in OSCC is associated with diffuse invasion and a high incidence of metastasis (2). In tumor cells, extracellular matrix metalloproteinase inducer [EMMPRIN (CD 147)] can stimulate the production of MMPs. It also participates in invasion and metastasis by facilitating tumor cell motility through the surrounding matrix (13). Cathepsin B, H and D have been strongly implicated in the invasion of OSCC, with over expression of these proteins thereby appearing to promote tumor growth and invasion (4). Cathepsin B and L are the two most important cysteine proteinases implicated in tumor cell invasion and metastasis (12).

Why does one patient with a certain type of cancer respond well to treatment, while other patients with histologically and anatomically identical disease do not? Why do some tumors grow more aggressively than others even though they are the same type of tumors? The key to resolving the enigma of individual tumor behavior clearly lies in understanding its molecular structure. cDNA microarray analysis is one of the strongest tools for mapping molecular signatures because with this technique, instead of working on a "one gene in one experiment", it is possible to observe the whole human genome on a single chip. Thus, researchers can have a better picture of the interactions among thousands of genes simultaneously. Briefly, with this technique it is possible to evaluate the gene expression profile of any type of cell or tissue.

In this study, 3 OSCC human cell lines, BHY, HN and HSC-3, were chosen for study based on their reported ability to invade adjacent bone or metastasize to cervical lymph nodes and/or distant organs. Also, a normal human nasal epithelial cell line (HNEC) was chosen as a control group. The working hypothesis of this study was that OSCC's that demonstrate different actions have different proteolytic enzyme, gene and pathway expression profiles. OSCC's that invade adjacent bone will have different expression profiles from OSCC's that metastasize to cervical lymph nodes and/or distant organs. Thus, identification of the gene expression differences of these three cell lines will help to determine which genes and pathways are responsible for lymph node or distant organ metastasis or bone invasion of OSCC. Genes, pathways or their related proteins that are associated with tumor metastasis or invasion will provide a basis for further studies investigating metastatic progression and local invasiveness of this tumor, and can be evaluated as therapeutic targets.

The four cell lines; BHY, HN, HSC-3 and HNEC were morphologically characterized and the level of differentiation and any possible organelle abnormalities studied using transmission electron microscopy. Micro positron emission tomography (PET)/computerized tomography (CT) imaging techniques were used to image the invasion and/or metastatic potential of each cell line in immuno-compromised *scid* mice. This technique also showed the differences in jaw bone invasion and distant organ metastasis of these three OSCC cell lines in a biological system. The 3 OSCC cell lines and HNEC were studied *in vitro* with both immunocytochemistry and flow cytometry to determine if MMP-1, MMP-2, MMP-3, MMP-9, and EMMPRIN and cathepsin B and L, were expressed. Finally, gene regulation of the three OSCC cell lines was investigated and compared to the HNEC cell line by using the cDNA microarray analysis method.

CHAPTER 2

LITRATURE REVIEW

2.1. Oral Squamous Cell Carcinoma (OSCC)

Oral squamous cell carcinoma (OSCC) is a malignant transformation of keratinocytes in stratified squamous epithelium (6). It is the most common cancer of the oral cavity (6, 9, 46, 52). This malignancy is an important public health problem worldwide with more than 500,000 new cases diagnosed each year (6). OSCC can metastasize to cervical lymph nodes (1, 2, 3, 9, 52) and distant organs (5, 9), and invade the jawbone which results in significantly decreased survival (6, 9).

The etiology of OSCC can be genetic from alterations of proto-oncogenes and tumor suppressor genes (25, 58, 70, 104, 107). Other common etiologies of OSCC are:

- Alcohol consumption and smoking (58, 104). Smoking and drinking alcohol in combination increases the risk of developing oral cancer by 15 times (6).
- Viral infections such as; Human Papilloma Virus (HPV), Herpes Viruses, Epstein Barr Virus (EBV), Human Immunodeficiency Virus (HIV).
- Candidiasis (58, 104).
- Exposure to high amounts of X-rays and UV rays (104).

Diagnosis of OSCC can be made by intraoral examination and biopsy, PET/CT imaging, and toluidine blue staining. Toluidine blue staining may permit early diagnosis of OSCC. The development of OSCC occurs in a stepwise fashion. It begins with dysplasia, progressing to carcinoma in situ, and finally to invasive and metastatic squamous cell carcinoma (10).

Clinically, early OSCC may present as a white lesion (leukoplakia), a red lesion (erythroplakia), or a combination of both (erythroleukoplakia). Superficial ulceration of the mucosal surface may develop. As the lesion progresses, it may become an exophytic mass with a fungating or papillary surface. Some tumors demonstrate an endophytic growth pattern that is characterized by a depressed, ulcerated surface with a raised, rolled border (6). Pain is not a reliable indicator because most oral cancers are asymptomatic, or may be associated with only minor discomfort in the early stages (6). Head and neck SCC mainly occurs in the oral cavity and larynx. Two thirds of the patients diagnosed with this tumor are in advanced stages (III and IV). OSCC is generally seen after the age of 40 and 2/3 of the cases occur in males (6).

Histopathologically, OSCC demonstrates larger nuclei and cell size, and prominent nucleoli. The nucleus-to-cytoplasm ratio is increased in OSCC. Hyperchromatic (excessively dark-staining) nuclei, dyskeratosis (premature keratinization of cells), and increased and / or aberrant mitotic activity are also common pathological characteristics of OSCC (6).

The treatment of OSCC is complex and multidisciplinary. Surgery, radiotherapy, and chemotherapy or the combinations of these are the mainstay of treatment (100). During surgical treatment, resection of the tumor with an uninvolved margin is vital to success (6). If there is invasion of underlying bone, resection of bone with its periosteum should be performed. Instead of surgery, radiation and chemotherapy may be employed. Multiple chemotherapeutic agents and combinations can be used. Platinum and 5-fluorouracil are the common drugs that have been used for chemotherapy. Following

surgical treatment, 6000-6400 cGy of external beam radiation therapy is commonly administered in daily 200 cGy fractions. (209).

The prognosis of OSCC is poor (9, 44). Early recurrence and rapid progression is common (3) and the 5-year survival rate is approximately 50% (93, 97). Twenty five percent of all patients develop a second cancer within 5 years from diagnosis (97). The prognosis of patients with OSCC is directly correlated with the histologic status of the cervical lymph nodes (9). Proteolytic degradation of the extracellular matrix (ECM) is required for tumor cells to invade the basement membrane, stromal matrix and vascular tissue (8, 33).

2.2. BHY, HN, HSC-3 and HNEC Cell Lines

The 3 OSCC cell lines chosen for this project included BHY, HN, and HSC-3. As a control group, normal human nasal epithelial cell (HNEC) was chosen.

2.2.1. BHY cell line

The BHY cell line was isolated from a 52-year-old Japanese male. The primary squamous cell carcinoma was located on the lower alveolus, and the tumor was highly differentiated. In this patient the tumor invaded the mandible but did not metastasize. Kawamata et al. showed that BHY cells actively invade mandible when injected into the masseter muscle of nude mice, but did not metastasize to cervical lymph nodes or any distant organs (1).

2.2.2. HN cell line

The HN cell line was isolated from a 60-year-old Japanese male. The primary squamous cell carcinoma was located on the soft palate, and was moderately differentiated. The tumor had metastasized to cervical lymph nodes, lungs and brain but

had not invaded bone. Kawamata et al. reported that HN cells injected into the masseter muscle of nude mice weakly invaded the surrounding masseter muscle but not the mandible; however, these cells did metastasize to cervical lymph nodes (1).

2.2.3. HSC-3 cell line

The HSC-3 cell line was isolated from a 64-year-old Japanese male. The primary squamous cell carcinoma was located on the tongue with lymph node metastasis, and was moderately differentiated. This cell line is highly metastatic to cervical lymph nodes (10, 23, 52).

2.2.4. HNEC cell line

The HNEC cell line was isolated from a 27-year-old Caucasian male, and represents a normal human nasal epithelial cell line.

2.3. Invasion and Metastasis of OSCC

Metastasis is the process by which cancers spread to regional and distant sites in the body (110). Invasion is the movement of cancer cells into the surrounding normal tissues. Both metastasis and invasion are multi-step processes that require a variety of genetic alterations in the tumor cells. Bone is one of the most common sites of tumor metastasis (7). In the oral cavity, OSCC frequently invades maxillary and/or mandibular bone. However, the metastatic and invasion mechanisms of OSCC are poorly understood. It has been shown that bone resorption by osteoclasts is an important step of bone invasion and metastasis in OSCC (14, 15, 16). Osteoclasts, rather than carcinoma cells, mediate bone destruction in carcinoma invasion and metastasis (7, 14, 15). In so doing, tumor cells promote differentiation and activation of osteoclasts (7). Osteoclast precursor cells are directly apposed to tumor cells in some instances, which means that tumor cells may

participate in the differentiation of osteoclasts. However, stromal cells or extracellular matrix (ECM) usually exist between these two cells (Figure 1¹). Direct contact of osteoblasts or stromal cells with stem cells is needed for osteoclast differentiation. Therefore, the interaction between stromal cells and osteoclast precursor cells is important in the process of osteoclast formation. Heparin-sulfate proteoglycan (HSPG) and fibronectin are the two representatives of the components of the extracellular matrix. HSPG can be observed in the stroma around the tumor nests, but not in the tumor cells. HSPG is also positive in the stroma away from the bone surface. HSPG binds a bone resorption-activating factor that is released from osteoblasts. Fibronectin shows the same pattern of localization as seen with HSPG. Since many osteoclast precursor cells can be observed in a stroma rich ECM component, they may play an important role in providing a micro-environment for osteoclast differentiation and activation (7). In addition, interleukin-6 (IL-6) secreted by oral cancer cells may enhance bone invasion of cancer cells mediated by induction and activation of osteoclasts (7, 20).

The proliferation and progression of cancer may be caused by abnormalities in various positive and negative cell cycle regulators (25, 68, 133). Cell cycle progression is positively regulated by multiple cyclins and cyclin-dependent kinases (CDKs). Cyclin/CDK complexes are activated through phosphorylation by the CDK-activating kinase, but are negatively regulated by a number of CDK inhibitors including p27 (25, 68). In oral carcinogenesis, p53 over-expression is an early event (25, 46, 68, 70). Loss of the p27 protein is also closely associated with early invasion and metastasis in OSSC. Aggressive cancers express low levels of p27 because of its decreased stability. Thus

¹ All figures are located in the Appendix B.

metastatic OCSS shows reduced expression of p27 in comparison with that in the primary lesion as well as in non-neoplastic mucosa (25, 26, 27, 28, 29, 30, 31, 32, 68).

Tumor growth, invasion and metastasis also depend upon angiogenesis, formation of new blood vessels from pre-existing endothelium (46, 51, 52, 55). Vascular endothelial growth factor (VEGF) (50, 51, 52, 53, 56) and fibroblast growth factor (FGF) (50) are considered the most important mediators in tumor angiogenesis. Shang et al. showed that VEGF concentration is increased in patients with OSCC (51). Moriyama et al. reported that the intensity of VEGF expression is significantly higher in metastatic positive patients than in metastatic negative patients with OSCC (53). VEGF promotes angiogenesis both *in vivo* and *in vitro* (46, 47, 50, 51). In addition to inducing cascades of angiogenic events, VEGF is also known as a vascular permeability factor (46, 50, 52, 56). When blood vessel permeability increases, VEGF permits creation of an extravascular fibril gel that supports endothelial cell growth and thus the tumor (50, 51). The tumor suppressor gene, p53 is a cell cycle regulator and DNA damage sensor. Loss of p53 function is associated with increased angiogenesis by upregulation of VEGF expression (46). There are 4 members of VEGF family, including VEGF A, B, C, and D. Shintani et al. found that VEGF A and B can be detected on both lymph node positive and negative cases, whereas VEGF C and D can only be detected in lymph node positive tumors. This suggests a possible relationship between the expression level of VEGF C and D and development of lymphatic tumor spread (47). Thrombospondin-1 (TSP-1) is a 450 kd glycoprotein synthesized and incorporated into ECM by numerous cell types and reported to suppress tumor growth and progression by its inhibition of angiogenesis. The level of TSP-1 is inversely associated with the pattern of tumor invasion and lymph node

metastasis. The reason for this might be that TSP-1 suppresses the proliferation and migration of vascular endothelial cells *in vitro*, and suppresses neovascularization *in vivo* (54).

In addition to VEGF, interleukin-8 (IL-8) is also a potent angiogenic factor and it plays an important role in cancer progression and metastasis (48, 125, 126). IL-8 is responsible for most of the angiogenic activity induced by human OSCC (49, 126). Lalla et al. showed that at a specific dose, fibrin causes upregulation of IL-8 expression in human OSCC cells *in vitro*, whereas it doesn't stimulate IL-8 formation in non-tumorigenic oral epithelial cells. This means that fibrin can be a pluripotent activator in OSCC. IL-8 transfected cells, show an upregulation in MMP-2 and MMP-7 levels that causes the enhancement of invasion and metastasis (48, 126). Beside this, perilymphatic recombinant interleukin-2 (IL-2) application combined with surgery and radiotherapy increases the disease free interval and survival of OSCC patients with T2-T4, N0-N3, M0 staging (127).

2.3.1. The effect of MMPs and EMMPRIN on invasion and metastasis of OSCC

Proteolytic enzymes play an important role in tumor invasion and metastasis by degrading the ECM (9, 13, 41, 42, 97). They are secreted by a variety of normal cells and tumor cells; however, the difference between normal and pathological processes is in the regulation of their activity (1). One of the groups of proteolytic enzymes is the matrix metalloproteinase (MMP) family, which is a family of zinc-dependent proteinases (9, 38, 42). The MMP family consists of 16 related members. Based on their structural and functional properties, they can be classified as collagenases, gelatinases, stromelysins, and membrane type MMPs (MT-MMPs) (9, 10, 42). In addition, there are 4 different

tissue inhibitor matrix metalloproteinases (TIMPs-1 to -4) that inhibit the function of MMPs (22). MMPs have specific functions in turnover of ECM. They play an integral role in the biological processes of development, tissue repair, and metastasis by regulating cell proliferation, differentiation, adhesion and migration. The production of these proteins by normal cells is regulated by growth factor and cytokines (42). It has been shown that they play a key role in promoting invasiveness of both normal and neoplastic cells (13, 21, 38, 42). Also, they play important roles in wound healing, tissue repair, bone resorption, angiogenesis and developmental tissue morphogenesis (10, 38, 42). Table 1² summarizes the role of MMPs in normal and pathological processes (21).

To establish invasion and metastasis, the first step is the active migration of cancer cells from their tissue of origin. This process involves the degradation of underlying basement membrane, which is made of collagens, laminin, and heparin sulfate proteoglycans (42, 44). OSCC can produce several collagen and gelatin-degrading enzymes such as MMP-1, MMP-2, MMP-7 and MMP-9 (1). Also, tumor cells can activate the proMMPs (zymogen forms) that are released mainly by the peritumor fibroblasts and/or the tumor cells themselves at the invasive edge of the tumor (1, 13). Several mechanisms can account for the secretion of MMPs and thus their mediation in tumor cell invasion. First, tumor cells can produce MMPs themselves, thus facilitating their own invasive behavior. Second, the neighboring stromal cells may produce the proteases required for tumor cell invasion. Third, an interaction between stromal and tumor cells may exist, resulting in increased MMP production and activation which enhances invasion (10, 44). After the tumor cells degrade the ECM, they enter the

² All tables are located in the Appendix A.

circulation. They then penetrate through the sub-endothelial basement membrane of lymphatic channels and capillary vessels (9, 44). This event largely depends on the activation of ECM degrading enzymes, such as MMPs that are produced by the tumor cells and the stromal cells (1, 9, 44). The MMPs that are synthesized by stromal cells are the proforms of MMPs. Then, the tumor cells receiving the proenzymes from the surrounding stromal cells activate them by their own MT1-MMP (44). Epidermal growth factor (EGF) has been shown to be a major constituent of serum to induce MMP-1, and to enhance ECM collagen degradation (2, 10). Collagenase MMP-1 initiates the degradation of type I collagen, which is one of the most abundant proteins of the ECM. Once cleaved, type I collagen denatures into gelatin and ultimate degradation of gelatin is accomplished by the action of gelatinases, which are MMP-2 and MMP-9 (9, 10, 44). Production of MMP-1 is required for complete collagen matrix degradation and is expressed in OSCC. Thus, MMP-1 appears to be a major factor in invasive OSCC (10). Another essential step in the invasive and metastatic nature of tumor cells is the degradation of type IV collagen in the basement membrane by MMP-2 and MMP-9 (9, 38, 41, 42, 44). However, tumor cells must also traverse interstitial stroma, composed of collagen III and I. Thus, to be invasive the tumor cells must be able to degrade interstitial collagen. Degradation of interstitial collagen is most effectively accomplished by collagenases MMP-1, MMP-8, MMP-13 and to some extent MMP-2 and membrane type 1 MMP (MT1-MMP) (10, 112).

Similar to other MMPs, MMP-2 is secreted in an inactive form (proMMP-2). Its conversion to an active form (MMP-2) is due to the MT1-MMP. In fact, proMMP-2 binds to TIMP-2 in combination with the MT1-MMP on the cell surface, forming a

ternary complex. Then, proMMP-2 in the complex is activated by adjacent MT1-MMP that is freed from TIMP-2 (43).

Hong et al. reported that the total activity of MMP-2 in OSCCs was 3.64 times higher than in normal mucosa, but in OSCC without metastasis it was similar to that in OSCC with metastasis (9). On the other hand, the total activity of MMP-9 in OSCCs was 2.81 times higher than in normal mucosa and in OSCC with metastasis, this was 1.54 times higher than when there was no metastasis (9). Although cancer cells that secrete MMP-2 and MMP-9 can become invasive, Hong et al. suggest that MMP-9 might play a more important role than MMP-2 during this process (9). According to the results of another study, OSCC patients with T3 and T4 tumors had significantly higher MMP-2 levels than T1 and T2 tumors. Also MMP-2 proportion in lymph node metastasis cases was significantly higher than that in non-lymph node metastasis cases. In contrast to MMP-2, the activated form/proform ratio of MMP-9 was very low, suggesting that MMP-9 is not activated in the matrix degrading of OSCC, although both MMP-2 and MMP-9 protein expression are present in the tumor cells (44). MMP-2 and MMP-9 are highly expressed in human OSCC with metastasis compared to normal oral keratinocytes (38, 97). MMP-2 and MMP-3 expression in OSCC is closely related to lymph node metastasis and vascular invasion (34). In addition, it has been reported that OSCC producing MMP-2 is prone to metastasize to lymph nodes more frequently than OSCC with no MMP-2 expression (35). Miyajima et al. showed that MMP-9 production is closely related to lymph node metastasis (36). Charous et al., however, found that there is no difference in MMP-2 and MMP-9 expression levels between primary and metastatic OSCC (37).

Besides MMP-2 and MMP-9, MMP-3 can also degrade collagen type IV. Thus, it is important to analyze the expression of MMP-3 in OSCC. EGF stimulates the production of MMP-3 coordinately with MMP-1 (2). Interestingly, the over expression of the EGF receptor (EGFR) is a remarkable property of squamous cell carcinoma (2, 56, 70). It is not clear, however, whether EGFR overexpression is implicated in the increased production of MMP-3 by oral squamous carcinoma. The expression of the EGFR has a significant correlation to the T-category of the primary tumor, pathological stage, and neck metastasis (2). This explains why tumors with EGFR over expression have a poor prognosis when compared to EGFR negative tumors (56, 133). On the other hand, there is a significant correlation between the expression of MMP-3 and pathological stage, mode of invasion and neck metastasis. EGF, platelet-derived growth factor (PDGF), interleukin-1 (IL-1), oncogenes such as *v-src* and *ras*, can induce the production of MMP-3. The more MMP-3 produced by oral carcinoma cells, the more diffusely and aggressively the tumor invades to adjacent normal tissue, including lymphatic and blood vessels (2).

E1AF is an ets-oncogene family transcription factor that is located on chromosome 17q21 and can upregulate the promoter activities of MMP-1, MMP-3 and MMP-9 in highly invasive OSCC. Cells transfected with an antisense E1AF expression vector cause down regulation of MMP-1, MMP-3, and MMP-9 and reduce the invasive ability of cancer cells. Therefore, this gene is closely associated with invasion and metastasis of cancer cells by activating MMP genes. It has been found that the incidence of E1AF expression is more frequent in invasive tumors than in expanding tumors. Also, E1AF mRNA can be detected in cancer cells invading into stromal connective tissue (8). Over

expression of the integrin $\alpha\beta 6$ (a receptor for fibronectin, vitronectin, tenascin and TGF- β latency-associated peptide) is found consistently in OSCC (116, 117, 123) whereas, it is not detectable in normal oral epithelium (113, 114, 115). The upregulation of integrin $\alpha\beta 6$ promotes OSCC invasion, which is associated with upregulation of urokinase-type plasminogen activator (uPA)/plasmin system, thus the proteases including MMP-9 and MMP-3 (113, 114). However, integrin $\alpha\beta 6$ does not activate collagenases (MMP-1, MMP-8 and MMP-13) but down regulates MMP-13 activation (112).

Extracellular matrix metalloproteinase inducer (EMMPRIN [CD147]) is a transmembrane glycoprotein that belongs to the immunoglobulin superfamily and can induce the production of MMPs and VEGF by both the stromal and tumor cells (13, 40, 134). It can also mediate a wide range of tumor-promoting molecular events that include acquisition of anchorage-independent growth and invasive phenotype by tumor cells as well as tumor-cell-induced angiogenesis (134). Bordador et al. showed that EMMPRIN is highly expressed in OSCC and may help to regulate OSCC invasion, and modulate the expression of MMP-2 (13). EMMPRIN is not only highly expressed in OSCC but also in oral premalignant cells. Even though the level of EMMPRIN is high in oral premalignant cells, they do not release it to the microenvironment as OSCC does. Primary and metastatic OSCC show strong cell surface expression of EMMPRIN. Its overexpression occurs at a very early stage of oral carcinogenesis and plays a contributing role in OSCC tumorigenesis. Abnormal high levels of EMMPRIN expression by cancer cells have been attributed to dysregulation of EGFR signaling (134). Generally fibroblasts produce low level of MMPs, however, tumor associated fibroblasts do produce high levels of MMPs

as they are stimulated or influenced by EMMPRIN (39). Chromosome 19p13.3 is the location for the EMMPRIN gene with the size of 10.8 kb (40).

BHY and HN OSCC cell lines both express a large amount of matrix-degrading enzymes, such as proMMP-1, proMMP-2, proMMP-9, MT1-MMP and uPA, which converts plasminogen to plasmin (1). Induction of tissue-type plasminogen activator (tPA) and uPA can mediate the activation of proMMP-2 and proMMP-9 in OSCC and thus enhance invasiveness (3, 97). In addition, BHY cells can secrete proMMP-7, while HN cells secrete active MMP-2 (1), which plays an important role in lymph node metastasis of OSCC (3). Thus, MMPs play an important role in the invasion and metastasis of oral cancer cells. The level of TIMP-1 mRNA in BHY cells and TIMP-2 mRNA in HN cells is low (1). Although both HN and BHY cells express MT1-MMP mRNA, the level of MT1-MMP is much lower than that in HT1080 cells, which is a human fibrosarcoma cell line (1).

Another OSCC cell line used in this study is HSC-3. In response to EGF, HSC-3 cells are able to degrade type I collagen matrix, suggesting that EGF can induce the secretion and activation of the type I collagen proteolytic enzyme MMP-1 (10). Both HSC-3 cells grown without EGF and with EGF can produce MMP-9, proMMP-2 and MMP-2. In the absence of EGF, the MMP-2 and the MMP-9 secreted from HSC-3 are not capable of degrading type I collagen. In HSC-3, EGF increases the secretion of collagenase, MMP-1. Then, MMP-1 changes collagen I to gelatin. The gelatinases, MMP-2 and MMP-9, complete the collagen I degradation by breaking down the gelatin (10). HSC-3 does not produce MMP-1 constitutively but it is up regulated in response to EGF and collagen I (10).

2.3.2. The effect of cathepsins on invasion and metastasis of OSCC

Cathepsins are another group of proteolytic enzymes that are responsible for tumor invasion and metastasis. There are 13 different cathepsins which are located in lysosomes. Cathepsin B, H, and L are expressed in all tissues, while cathepsin S is selectively expressed in antigen presenting cells. Cathepsins become mature enzymes either by other proteinases or by autocatalysis. Cathepsin K is highly expressed in osteoclasts and may play a major role in bone resorption. Also, there are low levels of cathepsin L, S and B in osteoclasts. Catalytic activity of cathepsins is dependent on a pH below 7.0. They are unstable at neutral and alkaline pH values. Cathepsins are associated with cancer, inflammation, rheumatoid arthritis, osteoarthritis, multiple sclerosis, muscular dystrophy, pancreatitis, liver disorders, lung disorders, diabetes, and myocardial disorders. Cathepsin B cleaves pro-urokinase type plasminogen activator (pro-uPA) and converts it to active form, which is uPA. uPA activates plasmin-plasminogen, which then participates in cellular invasion processes including tumor invasion. Increased expression of cathepsin B correlates with carcinoma progression. It has both endopeptidase and exopeptidase activity and can degrade multiple components of ECM including laminin, fibronectin, and type IV collagen. In normal cells cathepsin B is localized in lysosomes in the perinuclear region. In carcinoma cells lysosomes containing cathepsin B are localized both perinuclearly and at the cell periphery. Cathepsin L is a lysosomal cysteine proteinase with a major role in intracellular protein catabolism. Cathepsin L has endopeptidase activity and is involved in epidermal homeostasis (24).

Cystatins are specific endogenous inhibitors of lysosomal cysteine proteinases, namely cathepsins B, C, F, H, L, O and X. Vigneswaran et al. found that both cathepsin B

and L do not show any significant differences in their expression levels between metastatic and primary OSCC cell lines at mRNA and protein levels. However, they also found that the cystatin M gene is elevated 40-fold in a metastatic OSCC cell line compared to the primary OSCC cell line. Increase in cystatin M mRNA, results in a decrease in the cathepsin B to cystatins ratio in the metastatic cell line relative to the primary OSCC cell line. It is possible that cystatins, when over-expressed in tumor cells, may facilitate metastasis by interfering with TNF-related apoptosis inducing ligand (TRAIL). TRAIL is a type-II transmembrane protein that induces apoptotic cell death in tumor cells but not in normal cells. Over expression of cystatins in oral cancer cells can enhance their metastatic potential by rescuing them from NK-cell mediated apoptosis, an event mediated by inhibiting the pro-apoptotic function of cathepsin B in these cells. Cystatins may have bivalent effects in the progression of malignant tumors. On one hand, reduced expression of cystatins, when combined with over expression of cysteine proteases cathepsin B and/or L, can lead to deregulated proteolytic imbalance promoting tumor growth and invasion. However, over expression of cystatins, combined with normal expression levels of cathepsin B in tumor cells can augment their metastatic potential by protecting them against TRAIL-mediated apoptosis (12).

Cathepsin L has a major role in the process of bone resorption by activating osteoclasts (1). It is known that procathepsin L can be activated in acidic conditions. Thus, procathepsin L secreted from BHY cells can be activated at the point of bone invasion, and the activated cathepsin L might degrade the demineralized bone matrix (1). BHY cells express a large amount of procathepsin L, whereas HN cells don't express any detectable level of cathepsin L (1).

Kawasaki et al. found that cathepsin D and B expression are closely correlated with OSCC invasion and progression. They could not find any relation between cathepsin L expression and invasion of OSCC (4). Cathepsin B is active against large substrates such as laminin and fibronectin under physiological conditions of pH and temperature. It can degrade extracellular matrix components. Turk et al. showed that cathepsin B, H, and L are involved in cancer progression by either direct degradation of extracellular matrix or by activation of other proteases, such as uPA (45). A requirement for cathepsin D to be active is a value of pH <5.5; however, cathepsin B can work at neutral pH. Thus, cathepsin B mainly degrades the basement membrane (4).

2.4. Microarray Studies on OSCC

When oral mucosa is exposed to mutagens, OSCC may arise. This mainly happens if metabolism of carcinogen or pro-carcinogen, repairing mechanism of DNA damage, controlling mechanisms of cell signaling and cell cycling are defective (58, 70). Genetic damage affects many chromosomes and genes, and in some cases it ends up activating oncogenes and inactivating tumor suppressor genes (96). Cancer arises from damage to the DNA of genes located at various points on the short (p) or long (q) arms of a number of chromosomes. OSCC is a consequence of multiple molecular genetic events in many chromosomes and genes (58). The consequence of this chromosomal damage is cell dysregulation with disruption in cell signaling, the cell growth cycle, and mechanisms to repair cell damage or eliminate dysfunctional cells (58, 70, 94, 133). After the formation of such chromosomal changes, cell growth becomes autonomous, and invasive mechanisms develop leading to malignant lesions (58, 133).

Carcinogen residues bind to DNA or surrogate proteins and can cause carcinogen exposure. The most important carcinogens are aromatic amines found in tobacco smoke and cooked foods. Various enzymes known as xenobiotic-metabolising enzymes, are involved in the activation or degradation of carcinogens and pro-carcinogens. The most common of these enzymes are: alcohol dehydrogenase, cytochrome p450, glutathione-S-transferases, and N-acetyl transferase. Alcohol dehydrogenase catalyses the oxidative metabolism of a range of chemicals and activates the pro-carcinogens (58). Alcohol dehydrogenase type 3 genotypes appear predisposed to OSCC (59, 60, 61). Cytochrome p450 can activate many environmental pro-carcinogens by exposing or adding functional groups. Glutathione-S-transferase is involved in the detoxification of activated metabolites of carcinogens (58). This enzyme is also present in oral tissue (62). N-acetyl transferase deactivates carcinogenic aromatic amines through N-acetylation (58).

There is clear evidence of cellular DNA damage in patients with oral cancer. Damage to DNA can be repaired by several mechanisms, the main of which include nucleotide excision repair, base excision repair and *O*⁶-methylguanine DNA methyltransferase. DNA repair capacity varies between individuals as a result of inheritance, environmental factors, and physiological factors (58). In OSCC, several chromosomes, including those involved in the repair of DNA damages, show changes (63, 64, 65, 66, 67).

Cell signaling is important for messages to be able to pass from the plasma membrane to the nucleus. Harvey *ras* gene (*H-ras*) and epidermal growth factor receptor gene (*erbB1*) are two important genes involved in cell signaling, cell growth control and cancer (58, 124). *H-ras* gene may activate MMP-9 and MMP-13, which are involved in

angiogenesis of the tumor itself (135). It is reported that *Rab-1a*, which is a member of the *Ras* oncogene, is highly over expressed in squamous cell carcinoma of the tongue and oral premalignant lesions (109). The *H-ras* gene is located on chromosome 17p and is responsible for protein p21, which is an enzyme with guanosine triphosphatase activity. p21 acts like a signal transduction protein, transmitting mitogenic signals from the cell surface to the cell interior. *erbB1* which maps to 7p13-q22, encodes a receptor involved in cell signaling (58, 124). *H-ras* shows antitumorigenic response mediated by the up-regulation of both p14^{ARF} and p16^{INK4A}, which activate the tumor suppressor genes, p53 and retinoblastoma (Rb). The p16^{INK4A} tumor suppressor gene product acts as a negative regulator of cellular proliferation by interacting with CDK4 and inhibits its kinase activity (94, 118). Its inactivation is frequent (94, 106) and is one of the earliest events in head and neck cancers (118). p15^{INK4B} displays high homology to p16^{INK4A}, acts as an inhibitor of CDK4 and CDK6 and shows a tumor suppressor role. p15^{INK4B} is transcriptionally activated by transforming growth factor- β (TGF- β) (94) and is most frequently inactivated in OSCC (106).

The cell cycle consists of a series of phases that take place during cell division. By using a number of proteins, cell regulatory genes modulate the cell cycle in a highly sophisticated way (68). During cell division, the transition from one phase to the next is checked at a checkpoint before the cycle can progress. The cell cycle consists of a stationary G0 phase, which upon receipt of appropriate cell signals, is followed by G1, S, G2 phases, and mitosis. Checkpoint genes not only control transition between phases of the cell cycle but also coordinate cell cycle progression with cell signals. When DNA is damaged, there may be an arrest of the cell cycle which can facilitate DNA repair. G1

arrest prevents replication of a damaged DNA template and G2 arrest allows segregation of damaged chromosomes (58).

One of the main factors that regulates the cell cycle includes cyclins and their kinases that are called cyclin dependent kinases (69, 94). Protein kinase complex consists of a cyclin, CDK, and proliferating cell nuclear antigen (PCNA). Progression from the G0 phase of the cell cycle through the G1 phase is mediated by two CDK complexes, which are CDK4-cyclin D, and CDK2-cyclinE (69). These complexes are regulated by phosphorylation/de-phosphorylation. Cyclin D1 takes place in controlling of cell cycle by transiting the cells from G1 to S phases by regulating CDK-4 (119). Thus, cyclin and CDK are the most important regulators of cell cycle progression (58, 68, 94). The upregulation of cyclin D1 is commonly seen in OSCC, especially in patients with tobacco chewing history. Its' over expression is related to high cell proliferation activity, poor survival and progression of lymph node spread in OSCC. In mammalian cell cycle control, disorder takes place more commonly in G1 phase. Cyclin D1 expresses in G1 phase and accelerates the cell cycle progression. Human cyclin D1 maps to chromosome 11q13 (119). Another important pathway for cell proliferation is extracellular-signal regulated kinases (ERK MAPKs) pathway, which is one of the mitogen-activated protein kinases (MAPKs). The activation and over expression of ERK MAPKs play an important role in cancer progression (129).

Retinoblastoma (Rb), p21, p27 and p53 are the other factors that regulate the cell cycle (58, 68). Loss of the functional Rb gene is one of the initial genetic events in malignant transformation (108, 118). Rb is a key component of the G1 checkpoint (58, 68, 94). p21 binds to and inhibits cyclin/CDK complexes (58) to effect arrest of the G1/S

phase of the cell cycle (68). It is regulated by p53 in response to DNA damage (68, 94), interacting with PCNA to block its DNA synthesis function (70). A major effect of p53 is to block the cell cycle by stimulating the p21 gene (68). p27 also plays a role in G1 arrest induced by TGF- β in normal cells. It regulates proliferation by binding and inhibiting the G1 cyclin/CDK complexes. The product of the p53 gene is a main inhibitor of the cell cycle. It acts as a transcription factor and controls cell cycle progression and thus cell replication (58, 96). If a cell is damaged and repair is impossible, p53 triggers apoptosis (68). Loss of heterozygosity and mutation at the p53 gene are frequent events in OSCC (94, 105). Mutational inactivation of p53 has been reported to be the determinant of resistance to anticancer agents or radiation treatment (105). p53 blocks the cell cycle by switching on the GADD45 gene in cells when the cell is under stress. The GADD45 protein product binds PCNA and inhibits entry into the cell cycle S phase (58). Also, p14^{ARF} and p16^{INK4A} play active roles in the p53 and Rb growth-control pathway (95).

In OSCC, changes in chromosomes 3, 9, 11 and 17 are common (70, 96). The main targets of these changes are in chromosome 3, 3p24, 3p21.3, and 3p14, where the tumor suppressor genes are located; in chromosome 9, 9p21 where p16 is located; in chromosome 17, 17p13 where p53 is located. Over-expression of oncogenes, genes mainly involved in cell signaling like the ones on chromosome 11 and 17, are common in OSCC (70). In head and neck squamous cell carcinoma there are frequent structural rearrangements of the following chromosomal regions: 3q, 5p13-q11.2, 5q32-q34, 7p12-q11.2, 8p12-q12, 9p, 10p, 13p13-q12, 14q11.1-q11.2, 15p13-q11.2, 16p11.1-q11.1, 18q22-q23, and 22p13-q11.2. Loss of heterozygosity on regions of chromosomes 3, 4, 5, 6, 8, 9, 11, 13, 17, and 19 are common in head and neck squamous cell carcinoma (103,

107, 122), especially on the long arms of the loci (107). Chromosome 3 aberrations can be identified on both potentially malignant oral lesions (71, 72, 73) and OSCC (74, 75, 76, 77, 78, 79, 96). The short arm of this chromosome (3p) is often deleted in OSCC (80, 81). In 31% of OSCC cases there is an abnormality in the long arm of chromosome 4 (4q) at 4q25 (82, 83, 96) and 4q31-32.1 (84). Chromosome 5 anomalies can be seen in 25-73% of OSCC (77, 85, 86, 87, 107). There are changes at both 5q and 5p (88). These changes may be early in carcinogenesis, preceding p53 mutations (89). In chromosome 6, loss of heterozygosity can be found in 21-38% of OSCC on 6p (84, 90, 96) and 23-25% on 6q (84, 90). Genotypic changes in chromosome 7 have been described in OSCC, tissue adjacent to OSCC, and oral premalignant tissue. The EGFR, on 7p, is over expressed in epithelium adjacent to and in OSCC (70). Chromosome 8p deletions and loss of heterozygosity have been reported in 40 % of OSCC (70, 96). The most common chromosomal defect in OSCC can be seen on chromosome 9, specifically the loss of heterozygosity on 9p. Deletion of the long arms of chromosomes 10, 11 and 13 and frequent loss of heterozygosity have been identified in OSCC on 10q, 11q and 13q in 50% of cases (70, 107). A small number of OSCC appear to lose heterozygosity on 12p and 12q, and 16p and 16q. p53, which is located on chromosome 17, is a tumor suppressor gene with one of the highest frequency of mutations in cancers (70). Almost 60 % of OSCC cases have p53 gene mutations (70, 92). In carcinomas, the mutations of p53 are often the same in the primary tumor and lymph node metastasis and in immediately adjacent epithelium as well as in recurrences (70). Loss of heterozygosity at various genetic loci is a sign of losing tumor suppressor genes, as is microsatellite instability causing nucleotide expansion or deletion (111). Loss of heterozygosity has

been detected in OSCC mostly at 18p (91), 21q and with 73% at chromosome 22 (70). Allelic imbalance, especially on chromosome arms 3p, 9p, 11q, 13q and 17p, is an early event in oral carcinogenesis (70, 92, 111). In more advanced stages with invasion loss of heterozygosity of 6p, 8p, 8q, and 4q is commonly seen (111). Allelic loss on several chromosomes, together with the sequence loss of several tumor suppressor genes is consistent with the hypothesis that oral carcinogenesis involves multiple molecular steps (70, 92). Allelic imbalance can be detected in the plasma/serum DNA of OSCC, suggesting that circulating tumor-associated DNA in the blood of patients with OSCC can be a key determinant in predicting tumor recurrences or metastasis (106).

Only 10% of the genes encoded by the human genome are expressed in any given cell type. A small subset of these genes, with a role in normal housekeeping functions, are expressed in abundance, the vast majority are present in a very low copy number. This makes the task of detecting tissue specific genes much more difficult. It is essential to understand which genes are responsible for normal cell physiology and which ones are responsible for the disease process. In this regard identification of genes that are expressed in normal, precancerous, and cancer cells may help us discover the molecules that are related with tumor development, invasion and metastasis. Leethanakul et al. found that at least 168 novel genes are responsible for formation, metastasis, and invasion of OSCC. The most common and highly expressed genes in both normal oral squamous epithelium and OSCC are shown in table 2 and 3 (93).

A cluster of genes located at 1q21 region (involucrin, SPRR 1A, 1B, 2B, 3, NICE-1, S100 A1, 6, 8, 9, 14) are responsible for epidermal differentiation and are downregulated in OSCC compared to normal oral mucosa. Downregulation of these

genes is consistent with dedifferentiation of squamous epithelial cells, which is associated with malignant transformation. Another cluster of genes (tenascin X, dermatopontin, and collagen 14) downregulated in OSCC includes several extracellular matrix proteins regulating epithelial adhesion and collagen fibril formation. Genes that are upregulated in OSCC are associated with extracellular matrix function, interferon response, proliferation, and immune response. Also, genes implicated in invasion (snail homologue 2, met proto-oncogene), RAS signaling, (r-ras homologue 2), cell surface/extracellular matrix interaction (collagen type IV α_5 and α_6 , laminin γ_2 , α_3 , β_3 , integrin α_3 , α_6 , β_4 , β_6), and angiogenesis (VEGF C, placental growth factor) are highly expressed in OSCC (98, 128).

Irie et al. found that there are 86 genes that are upregulated and 67 genes downregulated in metastasis negative cases. However, in metastasis positive cases 183 genes are upregulated and 96 genes are downregulated. The prognosis of the patient is directly related to the expression level of the VEGF gene. When the expression level of this gene is elevated, the prognosis of the patient is worse. In metastatic cases the expression of neutrophil gelatinase-associated lipocalin (NGAL) is high. NGAL modulates MMP-9 activity, which is important during the formation of metastasis in OSCC (99).

In oropharyngeal squamous cell carcinoma, calgranulin B (CAGB), CD24, lymphoepithelial kazal-type-related inhibitor (LEKTI), zinc finger protein (ZNF-185), transglutaminase-3 (TGM3), ETS homololous factor (EHF) and headpin are downregulated. In contrast, periostin and human homologue of the Drosophila white gene (ABCG1) are found to be upregulated by microarray analysis (100). LEKTI and headpin

are known to be proteinase inhibitors and periostin and CD24 have been involved in cell adhesion. It is known that proteinases and cell adhesion molecules play important roles in the biological processes of cancer including cell adhesion and metastasis. TGM3 is involved in stabilizing the cell cornified envelope. This process causes the transition of keratinocytes to become corneocytes by apoptosis. Therefore the downregulation of TGM3 could interrupt a critical step to apoptosis, thereby tumor cell survival. ABCG1 belongs to the family of adenosine triphosphate-binding cassette transporters. Upregulation of this gene can cause the resistance to chemotherapy. The function of ZNF-185 is unknown, however, it may play a role in regulating cellular proliferation and differentiation (100). TNFRSF5, which encodes CD40, is involved in proapoptosis signals in cancer cells when they are treated with chemotherapeutic drugs (132).

Decorin is associated with negative regulation of cell growth. It has a prominent role in TGF- β and EGFR activation pathways. These pathways are important in cellular proliferation, angiogenesis, and immunomodulation. The expression level of this gene is extremely high in dysplastic oral epithelial cells and oral cancer cells (101). The GALNT6 and SMARCC2 genes, mapping to 12q13.13 and 12q13-q14, respectively, are upregulated with poor prognosis and metastasis in lung, and head and neck cancer patients. Upregulation of PRKAR2A and CLDN1 genes, which are located on chromosome 3 regions: 3p21.3 and 3q28-q29 respectively, is commonly seen in head and neck cancers. Gain of 3q is one of the earliest genetic markers for invasion and metastasis and correlates with a poor prognosis (102, 122).

Shimada et al. compared 4 tongue SCC patient tissues with normal tissues by using cDNA microarray technique. This comparison showed that 16 genes are highly

upregulated in all cases (Table 4) (109). Genes differentially regulated in metastases have been identified and the expression of 17 genes that is common to both primary tumors and metastasis have been found. Therefore primary tumors may carry expression patterns for the presence of lymph node metastasis or the potential for metastasis (110).

In OSCC the genes that are most commonly found with altered expression are those that encode cytoskeletal and extracellular matrix protein, inflammatory mediators, proteins involved in epidermal differentiation, and cell adhesion molecules. The downregulation of genes that encode ribosomal proteins, and upregulation of genes that encode MMPs and inflammatory response can be seen in almost every OSCC (111). In a study on 9 OSCC patients using microarray technique, 47 genes have altered expression with 28 being upregulated and 19 downregulated. The list of these genes is in table 5 (136).

Choi et al. reviewed more than 20 current articles that incorporated cDNA microarray analysis in the study of head and neck SCC. They identified 84 genes with common alterations; 32 of them are upregulated (Table 6), 33 of them are downregulated (Table 7) and 19 of them have conflicting expression data (Table 8) (111). Several major biological systems or pathways are altered in head and neck SCC. These include cell cycle control, MMP, and inflammatory response systems. Genes encoding for ECM and integral membrane proteins, proteins involved in epidermal development and differentiation, and cell adhesion molecules most frequently have altered expression in head and neck SCC (Table 9) (111).

There is a significant difference in macrophage content between normal epithelium and OSCC tissue. There is also a marked difference in the macrophage content of

primary OSCC tissue between patients with positive lymph nodes and patients with negative lymph nodes. Despite correlations with lymph node metastasis of OSCC, the macrophage count does not correlate directly with survival or recurrence (120).

Chen et al. found that the highest frequency of gene gain is for TP63, Serpine1, FGF4/FGF3, c-Myc, and DMD whereas the highest deletion is for Caspase8 and MTAP. The gained genes are mainly located on 17q21, 20q, 11q13, 3q27-29 and X chromosomes. Among these, gains of EGFR at 7p; FGF4/FGF3, CCND1 and EMS1 at 11q13; and AIB1 at 20q are significantly associated with lymph node metastasis (121).

The expression level of ECM glycoproteins tenascin and osteopontin increases during tumor progression. Tumor derived osteopontin is able to inhibit macrophage function and enhance survival of OSCC metastasis. Also, the expression level of arachidonate 5-lipoxygenase increases during OSCC progression and metastasis. However, 4 potential tumor suppressor genes, which are BRUSH-1, MXI1, oxidative stress response (OSR1) gene, and occluding, expression levels decrease (122). There is a great frequency of minichromosome maintenance protein 2 (MCM-2), which is essential for DNA replication, expression in surface layers of moderate/severe dysplasia and OSCC compared to benign keratosis/mild dysplasia. Thus, MCM-2 can be a marker for early detection of OSCC and dysplasia (130).

By using proteomic analysis Turhani et al. found that there were 14 proteins with increased expression and 6 proteins with decreased expression in OSCC (Table 10). These proteins have various functions. They are involved in cellular transport, like HSP27 and heat shock 20 kDa like protein p20; in regulatory functions, like creatine kinase, pyruvate kinase M1, and NME1; and glycolic pathway, like phosphoglucerate

mutase 1. Also, some of the proteins are highly tissue specific, like galectin-7, which is involved in cell-cell and cell-matrix adhesion (131).

CHAPTER 3

MATERIAL AND METHODS

3.1. Cell Lines and Cultures

3.1.1. HNEC cell line

The cryopreserved HNEC cell line was obtained from PromoCell, Germany. This cell line was isolated from a 27-year-old Caucasian male. Five ml of Airway Epithelial Cell Growth Medium (PromoCell) with 100µg/ml streptomycin and 100 U/ml penicillin was incubated in a 25 cm² corning cell culture flask with a vent cap (Fisher) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C for 30 minutes. The frozen cells were thawed in a 37°C water bath until about 90% of the contents were thawed. The tube was removed from the water bath and agitated until all the contents were completely thawed. 250,000 to 375,000 suspended cells were distributed in the pre-incubated 5ml medium in a 25cm² cell culture flask and incubated for 5 to 6 days in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. After the first 24 hours, 80% of the cells were adhered to the surface of the flask, and the medium was replaced with the fresh 30 minute pre-incubated airway epithelial cell growth medium with 100µg/ml streptomycin and 100 U/ml penicillin. Replacement of medium was then repeated every 2 to 3 days. After 5 to 6 days the cell culture was examined under an inverted microscope. If the monolayer was sub-confluent (60-80%) and many mitotic figures were visible, cells were sub-cultured. During sub-culturing HepesBSS, trypsin and trypsin neutralizing solution (TNS) at room temperature were used. First, the medium was replaced with 5 ml HepesBSS and cells were washed for 30 seconds by gently swirling the culture flask.

Then, the HepesBSS was removed and the cell monolayer was covered with 2.5ml trypsin/EDTA solution and incubated at room temperature until the cells were released from the floor of flask. When the cells were completely detached, 2.5ml TNS was added. The cell suspension was removed, placed into a centrifuge tube and centrifuged at about 220xg for 4 minutes at room temperature. Supernatant was removed and 1.5ml pre-incubated medium was added and re-suspended. Cell suspension was distributed in the usual way and in the required density as stated above into the new flasks with pre-incubated cultures. The medium replacement and incubation were scheduled as mentioned previously.

3.1.2. BHY cell lines

The cryopreserved BHY cell line was obtained from DSMZ, Germany. This cell line was isolated from a 52-year-old Japanese male with highly differentiated squamous cell carcinoma of the lower alveolus that was highly invasive to the mandibular bone and the muscle layer of the oral floor; however, it did not metastasize (1). Frozen cells were thawed in a 37°C water bath. Approximately 1×10^6 cells were cultivated in 75cm² corning cell culture flask with a vent cap in a humidified atmosphere of 95% air and 5% CO₂ at 37°C for 3 to 5 days. Fifteen ml Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum, 100 µg/ml streptomycin and 100 U/ml penicillin was used as the medium. Following the formation of a monolayer at the bottom of the flask, cells were sub-cultured. Trypsinization was performed as follows: Media was poured off of the flask into a 15ml centrifuge tube. Three ml of trypsin/EDTA was placed in the flask and swirled and poured into a centrifuge tube. Five ml fresh trypsin/EDTA was placed into the flask and the flask was incubated in humidified atmosphere of 95% air and 5% CO₂ at

37°C for 3 minutes. The warmth of the incubator enhanced the action of trypsin. At the end of three minutes the flask was removed from the incubator and gently agitated to aid in the dispersal of the cells. 6-8ml of medium from the centrifuge tube was placed back into the flask. The serum in the media stopped the reaction of trypsin. Then the media containing the cells was transferred back into the 15ml centrifuge tube and centrifuged at 1200 rpm for 4 minutes. Supernatant was removed and 5ml medium was added and re-suspended. The cell suspension was distributed in the usual way and in the required density into the new 75cm² flasks with 15 ml medium.

3.1.3. HN cell line

The cryopreserved HN cell line was obtained from DSMZ, Germany. The HN cell line was isolated from a 60-year-old Japanese male with moderately differentiated squamous cell carcinoma of the soft palate that metastasized to cervical lymph nodes, lungs and brain, but did not invade bone (1). The same media, number of cells, incubation conditions, cell culturing and trypsinization method was used as stated in the BHY cell line. However, the incubation period for this cell line was 5 days.

3.1.4. HSC-3 cell line

The cryopreserved HSC-3 cell line was obtained from Japan Health Science Research Resources Bank (HSRRB) The HSC-3 cell line was isolated from a 64-year-old Japanese male. The primary tumor was on the tongue with lymph node metastasis, and was moderately differentiated. This cell line is highly metastatic (10, 23, 52). Approximately 1×10^6 cells were cultivated in a 25cm² corning cell culture flask with a vent cap. Cells were cultivated in 5ml Eagle's Minimal Essential Medium (EMEM) with 10% calf serum, 100µg/ml streptomycin and 100 U/ml penicillin in a humidified

atmosphere of 95% air and 5% CO₂ at 37°C for 7 days. The same cell culturing and trypsinization method was used as stated in the BHY cell line. However, for the first trypsinization instead of 3ml, 1.5ml was used and for the second trypsinization instead of 5ml, 2.5ml trypsin was used.

3.2. The Invasion and/or Metastatic Characteristics of OSCC Cell Lines

All three OSCC cell lines have different invasion and/or metastatic characteristics (Figure 2). Micro positron emission tomography (PET)/computerized tomography (CT) imaging techniques were used to image the invasion and/or metastatic capability of each of the cell lines in *scid* mice. The advantage of micro-imaging technology is the ability to monitor tumor growth in vivo. Immuno-compromised *scid* mice were used to show the differences in jaw bone invasion and distant organ metastasis of these three different OSCC cell lines in a biological system. 5 study groups each with 3 *scid* mice were involved in the study. These 5 groups were: BHY group, HN group, HSC-3 group, human normal nasal epithelial cell line (HNEC) group, and Matrigel only group. The OSCC cell lines and HNEC cell line were induced in the left masseter muscles of the *scid* mice by injecting a suspension of 1×10^7 cells in Matrigel with a 26G needle. Control mice received matrigel only in their left masseter muscles. The HNEC group served as a non-OSCC control group, and the Matrigel only group was included in the study to show that this gel does not have any effect on the formation of OSCC. Tumors were allowed to grow for 30 days. On day 30, mice were anesthetized and the tumor characteristics were evaluated using microPET/CT imaging. Mice were administered 300 μ Ci of [¹⁸F] fluoro-D-glucose (¹⁸FDG) i.p. and 30 minutes thereafter received an i.v. injection of vascular CT-contrast agent. The animals were euthanized using an overdose of isoflurane

inhalation and imaged by micro PET and microCT. MicroPET images were acquired using a P4 or Focus 220 imaging system, followed by a microCT scan using a MicroCAT II imager. To reduce the experimental stress and discomfort associated with tumor formation on the left cheek of the mice, they were put on a soft food diet (ground lab blocks mixed with water). The clinical conditions of animals were monitored 3 times each day and the tumors were never allowed to develop more than 0.5 cm in diameter. Their eating and drinking habits and activity levels were observed 3 times daily. Mice that developed infection at the injection site, did not eat for 1-2 days, or had tumor growth greater than 0.5 cm in diameter were excluded from the study.

3.3. Immunocytochemistry

After removal of the monolayer of cells by trypsinization, samples from each cell line were transferred to microscopic slides by cytopinning for 5 minutes at 500 r.p.m. Slides were fixed in acetone for 10 minutes and then air dried. Cells were rinsed in 10mM phosphate-buffered saline (PBS) for 2 minutes for 3 times. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide and then cells were washed with PBS for 2 minutes 3 times. Samples were incubated in 5% non-immune goat serum for 10 minutes to block non-specific proteins. All samples were washed with PBS solution for 2 minutes 3 times prior to incubation with the primary antibodies. For each primary antibody, one sample from each cell line was incubated with that primary antibody and rinsed in PBS for 5 minutes 3 times. All samples were then incubated with a proper secondary antibody and rinsed in PBS for 5 minutes 3 times. Finally a strepABComplex (Vector) was applied and samples were stained with diaminobenzidine for 10-12 minutes and rinsed with distilled water (Vector). So as to obtain acceptable

contrast and stain, the nucleus of cells slides were placed in gill hematoxylin for one minute. For each cell line a negative control sample was prepared which received all of the steps in the immunostaining process except for application of primary antibody.

The following primary and secondary antibodies were used:

- 1) For MMP-1; rabbit anti-human MMP-1 polyclonal primary antibody 1:10 (Chemicon International) and anti-rabbit Ig G secondary antibody ABC Kit (Vector)
- 2) For MMP-2; rabbit anti-human MMP-2 polyclonal primary antibody 1:10 (Chemicon International) and anti-rabbit Ig G secondary antibody ABC Kit (Vector).
- 3) For MMP-3; rabbit anti-human MMP-3 polyclonal primary antibody 1:10 (Chemicon International) and anti-rabbit Ig G secondary antibody ABC Kit (Vector).
- 4) For MMP-9; goat anti-human MMP-9 polyclonal primary antibody 1:25 (R&D Systems) and anti-goat Ig G secondary antibody ABC Kit (Vector).
- 5) For cathepsin L; goat anti-human cathepsin L polyclonal primary antibody 1:15 (R&D Systems) and anti-goat Ig G secondary antibody ABC Kit (Vector).
- 6) For cathepsin B; rabbit anti-human cathepsin B polyclonal primary antibody 1:15 (Serotec) and anti-rabbit Ig G secondary antibody ABC Kit (Vector).
- 7) For EMMPRIN; mouse anti-human EMMPRIN monoclonal primary antibody 1:20 (Chemicon International) and anti-mouse Ig G secondary antibody ABC Kit (Vector).

Slides were evaluated microscopically (Leica Leitz DM 12B) and using the SPOT advanced imaging program. The original magnifications were 100x and 200x.

Immunostaining was scored according to the staining of HNEC cell line. All proteolytic enzyme expressions of HNEC were considered normal levels of expression, and OSCC

cell lines proteolytic enzyme expressions were measured accordingly. The same intensity in tumor cells as normal epithelium cells was designated by (-), less than 10% stronger intensity in tumor cells than normal epithelium cells was shown by (+), between 10% to 50% stronger intensity in tumor cells than normal epithelium cells was shown by (++), between 50% to 90% stronger intensity in tumor cells than normal epithelium cells was shown by (+++) and more than 90% stronger intensity in tumor cells than normal epithelium cells was shown by (++++).

3.4. Flow Cytometry

Monolayer cells were removed from the flasks by trypsinization. Five groups, each with 1×10^5 cells in 0.1 ml of medium, from each cell line were incubated with the 10 μ l MMP-1, MMP-2, MMP-3, MMP-9 and EMMPRIN flow antibodies for 20 minutes at room temperature in the dark for surface staining. The following flow antibodies were used:

1) For MMP-1; mouse anti-human MMP-1 phycoerythrin conjugated monoclonal antibody, R&D Systems.

2) For MMP-2; mouse anti-human MMP-2 phycoerythrin conjugated monoclonal antibody, R&D Systems.

3) For MMP-3; mouse anti-human MMP-3 phycoerythrin conjugated monoclonal antibody, R&D Systems.

4) For MMP-9; mouse anti-human MMP-9 fluorescein conjugated monoclonal antibody, R&D Systems.

5) For EMMPRIN; mouse anti-human EMMPRIN phycoerythrin conjugated monoclonal antibody, Chemicon International.

All samples were washed with 2ml PBS, centrifuged at 1200 rpm for 4 minutes and the supernatant was removed. Samples of each cell line that were incubated with MMP-1, MMP-2, MMP-3 and EMMPRIN antibodies were also incubated with 50µl of pholloidin/fix and perm solution (15µl pholloidin into 0.5 ml fix and perm solution); however, samples of each cell line that were incubated with MMP-9 were only incubated with fix and perm solution for 20 minutes at room temperature in the dark. All samples were washed with a 2ml permeabilization/wash buffer. Samples were incubated with 10µl of each antibody for 30 minutes at room temperature. Finally, samples were washed with buffer, centrifuged for 4 minutes at 1200 rpm and supernatants were removed. 0.5 ml formalin (1% formaldehyde) was added and each sample was analyzed by flow cytometry. A sample from each cell line was prepared and treated only with buffer and 1% formaldehyde as a control group. From each sample 5000 cells were counted by flow cytometry.

3.5. Ultrastructural Characterization of Three OSCC Cell Lines

Each of the 3 cell lines was grown to confluence in culture flasks. The culture medium was decanted and the cells were immediately overlaid with 2.5% glutaraldehyde in cacodylate buffer, pH 7.2. The cells were fixed overnight at 4⁰C, and post fixed in 2% aqueous osmium tetroxide for 2 hrs. Cells were dehydrated in a graded acetone series and propylene oxide and embedded with Spurr's plastic. The embedded cells were cut out of the flasks with a jeweler's saw, mounted on plastic studs, and cut for transmission electron microscopy (TEM) analysis. Cells were stained with uranyl acetate and lead citrate and examined with a Hitachi H-800 TEM.

3.6. Total RNA Extraction From The Cell Lines

Total RNA extraction was performed using a RNeasy Midi Kit (Qiagen). Each of the 4 cell lines was grown in proper culture flasks and media. From each cell line approximately 1×10^8 cells were used for total RNA extraction. Cells were trypsinized as mentioned previously. Cells were pelleted by centrifugation at 300xg for 5 minutes. Supernatant was aspirated and cells were disrupted by addition of 4ml buffer RLT with 40 μ l β -ME. The cell pellets were loosened by flicking the tube. Cells were homogenized by vortexing the samples for 10 seconds, and passing the lysates at least 5-10 times through 18-20-gauge needle. 4ml 70% ethanol was added to homogenized lysates and mixed thoroughly by shaking vigorously. Samples were applied to midi columns placed in 15ml centrifuge tubes and centrifuged for 5 minutes at 3000-5000xg. The flows that passed through the column were discarded. After centrifugation, 4ml buffer RW1 was added to the columns and centrifuged for 5 minutes at 3000-5000xg to wash the columns. Columns were further washed twice by adding 2.5ml buffer RPE and centrifuging for 2 minutes at 3000-5000xg. The RNeasy columns were removed from the centrifuge tubes and transferred to new 15ml centrifuge tubes. Finally, 250 μ l of RNase-free water were directly applied onto the columns and let stand for 1 minute. Tubes were then centrifuged for 3 minutes at 3000-5000xg in order to obtain the total RNA from the columns. This final process was performed twice to be able to get a higher amount of total RNA. Total RNAs were stored at -80°C in RNA Storage Solution (Ambion) together with SUPERase RNase Inhibitor (Ambion) with a concentration of 1:40.

3.7. Checking The Quality and Quantity of Total RNA.

After the extraction of total RNA, 3µl total RNA from each cell line was diluted with distilled water. The dilution factor was 1:100. The diluted sample was used to measure the quantity of RNA with a spectrophotometer at 230nm-300nm. The following formula was used to find the quantity of total RNA:

$$\text{Dilution factor} * \text{Optimal Density at } A_{260} * 40\mu\text{g/ml}.$$

Aldolase and β-actin genes, which are housekeeping genes, and SuperScript III First-Strand Synthesis System for RT-PCR Kit (Invitrogen) was used to check the quality of total RNA of each cell line.

3.7.1. Synthesis of cDNA from total RNA

For each cell line, 1µl of oligo(dT)₂₀ (50µM), 5µg total RNA, 1µl 10mM dNTP mix (10mM each dATP, dGTP, dCTP, dTTP at neutral pH), 1µl of random decomer and sterile distilled water to 13µl were added to a nuclease-free microcentrifuge tube. This mixture was heated to 65°C for 5 minutes and incubated on ice for at least 1 minute. The contents of the tube were collected by centrifugation and 4µl 5X First-Strand buffer, 1µl 0.1M DTT, 1µl RNaseOUT Recombinant RNase Inhibitor (Invitrogen), and 1µl of SuperScript III RT (200 units/µl) was added. By pipetting gently up and down the contents were mixed and incubated at 25°C for 5 minutes. Then, a further incubation at 50°C for 90 minutes was done. The reaction was inactivated by heating the mixture at 70°C for 15 minutes. The synthesized cDNA was used as a template for amplification in PCR.

3.7.2. PCR reaction

50µl of PCR reaction for each cell line was prepared as follows: 5µl 10X PCR buffer [200mM Tris-HCl (pH 8.4), 500 mM KCl], 1.5µl 50mM MgCl₂, 1µl 10mM dNTP mix, 1µl amplification primer 1, 1µl amplification primer 2, 0.4µl *Taq* DNA polymerase (5U/µl), 2µl cDNA, and 38.1 autoclaved distilled water were added in a PCR reaction tube and mixed gently. Denaturation of cDNA was done at 94°C for 2 minutes. Annealing temperature was 88°C for aldolase gene reaction and 62°C for β-actin gene for 30 seconds. 30 cycles of PCR reaction was performed. The PCR product of each cell line was run on a gel in order to observe the expression of aldolase and β-actin genes.

The primers of the aldolase gene are 5'-GGAATTCCATATGCCCTACCAATATCCAGC-3', and 5'-GGAATTCGGATCCTTAATAGGCGTGGTTAG-3. The primers of the β-actin gene are 5'-TCCTGGCCTCACTGTCCAC-3', and 5'-GTCCGCCTAGAA GCACTT-3'.

3.8. Synthesis of mRNA From Total RNA

PolyATtract® mRNA Isolation Systems (Promega) was used to extract the mRNA from the total RNA. This procedure is designed for use with 1-1.5mg of total RNA. In a sterile, RNase-free 3ml tube, 1-1.5mg of total RNA and RNase-Free Water to a final volume of 2.43ml were combined. The tube was placed in a 65°C heating block for 10 minutes. 10µl of the Biotinylated-Oligo(dT) Probe and 60µl of 20X SSC solution were added to the total RNA and mixed gently and incubated at room temperature until completely cooled. This is the annealing reaction.

Stock solutions of 0.5X SSC (0.125ml of 20X SSC with 4.875ml of RNase-Free Water) and 0.1X SSC (50µl of 20X SSC with 9.95ml of RNase-Free Water) were

prepared. Streptavidin-paramagnetic particles, which come with the kit, were rinsed 3 times with an equal volume of 0.5X SSC. Streptavidin-paramagnetic particles were resuspended by flicking the bottom of the tube until they were completely dispersed and captured by placing the tube in a magnetic stand for 30 seconds. Washed streptavidin-paramagnetic particles were resuspended in 0.5ml of 0.5X SSC.

The entire contents of the annealing reaction were added to the tube containing the washed streptavidin-paramagnetic particles. This mixture was incubated at room temperature for 10 minutes and gently mixed by inverting every 1-2 minute. Streptavidin-paramagnetic particles were captured using a magnetic stand and the supernatant was carefully removed without disturbing the streptavidin-paramagnetic pellet. The particles were washed four times with 0.1X SSC by gently flicking the bottom of the tube. After the final wash the supernatant was removed as much as possible without disturbing the streptavidin-paramagnetic particles. To be able to elute mRNA, the final streptavidin-paramagnetic pellet was resuspended in 1.0ml of the RNase-Free water. Streptavidin-paramagnetic particles were magnetically captured and eluted mRNA was transferred to 2ml tube. After synthesis of mRNA from each cell line, cDNA Synthesis System (Promega) was used to synthesize cDNA from mRNA.

3.9. cDNA Microarray Analysis

Each tumor cell line was compared with the HNEC cell line by using the cDNA microarray technique. Operon Human Version 3.0 microarray slides, which contains 25,000 human genes, were used for this study (Figure 3). cDNA Synthesis System (Promega) was used to synthesize cDNA from mRNA. From each cell line, 5 µg of cDNA was labeled and processed to hybridization. To be able to eliminate the Cy dye

bias reverse labeling was performed. Basically, for each tumor cell vs. HNEC cell, the cancer cells were first labeled as Cy5 dye and HNEC as Cy3 dye, and then the HNEC cell line as Cy3 dye and the tumor cell lines as Cy5 dye. Two microarray hybridizations were performed in parallel for each group. Reverse labeling has been proved to be important in improving data correlation between the samples and data quality as shown by statistical analysis. Labeled tumor cell lines and HNEC cell line cDNAs were hybridized on microarray slides by using the hybridization buffer. All slides were washed by using the washing buffer and scanned in a GenePix 4000 scanner (Axon Instrument). The images were saved for further data analysis.

Typically, microarray data analysis includes image processing, data normalization, statistical analysis, data cluster and visualization, and data management. The microarray image was processed using the GenePix Pro software. The spots with fifty percent of pixels higher than two times background standard deviation were filtered to be positive and subjected to further analysis. Both bulk normalization and Lowess normalization were applied to analyze our data. With bulk normalization, we averaged all the filtered spot for each channel, and used the ratio to normalize the signal ratio at each spot. Bulk normalization is enough for most of the applications; however, we also considered using Lowess normalization, in which intensity dependent dye bias can be removed.

Self-organizing maps and hierarchy clustering were performed to further understand gene function and interaction. We clustered the data by gene, which gives expression patterns among the different OSCC cell lines and helps to understand the gene interaction as well as shared pathways. We also clustered the data by OSCC cell lines, which provides the information about how each cell line is relevant to one another at the global

gene expression level. Based on the expression levels of the genes in microarray analysis, different pathways were analyzed on each OSCC cell lines by using Kyoto Encyclopedia of Gene and Genomes (KEGG).

In order to narrow down specific genes, we used the ANOVA model to obtain the estimates and confidence intervals for all genes in each experiment set. For the genes of interest, the confidence intervals will help us to decide whether to choose this gene for further study or not. All of the above data analysis can be done in Bioconductor.

Microarray data was confirmed by real-time PCR. We designed gene specific primers for genes of interest and performed the SYBR based RT-PCR to confirm the relative abundance of gene expression as observed in the microarray analysis. Ubiquitin was used as internal control. Standard curve analysis and an internal control were applied to obtain the absolute quantification.

3.10. Real Time PCR Analysis

The results of cDNA microarray analysis were confirmed to the most highly expressed three genes from each cell line by using Real Time PCR analysis. The expression level of MAGEA9, Q96AL8, and GFI1 genes in BHY cell line; NMES1, MAGEA1 and Q96AL8 genes in HN cell line; and MAGEA8, MAGEA1 and EREG genes in HSC-3 were compared with the expression levels of these genes in HNEC cell line. Glucose-6-phosphate gene was used as the control gene.

The same total RNA samples, which were extracted from the cell lines for cDNA microarray analysis, were used for Real Time PCR analysis. Before the synthesis of cDNA from total RNA, the qualities of total RNA samples were examined by running the samples in the gel (Figure 4a). Synthesis of cDNA from total RNA was performed and

the quality and quantity of cDNA was checked by running the samples in the gel with their glucose-6-phosphate gene expression levels (Figure 4b).

The total Real Time PCR reaction was 25 μ l, which contained 5 μ l of cDNA and 20 μ l of the mixture. The Real Time PCR mixture was prepared as follows: 8 μ l primer mixture, 80 μ l IQSM (IQ Supermix, BioRad) and 72 μ l water. Human GFI1, MAGEA8, MAGEA1, MAGEA9, EREG, and NMES1 Real Time PCR primer sets (Superarray) were used. The forward and reverse primer of Q96AL8 gene was set up by using Blast Sequencing Computer Program. BioRad Real Time PCR machine iQ5 was used to run the 95°C, 15 minutes; 40 cycles of (95°C, 30 sec; 55°C, 30 sec; and 72°C, 30 sec) reaction. iQ5 RT-PCR data analyzing computer program was used to analyze the results.

The forward and reverse primer sequences used for the Real Time PCR reactions were as follows:

MAGEA1 forward primer sequence: 5'GGCCATTCTTCACTCTGAAGA3'.

Reverse primer sequence: 5'TTGAACAATTCCAAAAGAGAACAAAG3'.

MAGEA8 forward primer sequence: 5'GTTCTGTTCTATTGGGCGATTT3'.

Reverse primer sequence: 5'CCATTAGAAGGAACATTGGAACAA3'.

MAGEA9 forward primer sequence: 5'GGCATGTCCTTCTGTTCCAT3'. Reverse primer sequence: 5'GGAGCTTGGCTCTTCCTCTT3'.

Q96AL8 forward primer sequence: 5'CAACAGTGTGTTTCGTCAAAGG3'.

Reverse primer sequence: 5'CCACATGGCAGCCAGGAT3'.

EREG forward primer sequence: 5'CAGGCAGTTATCAGCACAAC3'. Reverse primer sequence: 5'CACAGGATTCCATACCCAAG3'.

NMES1 forward primer sequence: 5'GGTTCAAATGTATTTTCTCCCAT3'.

Reverse primer sequence: 5'TTTGGGCTCTGGATAAGGAAT3'.

GFI1 forward primer sequence: 5'TTCTCTCGCTGCGGAGTCT3'. Reverse
primer sequence: 5'AGGCACTAGAAATGACTTGA3'.

CHAPTER 4

RESULTS

4.1. The Invasion and/or Metastasis Characteristics of OSCC Cell Lines

PET/ CT imaging techniques were used to image the invasion and/or metastatic capacities of each OSCC cell line in immuno-compromised *scid* mice. The 5 study groups included, BHY, HN, HSC-3, HNEC, and Matrigel only groups, with 3 *scid* mice each in the study. The HNEC group served as a non-OSCC control group, and the Matrigel only group was included in the study to show that this gel does not have any effect on formation of OSCC.

The PET/CT images of HNEC and Matrigel only groups showed no evidence of tumor formation in any of the mice in these groups (Figures 5, 6, and 7). The PET images of these two groups showed no activity at the injection area due to the Matrigel or HNEC presence (Figures 5 and 6). Thus, these two different groups of animals served as reliable control groups.

All three mice of the BHY cell line group showed invasion of the OSCC to the left mandibular bone in the images (Figures 8 and 9). However, only two mice of the HN group showed lymph node and distant organ metastasis, such as lung and kidney (Figures 10 and 11). The other mouse in HN group had cervical lymph node metastasis of the OSCC. The HSC-3 cell line group represented lymph node metastasis in all three mice in the group (Figures 12 and 13). The results of the imaging study matched with the characteristics of the OSCC cell lines that have been reported (1, 10, 23, 52).

4.2. Immunocytochemistry

Except for the MMP-9 expression of HSC-3, seven proteolytic enzyme expressions of OSCC cell lines were higher than HNEC. The expression levels of proteolytic enzymes in the OSCC cell lines are shown in Table 11.

MMP-1 was expressed with 50-90% stronger intensity in OSCC cell lines, than in HNEC (Figure 14). MMP-2 was expressed with more than 90% stronger intensity in HN and HSC-3 than HNEC; however, in BHY this percentage was 83 (Figure 15). HSC-3, HN, and BHY showed 95%, 81%, and 73% stronger MMP-3 intensity than HNEC, respectively (Figure 16). MMP-9 expression of HSC-3 was the same with HNEC, HN had moderate MMP-9 expression (Figure 17). BHY showed the strongest EMMPRIN and MMP-9 expression with 93% and 58% respectively (Figure 18). Cathepsin B expressions of OSCC cell lines were higher than 90% (Figure 19). Cathepsin L expression was the highest in HSC-3 cell line with 96% and the lowest in BHY cell line with 72% (Figure 20).

4.3. Flow Cytometry

Five thousand cells were evaluated from each cell line using flow cytometry. The percentages of antibody-stained cells are shown in Table 12. MMP-1, MMP-2 and MMP-3 were highly expressed in all three OSCC cell lines and the HNEC cell line (Figure 21). However, MMP-9 and EMMPRIN expression levels were highest in the BHY cell line (Figure 22). MMP-9 expression level in the BHY cell line was significantly higher, compared to the HNEC and HSC-3 cell lines. Also, the HN cell line showed elevated MMP-9 expression compared to HNEC and HSC-3 cell lines but it wasn't as high as in the BHY cell line (Table 12).

Increased MMP-9 and EMMPRIN expression may play a role in bone invasion by OSCC. According to flow cytometry results 97-99.9% of OSCC and HNEC cells were stained with the MMP-1, MMP-2 and MMP-3 antibodies; however, immunocytochemistry staining for the same proteolytic enzymes showed stronger intensity in tumor cells than HNEC cells. Thus, normal epithelial cells and tumor cells secrete MMP-1, MMP-2 and MMP-3 but the difference is the regulation of their activity.

4.4. Ultrastructural Characterization of Three OSCC Cell Lines

At the ultrastructural level, the BHY cells were the most highly differentiated of the 3 OSCC cell lines (Figure 23). There was interdigitation of adjacent cells and numerous cell junctions were present. HN cells had cytoplasmic projections, but did not show interdigitation of adjacent cells. Cell junctions were often seen between adjacent cells (Figure 24). The HSC-3 cells in culture were smaller than the other 2 OSCC cell lines. They possessed cytoplasmic projections but did not show any sign of interdigitation. Cell junctions were rarely seen (Figure 25). The control HNEC cell line showed obvious squamous cell features including interdigitation, numerous cell junctions, and occasional cytoplasmic tonofilaments (Figure 26).

4.5. The Quality and Quantity of Total RNA

After total RNA extractions from the cell lines, 3µl total RNA from each cell line was diluted with distilled water, and observed for the quality and quantity of RNAs. The quantity measurements of total RNA at spectrophotometer at 260nm and calculation of total amount of each cell line total RNAs are as follows:

Dilution factor X Optimal Density at A_{260} X 40µg/ml

For the BHY cell line: the value of optimal density at 260nm at spectrophotometer is 0.16 (figure 27) and the dilution rate is 1:100. Thus, the amount of total RNA in 1 ml is $0.16 \times 40 \times 100 = 640 \mu\text{g/ml}$. The total volume of BHY total RNA was 2ml, so the total amount for this cell line was $640 \mu\text{g/ml} \times 2\text{ml} = 1280 \mu\text{g}$.

For the HN cell line: the value of optimal density at 260nm at spectrophotometer is 0.15 (figure 28) and the dilution rate is 1:100. Thus, the amount of total RNA in 1 ml is $0.15 \times 40 \times 100 = 600 \mu\text{g/ml}$. The total volume of HN total RNA was 2ml, so the total amount for this cell line was $600 \mu\text{g/ml} \times 2\text{ml} = 1200 \mu\text{g}$.

For the HSC-3 cell line: the value of optimal density at 260nm at spectrophotometer is 0.08 (figure 29) and the dilution rate is 1:100. Thus, the amount of total RNA in 1 ml is $0.08 \times 40 \times 100 = 320 \mu\text{g/ml}$. The total volume of HSC-3 total RNA was 4ml, so the total amount for this cell line was $320 \mu\text{g/ml} \times 4\text{ml} = 1280 \mu\text{g}$.

For the HNEC cell line: the value of optimal density at 260nm at spectrophotometer is 0.4 (figure 30) and the dilution rate is 1:100. Thus, the amount of total RNA in 1 ml is $0.4 \times 40 \times 100 = 1600 \mu\text{g/ml}$. The total volume of HNEC total RNA was 2ml, so the total amount for this cell line was $1600 \mu\text{g/ml} \times 0.75\text{ml} = 3200 \mu\text{g}$.

cDNA of each cell line was synthesized from total RNA and aldolase and β -actin genes were run in PCR in order to check the quality of total RNA of each cell line. All cell lines showed the expression of both housekeeping genes on the electrophoresis gel (Figure 31).

4.6. cDNA Microarray Analysis of Three OSCC Cell Lines

The genetic structure comparison of tumor cell lines with HNEC cell line by using the cDNA microarray technique showed that each OSCC cell line has its unique gene

expression structure. After the normalization process each OSCC cell line demonstrated different upregulated and downregulated genes. The BHY cell line expressed 139 upregulated genes and 117 downregulated genes (Table 13 and 14). The HN cell line expressed 214 upregulated and 262 downregulated genes (Table 15 and 16). Finally, the HSC-3 cell line expressed 128 upregulated and 117 downregulated genes (Table 17 and 18).

The clusterization of data showed that there were 13 genes that are upregulated and 83 genes that were downregulated in all three OSCC cell lines (Figures 32 and 33). Each cell line was compared with the other two cell lines based on the most upregulated and the most downregulated 20 genes of that cell line. This permitted an understanding of the genetic differences between these three cell lines better and thus the most responsible genes that are related to the formation of their genetic characteristics. Figure 34 and table 19 shows the 20 most upregulated and downregulated genes in the BHY cell line and the expression levels of these 20 genes in the other two OSCC cell lines, HN and HSC-3. Figure 35 and table 20 shows the 20 most upregulated and downregulated genes in the HN cell line and the expression levels of these 20 genes in the other two OSCC cell lines, BHY and HSC-3. And figure 36 and table 21 shows the 20 most upregulated and downregulated genes in the HSC-3 cell line and the expression levels of these 20 genes in the other two OSCC cell lines, BHY and HN.

There were 57 genes that were upregulated and 42 genes that were downregulated in both BHY and HN cell lines (Figure 37). The comparison of BHY with HSC-3 showed that there were 24 upregulated genes and 49 downregulated genes on both BHY

and HSC-3 cell lines (Figure 38). Finally, there were 24 common upregulated and 44 downregulated genes in HN and HSC-3 cell lines (Figure 39).

Based on the expression levels of the genes in the microarray analysis, different pathways were analyzed on each OSCC cell line by using the Kyoto Encyclopedia of Gene and Genomes (KEGG). The pathway analysis was done based on the number of genes that were upregulated or downregulated within the pathway, the importance of the upregulated or downregulated genes for that specific pathway (impact factor) and how significant the change was of upregulated or downregulated gene expression (p values). First, both upregulated and downregulated genes' p values were considered in pathway analysis. Seven, 9 and 8 pathways were influenced in BHY (figure 40), HN (figure 43) and HSC-3 (figure 46) cell lines respectively ($p < 0.05$). When only upregulated genes' p values were evaluated 8, 10, and 3 pathways were upregulated in BHY (figure 41), HN (figure 44) and HSC-3 (figure 47) cell lines respectively ($p < 0.05$). Finally, only downregulated genes' p values were evaluated and 3, 9, and 6 pathways were downregulated in BHY (figure 42), HN (figure 45) and HSC-3 (figure 48) cell lines respectively ($p < 0.05$).

4.7. Real Time PCR Analysis

Prior to cDNA synthesis, the quality of the extracted total RNA was determined using gel electrophoresis (Figure 4a). Also, the quality and quantity of cDNA, used for Real Time analysis, was checked by measuring the glucose-6-phosphate gene expression levels (Figure 4b).

The Real Time PCR analysis results confirmed the cDNA microarray analysis results (Figure 49). The upregulation values of MAGEA9, Q96AL8 and GF11 genes in

the BHY cell line were 3.34, 5.18 and 7.06 respectively. The upregulation values of NMES1, MAGEA1 and Q96AL8 genes in the HN cell line were 8.33, 3.58, and 10.67 respectively. Finally, the upregulation values of MAGEA8, MAGEA1 and EREG genes in the HSC-3 cell line were 1.63, 0.90, and 3.71 respectively. When we compare the results of the Real Time-PCR with the results of the cDNA microarray analysis, the only questionable upregulation in the cDNA microarray analysis was the expression level of the MAGEA1 gene in the HSC-3 cell line with an error bar of 0.89 (Figure 49). However, all others have reasonable error bars as seen in figure 49, which means that the cDNA microarray analysis results in this study were reliable. The p values of all the Real Time PCR analysis results were <0.0001 except the value of the MAGEA1 gene in the HSC-3 cell line.

CHAPTER 5

CONCLUSIONS & RECOMENDATIONS

5.1. The Invasion and/or Metastatic Characteristics of OSCC Cell Lines

This part of the study examined the invasion and/or metastatic potentials of three different OSCC human cell lines, using micro positron emission tomography (PET) and computerized tomography (CT) imaging. The advantage of micro-imaging technology is the ability to monitor tumor growth in vivo. PET/CT revealed good image quality and allowed us to evaluate and correlate the metabolic alterations of cancer with anatomical findings. Thus, it is a good imaging technique of the tumors. PET is a method by which cellular and molecular events can be followed by using injected radiolabelled molecular probes. The addition of CT to PET is clearly helpful for a better anatomic delineation of the tumor, treatment monitoring, prognosis assessment and radiation treatment planing.

Immunocompromised *scid* mice were used to show the differences in jaw bone invasion and distant organ metastasis of the three different OSCC cell lines in a biological system. Three animals per group was the minimum number required to demonstrate infiltrative bone lesions or metastases. There are three anatomical areas to form a tumor in the oral cavity. They are tongue, floor of the mouth and cheek (137, 138). The masseter muscle is the ideal anatomical area for tumor growth due to its proximity to the jaw bones. Thus, we injected the cell lines into the masseter muscle. 1×10^7 cells was the minimum acceptable amount to inject in each mouse according to the literature (139). As tumors in animals were clearly visualized by FDG-PET/CT (138, 140), we decided to use ^{18}F FDG as the tracer. ^{18}F FDG is actively taken up and accumulates

in cancer cells (141). To the best of our knowledge, micro positron emission tomography (PET)/computerized tomography (CT) imaging has not been used to show the invasion and metastatic capacity of these three OSCC cell lines.

The results of this experiment provided important information on the invasive and metastatic capacity of different OSCC cell lines that matched with their reported characteristics. It was determined that OSCC development can result in jaw bone invasion and/or distant organ metastasis. The BHY cell line invaded the mandible when they were injected in the masseter muscle of the *scid* mice (figure 8). This was also proven by Kawamata et al. (1). However, the HN cell line showed metastasis to cervical lymph nodes and distant organs both in our study and according to the literature (figure 10 and 11) (1). The characteristics of the HSC-3 cell line in our study correlated with other reported studies (figure 12 and 13) (10, 23, 52). As we found differences based on the invasion and/or metastasis of these cell lines, we focused on the expression levels of proteolytic enzymes by using immunocytochemistry and flow cytometry, and gene regulations of these three OSCC cell lines by using the cDNA microarray technique. Thus, we might determine the genes that are upregulated or downregulated in the process of metastasis or bone invasion of OSCC.

5.2. Characterization of The Oral Squamous Cell Carcinoma Cells

MMPs play an important role in the invasion and metastasis of oral cancer. OSCC can induce the secretion of type I collagen proteolytic enzymes, most notably MMP-1 (10). MMP-1 breaks down collagen I to gelatin. Then gelatinases, which are MMP-2 and MMP-9, complete the collagen I degradation by breaking down gelatin (10). Although

cancer cells that secrete MMP-2 and MMP-9 can invade bone, it is thought that MMP-9 plays a more important role than MMP-2 during this process (9).

Several studies have focused on the effects of MMP-1 (1, 10), MMP-2 (1, 9, 10, 17), MMP-3 (2, 17) and MMP-9 (1, 9, 10, 19) along with cathepsin B, H, L, and D (1, 4) on the ability of OSCC to invade bone. Production of MMP-1 and MMP-9 appear to play a role in the invasion of OSCC to bone (8, 10) and our results substantiate this finding. These two metalloproteinases are necessary for the complete collagen matrix degradation of bone, and have been shown to be expressed by OSCC (10). According to Hong et al. (9) and our study, although MMP-2 was highly expressed in OSCC it is not related to invasion. Over expression of MMP-3 in OSCC is associated with invasion and a high incidence of metastasis (2). Also, our results showed that MMP-3 has a stronger intensity in tumor cells than in HNEC cells. Cathepsin B and L are the two most important cysteine proteinases implicated in tumor cell invasion and metastasis (12). This study showed that all three OSCC cell lines highly express both cathepsin B and L. EMMPRIN is highly expressed in OSCC and may regulate OSCC invasion, and modulate the expression of MMP-2 (13). We also observed that EMMPRIN is highly expressed in tumor cells, particularly in BHY cell lines.

MMP-2 expression is significantly higher in cases of lymph node metastasis than that in non-lymph node metastasis cases (34, 35, 44). This may explain the observation of the highest expression of MMP-2 in HN and HSC-3 cell lines. MMP-9 production is also closely related to lymph node metastasis (36) however, Charous et al. (37) found that there is no difference in MMP-2 and MMP-9 expression levels between primary and

metastatic OSCC. We believe that higher levels of MMP-2 might correlate with lymph node metastasis of OSCC.

MMP-3 can degrade type IV collagen. Thus, it is important to analyze the expression of MMP-3 in OSCC. EGF stimulates the production of MMP-3 coordinately with MMP-1 (2). Interestingly, the over-expression of epithelial growth factor receptor (EGFR) is a remarkable property of squamous cell carcinoma (2, 56, 58). It is not clear, however, whether EGFR overexpression is implicated in the increased production of MMP-3 by OSCC (2).

This study did not reveal any significant differences in the expression levels of cathepsin B and L in the three OSCC cell lines. Cathepsin L has a major role in the process of bone resorption by activating osteoclasts (1). Kawasaki et al. (4) found that cathepsin B expression is closely correlated with OSCC invasion and progression; however, they could not find any relationship between cathepsin L expression and invasion of OSCC. Turk et al. (45) showed that cathepsin B, H, and L are involved in cancer progression by either direct degradation of extracellular matrix or by activation of other proteases, such as uPA.

In conclusion, proteolytic enzymes appear to play an important role in OSCC invasion and metastasis. Increased expression of MMP-9 and EMMPRIN may be associated with bone invasion of OSCC. We believe, however, that there are more genes and related proteins involved in the bone invasion pathway of OSCC. Future studies will refine gene profiles of OSCC's with different biologic behavior leading to targeted clinical treatments.

5.3. cDNA Microarray Analysis of The Three OSCC Cell Lines and Comparison of The Results With Real Time PCR Analysis

cDNA microarray analysis helped us better understand which genes are involved in bone invasion, lymph node metastasis and distant organ metastasis of OSCC. Regulation of gene expression is the cellular control via the functional product of the gene. It is the basis for cellular differentiation, morphogenesis and formation of the most significant pathology, such as cancer.

5.3.1. The quality and quantity of total RNA

Before the cDNA was synthesized from the total RNA, spectrophotometry was used to be ensured that there was enough total RNA. The quantity and the quality of the total RNA was important. Thus, aldolase and β -actin genes were two good housekeeping genes to use for checking the quality of total RNA. The first step was the synthesis of the cDNA from the total RNA and the second step was running a PCR reaction using the proper primers for aldolase and β -actin genes in order to observe their expressions (Figure 31).

A housekeeping gene is typically a constitutive gene that is transcribed at a constant level. Its products are needed for maintenance of the cell and its expression level is unaffected by experimental conditions. Thus, the expression levels of housekeeping genes are a good source of evaluation of the quality of the total RNA. So far 575 housekeeping genes have been identified (142). Aldolase and β -actin genes are in the group of the most commonly used housekeeping genes (143). The major function of the aldolase gene is in sugar metabolism in the cell in which they are expressed. Thus, this

gene is important in cell motility. The β -actin gene is one of the six different actin isoforms that is involved in cell motility, structure and integrity.

Column 1 in figure 31 did not show any band as it was the negative control group. However column 2, which was the positive control group, and columns 3 to 6, which were BHY, HNEC, HN and HSC-3 cDNA samples respectively, showed similar bands corresponding to the 161 kb molecular weight of the aldolase gene. Column 7 with the β -actin negative group did not show any band. Columns 8 to 12, which were β -actin positive, BHY, HNEC, HN and HSC-3 cDNA samples respectively, showed the same bands with 42 kb which is the molecular weight of that gene. The gel electrophoresis in figure 31 showed that the total RNA samples that were used for cDNA microarray experiment was in good quality.

5.3.2. cDNA microarray analysis of three OSCC cell lines

Good quality and quantity of total RNA allowed us to obtain pure cDNA samples from each tumor cell lines and the HNEC control cell line. Each OSCC cell line cDNA was compared with the HNEC cell line based on the expression levels of 25,000 genes using the cDNA microarray technique. Operon Human Version 3.0 microarray slides were used for the cDNA microarray experiment. Any single microarray output is subject to substantial variability. Thus, the comparison of gene expression of each tumor cell lines was repeated four times using four microarray slides. By pooling data from replicates, we can provide more variable analysis of gene expression data. Based on the literature, in a microarray experiment at least 3 replicates produce more consistent and reliable findings (144), however, we preferred to do 4 replicates.

It has been shown that some genes have more affinity to Cy3- or Cy5 dyes (145). Reverse labeling has been proved to be important in improving data correlation, thus each group of cDNA was reverse labeled. Reverse labeling helped us to eliminate gene dye affinity error. Also, the Lowess normalization method was used in order to remove intensity dependent dye bias.

Based on the cDNA microarray results, each OSCC cell line has a unique gene expression structure. The HN cell line had the highest amount of gene alteration of all three OSCC cell lines with 214 upregulated and 262 downregulated genes (Table 15 and 16). The BHY and HSC-3 cell lines had almost the same amount of upregulated and downregulated genes. This correlates with the metastatic and invasion capacities of these three cell lines (Figure 2). According to Leethanakul et al. study (93) the 23 genes that are most commonly expressed in normal oral squamous epithelium (table 2) were neither upregulated nor downregulated in any of the three OSCC cell lines. However, according to the same study the two genes of the 23 genes (table 3) that are most commonly expressed in OSCC were downregulated only in the HN cell line (table 16) and neither upregulated or downregulated in the BHY and HSC-3 cell lines. These two genes are RPS6KA2 and GSTP1. RPS6KA2 is a ribosomal protein S6 kinase, 90kDa, polypeptide 2 and located at the 6q27. This gene is highly expressed in lung tissue and skeletal muscle. It may play an essential regulatory role in protein synthesis during cell proliferation. It was shown that this gene is downregulated in renal cell carcinomas (146). GSTP1 is glutathione S-transferase and located at 11q13. Its function is the conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic

electrophiles. Increased risk for oral cancer was observed in individuals who are homozygous for the GSTP1 gene and this risk increases with smoking (147).

Shimada et al. found 16 genes that were upregulated in tongue cancer (table 4). Only one of these genes, which is KIAA, was upregulated in our study in the HN cell line (table 15), which is the most aggressive of the three OSCC cell lines. KIAA was found to be upregulated in OSCC tissues. The KIAA family of proteins bear a trans-membrane region capable of binding to other KIAA protein containing domains (such as PDZ, SH3, rhoGEF, and spectrin) known to be localized to sub-membranous sites indicating that they participate in cellular junction formation, receptor or channel clustering, and intracellular signaling events (148). Upregulation of KIAA may be involved in the late stages of OSSC.

Tomioka et al. reported that 19 genes were downregulated in 9 patients with OSCC (136). CDH11, PAM, and TM4SF10 are the three genes of those 19 genes that were downregulated according to our results. CDH11 was downregulated in both HN and HSC-3 cell lines (table 16 and 18). CDH 11 is the cadherin 11 gene and has the chromosomal position of 16q22.1. Cadherins are calcium dependent cell adhesion proteins. They preferentially interact with themselves in a homophilic manner in connecting cells; cadherins may thus contribute to the sorting of heterogeneous cell types. CDH11 is also downregulated in ameloblastoma (149). PAM (peptidylglycine alpha-amidating monooxygenase) was only downregulated in the HN cell line. This gene is located at 5q14-q21. There is no study that has been performed that examines the relationship of this gene with OSCC. Gene TM4SF10, transmembrane 4 superfamily

member 10, was downregulated in all three OSCC cell lines. Its location is on the short arm of human chromosome X at p21.1 (150).

Choi et al. listed 32 genes (table 6) that were upregulated in head and neck SCC (111). COL1A1, COL4A1, COL4A2, and COL5A1 are four of these genes (111, 151). According to our study, however, COL7A1, COL8A1, COL4A1, and COL16A1 were downregulated in the HN cell line (table 16). In the BHY cell line COL5A1 was upregulated (table 13) and COL8A1 was downregulated (table 14) and in the HSC-3 cell line COL4A1 was downregulated (table 18). The LY-2 cell line which was isolated from lymph node metastases of OSCC expressed down regulation of the COL1A2 and COL4A1 genes (152). This correlates with our HSC-3 cell line gene expression results. COL4A1 is collagen, type IV, alpha 1 and located at 13q34. Type IV collagen is the major structural component of glomerular basement membranes, forming a meshwork together with laminins, proteoglycans and entactin/nidogen. COL5A1 is collagen, type V, alpha 1. Type V collagen is a member of group I collagen (fibrillar forming collagen). It is a minor connective tissue component of nearly ubiquitous distribution. Type V collagen binds to DNA, heparan sulfate, thrombospondin, heparin, and insulin. The genomic location of the gene is 9q34.2-q34.3. COL8A1 is collagen, type VIII, alpha 1. The molecular function of this gene is unknown. The genomic location of the COL8A1 is 3q12.3. COL1A2 is collagen, type I, alpha 2. Its genomic location is 7q22.1. Type I collagen is a member of group I collagen (fibrillar forming collagen). COL1A2 are specifically repressed by EGFR signaling, thereby facilitating oncogenic transformation. Therefore, it is likely that COL1A2 interferes with the progression of human carcinomas expressing aberrant forms of one or more EGFR family receptor tyrosine kinases (153).

COL1A1 is collagen, type I, alpha 1 with location of 17q21.33. Type I collagen is a member of group I collagen (fibrillar forming collagen). COL7A1 is collagen, type VII, alpha 1. The genomic location of this gene is 3p21.1. It is a stratified squamous epithelial basement membrane protein that form anchoring fibrils which may contribute to epithelial basement membrane organization and adherence by interacting with extracellular matrix proteins such as type IV collagen. COL5A2, COL3A1, COL10A1, COL4A1, FN1, and INHBA were well correlated with periostin expression. Higher expression of periostin was frequently observed in HNSCC tissues compared with normal tissues. By evaluation of periostin expression in HNSCC patients, a strong correlation of periostin expression with FAP, SULF1, COL5A2, COL3A1, COL10A1, COL4A1, FN, and INHBA was observed (154). However, in our study none of these genes and periostin were upregulated in any of the three OSCC cell lines.

Other upregulated genes that were listed by Choi et al. (table 6) were FN1, ITGA6, IL-6, IL-8, POSTN, SERPINE1, SOD2, and THBS2. FN1, which is fibronectin 1, is located at 2q34. Fibronectin binds cell surfaces and various compounds including collagen, fibrin, heparin, DNA, and actin. It is involved in cell adhesion, cell motility, wound healing, and maintenance of cell shape. The upregulation of this gene in head and neck SCC was shown (151), however, according this study this gene was downregulated in all three OSCC cell lines (tables 14, 16 and 18). Neither upregulation nor downregulation of ITGA6 was observed in this study, whereas the downregulation of ITGB6 in BHY and HSC-3 cell lines were recorded (table 16 and 18). ITGB6 is the integrin beta-6 precursor that is Located at 2q24.2. It is a fibronectin receptor for carcinoma cells and plays a critical structural role in the hemidesmosome of epithelial

cells (155). Both IL-6 (interleukin 6) and IL-8 (interleukin 8) genes were upregulated in the three OSCC cell lines in this study (tables 13, 15, and 17). The genomic location of IL-6 is at 7p.21. It is a cytokine with a wide variety of biological functions: it plays an essential role in the final differentiation of B-cells into Ig-secreting cells, it induces myeloma and plasmacytoma growth. It is also involved in the regulation of immune response, hematopoiesis, platelet production, acute phase reaction and bone resorption (susceptibility factor for osteopenia), playing a role in the aggressiveness of non-Hodgkin's lymphoma by stimulating MMP-2 and MMP-9. IL-8, which is located at location 4q13-q21, is a chemotactic factor that attracts neutrophils, basophils, and T-cells, but not monocytes. It is released from several cell types in response to an inflammatory stimulus. IL-6 and IL-8 are important cytokines involved in tumor growth and angiogenesis in a variety of cancers, including OSCC (49, 126). The impact of IL-6 and IL-8 expression in cancer cell lines has been reported. But the in vivo relationship between the IL-6/IL-8 expression and clinical parameters is still unknown (156). POSTN (periostin) is located at 13q13.3. It binds to heparin and induces cell attachment and spreading and plays a role in cell adhesion. Periostin overexpression enhances invasiveness in oral cancer as its expression is well correlated with the invasion pattern and metastasis. Moreover, blood vessel density of periostin-positive cases was higher than those of periostin-negative cases (157), however, in this study it was downregulated (tables 14, 16 and 18). SERPINE1 (serpin peptidase inhibitor, clade E member 1) is located at 7q21.3-q22. Both SERPINE1 and SOD2 expression increase in OSCC with a poor prognosis (121). These two genes were only upregulated in the HN cell line (table 15) that has the most aggressive metastatic behavior. No THBS2 upregulation was found

in this study, but downregulation of the THBS1 gene was observed in the HSC-3 cell line (table 18). The THBS1 gene product is an adhesive that mediates cell-to-cell and cell-to-matrix interactions. It can bind to fibrinogen, fibronectin, laminin, type V collagen and integrins. Downregulation of this gene was observed in human lung and breast cancer cell lines and associated with the malignant progression of murine melanoma cell lines. Although the role of THBS1 in cancer progression is not yet clear, it is suggested that production of THBS1 by tumor cells is correlated with a reduction in angiogenesis (158).

Choi et al. also listed the 33 most common downregulated genes of head and neck SCC (table 7) (111). AIM1, ALDH9A1, ANXA1, DUSP5, IL1RN, KRT13, KRT15, KRT4, and ZNF185 are some of them. We did not appreciate any remarkable change in expression level of the AIM1 gene, but the expression level of AIM2 (Absent in melanoma 2) gene, located at 1q22, was upregulated according to this study's results (table 13, 15, and 17). Although AIM2 is known to inhibit fibroblast cell growth *in vitro*, its antitumor activity has not been shown. The expression level of ALDH9A1 gene was not downregulated but ALDH1A3 (aldehyde dehydrogenase 1 family, member A3) gene which is located at 15q26.3, was downregulated in HN cell line (table 16). The ALDH family consists of 16 different enzymes in the human. Several of these have been characterized in recent years but for many, a specific function remains to be revealed. The function for ALDH1A3 may be a back-up enzyme in retinoid metabolism. Apart from ALDH1A3, no true retinoid-metabolising enzyme was identified. Downregulation of the ANXA1 gene in OSCC was shown previously (table 7) (111). Our study confirmed the downregulation of the ANXA6 (annexin A6) gene in the HN cell line (table 16). The location of this gene is 5q32-q34. This gene may regulate the release of Ca^{2+} from

intracellular stores. ANXA1 is overexpressed in breast cancer and hepatocellular carcinoma but markedly down-regulated in esophageal, prostate, gastric carcinomas and HNSCC. The loss of the ANXA1 protein expression is an early event in the initiation of premalignant lesions in HNSCC. Also, possible tumor suppressor roles have been proposed for the ANXA6 gene in melanoma and squamous cell carcinoma (159).

DUSP6, which was downregulated in the HN cell line (table 16), is located at 12q22-q23. Its function is to inactivate MAP kinases. There are five studies that have been performed regarding the expression levels of DUSP6 in head and neck SCC. Four of these studies showed downregulation of this gene whereas one of them showed the upregulation of the gene (table 8) (111). Of the down-regulated genes, DUSP1, which has known proliferation and differentiation functions, has been shown previously to be down-regulated in follicular carcinomas and in advanced epithelial ovarian cancer. Individual genes that differentiate between papillary and follicular carcinomas are the DUSP6 and DUSP5. DUSP5 has been reported previously to be upregulated in premalignant lesions and underexpressed in invasive pancreatic carcinoma (160). IL1RN was only downregulated in the HN cell line (table 16). This gene (interleukin 1 receptor antagonist) is located at 2q14.2. It inhibits the activity of IL-1 by binding to its receptor, however it has no IL-1 like activity. It is downregulated in OSCC (151). KRT23 in the BHY cell line (table 14); KRT8, KRT16, and KRT6B in the HN cell line (table 16) and KRT23, KRT15, and KRT6B (table 18) were downregulated in the HSC-3 cell. There are six studies that have been performed regarding the expression levels of KRT16 in head and neck SCC. Four of these studies showed downregulation of this gene whereas two of them showed the upregulation of the gene (table 8) (111). KRT genes are basal cell

epithelial markers. Immortal OSCC show increased expression of genes encoding several cell cycle regulators, compared with mortal OSCCs (e.g., CCNA2, CCNB1, CCNB2; CDC2, CDC6, CDC20, CDCA7; CDKN3; several MCMs; PCNA; BIRC5), but reduced expression of genes encoding terminal differentiation markers such as several keratins, involucrin (IVL), and the family of small proline-rich proteins (SPRR), as well as the S100 family of calcium-binding motility proteins. In contrast, a variety of adhesion, invasion, and extracellular matrix proteins are upregulated in mortal OSCC (161). ZNF25 and ZNF625 gene were downregulated in the BHY cell line and ZNF569, ZNF606 and ZNF83 genes were downregulated in the HSC-3 cell line. ZNF genes are located at 19q. These genes are involved in the regulation of cellular proliferation, differentiation and transcriptional regulation. Downregulation of ZNF 185 in head and neck cancers has been shown previously (100).

Other common genes that are altered in head and neck SCC are MMP-1, MMP-2, MMP-3, MMP-9, MMP-10, MMP-13, MMP-25, LAMP3, LAMA3, LAMB3, LAMC2, CDH2, CXCL6 and HRAS . MMP-13 is an important matrix metalloproteinase, which is known also as collagenase 3. The collagenases can mediate tumor invasion through several mechanisms, which include constitutive production of enzymes by the tumor cells, induction of collagenase production in the neighboring stromal cells, and interactions between tumor/stromal cells to induce collagenase production by one or both cell types. Thus, evidence indicates that elevated expression of the interstitial collagenases is associated with a poor prognosis in a variety of cancers, and therefore, these MMPs can serve as a marker of tumor progression. Human MMP-13 was first identified in breast carcinoma. Squamous cell carcinomas of the head and neck, in

particular, are noted for their propensity to rapid progression and poor clinical outcome. In these tumors, MMP-13 has been seen mostly in tumor cells at the invading front. Only malignant squamous cell tumors, but not premalignant and benign lesions, express MMP-13, supporting the concept that MMP-13 can serve as a prognostic marker in these carcinomas with characteristically poor outcomes (162). Recently, MMP-1 has been described in a wide variety of advanced cancers. Some reports document MMP-1 production by tumor cells and correlate this with the invasive potential of the tumors, whereas other investigations support the belief that MMP-1 is predominantly expressed by the surrounding stromal fibroblasts. Tumor cells are constantly changing both their genotype and their phenotype as they respond to genetic, physiological, and pharmacological pressures. As a result of this genetic instability, errors in gene regulation accrue as the tumor continues to metastasize and progress (162). Many matrix metalloproteinases (MMPs), such as MMP-1, -2, -7, -9, -13, and -14 have been implicated in cancer invasion. The absence of MMP-7, -9 and -12 from epithelial cells may serve as a good prognostic marker of non-invasive oral carcinoma (163). Blocking the activity of invasion-specific MMPs might offer novel therapeutic modalities in early-stage oral carcinoma. The tissues of OSCCs with lymph node metastasis exhibited increased expression levels of MMP-1, MMP-3, uPA, integrin- α 3, paxillin, tenascin C and IL-6 transcripts. Functional gene groups of proteolytic enzymes and integrin-related molecules are involved in cervical lymph node metastasis. MMP-1 and integrin-3 in particular can provide an accurate biomarker system for predicting the risk of cervical lymph node metastasis in OSCC (164). Previous studies have indicated that E1AF transactivates multiple MMP genes, such as MMP-1, MMP-3 and MMP-9, and plays an

important role in tumor invasion. Expression of MMP-1, MMP-9 and E1AF has been found to be correlated with the invasive potential of oral squamous cell carcinoma (165). According to our cDNA microarray analysis study in the BHY cell line MMP-1, MMP-3, MMP-9 and MMP-10 (table 13); in HN cell line MMP-2, MMP-13, and MMP-25 (table 15); and in HSC-3 cell line MMP-2 and MMP-3 (table 17) were highly expressed.

LAMP3, lysosomal-associated membrane protein 3, is another gene that is altered in OSCC. Its location is 3q27 and it may change the lysosome function after the transfer of peptide-MHC class II molecules to the surface of dendritic cells. LAMP3 expression is upregulated in laryngeal squamous cell carcinoma (166). LAMP3 might also play an important part in metastasis (167). In our study this gene was upregulated both in the BHY and HN cell lines (table 13 and 15). LAMA3, laminin alpha 3, gene is located at 18q11.2. It mediates the attachment, migration and organization of cells into tissues during embryonic development by interacting with other extracellular matrix components. Functions of this gene include adhesion or migration, depending on whether or not it has been proteolytically remodeled by MMPs such as MMP-2 and/or MT1-MMP. This gene expression upregulates in wound sites (168). It has an important role in regulating tissue organization, gene expression, and survival of epithelium (169). A number of reports suggest its involvement in the spread and metastatic activities of cancer cells (170). The HN cell line was the only one of the three cell lines that showed upregulation of LAMA3 gene (table 15). CDH2, cadherin 2, type 1, is located at 18q11.2. It is one of the first to be considered as an invasion-promoter gene. The upregulation of this gene is common in diffuse type gastric cancer and lobular breast cancer (171). This gene was only upregulated in the HN cell line (table 15). CXCL6, chemokine (C-X-C

motif) ligand 6, is located at 4q21. Mortal SCC cultures express a variety of chemokines in culture, such as IL-8, CXCL5, CXCL6, CXCL7, and CCL20, in contrast to immortal SCCs (161). This may be one reason why this gene is only upregulated in HN cell lines. HRAS, Harvey-ras, gene is involved in cell signaling, cell growth, and cancer (58, 124). This gene was upregulated in BHY and HN cell lines.

Based on this study OSCC involves multiple molecular genetic events that take place on many chromosomes and genes. Also this study showed that the loss of heterozygosity on regions of chromosomes 3, 4, 5, 6, 8, 9, 11, 13, and 19 is common in OSCC. The genes that showed alterations in this study have a role in extracellular matrix degradation, cell adhesion, epidermal development and differentiation, signal transduction, transcriptional regulation, cell growth and proliferation, proteolysis, basement membrane degradation, and cytoskeleton.

Thirteen of the 20 most upregulated BHY genes were upregulated, and the other 7 were neither upregulated nor downregulated in the HN cell line. However, only 3 of the 20 most upregulated BHY genes were upregulated, and the other 17 were neither upregulated nor downregulated in the HSC-3 cell line. When the 20 most downregulated BHY genes were evaluated, 11 of them in the HN cell line and 14 of them in HSC-3 cell lines were also downregulated. The other 9 in the HN, and the other 6 in the HSC-3 cell lines were neither upregulated nor downregulated (table19). Twelve of the 20 most upregulated HN genes were upregulated, 7 of them were neither upregulated nor downregulated and one of them was downregulated in the BHY cell line. However, only 5 of the 20 most upregulated HN genes were upregulated, 14 of them were neither upregulated nor downregulated and one of them was downregulated in the HSC-3 cell

line. When the 20 most downregulated HN genes were evaluated, 9 of them in the BHY and 10 of them in HSC-3 cell lines were also downregulated. Ten of them were neither upregulated nor downregulated and one of them was upregulated in the BHY cell line. Ten genes of the 20 most downregulated HN genes were neither upregulated nor downregulated in HSC-3 cell lines (table 20). Ten of the 20 most upregulated HSC-3 genes were upregulated, and the other 10 were neither upregulated nor downregulated in the BHY cell line. Nine of the 20 most upregulated HSC-3 genes were upregulated, and the other 11 were neither upregulated nor downregulated in the HN cell line. When the 20 most commonly downregulated HSC-3 genes were evaluated, 13 of them in BHY and 14 of them in HN cell lines were also downregulated. The other 7 of them in BHY, and the other 6 of them in HN cell lines were neither upregulated nor downregulated (table 21). These comparisons and the microarray gene clusterizations in figures 34, 35, and 36 showed that the upregulated genetic structure of the BHY cell line was more similar to the upregulated genetic structure of the HN cell line (Figure 34A and 35A). Whereas the upregulated genetic structure of the HSC-3 cell line showed more similarity to the upregulated genetic structure of the BHY cell line (Figure 36A). Based on the gene clusterization, the downregulated genetic structure of the BHY cell line was more similar to the downregulated genetic structure of the HSC-3 cell line (Figure 34B, 35B and 36B).

The results of this study represented thirteen genes that were upregulated in all of the three OSCC cell lines (figure 32). Twelve of them were known genes whereas one of them has not been previously described. Basically, the functions of these genes are: mitotic progression and chromosome segregation, apoptosis inhibition, cell growth, transduction and intercellular transmission of polarity information during tissue

morphogenesis and/or in differentiated tissues, embryonal development and tumor transformation and progression, condensation of nucleosome chains into higher order structures.

There were 83 genes that were downregulated in all three OSCC cell lines (figure 33). Most of the genes have vital functions for cell function. Some of these 83 genes, such as EVA1, DSC2, POSTN, CSPG2, SRPX, ANGPTL4, FN1, ITGAV, COL8A1 and AMPH have important functions related to cell-cell or cell-basement membrane interaction (figure 33). Downregulation of such genes in malignancies helps the tumor cells to spread to surrounding tissues and organs. The EVA1 gene is involved in the formation of homotypic adhesion molecules that help the cells attach to the extracellular matrix. The DSC-2 gene is a component of intercellular desmosome junctions. It is involved in the interaction of plaque proteins and intermediate filaments mediating cell-cell adhesion. It may also contribute to epidermal cell positioning by mediating differential adhesiveness between cells. POSTN induces cell attachment and adhesion. The CSPG2 gene may play a role in intercellular signaling and in connecting cells with the extracellular matrix. It may take part in the regulation of cell motility, growth and differentiation. The SRPX gene is also involved in cell-cell adhesion. The ANGPTL4 gene inhibits proliferation and migration of cells and reduces vascular leakage. FN1 is involved in cell adhesion, cell motility, wound healing, and maintenance of cell shape. Inhibition of the gene mediates inhibition of cell adhesion. ITGAV has an important role in mediating cell-adhesion to extra cellular matrix or to other cells, through hetero-dimerization and connecting to the cytoskeleton and various signaling molecules within cells. COL8A1 has a role in the formation of the major component of the basement

membrane. The AMPH gene may participate in mechanisms of regulated exocytosis in synapses. It may control the properties of the membrane associated cytoskeleton.

Another group of genes, SRFP1, KFL10, NDN, that play a major role of cell growth regulation was also within this 83 downregulated gene list (figure 33). SRFP1 has a role in cell growth regulation and differentiation. KFL10 is involved in the regulation of cell growth by inhibition. The NDN gene is another growth suppressor that facilitates the entry of the cell into cell cycle arrest. Functionally it is similar to the retinoblastoma protein as it binds to and represses the activity of cell-cycle- promoting proteins.

Some of the genes involved with transportation were downregulated in all three OSCC cell lines. These genes are GJB6, SLC2A1, FOLR3, ANXA6, and RAB4B. GJB6 has a role in forming one gap junction that consists of a cluster of closely packed pairs of transmembrane channels, the connexons, through which materials of low molecular weight diffuse from one cell to a neighboring cell. SLC2A1 is the glucose transporter in the cell. FOLR3 mediates delivery of 5-methyltetrahydrofolate to the interior of cells and ANXA6 regulates the release of Ca^{2+} from intracellular stores. The RAB4B gene is effective in protein transport within the cell.

Genes of immune response such as SERPINB3, SEPP1, and IL1F9 were also within the 83 commonly downregulated genes in the three OSCC cell lines (figure 33). The SERPINB3 gene may act as a protease inhibitor to modulate the host immune response against tumor cells. SEPP1 might be responsible for some of the extracellular antioxidant defense properties. IL1F9 could constitute part of an independent signaling system analogous to interleukin-1 alpha, beta receptor agonist and interleukin-1 receptor type I, that is present in epithelial barriers and takes part in the local inflammatory response.

The RGS17 and PTPNS1 genes were downregulated signal transduction inhibitor genes within the 83 downregulated genes (figure 33). RGS17 inhibits signal transduction by increasing the activity of G protein. PTPNS1 plays an important role in signal transduction cascades.

Other listed genes have various different functions in the cell. Some of the important ones are CFHL1, SPRR1B, RHBDL2, MAF, and CALD1. The CFHL1 gene might be involved in complement regulation and can associate with lipoproteins and may play a role in lipid metabolism. The SPRR1B gene produces a keratinocyte protein that first appears in the cell cytosol. RHBDL2 is involved in regulated intramembrane proteolysis. MAF has a role in transcription and the CALD1 gene plays an essential role during cellular mitosis and receptor capping.

5.3.3. Pathway analysis of three OSCC cell lines based on cDNA microarray results

Pathway analyses of each OSCC cell line was performed to be able to better understand the invasion, lymph node metastasis and distant organ metastatic mechanisms of OSCC. Pathway upregulation in cell lines was most pronounced in metabolic pathways including cell nucleotide metabolism and oxidative phosphorylation. Signaling pathways involved in adhesion and communication of cultured cancer cells were downregulated. Based on the expression levels of the genes in the microarray analysis, different pathways were analyzed on each OSCC cell line by using the Kyoto Encyclopedia of Gene and Genomes (KEGG). The pathway analysis was performed based on the criteria that were described before in chapter 4.

5.3.3.1. BHY cell line

Seven pathways based on the p values ($p < 0.05$) of both upregulated and downregulated genes (Figure 40), 8 pathways based on the p values ($p < 0.05$) of only the upregulated genes (Figure 41) and 3 pathways based on the p values ($p < 0.05$) of only downregulated genes (Figure 42) were influenced in the BHY cell line. The most important pathways that were upregulated in the BHY cell line were; regulation of actin cytoskeleton, TGF-beta signaling pathway, dentatorubropallidoluysian atrophy, hedgehog signaling pathway, focal adhesion, Jak-STAT signaling pathway, Parkinson's disease, and the mitogen-activated protein kinase (MAPK) signaling pathway.

In regulation of the actin cytoskeleton pathway, the actin cytoskeleton mediates a variety of essential biological functions in the cell. In addition to providing a structural framework around which cell shape and polarity are defined, its dynamic properties provide the driving force for cells to move and to divide. Members of the Rho family of small guanosine triphosphatases have emerged as key regulators of the actin cytoskeleton, and furthermore, through their interaction with multiple target proteins, they ensure coordinated control of other cellular activities such as gene transcription and adhesion (172). Actomyosin-mediated cell retraction and formation of intercellular gaps occurs through this pathway (173). Actin filaments play important roles in mitosis, cell signaling, and motility. Thus they are the targets of anti-cancer drugs. These drugs target microtubules, and actin filaments to inhibit cell proliferation by blocking mitosis at the mitotic checkpoint and inducing apoptosis (174). Activation of cell surface proteins implicated in cell adhesions can cause the alteration of the actin cytoskeleton, which is required for the function of cell-to-cell or cell-to-extracellular matrix interactions. The

actin cytoskeleton is a dynamic structural entity and constantly undergoes assembly or disassembly. The assembled actin filaments can be further cross-linked to form either actin bundles or actin meshwork. The over activation of this system can cause bone metastasis in breast cancer (175). This pathway was upregulated in all three OSCC cell lines.

TGF-beta signaling pathway activation is common in malignancies. Carcinomas often secrete excess TGF- β 1 and respond to it by enhanced invasion and metastasis. Pre-treatment of adenocarcinoma cells with TGF- β 1 *in vitro* enhanced their subsequent metastatic ability when injected into rats. The TGF- β -treated carcinoma cells became more invasive *in vitro*, and this was attributed to increased expression of gelatinases (or matrix metalloproteases), enhancing tumor cell migration and invasion. TGF- β 1 not only has transforming potential, but can also drive malignant progression, invasion and metastasis both *in vitro* and *in vivo*. However TGF- β can act as both a tumor suppressor and as a significant stimulator of tumor progression, invasion and metastasis. At the initial stage of the cancer TGF- β acts as a tumor suppressor whereas as the tumor progresses, genetic changes allow TGF- β to stimulate tumor progression by its pleiotropic activities on both the cancer cells and on non-malignant stromal cell types of the tumor (176). This may be a clue that the bone invasion phase of the OSCC is an advanced phase of the cancer.

Dentatorubropallidolusian Atrophy (DRPLA) affects both the mind and body. It is characterized by abrupt muscle jerking, involuntary movements, and eventual dementia. The protein product of the DRPLA gene is called atrophin-1. Although its function is unclear, the leading theory is that atrophin-1 is involved in the pathway that helps insulin

take effect in the body's cells. Since insulin helps determine how cells utilize their energy, it is essential that this pathway work smoothly so that cells can function efficiently (177). There are some cases in the literature with DRPLA and where patients died due to lung cancer or prostate cancer, however there is no proven relation between this pathway and cancer.

The Hedgehog signaling pathway is involved in the mechanisms that control cell proliferation and cell differentiation during morphogenesis of the endochondral skeleton of vertebrates. The Hedgehog signaling pathway has a role in the control of chondrocyte maturation by way of feedback control of a second secreted factor which is parathyroid hormone-related protein (PTHrP). Patients with solid tumors and hypercalcemia have increased serum PTHrP. Its production by a tumor is associated with increased tumor size and rate of growth. This pathway coordinates diverse aspects of skeletal morphogenesis through PTHrP-dependent and independent processes. Over expression of PTHrP in chondrocytes causes a delay in chondrocyte maturation and bone formation (178). Tumor necrosis factor (TNF) enhances PTHrP-induced hypercalcemia which causes bone resorption. This effect is due to the increased production of committed osteoclast precursors with a subsequent increase in osteoclastic bone resorption. It was shown that blockage of this pathway suppresses the growth of breast carcinoma cells (179). The upregulation of this pathway is a common process for OSCC (180).

The focal adhesion pathway has a role in mechanical adhesion of the cells to the extracellular matrix which is important for cell growth, survival and migration. Analysis of primary human prostate cancer cells isolated from 16 surgical specimens showed that these cells expressed the focal adhesion pathway, whereas normal prostate epithelial cells

did not. Also, PC3, which is a prostate cancer cell line was found to invade mature bone and highly express focal adhesion pathway (181). However, to the best of our knowledge there has been no study relating invasion of OSCC to bone and this pathway.

The Jak-STAT signaling pathway was another pathway that was upregulated in the BHY cell line. A wide variety of extracellular signals activate the signal transducers and activators of transcription (STAT) class of transcription factors. Many cytokines, lymphokines, and growth factors signal through a related superfamily of cell surface receptor tyrosine kinases that are associated with and activate Janus kinases (JAKs) (182). The urokinase-type plasminogen activator receptor (uPAR) generates a proteolytic cascade important for matrix degradation in tumor invasion and tissue remodeling. Binding of uPA to uPAR at the cell surface stimulated cell migration. In contrast to normal cells, malignant tumor cells usually overexpress uPAR. uPAR, together with its signaling functions, coordinate tumor cell adhesion and invasive migration through the extracellular matrix (ECM) and to determine whether tumor cells will, or will not, grow *in vivo*. The Jak-STAT signaling pathway is one of the pathways that can be activated by the uPA- uPAR interaction (183). The activation of this pathway has been shown to be involved in bone metastasis in prostate cancer (184). One other role of this pathway is activation of MMP which has a role in bone invasion of OSCC. Upregulation of MMPs in the BHY cell line can be due to the activation of this pathway.

The mitogen-activated protein kinase signaling pathways transduce a large variety of external signals, leading to a wide range of cellular responses, including growth, differentiation, inflammation and apoptosis. It is thought that the balance between distinct MAPK pathways regulates cell growth, differentiation, survival, and death. They also

have a role in the regulation of MMP gene expression, especially MMP-1 and MMP-3. In addition to MMP-1 and MMP-3, increased transcriptional activity of MMP-9 can be regulated by the MAPK signaling pathway in malignant cells. Invasive cancer cells and stromal cells at the invading edge of tumors are under physical stress, such as hypoxia and hyperthermia. Stress activates MAPKs which is responsible for regulation of MMP expression (185). Inhibition of the MAPK pathway could serve as a therapeutic target to specifically prevent tumor cell invasion.

Based on the downregulated genes in BHY cell lines there were 3 downregulated pathways ($p < 0.05$) (Figure 42). These pathways were olfactory transduction, taste transduction and ECM-receptor interaction pathways. No relation was found in the literature between OSCC and either the olfactory or the taste transduction pathway. Pathways that were downregulated in cell lines compared to tumor and normal tissue included cell communication, cell adhesion molecules (CAMs), and ECM-receptor interaction. Degradation of ECM is one of the essential steps in invasion and metastasis of the tumor (186). Integrins and growth factors play an important part in maintaining tissue architecture. Disruption of the balance of signaling results in dramatic changes in the way cells interact with each other and with the extracellular matrix, leading to cancer. The extracellular matrix itself plays a central role in coordinating these signaling processes. Downregulation of this pathway helps tumor cells migrate to distant organs thus, metastasis and invasion. ECM-receptor interaction was found to be of relevance to head and neck SCC (187). This pathway was not only downregulated in the BHY cell line but also in the HN and HSC-3 cell lines according to the results of this study.

5.3.3.2. HN cell line

Ten different pathways showed upregulation in the HN cell line ($p < 0.05$) (Figure 44). Seven of them, which are MAPK signaling pathway, regulation of actin cytoskeleton, antigen processing and presentation, focal adhesion, cell cycle, calcium signaling pathway, and axon guidance, were closely related with invasion and metastasis of the cancer.

As previously explained, MAPK pathway regulates cell growth, differentiation, survival, and death. This pathway was also upregulated in the BHY and HSC-3 cell lines. As it regulates MMP expression, this pathway may be involved in distant organ and lymph node metastasis of OSCC. Transfection studies indicate that many genes can induce metastatic ability, including familiar oncogenes such as RAS and MYC that have a role in the MAPK signaling pathway. The metastatic process requires a complex set of cellular functions such as changes in adhesion, and initiation of motility and extracellular matrix proteolysis, which culminates in the shedding of cells into the circulation either directly or via the lymphatics. A hyperactive Raf/MAPK pathway is required for epithelial mesenchymal transition that correlates with metastasis (188). This pathway was upregulated in all three OSCC cell lines. This may mean that the MAPK signaling pathway is one of the major pathways related to OSCC invasion and metastasis.

Regulation of the actin cytoskeleton was another important pathway that was upregulated in all three OSCC cell lines. As it was mentioned before, regulation of the actin cytoskeleton provides the driving force for cells to move and to divide. Cell-cell adherence junctions, the most common type of intercellular adhesions, are important for maintaining tissue architecture and cell polarity, and can limit cell movement and

proliferation. Cell-cell adherence junctions assemble via homophilic interactions between the extracellular domains of calcium-dependent cadherin receptors on the surface of neighboring cells. The cytoplasmic domains of cadherins bind to the submembranal plaque proteins β -catenin or plakoglobin (γ -catenin), which are linked to the actin cytoskeleton via α -catenin. The transmembrane assembly of cadherin receptors with the cytoskeleton is necessary for the stabilization of cell-cell adhesions and normal cell physiology. Malignant transformation is often characterized by major changes in the organization of the cytoskeleton, decreased adhesion, and aberrant adhesion-mediated signaling. Disruption of normal cell-cell adhesion in transformed cells may contribute to enhance tumor cell migration and proliferation, leading to invasion and metastasis. This disruption can be achieved by downregulating the expression of cadherin or catenin family members or by activation of signaling pathways that prevent the assembly of cell-cell adherence junctions such as the actin cytoskeleton. Thus, regulation of the actin cytoskeleton is important in cell-cell adherence junction (173).

Antigen processing and presentation are processes that occur within a cell that result in fragmentation (proteolysis) of proteins, association of the fragments with major histocompatibility complex (MHC) molecules, and expression of the peptide-MHC molecules at the cell surface where they can be recognized by the T cell receptor. However, the path leading to the association of protein fragments with MHC molecules differs for class I and class II MHC. MHC class I molecules present degradation products derived from intracellular (endogenous) proteins in the cytosol. MHC class II molecules present fragments derived from extracellular (exogenous) proteins that are located in an intracellular compartment. Dendritic cells are antigen-presenting cells with a unique

ability to cross prime T cells and generate strong anti-tumor responses. Reichert et al. reported that dendritic cell infiltrates were low in 20% of OSCC specimens, intermediate in 42% of OSCC specimens, and high in 37% of OSCC specimens (189). The upregulation of this pathway only in the HN cell line may suggest that the immune system reacts against OSCC in advanced stages of the tumor.

The focal adhesion pathway serves as the mechanical linkage to the ECM, and as a biochemical signalling hub to concentrate and direct numerous signaling proteins at sites of integrin binding and clustering. The activation of this pathway may cause metastasis by OSCC (23). Integrins are cell surface receptors that mediate the adhesion of cells to the extracellular matrix. They are essential for tissue organization and survival. They allow normal, nontransformed cells to sense that they are adhered to the extracellular matrix, thus providing a cell survival signal. Focal adhesion kinase (FAK) becomes phosphorylated and activated during integrin-mediated cell adhesion. Focal adhesion kinase is a signal transducer of integrins. Enhanced expression of focal adhesion kinase in oral carcinoma cells may cause growth and increased invasive potential of the primary oral tumor (190). High expression of this pathway may be associated with distant metastasis of OSCC.

The cell cycle pathway is the series of events in a eukaryotic cell between one cell division and the next. The cell cycle consists of four distinct phases: G₁ phase, S phase, G₂ phase (collectively known as interphase) and M phase. M phase is itself composed of two tightly coupled processes: mitosis, in which the cell's chromosomes are divided between the two daughter cells, and cytokinesis, in which the cell's cytoplasm physically divides. Cells that have temporarily or reversibly stopped dividing are said to have entered a state

of quiescence called G_0 phase. Some cells may permanently stop dividing due to age or accumulated DNA damage. Some cell types in mature organisms, such as parenchymal cells of the liver and kidney, enter the G_0 phase semi-permanently and can only be induced to begin dividing again under very specific circumstances; other types, such as epithelial cells, continue to divide throughout an organism's life. The G_1 phase is a period in the cell cycle during interphase, before mitosis, cytokinesis and the S phase. For many cells, this phase is the major period of cell growth during its lifespan. During this stage new organelles are being synthesized, so the cell requires both structural proteins and enzymes, resulting in great amounts of protein synthesis. The metabolic rate of the cell is high. The S phase, designated by DNA synthesis, is a period in the cell cycle during interphase, between G_1 phase and the G_2 phase. Following G_1 , the cell enters the S stage, when DNA synthesis or replication occurs. At the beginning of the S stage, each chromosome is composed of one coiled DNA double helix molecule, which is called a chromatid. At the end of this stage, each chromosome has two identical DNA double helix molecules, and therefore is composed of two sister chromatids. During the S phase, the centrosome is also duplicated. Damage to DNA often occurs during this phase. DNA repair is initiated following the completion of replication. The G_2 phase is the third, final, and usually the shortest subphase during interphase within the cell cycle. It follows successful completion of DNA synthesis and chromosomal replication during the S phase. The G_2 phase prepares the cell for mitosis (M phase) which is initiated by prophase. The G_2 checkpoint determines if the cell can proceed to the M phase and divide. The G_2 checkpoint prevents cells from entering mitosis with DNA damage since the last division, providing an opportunity for DNA repair and stopping the proliferation

of damaged cells. Because the G₂ checkpoint helps to maintain genomic stability, it is an important focus in understanding the molecular causes of cancer. Mitosis (M phase) is the process by which a cell separates its duplicated genome into two identical halves. It is generally followed immediately by cytokinesis which divides the cytoplasm and cell membrane. This results in two identical daughter cells with a roughly equal distribution of organelles and other cellular components. Mitosis and cytokinesis together is defined as the M phase of the cell cycle, the division of the mother cell into two daughter cells, each the genetic equivalent of the parent cell. There are two key classes of regulatory molecules that determine a cell's progress through the cell cycle: cyclins and cyclin-dependent kinases (191). The cyclin family members interact with CDKs, and these complexes are required to pass through specific phases of the cell cycle. D-type cyclins interact with CDK4 and CDK6 and are necessary for G₀/G₁ transition. Cyclin E binds to CDK2 and mediates S phase entry. The cyclin A/CDK2 complex regulates passage through the S-phase. Later, in conjunction with CDC 2, cyclin A also induces the G₂ phase of the cell cycle. Cyclin B1 and CDC2 trigger the molecular events associated with mitosis (192). Cell cycle pathway upregulation is common in cancers with high metastatic rates. Cellular proliferation depends on the rates of both cell division and cell death. All tumors have decreased cell death and increased cell proliferation. Genetic changes may cause the loss of apoptosis. This change is a major component of tumorigenesis. Many of the gene products that appear to control apoptotic tendencies are regulators of cell cycle progression; thus, cell cycle control and cell death appear to be tightly linked processes. p53 protein is an example of a gene product which affects both cell cycle progression and apoptosis. The ability of p53 overexpression to induce

apoptosis may be a major reason why tumor cells frequently disable p53 during the transformation process. Unfortunately, the same genetic changes which cause loss of apoptosis during tumor development, may also result in tumor cell resistance to anti-neoplastic therapies which kill tumor cells by apoptosis (46, 58, 68). The upregulation of both cyclins and cyclin-dependent kinase genes were observed in the HN cell line. Cyclins and cyclin-dependent kinases could be another two groups of genes that are responsible for metastasis of OSCC.

Calcium signaling pathway is another activated pathway in the HN cell line. It has been shown that prostate cancer frequently metastasizes to bone. Elevated extracellular Ca^{+2} can enhance proliferation of skeletal metastases (193). Increased Ca^{+2} signaling pathway plays an important role in cancer progression and metastasis (194).

The axon guidance pathway is an important process in the development of the nervous system. It was shown that semaphorin 4D (Sema4D) protein, which is responsible in the development of the central nervous system and regulates axonal growth, is highly expressed in cell lines derived from head and neck squamous cell carcinomas. Sema4D stimulates endothelial cell migration and angiogenesis (195). A similar pattern was observed in malignant cells from prostate, colon, breast, and lung cancer tissues (196, 197). Axon guidance was highly upregulated only in the HN cell line. This suggests that upregulation of this pathway may occur in advance stages of OSCC and may have a role in metastasis of OSCC.

Based on the downregulated genes in the HN cell lines there were 9 downregulated pathways ($p < 0.05$) (Figure 45). These pathways were Hedgehog signaling pathway, circadian rhythm, olfactory transduction, Wnt signaling pathway, notch signaling

pathway, ECM receptor interaction, insulin signaling pathway, taste transduction, and amyotrophic lateral sclerosis.

The hedgehog signaling pathway was a downregulated pathway in this cell line. The Wnt and hedgehog signaling pathways have long been known to direct growth and patterning during embryonic development. There are some studies that show the activation of the hedgehog signaling pathway in various types of malignancies, however, it remains unclear whether this pathway is activated in all cancer types (179). This pathway was upregulated in the BHY cell line whereas it was downregulated in the HN cell line. Thus, this pathway may be involved in bone invasion but not metastasis.

Circadian rhythm is a roughly-24-hour cycle in the physiological processes of living beings. Jones et al. reported the expression of *per-1*, which is a circadian rhythm gene, in oral dysplasias and OSCC for the first time (198). There was significantly reduced expression of *per-1* protein in OSCC compared to normal epithelial controls and dysplasias based on Jones et al. study (198). Downregulation of this pathway in the HN cell line was also a result of this study. Thus, circadian rhythm may play a role in oral carcinogenesis. Circadian rhythm is a changeable process during the day. It was proven that there is a statistically significant variation in the nuclear expression of all of normal oral epithelial cell cycle associated proteins during a day. The time of day for maximum p53 expression for normal epithelial cell (late G₁ phase) is in the morning. This is followed 4 hours later by maximum cyclin E expression (G₁/S phase), and 5 hours later by maximum cyclin A expression (G₂ phase), whereas the maximum expression for cyclin B1 (M phase) is 10 hours later in the evening. The importance of this information for cancer treatment is the timing of chemotherapy. Several chemotherapeutic drugs have

cell cycle phase dependent effects. The greatest radiosensitivity for cells is in G₂ and M phases, which occurs in the late afternoon/evening. Thus, the timing of chemotherapy and radiotherapy during the day might be important in terms of the effectiveness of the treatment (199).

Wnt signaling pathway proteins form a family of highly conserved secreted signaling molecules that regulate cell-to-cell interactions during embryogenesis. Leethanakul et al. found that most head and neck SCC overexpress members of the Wnt and notch growth and differentiation regulatory system (200). However, Yeh et al. concluded that Wnt pathway related genes play a very limited role in the development of OSCC (201). Deregulation of the Wnt pathway has been shown to occur by several different mutational mechanisms in human cancers (202).

The notch signaling pathway is a gene regulatory pathway involved in multiple differentiation processes. The notch protein sits like a trigger penetrating the cell membrane, with part of it inside and part outside. Proteins when binding to the exterior part cause the release of a portion of the interior part, which then makes its way to the cell nucleus to alter gene expression. This pathway is a highly conserved pathway for cell-cell communication. It is involved in the regulation of cellular differentiation, proliferation, and specification. According to a study MAPK in head and neck SCC cells triggered notch signaling activation in neighboring endothelial cells and promotes angiogenesis (203). The Wnt, TGF-beta, BMP, Notch, and Par polarity pathways are the major players in homeostatic control of the adult epithelium. Several hereditary cancer syndromes deregulate these same signaling cascades through mutation. It was shown that JAGGED1, a NOTCH receptor ligand, is significantly more highly expressed in

metastatic prostate cancer as compared with localized prostate cancer or benign prostatic tissues (204). However this pathway was downregulated in the HN cell line based on the cDNA microarray analysis performed in this study.

ECM receptor interaction was another downregulated pathway in the HN cell line. As explained previously in the BHY cell line, ECM receptor interaction has a role in integrins and growth factors signaling process and was downregulated in all three OSCC cell lines. No correlations have been found between the formation of OSCC and other downregulated pathways, including insulin signaling pathway, taste transduction, and amyotrophic lateral sclerosis, in the HN cell line.

5.3.3.3. HSC-3 cell line

The 3 upregulated pathways in the HSC-3 cell line ($p < 0.05$) (Figure 47), were regulation of actin cytoskeleton, MAPK signaling pathway, and Toll like receptor signaling pathway, and were closely related with the invasion and metastatic potential of the cancer. The MAPK signaling pathway and regulation of actin cytoskeleton pathway was not only upregulated in HSC-3 cell line but also in BHY and HN cell lines as described previously. This may mean that the MAPK signaling pathway and regulation of actin cytoskeleton pathway are the major pathways related to OSCC invasion and metastasis. Toll like receptor signaling pathway was the only pathway that was only upregulated in HSC-3 cell line. This pathway is a novel pathway that engages the apoptotic machinery (205). Toll-like receptors recognize many pathogens that are involved in the induction of anti-cancer immunity. TLR4 signaling, which is a member of toll-like receptor, is intimately involved in anti-cancer immunity (206).

Six pathways were downregulated in the HSC-3 cell line ($p < 0.05$) (Figure 48).

These pathways were ECM-receptor interaction, olfactory transduction, taste transduction, Huntington's disease, GAP junction and cell adhesion molecules. The ECM-receptor interaction pathway has a role in integrins and growth factors signaling process and was downregulated in all three OSCC cell lines. Thus, this pathway could be a common pathway that downregulates during the invasion and metastatic process of OSCC.

The gap junction is a junction between certain cell-types that allows different molecules and ions to pass freely between cells. The junction connects the cytoplasm of adjacent cells. Cell-cell interaction via gap junctions is a key factor in tissue homeostasis as they mediate intercellular communication in control of cell growth. Growth rates of tumor cells are negatively correlated with strength of intercellular communication. Thus, alteration of the gap junction may cause pathology (207). Downregulation of this pathway may cause tumor cells to move freely and penetrate into lymph vessels, thus causing lymph node metastasis.

Cell adhesion molecules are proteins located on the cell surface involved with the binding with other cells or with the ECM. These molecules have a role in cell division, migration, and differentiation. The expression of cell adhesion molecules is normally tightly regulated, thereby controlling cell proliferation, mobility, differentiation, and survival. Many of these processes are misregulated in malignant tumors, and it has been shown that many of the characteristics of tumor cells are attributable to the abnormal expression or function of cell adhesion molecules (208). Like the Gap junction pathway

inhibition, the inhibition of the cell adhesion molecules might help tumor cells to metastasize to lymph nodes.

No correlation between OSCC and olfactory transduction, taste transduction, and Huntington's disease pathway whatsoever has been found in the literature. Interestingly olfactory and taste transductions were downregulated in all the three OSCC cell lines.

5.3.4. Real Time PCR analysis

Real Time PCR analysis helps to determine the quantitative differences in mRNA expression. This method is the most sensitive method and can discriminate closely related mRNAs. Thus, this makes the technique an excellent one to confirm the results of cDNA microarray results. The most upregulated three genes from each cell line were chosen to run the Real Time PCR. The results of cDNA microarray analysis correlated with Real Time PCR results (figure 49), which makes the results of cDNA microarray analysis reliable.

5.4. Recommendations

The results of this study will help to better understand the invasion and metastatic mechanisms of OSCC, however, further studies need to be performed. Studies that will focus on the specific pathways that are upregulated and downregulated in these three OSCC cell lines may be a part of the future studies. Based on the results of this study the pathways that needs to be focused on are cell cycle, Jak-STAT, regulation of actin cytoskeleton, MAPK signaling, hedgehog signaling, focal adhesion, Wnt signaling and notch signaling pathways. The effect of chemotherapeutic drugs on these pathways may be helpful to make a better drug choice in the clinic.

This study focused on the gene expression profile of three OSCC cell lines with different invasion and metastatic capacities. Another study may be performed by investigating the protein expression profile of these cell lines. Thus, invasion and metastasis of OSCC can be understood in greater detail and also a better drug target can be identified. Proteomics might be an excellent means for such a study.

The cDNA microarray technique with laser microdissection can also be used in the clinic by using patients' tissue samples. Instead of using cell lines, by using tissue samples more accurate gene expression profile of OSCC can be determined. Also, a gene data bank can be formed which may assist in determining a better target gene to treat the OSCC. In addition to this, it may find a gene for early diagnosis of OSCC. If a gene data bank can be formed, based on the gene expression profile of each patient a better assumption can be done about the prognosis of the patients.

APPENDICES

APPENDIX A: TABLES

Table 1: Normal and pathological processes involving MMPs (21).

Pathological	Normal
1.Tissue Destruction Rheumatoid arthritis Osteoarthritis Cancer invasion Cancer metastasis Ulcer Periodontal diseases	1.Development •Blastocyst implantation •Embryonic development •Nerve growth •Bone growth •Enamel maturation •Primary tooth root resorption
2.Fibrotic Diseases Liver cirrhosis Fibrotic lung diseases Otosclerosis Atherosclerosis Multiple sclerosis	2.Reproduction •Endometrial cycling •Graafian follicle rupture •Cervical dilatation •Postpartum uterine involution •Mammary gland morphogenesis •Mammary gland involution •Rupture of fetal membrane
3.Weakening of Matrix Dilated cardiomyopathy Epidermolysis bullosa Aortic aneurysm	3.Maintenance •Remodeling of bone •Hair follicle cycle •Wound healing •Angiogenesis •Apoptosis •Macrophage function •Neutrophil function

Table 2: Common highly expressed genes in normal oral squamous epithelium (93).

Symbol of gene	Description
GTT1	GTT1 protein
RPL12	Ribosomal protein L12
EMP1	Epithelial membrane protein 1
CSTB	Cystatin B (stefin B)
OCIA	Ovarian carcinoma immunoreactive antigen
PRKWNK1	Protein kinase, lysine deficient 1
BRF1	Butyrate response factor 1 (EGF-response factor 1)
UBA2	SUMO-1 activating enzyme subunit 2
H4FG	H4 histone family, membrane G
ECM1	Extracellular matrix protein 1
RPS29	Ribosomal protein S29
TGM3	Transglutaminase 3 (E polypeptide, protein-glutamine-gamma-glutamyltransferase)
KIAA0336	K1AA0336 gene product
FLJ11090	Hypothetical protein FLJ11090
IK	IK cytokine, down-regulator of HLA II
ATP1B3	ATPase, Na ⁺ /K ⁺ transporting, beta 3 polypeptide
LOC55893	Papillomavirus regulatory factor PRF-1
FLJ13081	Hypothetical protein FLJ 13081
IVL	Involucrin
ATP2A2	ATPase, Ca ⁺⁺ transporting, cardiac muscle, slow
FLJ10375	Hypothetical protein FLJ 10375
STAG2	Stromal antigen 2
PLEC1	Plectin 1, intermediate filament binding protein

Table 3: Common highly expressed genes in OSCC (93).

Symbol of gene	Description
RPL37A	Ribosomal protein L37a
ACTB	Actin, beta
H3F3A	H3 histone, family 3A
S100A7	S100 calcium-binding protein A7 (psoriasin 1)
NFE2L2	Nuclear factor (erythroid-derived 2)-like 2
RPL21	Ribosomal protein L21
RPS6	Ribosomal protein S6
GTF2I	General transcription factor II
HMG1	High-mobility group (nonhistone chromosomal) protein
UCH37	Ubiquitin C- terminal hydrolase UCH37
LAMR1	Laminin receptor 1 (67 kD, ribosomal protein SA)
GSTP1	Gluthathione S-transferase pi
CTNNA1	Catenin (cadherin-associated protein), alpha1
LOC51142	16.7 Kd protein
IL4R	Interleukin 4 receptor
FXYD3	FXYD domain-containing ion transport regular 3
SPRR2A	Small protein-rich protein 2A
ANXA5	Annexin A5
RPS21	Ribosomal protein S21
QP-C	Low molecular mass ubiquinone-binding protein (9.5 kD)
RPL4	Ribosomal protein L4
D6S81E	HLA-B associated transcript-1
LEPR	Leptin receptor

Table 4: 16 genes that are highly upregulated in tongue SCC cases (109).

Gene	Molecular Function	Chromosomal Position
Hypothetical protein	Unknown	Chromosomes 19
IGHM	Immune system	14q32.33
SPONI	Extracellular Matrix protein	11p14-p15.2
PKM2	Catalyses the production of phosphoenolpyruvate	15q22
IGKC	Immune system	2p12
KIAA0007	Unknown	2p23.2
C3orf6	Unknown	Chromosomes 3
Hypothetical protein	Unknown	6p22.3
Hypothetical protein MGC33424	Unknown	Unknown
P4HB	Protein disulphide isomerase	17q25
CALR	Calciumstrage, transcription co-repressor	19p13.3-p13.2
CAPZB	Actin Binding	1pG6.1
Rabla (RAS oncogene family)	RAB small monomeric GTPase	2p14
RALBPI	GTPase activator	1sp11.3
3ERPINF1	Serine protease inhibitor	17p13.1

Table 5: Upregulated and downregulated gene list of 9 OSCC patients (136).

Upregulated Genes		Downregulated Genes	
Category	Gene	Category	Gene
Adhesion	CDH3	Apoptosis	MAL
Adhesion	TGFB1	Biosynthesis	IMPDH2
Apoptosis	FADD	Biosynthesis	MRPL16
Apoptosis	LGALS1	Biosynthesis	RPL15
Biosynthesis	C20orf3	Cell cycle	DUSP1
Cell cycle	DKC1	Metabolism	ALDH9A1
Cell cycle	SH3BP4	Metabolism	DPYSL2
Growth	PLAU	Metabolism	H11
Growth	TGF	Metabolism	MGLL
Metabolism	BRS3	Metabolism	PAM
Metabolism	FLJ13855	Transcription	FOS
Transcription	EN1	Transcription	NOTCH1
Transcription	HSF1	Transcription	SF1
Transcription	HMGB1	Other	GPX3
Transcription	MCM4	Other	IK
Transcription	RFX5	Other	VAV3
Transcription	TEAD4	Unknown	GLTSCR2
Other	ARPC1B	Unknown	LLGL2
Other	DSS1	Unknown	TXNIP
Other	IFI30		
Other	NXN		
Unknown	CAV1		
Unknown	FLJ13031		
Unknown	GPCR41		
Unknown	LAPTM4B		
Unknown	LYRIC		
Unknown	MGC2477		
Unknown	MGC45714		

Table 6: Common upregulated genes in head and neck SCC (111).

Upregulated genes name	Symbol
Cadherin 11, type 2, OB-cadherin (osteoblast)	CDH11
Cathepsin L	CTSL
Chemokine (C-X-C motif) ligand 10	CXCL10
Chemokine (C-X-C motif) ligand 1 (melanoma growthstimulating activity, alpha)	CXCL1
Collagen, type I, alpha 1	COL1A1
Collagen, type IV, alpha 1	COL4A1
Collagen, type IV, alpha 2	COL4A2
Collagen, type V, alpha 2	COL5A2
Fibronectin 1	FN1
Integrin, alpha 6	ITGA6
Integrin, beta 4	ITGB4
Interferon, alpha-inducible protein (clone IFI-6-16)	G1P3
Interleukin-6 (interferon, beta 2)	IL6
Interleukin-8	IL8
Matrix metalloproteinase 1 (interstitial collagenase)	MMP1
Matrix metalloproteinase 3 (stromelysin 1, progelatinase)	MMP3
Matrix metalloproteinase 10 (stromelysin 2)	MMP10
Matrix metalloproteinase 12 (macrophage elastase)	MMP12
Microfibrillar-associated protein 2	MFAB2
Parathyroid hormone-like hormone	PTH1H
Periostin, osteoblast-specific factor	POSTN
Profilin 2	PFN2
ras homolog gene family, member C	RHOC
Runt-related transcription factor 1 (acute myeloid leukemia 1; aml1 oncogene)	RUNX1
Secreted phosphoprotein 1 (osteopontin, bone sialoproteinI, early T-lymphocyte activation 1)	SPP1
Secreted protein, acidic, cysteine rich (osteonectin)	SPARC
Serine (or cysteine) proteinase inhibitor, clade E, member 1	SERPINE1
Superoxide dismutase 2, mitochondrial	SOD2
Tenascin C (hexabrachion)	TNC
Thrombospondin 2	THBS2
Transforming growth factor, beta-induced, 68 kD	TGFBI
Trophoblast glycoprotein	TPBG

Table 7: Common down regulated genes in head and neck SCC (111).

Downregulated genes name	Symbol
Absent in melanoma 1	AIM1
Actin binding LIM protein 1	ABLIM1
Aldehyde dehydrogenase 9 family, member A1	ALDH9A1
Annexin A1	ANXA1
BENE protein	BENE
Carcinoembryonic antigen-related cell adhesion molecule 1	CEACAM1
Clusterin	CLU
Creatine kinase, mitochondrial 1 (ubiquitous)	CKMT1
Cyclin G2	CCNG2
Cystatin B	CSTB
Deoxyribonuclease I-like 3	DNASE1L3
Dual specificity phosphatase 5	DUSP5
Envoplakin	EVPL
Epithelial membrane protein 1	EMP1
ECM protein 1	ECM1
Hydroxyprostaglandin dehydrogenase 15-(NAD)	HPGD
Interleukin-1 receptor antagonist	IL1RN
Keratin 13	KRT13
Keratin 15	KRT15
Keratin 4	KRT4
KIAA0089 protein (GPD1L: glycerol-3-phosphate dehydrogenase 1-like)	GPD1L
Mal, T-cell differentiation protein	MAL
Monoamine oxidase B	MAOB
Monoglyceride lipase	MGLL
Neuromedin U	NMU
Paired-like homeodomain transcription factor 1	PITX1
Periplakin	PPL
Sciellin	SCEL
Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 1	SERPINB1
Serine protease inhibitor, Kazal type 5	SPINK5
Tetranectin (plasminogen binding protein)	TNA
Transglutaminase 3 (E polypeptide, protein-glutaminegamma-glutamyltransferase)	TGM3
Zinc finger protein 185 (LIM domain)	ZNF185

Table 8: List of genes with conflicting expression data in head and neck SCC (111).

Genes with conflicting expression data	Number of studies	Symbols
Carcinoembryonic antigen-related cell adhesion molecule 5	5 down, 1 up	CEACAM5
Collagen, type I, alpha 2	9 down, 1 up	COL1A2
Cystatin A (stefin A)	6 down, 1 up	CSTA
Dual specificity phosphatase 6	4 down, 1 up	DUSP6
FAT tumor suppressor homolog 1	6 down, 1 up	FAT
Fibroblast activation protein, alpha	4 down, 1 up	FAP
Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	5 down, 1 up	ITGA3
Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	4 down, 1 up	ITGB1
Keratin 16 (focal nonepidermolytic palmoplantar keratoderma)	4 down, 2 up	KRT16
Keratin 17	5 down, 2 up	KRT17
Laminin, alpha 3	4 down, 2 up	LAMA3
Laminin, beta 3	5 down, 1 up	LAMB3
Laminin, gamma 2	6 down, 1 up	LAMC2
Lumican	4 down, 1 up	LUM
Plasminogen activator, urokinase	10down, 1 up	PLAU
Tumor necrosis factor, alpha-induced protein 3	5 down, 1 up	TNFAIP3
TYRO3 protein tyrosine kinase	4down, 1 up	TYRO3
v-fos FBJ murine osteosarcoma viral oncogene homolog	3 down, 2 up	FOS
v-myc myelocytomatosis viral oncogene homolog (avian)	4 down, 1 up	MYC

Table 9: Ontology of altered genes in head and neck Carcinoma (111).

Ontology	Number of genes identified	Genes
Extracellular matrix	22	COL1A1, COL1A2, COL4A1, COL4A2, COL5A2, ECM1, FN1, LAMA3, LAMB3, LAMC2, LUM, MFAP2, MMP1, MMP3, MMP10, MMP12, POSTN, SPARC, SPP1, TGFB1, THBS2, TNC
Cell adhesion	20	CDH11, CEACAM1, CEACAM5, FAT, FN1, IL8, ITGA3, ITGA6, ITGB1, ITGB4, LAMA3, LAMB3, LAMC2, POSTN, SPP1, TGFB1, THBS2, TNC, TPBG, TYRO3
Integral to membrane	16	BENE, CDH11, CEACAM1, CEACAM5, EMP1, FAP, FAT, G1P3, ITGA3, ITGA6, ITGB1, ITGB4, MAL, MAOB, TPBG, TYRO3
Epidermal development/differentiation	12	COL1A1, EMP1, EVPL, KRT13, KRT15, KRT16, KRT17, LAMA3, LAMB3, LAMC2, PTHLH, SCEL, TGM3
Signal transduction	11	ANXA1, CXCL1, CXCL10, IL6, IL8, LAMA3, MAL, NMU, PLAUI, RHOC, TYRO3
Ca ²⁺ binding	10	ANXA1, CDH11, DNASE1L3, FAT, MMP1, MMP3, MMP12, SPARC, TGM3, THBS2
Cytoskeleton	10	ABLIM1, CXCL1, EVPL, KRT4, KRT13, KRT15, KRT16, KRT17, PFN2, PPL
Cell growth and proliferation	9	CXCL1, CXCL10, EMP1, IL6, IL8, KRT16, MYC, PTHLH, TGFB1
Inflammation	9	ANXA1, CXCL1, CXCL10, FOS, IL1RN, IL8, MGLL, SPINK5, SPP1
Zn ²⁺ binding	9	ABLIM1, MMP1, MMP3, MMP10, MMP12, RUNX1, SCEL, TNFAIP3, ZNF185
Cell motility/chemotaxis	8	ANXA1, CXCL1, CXCL10, IL8, MMP12, PLAUI, SPP1, TPBG
Proteolysis	7	CTSL, FAP, MMP1, MMP3, MMP10, MMP12, PLAUI
Basement membrane	6	COL4A1, COL4A2, LAMA3, LAMB3, LAMC2, SPARC
DNA binding	6	DNASE1L3, FOS, MYC, PITX1, RUNX1, TNFAIP3
Skeletal development	6	CDH11, COL1A1, COL1A2, PITX1, POSTN, TNA
Transcriptional regulation	6	FOS, MYC, PITX1, RUNX1, SOD2, TNFAIP3
Redox activity	5	ALDH9A1, GPD1L, HPGD, MAOB, SOD2

Table 10: List of proteins with increased and decreased expressions in OSCC (131).

Proteins with increased expression	Proteins with decreased expression
1- Cofilin 2- C-reactive protein 3- Creatine kinase, M-chain 4- Cytokeratin 6B 5- Fatty acid-binding protein 6- Galectin-7 7- Glutathione-S-transferase 8- HSP27 9- Keratin type II 10- NME1 11- Phosphoglucerate mutase 1 12- Pyruvate kinase M1 13- Retinoic acid-binding protein II 14- SCCA-1	1- Annexin 1 (ANXA1) 2- Heat shock 20 kDa like protein p20 3- Myosin light chain 1 4- Myosin light chain 2 5- Myosin light chain 3 6- Plakoglobin

Table 11: The expression levels of proteolytic enzymes by using immunocytochemistry.

Proteolytic Enzymes	OSCC Cell Lines		
	BHY	HN	HSC-3
MMP-1	+++	+++	+++
MMP-2	+++	++++	++++
MMP-3	+++	+++	++++
MMP-9	+++	++	-
EMMPRIN	++++	+++	+++
Cathepsin B	++++	++++	++++
Cathepsin L	+++	+++	++++

(-) Shows the same intensity in tumor cells as normal epithelium cells; (+) less than 10% stronger intensity in tumor cells than normal epithelium cells; (++) between 10% to 50% stronger intensity in tumor cells than normal epithelium cells; (+++) between 50% to 90% stronger intensity in tumor cells than normal epithelium cells; (+++++) more than 90% stronger intensity in tumor cells than normal epithelium cells.

Table 12: The expression levels of proteolytic enzymes in three different OSCC cell lines by using flow cytometry.

Proteolytic Enzymes	OSCC Cell Lines			
	HNEC	BHY	HN	HSC-3
MMP-1	97% +	99.8% +	99.9% +	99.9% +
MMP-2	98.8% +	99.1% +	99.6% +	99.9% +
MMP-3	98.4% +	99.9% +	99.8% +	99.9% +
MMP-9	0.5 % +	33.3% +	18.6% +	0.05% +
EMMPRIN	70.1% +	85.8% +	67.5% +	73.4% +
(-) Control (No staining)	99.9% -	95.3% -	98.1% -	99.8% -

Table 13: 139 upregulated genes in BHY cell line. The gene list is from the most upregulated gene to least upregulated gene.

Name of the upregulated gene in BHY cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
H300004818_MAGEA9	2.9835	0.740776	0.248291	4	7.909026
H200001373_Q96AL8_HUMAN	2.9755	0.472755	0.158883	4	7.86529
H200005842_GFI1	2.75875	0.799114	0.289665	4	6.768096
opHsV0400005829_OAS1	2.6725	0.637914	0.238695	4	6.37533
H200007111_NP_689700.1	2.60575	0.633773	0.243221	4	6.087079
H200005801_MAGEA1	2.55525	0.884437	0.346125	4	5.877693
opHsV0400002611_PRAME	2.5525	0.286732	0.112334	4	5.8665
opHsV0400006088_NOV	2.5515	1.062371	0.416371	4	5.862435
H200011702_HOXA10	2.54125	0.590219	0.232255	4	5.820931
H200012712_BIRC3	2.3735	0.457046	0.192562	4	5.181968
H200004378_MAGEA8	2.36275	1.281263	0.542276	4	5.143499
H200000112_DLX2	2.354	0.63516	0.269822	4	5.112397
H200003290_DEPDC1B	2.3485	0.45264	0.192736	4	5.092945
H200000421_DPYD	2.30175	0.37118	0.16126	4	4.930555
H200000404_IRK2_HUMAN	2.298	0.313003	0.136207	4	4.917755
opHsV0400006060	2.2975	0.500092	0.217668	4	4.916051
H200011805_NMES1_HUMAN	2.2625	0.600718	0.265511	4	4.798222
H300010220_ID2	2.253	0.213936	0.094956	4	4.76673
H200005149_NP_116262.2	2.2435	0.25993	0.115859	4	4.735445
H200006112_NP_006811.1	2.21625	0.780182	0.352028	4	4.64684
opHsV0400002553_MAGEA11	2.20675	0.859448	0.389463	4	4.616342
H200010620_IL6	2.1545	0.45013	0.208925	4	4.452143
H200013568_COL5A1	2.13175	0.640914	0.300651	4	4.382488
H200015644_GDA	2.114	0.824693	0.39011	4	4.328899
opHsV0400011773	2.09725	0.642212	0.306216	4	4.27893
H200008768_FGF2	2.096	0.885641	0.422539	4	4.275224
opHsV0400000818_IL8	2.09075	0.32249	0.154246	4	4.259695
opHsV0400008521	2.0825	0.909608	0.436787	4	4.235405
H300018275_NP_620711.2	2.07625	0.734066	0.353554	4	4.217096
H200004597_XM_374020.2	2.0475	0.611487	0.298651	4	4.13389
opHsV0400006084_MAGEB2	2.033	0.494992	0.243478	4	4.09255
H300009117_OSR2	2.01225	0.586449	0.291439	4	4.034109
H200008845_STMN3	2.0065	0.517001	0.257663	4	4.018062
H200011948_EREG	2.0015	0.586814	0.293187	4	4.004161
opHsV0400006083_MAGEA4	1.9765	0.681241	0.344671	4	3.935372

Table 13 continued

Name of the upregulated gene in BHY cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
H200010289_C6orf139	1.97175	0.330586	0.167661	4	3.922436
H300000884	1.96425	0.607126	0.309088	4	3.902098
H300006722_NP_003513.1	1.96325	0.456425	0.232484	4	3.899394
H300021964_SLCO1B3	1.93075	0.370317	0.1918	4	3.812533
H200005669_LY96	1.922	0.493631	0.256832	4	3.78948
H200012463_MAGEC2	1.917	0.726043	0.378739	4	3.77637
H300006646_ZNF326	1.90925	0.249361	0.130607	4	3.756138
H200000708_MMP13	1.908	0.726138	0.380576	4	3.752885
H200006097_ID1	1.901	0.40408	0.212562	4	3.73472
H200002977_NP_065726.1	1.88775	0.296147	0.156878	4	3.700576
H300020557_GLMN	1.88725	0.507303	0.268805	4	3.699294
opHsV0400007188	1.8805	1.083741	0.576304	4	3.682026
H200016568_CLDN14	1.8765	0.718913	0.383114	4	3.671832
H300008891_IFIT1	1.8465	0.7824	0.423721	4	3.596267
H200011533_KLF2	1.846	0.510175	0.276368	4	3.595021
H200006581_XK	1.84475	0.958007	0.519315	4	3.591907
H200017806_TDRD7	1.8415	0.569578	0.309301	4	3.583825
H200003668_HERC5	1.83375	0.526164	0.286933	4	3.564624
H300006703_LY6K	1.8335	0.639148	0.348595	4	3.564007
H300009194_ABHD7	1.8225	0.878164	0.481846	4	3.536936
H300002344_NP_777601.2	1.8175	0.603638	0.332126	4	3.524699
opHsV0400003040_LCK	1.81625	1.093485	0.602056	4	3.521646
H300018519_IRF7	1.7895	0.259885	0.145228	4	3.456951
H300015308	1.786	0.648679	0.363202	4	3.448574
H300009179_MAGEA9	1.77325	0.761064	0.429192	4	3.418231
H200005573_CALB1	1.765	1.087427	0.616106	4	3.39874
opHsV0400008078	1.7645	0.641701	0.363673	4	3.397562
H200006891_NR4A2	1.73525	0.893226	0.514754	4	3.329372
H200004856_BAL_HUMAN	1.732	0.407424	0.235233	4	3.32188
opHsV0400011488	1.729	0.991203	0.573281	4	3.31498
H200005324_FYB	1.72525	0.471569	0.273334	4	3.306374
H200001951_LAMP3	1.7175	0.474952	0.276537	4	3.28866
H200007266_LHX1	1.7165	0.637868	0.371609	4	3.286382
opHsV0400001371_PTE2B_HUMAN	1.7125	0.549354	0.320791	4	3.277282
H200002223_TXNRD1	1.70925	0.277703	0.162471	4	3.269908
H200014971_HELIS	1.70325	0.459937	0.270035	4	3.256337
H200006072_RRM2	1.70275	0.284603	0.167143	4	3.255209

Table 13 continued

Name of the upregulated gene in BHY cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
H200005594_CC14A_HUMAN	1.7	0.761705	0.448062	4	3.24901
H300013260_DPYD	1.7	0.677127	0.39831	4	3.24901
H300015190_BIRC3	1.6985	1.104304	0.650165	4	3.245633
H200004706_FOXF2	1.6945	0.947529	0.559179	4	3.236647
H200006824_FZD2	1.6905	0.503315	0.297731	4	3.227685
opHsV0400007038	1.68775	0.459065	0.271998	4	3.221539
H200008051_ANKRD10	1.68475	0.396982	0.235633	4	3.214847
H300019089_NP_060408.2	1.68	0.448588	0.267017	4	3.20428
opHsV0400003583_BRI3BP	1.67925	0.488002	0.290607	4	3.202614
H300007499_HRASLS3	1.66875	0.61295	0.367311	4	3.17939
H200017104_NP_001007528.1	1.6475	0.402604	0.244372	4	3.132903
H300000210_IFIT2	1.64375	0.614255	0.373691	4	3.12477
opHsV0400002734_NP_848664.1	1.641	0.426324	0.259795	4	3.118819
opHsV0400003034_KLK10	1.63875	0.462452	0.282198	4	3.113959
H200013657_NP_076942.1	1.634	0.45373	0.277681	4	3.103723
H200015606_IL7R	1.62775	0.323239	0.19858	4	3.090307
H200020534_TRIM6	1.61625	0.286686	0.177377	4	3.065771
opHsV0400010212	1.61425	0.797001	0.493728	4	3.061524
H200017773_GTPBP1	1.6135	0.556646	0.344993	4	3.059933
H300013986_PSG10	1.613	0.520234	0.322525	3	3.058873
H300018167_AMID	1.6125	0.512751	0.317985	4	3.057813
H300021783_NP_149081.1	1.60925	0.356157	0.221318	4	3.050932
H200005813_IL7	1.59975	0.409299	0.255852	4	3.030908
opHsV0400003905_IL7R	1.597	0.760413	0.476151	4	3.025136
H200004431_MLF1IP	1.58425	0.551219	0.347937	4	2.998519
opHsV0400000049_XP_291989.3	1.58175	0.30548	0.193128	4	2.993327
H200007026_GBG11_HUMAN	1.579	0.385193	0.243948	4	2.987627
H200000576_MMP10	1.578	0.272033	0.172391	4	2.985557
opHsV0400013002	1.57575	0.443983	0.28176	4	2.980904
H300018946_DR1	1.56675	0.381593	0.243557	4	2.962366
H300022249_GP110_HUMAN	1.56	0.391751	0.251122	4	2.948538
H200007011_MMP1	1.534	0.649902	0.423665	4	2.895876
opHsV0400010717	1.53275	0.676085	0.441093	4	2.893368
opHsV0400007760	1.532	0.597638	0.390103	4	2.891865
H200013536_C20orf29	1.521	0.718596	0.47245	4	2.869899
H200013521_DDX58	1.513	0.858988	0.567738	4	2.854029
H200007019_MMP3	1.5075	0.368524	0.24446	4	2.843169

Table 13 continued

Name of the upregulated gene in BHY cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
H300001749_AQP11	1.50725	0.380475	0.25243	4	2.842677
H300016431_CT022_HUMAN	1.504	0.343714	0.228533	4	2.83628
H300009288_NP_653185.1	1.5	0.611704	0.407803	4	2.828427
H300002737_GBP4	1.497	0.772955	0.516336	4	2.822552
H200020305_PARP10	1.492	0.318087	0.213195	4	2.812786
opHsV0400005063	1.49025	0.467101	0.313438	4	2.809377
opHsV0400005091_ACYP1	1.489	0.505898	0.339757	4	2.806943
H200008827_NP_982274.1	1.48725	0.498681	0.335304	4	2.803541
H200005110_SULT1E1	1.4835	1.056875	0.71242	4	2.796263
H300007730_MAGEA6	1.48175	0.313321	0.211454	4	2.792873
H300008499_IRF1	1.4795	0.368059	0.248773	4	2.788521
H300015276_Q96H22_HUMAN	1.4765	0.432985	0.293251	4	2.782728
H200004209_RAMP1	1.4745	0.362929	0.246137	4	2.778873
H200006368_OCTN1_HUMAN	1.47375	0.659172	0.447275	4	2.777429
H300018045_PRIM1	1.47375	0.120801	0.081969	4	2.777429
opHsV0400009665	1.4735	0.544974	0.36985	4	2.776948
H300013149_CDKN3	1.46925	0.718136	0.488777	4	2.768779
H300021781_CENPA	1.46525	0.37843	0.25827	4	2.761113
H200003417_RAD51AP1	1.46175	0.479901	0.328306	4	2.754423
H300000747	1.4615	0.462733	0.316615	4	2.753945
H200005018_GATA6	1.45775	0.375212	0.257391	4	2.746796
opHsV0400008467	1.453	1.443222	0.99327	3	2.737768
opHsV0400007148	1.451	0.798016	0.549977	4	2.733975
opHsV0400000522_NP_689663.1	1.4505	0.945205	0.651641	4	2.733028
H200011351_AIM2	1.44825	0.49706	0.343214	4	2.728768
H300008871_NP_001008708.1	1.447	0.398061	0.275094	4	2.726405
H300019216_SPAG1	1.44525	0.81659	0.565016	4	2.7231
H200007081_SLCO2A1	1.44425	1.022917	0.708268	4	2.721213
opHsV0400003953_HIST1H1C	1.43925	0.426934	0.296637	4	2.711799
H200010772_GPR160	1.43425	0.350812	0.244596	4	2.702416

Table 14: 117 downregulated genes in BHY cell line. The gene list is from the least downregulated gene to most downregulated gene.

Name of the downregulated gene in BHY cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
H300000194_CSPG2	-1.5935	0.414519	-0.26013	4	0.331367
H200001115_ZNF25	-1.60125	1.06914	-0.66769	4	0.329591
opHsV0400000079Q6NZ63_HUMAN	-1.60525	1.161	-0.72325	4	0.328679
H200005961_DPYSL3	-1.60625	1.348248	-0.83938	4	0.328451
H300012616_ANPEP	-1.6075	1.109451	-0.69017	4	0.328167
H200005275_KLK11	-1.60875	0.747015	-0.46434	4	0.327882
H200002000_RHBDL2	-1.60875	0.80247	-0.49882	4	0.327882
H300013882_AES	-1.6105	0.222317	-0.13804	4	0.327485
H300017884_SLC37A2	-1.6105	0.830395	-0.51561	4	0.327485
H300000561_CBS	-1.611	0.79006	-0.49042	4	0.327371
opHsV0400003752	-1.61333	1.595829	-0.98915	3	0.326842
opHsV0400008056	-1.61425	1.405615	-0.87075	4	0.326635
H300009655	-1.62125	0.757105	-0.46699	4	0.325054
H200019593_ZNF626	-1.62825	1.358063	-0.83406	4	0.32348
H300002980	-1.63125	0.475368	-0.29141	4	0.322808
H300005674_Q8IZ15_HUMAN	-1.63375	0.569106	-0.34834	4	0.322249
opHsV0400012118	-1.63425	0.388394	-0.23766	4	0.322138
H200017197_HSD11B1	-1.63925	0.945894	-0.57703	4	0.321023
H300013100_SLC4A3	-1.64067	1.063076	-0.64795	3	0.320708
H200012598_LRRN1_HUMAN	-1.64725	1.302948	-0.79098	4	0.319248
H200010619_CNGB1	-1.65375	1.394051	-0.84296	4	0.317813
opHsV0400000628_ARTS1_HUMAN	-1.655	1.123805	-0.67904	4	0.317538
opHsV0400010774	-1.65525	0.921909	-0.55696	4	0.317483
H300006889_XP_059037.4	-1.65725	0.827554	-0.49935	4	0.317043
opHsV0400001923_IDH2	-1.66275	0.145039	-0.08723	4	0.315837
H200003299_SLC16A4	-1.6675	0.557852	-0.33454	4	0.314798
H300018412_MGST1	-1.67525	0.171506	-0.10238	4	0.313112
H200006867_SHC1	-1.67525	0.206479	-0.12325	4	0.313112
H200009585_NP_001007190.1	-1.68125	0.641676	-0.38167	4	0.311812
H200006995	-1.69275	0.925138	-0.54653	4	0.309337
H200013065	-1.6945	0.3687	-0.21759	4	0.308962
opHsV0400002007_NM_000407.3	-1.69925	0.446025	-0.26248	4	0.307946
H200004912_GJB6	-1.70075	0.667052	-0.39221	4	0.307626
H200011169_TCF7L1	-1.7025	0.381282	-0.22395	4	0.307253
H200001141_EFEMP2	-1.71625	0.77124	-0.44938	4	0.304339

Table 14 continued

Name of the downregulated gene in BHY cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
H200012556_THY1	-1.718	0.763368	-0.44434	4	0.30397
H200007876_SLC2A1	-1.73925	0.295673	-0.17	4	0.299525
opHsV0400011936	-1.7445	1.036602	-0.59421	4	0.298437
H200011421_CDKN1C	-1.762	1.210316	-0.6869	4	0.294839
opHsV0400007762	-1.76267	1.587394	-0.90056	3	0.294703
H200005983_Q6PJ56_HUMAN	-1.7635	0.973849	-0.55223	4	0.294533
H300009738_Q14922_HUMAN	-1.76775	1.295378	-0.73278	4	0.293666
opHsV0400003654_NP_001004313.1	-1.76925	1.763672	-0.99685	4	0.293361
H200015626_RRAGD	-1.78425	1.706914	-0.95666	4	0.290327
H200013543_Q96MI4_HUMAN	-1.7895	1.351199	-0.75507	4	0.289272
H200001865_TNS	-1.79925	1.745972	-0.97039	4	0.287324
opHsV0400002303_NDN	-1.818	0.954687	-0.52513	4	0.283614
opHsV0400002365_PTPRM	-1.82	0.215937	-0.11865	4	0.283221
H200013711_BTEB1_HUMAN	-1.82125	0.492802	-0.27058	4	0.282976
H300008702_ITGB6	-1.8265	0.969306	-0.53069	4	0.281948
H200000104_ADH7	-1.8265	1.742103	-0.95379	4	0.281948
H200008152_ODZ2	-1.8325	0.203169	-0.11087	4	0.280778
H200004216_PLXDC2	-1.84025	0.7515	-0.40837	4	0.279273
H300014602_IL1R1	-1.8415	1.208037	-0.65601	4	0.279032
H200014313_BEXL1	-1.86125	0.515799	-0.27712	4	0.275238
H200015831_THAP2_HUMAN	-1.86175	1.36207	-0.73161	4	0.275142
H200000465_CD1D	-1.862	1.781241	-0.95663	4	0.275095
H200003796_FNDC4	-1.865	0.738472	-0.39596	4	0.274523
H200004211_COBL	-1.871	1.174565	-0.62777	4	0.273384
opHsV0400006329_PALMD	-1.8845	1.320225	-0.70057	4	0.270838
H200009642_ITGAV	-1.88625	0.352733	-0.187	4	0.270509
H200000047_IGFBP2	-1.89925	0.382519	-0.20141	4	0.268083
H200013755_KLK7	-1.90075	0.692081	-0.36411	4	0.267804
H200011925_COL8A1	-1.90625	0.820488	-0.43042	4	0.266785
H200005569_LIPG	-1.90825	0.367554	-0.19261	4	0.266416
opHsV0400005248_FAM46B	-1.91225	0.347777	-0.18187	4	0.265678
H200000073_GALC	-1.913	0.965822	-0.50487	4	0.26554
H300000923_POSTN	-1.91725	0.686764	-0.3582	4	0.264759
opHsV0400012721	-1.92225	0.923278	-0.48031	4	0.263843
H200005130	-1.928	1.441162	-0.74749	4	0.262793
H200005198_GALNT5	-1.93175	1.069411	-0.5536	4	0.262111
opHsV0400012479	-1.941	1.744599	-0.89881	4	0.260436

Table 14 continued

Name of the downregulated gene in BHY cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
H200008006_SEMA3C	-1.948	0.383193	-0.19671	4	0.259175
H200012177_RAB4A	-1.992	0.911509	-0.45758	4	0.25139
opHsV0400006127_TNFRSF6B	-2.00025	0.446626	-0.22329	4	0.249957
H200000491_PRG1	-2.001	0.48489	-0.24232	4	0.249827
opHsV0400006528	-2.00375	1.463535	-0.7304	4	0.249351
H300003568_CPS1	-2.016	1.306051	-0.64784	4	0.247243
H200004031_NP_945120.1	-2.022	0.617709	-0.30549	4	0.246217
H200001783_PLEA4_HUMAN	-2.061	0.715854	-0.34733	4	0.23965
opHsV0400011552	-2.0615	0.559359	-0.27134	4	0.239567
H200016573_ADPN	-2.08425	0.67585	-0.32427	4	0.235819
opHsV0400003414_GSPT2	-2.097	1.624817	-0.77483	4	0.233744
H200001094_NP_057702.1	-2.1075	0.738391	-0.35036	4	0.232049
H300021562_FN1	-2.1185	0.873318	-0.41223	4	0.230286
opHsV0400010233	-2.1275	1.897333	-0.89181	4	0.228854
opHsV0400011241	-2.13425	1.663044	-0.77922	4	0.227786
H300006196_MAF	-2.13725	0.559296	-0.26169	4	0.227313
H200020242_C9orf26	-2.151	0.471467	-0.21918	4	0.225156
H200013413_LRP3	-2.151	0.63614	-0.29574	4	0.225156
H200011379_NPDC1	-2.17125	0.710231	-0.32711	4	0.222018
H300006645_Q8IYY5_HUMAN	-2.18875	0.303208	-0.13853	4	0.219341
H300010137_CALML3	-2.208	0.427549	-0.19364	4	0.216434
H300006447_MT1K	-2.23625	1.764254	-0.78893	4	0.212237
H200001726_KRT23	-2.26975	0.479364	-0.2112	4	0.207366
H200009653_RAB4A	-2.2805	0.739566	-0.3243	4	0.205826
H300016644_WASPIP	-2.2925	1.087845	-0.47452	4	0.204121
H300018293_SLC38A5	-2.29475	0.431198	-0.18791	4	0.203803
H200001823_ARMCX1	-2.299	0.708874	-0.30834	4	0.203204
H200011330_RKHD3	-2.3195	1.852087	-0.79849	4	0.200337
H300021749_FOLR3	-2.33525	0.541148	-0.23173	4	0.198162
H200001408_SFRP1	-2.35767	0.264814	-0.11232	3	0.195106
H300021145_ANGPTL4	-2.3915	0.448694	-0.18762	4	0.190584
H200016276_ESPN	-2.4205	1.3472	-0.55658	4	0.186791
H300016617_THY1	-2.42475	1.65256	-0.68154	4	0.186242
H200018944_KLF8	-2.425	0.698834	-0.28818	4	0.18621
H200005423_Q9H7B6_HUMAN	-2.43525	1.371003	-0.56298	4	0.184891
H200001686_TM4SF10	-2.5915	1.26025	-0.4863	4	0.165913
H200004832_C3orf14	-2.64375	1.162073	-0.43955	4	0.160012

Table 14 continued

Name of the downregulated gene in BHY cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
H200011635_LXN	-2.689	1.396491	-0.51933	4	0.155071
opHsV0400010653	-2.73375	1.220994	-0.44664	4	0.150335
H200005947_GJA1	-2.948	0.575297	-0.19515	4	0.129588
H200017869	-2.99633	2.483282	-0.82877	3	0.125318
H200014307_FADS2	-3.02275	0.370271	-0.12249	4	0.123044
H300017162_Q9H7B6_HUMAN	-3.079	0.937163	-0.30437	4	0.118339
opHsV0400010227_Q96NA9HUMAN	-3.3075	0.735836	-0.22248	4	0.101005
H300000043_RGS17	-3.42475	1.15765	-0.33802	4	0.093121

Table 15: 214 upregulated genes in HN cell line. The gene list is from the most upregulated gene to least upregulated gene.

Name of the upregulated gene in HN cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
H200011805_NMES1_HUMAN	3.61425	0.344629	0.095353	4	12.2461
H200005801_MAGEA1	3.4955	0.938984	0.268627	4	11.27847
H200001373_Q96AL8_HUMAN	3.39425	0.559974	0.164977	4	10.51407
opHsV0400013404	3.1775	0.278336	0.087596	4	9.04738
H200004165_FSTL5	2.96775	0.964376	0.324952	4	7.823152
opHsV0400005829_OAS1	2.86425	0.337914	0.117976	4	7.281572
H200010620_IL6	2.84925	0.652263	0.228924	4	7.206256
H200010289_C6orf139	2.83925	0.44019	0.155037	4	7.156479
H300005969_COX7B2	2.744	1.244172	0.453416	4	6.699252
opHsV0400002611_PRAME	2.72925	0.998202	0.365742	4	6.631108
H300006342_NP_775969.1	2.69225	0.843384	0.313264	4	6.463206
H200010916_NP_114156.1	2.59475	0.43994	0.16955	4	6.040843
H200012712_BIRC3	2.52475	0.986658	0.390795	4	5.754737
H300006447_MTIK	2.503	0.519416	0.207517	4	5.66863
H200007081_SLCO2A1	2.49425	1.343249	0.538538	4	5.634353
H200002977_NP_065726.1	2.46325	0.945773	0.383953	4	5.514576
H200004279_ID4	2.42325	0.553582	0.228446	4	5.36378
H200008845_STMN3	2.3605	0.871202	0.369075	4	5.135483
H300022894_NP_002116.2	2.34725	0.319804	0.136246	4	5.088534
opHsV0400000818_IL8	2.341	1.005232	0.429403	3	5.066537
H200011575_BSPRY	2.33875	1.31912	0.564028	4	5.058641
H300022533_HLA-J	2.312	0.155681	0.067336	4	4.96571
opHsV0400003040_LCK	2.278	1.654258	0.726189	4	4.850051
H200001951_LAMP3	2.26225	0.660399	0.291921	4	4.797391
H200010376_CYP24A1	2.2595	0.469224	0.207667	4	4.788255
H200017806_TDRD7	2.24575	0.4908	0.218546	4	4.742836
H200015633_SLC16A9	2.2445	0.649934	0.289567	4	4.738728
H300000210_IFIT2	2.2225	0.6966	0.313431	4	4.667015
H200003668_HERC5	2.205	0.464972	0.210872	4	4.610745
H200006478_ACTH_HUMAN	2.1895	0.428789	0.195839	4	4.561474
H300006703_LY6K	2.18225	0.368532	0.168877	4	4.538608
H200006112_NP_006811.1	2.17775	0.582087	0.267288	4	4.524474
H200011702_HOXA10	2.155	1.423344	0.660485	4	4.453686
H200005362_NP_115724.1	2.13875	1.128071	0.527444	4	4.403803
H200000708_MMP13	2.135	1.48257	0.694412	4	4.392371

Table 15 continued

Name of the upregulated gene in HN cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
opHsV0400003753_PLSCR1	2.128	0.567288	0.266583	4	4.371111
opHsV0400006084_MAGEB2	2.12175	1.118117	0.526979	4	4.352216
H300016329_MMP25	2.12	0.43629	0.205797	4	4.346939
H200013521_DDX58	2.1155	0.525671	0.248485	4	4.333402
H200011983_Q9BT26_HUMAN	2.11175	0.947054	0.448469	4	4.322153
H200006368_OCTN1_HUMAN	2.0795	0.933586	0.448947	4	4.226607
H300022436_HLA-DRB3	2.07225	0.396335	0.191258	4	4.20542
opHsV0400012005	2.06625	1.134699	0.549159	4	4.187967
opHsV0400008956	2.06575	0.416258	0.201505	4	4.186516
H200006072_RRM2	2.059	0.43027	0.208971	4	4.166974
H200001243_TRHDE_HUMAN	2.05475	1.08619	0.528624	4	4.154716
H300008891_IFIT1	2.05375	0.571091	0.278072	4	4.151838
H300001406_CNN1	2.04075	0.963906	0.472329	4	4.114594
H300001612_PCOLCE2	2.038	0.648625	0.318265	4	4.106758
opHsV0400008211_XM_375042.2	2.02375	1.433107	0.708144	4	4.066394
opHsV0400006097_PTPRR	2.00625	1.242772	0.61945	4	4.017366
H300018045_PRIM1	2.002667	0.267808	0.133726	3	4.0074
H300001976_NMI	1.99775	0.512455	0.256516	4	3.993767
H200014971_HELLS	1.992	0.413372	0.207516	4	3.977881
H300018428_BID	1.981	0.522077	0.263542	4	3.947666
H200011914_CDC45L	1.961	0.197314	0.100619	4	3.893317
H200010772_GPR160	1.95375	1.3282	0.679821	4	3.873801
H200004597_XM_374020.2	1.95175	0.752455	0.385528	4	3.868435
opHsV0400006060	1.9475	0.915296	0.469985	4	3.857056
H200007582_CXCL6	1.943	0.714921	0.367947	4	3.845044
H300010511_SOD2	1.943	0.611604	0.314773	4	3.845044
opHsV0400003953_HIST1H1C	1.939	0.293976	0.151612	4	3.834398
H200014073_MCM6	1.93275	0.407792	0.210991	4	3.817822
H300010084_FBXO27	1.928	0.424222	0.220032	3	3.805273
H200015506_CDCA1	1.927	0.807785	0.419193	4	3.802636
H300007731	1.924	0.499857	0.259801	4	3.794737
H300012772_C10orf94	1.91325	1.093819	0.571708	4	3.766566
opHsV0400011941	1.896	0.817471	0.431156	4	3.721799
opHsV0400012784	1.8955	0.275088	0.145127	4	3.720509
H300001248_PROM1	1.8955	1.149884	0.606639	4	3.720509
H200005842_GFI1	1.8945	1.099902	0.580576	4	3.717931
H200011351_AIM2	1.887	0.542437	0.28746	4	3.698653

Table 15 continued

Name of the upregulated gene in HN cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
H300006205_S100P	1.88575	0.585973	0.310737	4	3.69545
H200016569_PCDHB2	1.884	0.913877	0.485073	4	3.69097
H200012959	1.88175	0.65093	0.345918	4	3.685218
H200000046_CDH2	1.877	1.130349	0.60221	4	3.673105
H200004431_MLF1IP	1.86075	0.273527	0.146998	4	3.631964
opHsV0400006094_HHEX	1.85425	0.975674	0.526183	4	3.615637
opHsV0400006196	1.849333	0.678552	0.366917	3	3.603336
H300009187_PSMB9	1.84125	0.322845	0.17534	4	3.583204
H200004856_BAL_HUMAN	1.83875	0.337235	0.183405	4	3.577
H300001485_HLA-DPA1	1.833	0.716288	0.390773	4	3.562772
opHsV0400003771_HB2K_HUMAN	1.82175	0.347382	0.190686	4	3.535097
H200006657_MEST	1.81925	0.457504	0.251479	4	3.528977
H200015731_CCL5	1.79575	0.493276	0.274691	4	3.471959
H300019551_UGCGL2	1.79525	0.490762	0.273367	4	3.470756
H200006581_XK	1.79175	1.672909	0.933673	4	3.462346
H300009288_NP_653185.1	1.7885	0.303107	0.169475	4	3.454555
H300022475_CRYZL1	1.78575	0.435806	0.244047	4	3.447977
H200010475_PFDN4	1.78425	0.368762	0.206676	4	3.444394
H200018923_SOD2	1.7835	0.363238	0.203666	4	3.442603
H300021066_Q96H22_HUMAN	1.7805	0.407449	0.22884	4	3.435452
H200020305_PARP10	1.78	0.568335	0.319289	4	3.434262
H300002968_SYT14	1.77975	1.597834	0.897786	4	3.433667
H200013760_KIAA1524	1.77825	0.540236	0.303802	4	3.430098
H300018167_AMID	1.777	0.953326	0.53648	4	3.427128
H200010704_CPVL	1.77575	0.52984	0.298376	4	3.42416
H200000491_PRG1	1.76525	0.284848	0.161364	4	3.399329
H300007730_MAGEA6	1.76425	1.020558	0.578466	4	3.396974
H200014902_Q96RF1_HUMAN	1.764	1.100442	0.623833	3	3.396385
H300011169_GPR160	1.75625	1.453927	0.827859	4	3.378189
opHsV0400003583_BRI3BP	1.751	0.992618	0.566887	4	3.365918
H200004874_HS3ST3A1	1.74225	0.75636	0.434128	4	3.345565
H200008652_ICMT	1.73875	0.232372	0.133643	4	3.337459
H200017455_NP_060582.1	1.73075	0.5441	0.314372	4	3.319003
H200005223_NP_996794.1	1.72925	0.510066	0.294963	4	3.315554
H200016778_FBXO5	1.729	0.526669	0.304609	4	3.31498
H300020866_Q7Z545_HUMAN	1.728	0.976577	0.565149	4	3.312683
H200000405_DCLRE1A	1.72375	0.645755	0.374622	4	3.302938

Table 15 continued

Name of the upregulated gene in HN cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
H200001011 NP_054895.1	1.71825	0.833375	0.485014	4	3.29037
H200002434 QKI	1.7125	0.643398	0.375707	4	3.277282
H200002164 ZC3HDC1	1.71025	0.301587	0.176341	4	3.272175
H300001924 ATAD2	1.70625	0.622159	0.364635	4	3.263115
H200019857 NCOA7	1.69975	0.495825	0.291705	4	3.248447
opHsV0400004769Q9NT91_HUMAN	1.6965	0.531851	0.313499	4	3.241137
H300002301 HES4	1.68925	0.313784	0.185753	4	3.22489
H200000203	1.686	0.146806	0.087074	4	3.217633
opHsV0400001953_XP_495896.1	1.683333	1.325827	0.78762	3	3.211692
H300015276 Q96H22_HUMAN	1.67325	0.295831	0.176801	4	3.189323
H200012301 TRIM36	1.672	0.745459	0.445849	4	3.18656
H200011999 OIP5	1.66975	0.565244	0.33852	4	3.181595
H200011533 KLF2	1.6645	0.628264	0.377449	4	3.170038
H200008556 IFIT3	1.65625	0.746136	0.450497	4	3.151962
opHsV0400000541 TMED4	1.65525	0.339298	0.204983	4	3.149778
H200017505 FANCL	1.6525	0.397534	0.240565	4	3.143779
H200000621 ANGPT1	1.64775	1.626822	0.987299	4	3.133446
H300015308	1.6395	0.224063	0.136666	4	3.115578
H200006611 QPCT	1.63775	0.402911	0.246015	4	3.111801
H300009117 OSR2	1.63625	0.558735	0.341473	4	3.108568
H300001290 SUNC1	1.634	0.527525	0.322843	3	3.103723
H200007111 NP_689700.1	1.6325	1.570719	0.962156	4	3.100498
H300017820 GLS	1.63125	0.453618	0.27808	4	3.097813
opHsV0400012444	1.63	0.557309	0.341907	3	3.09513
opHsV0400002734 NP_848664.1	1.62675	0.832261	0.51161	4	3.088165
H300003538 Q9NYD4_HUMAN	1.6265	1.258498	0.773746	4	3.08763
opHsV0400004371 HLA-DRA	1.62475	0.737985	0.454215	4	3.083887
H200009565 IFIH1	1.6205	0.799292	0.493238	4	3.074816
opHsV0400000732_C6orf148	1.61975	1.121724	0.692529	4	3.073218
H200006282 SERPINA5	1.619	1.310318	0.809338	4	3.071621
H200006302 MAL2	1.61775	0.421879	0.260781	4	3.06896
H200000169 TACSTD1	1.6175	0.876741	0.542035	4	3.068429
H300007499 HRASLS3	1.6165	0.663349	0.410361	4	3.066302
H200005162 BAG2	1.61025	0.6921	0.429809	4	3.053047
H300015490 TRIM14	1.60975	0.854371	0.530748	4	3.05199
H300015757 PPM2C	1.6095	0.64351	0.39982	4	3.051461
H200014344 IL27B_HUMAN	1.60625	1.055639	0.657207	4	3.044594

Table 15 continued

Name of the upregulated gene in HN cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
H200004487_DKK1	1.60575	0.596592	0.371535	4	3.043539
H300005881_CTCFL	1.60425	1.235159	0.769929	4	3.040377
H300020304_PLAC8	1.5955	0.829325	0.51979	4	3.021992
H200016697_EMILIN2	1.5935	1.428029	0.896159	4	3.017806
H200010947_POLE2	1.581	0.423762	0.268034	4	2.991772
H200005687_CDC6_HUMAN	1.58025	0.908132	0.574676	4	2.990217
opHsV0400000033_USP18	1.57925	0.465642	0.29485	4	2.988145
H200006576_RNF144	1.5765	0.782792	0.496538	4	2.982454
H300019419_SP110	1.566	0.855142	0.546068	4	2.960827
H200007266_LHX1	1.566	1.433402	0.915327	4	2.960827
H200000387_MSX1	1.565	0.862229	0.550945	4	2.958775
opHsV0400006517_LIMS3	1.56475	1.375443	0.879017	4	2.958262
H200006824_FZD2	1.56425	0.881607	0.563597	4	2.957237
H200004906_CDCA8	1.558	0.622277	0.399408	4	2.944454
H200019588_CDC2	1.54775	0.564203	0.364531	4	2.923608
H200005202_OAS3	1.54725	0.767262	0.495888	4	2.922595
H200012133_OASL	1.544	0.360654	0.233584	4	2.916019
H300020240_Q7Z7J6_HUMAN	1.54275	1.035721	0.671348	4	2.913493
H200006623_SEMA4D	1.5405	0.920567	0.597577	4	2.908953
H300001211_ARVCF	1.53925	0.769145	0.499688	4	2.906434
opHsV0400007148	1.53675	0.901378	0.586548	4	2.901402
H200000423_RPA3	1.53675	0.384891	0.250458	4	2.901402
opHsV0400003019_NP_115749.2	1.5365	0.639597	0.416269	4	2.900899
H200011709_DEK	1.5345	0.681863	0.444355	4	2.89688
H200009876_EEF1E1	1.53275	0.359703	0.234678	4	2.893368
opHsV0400007760	1.52125	0.468175	0.307757	4	2.870396
opHsV0400004148_BGN	1.5195	1.341237	0.882683	4	2.866917
H200002273_NP_620134.2	1.5185	0.378022	0.248944	4	2.86493
H300002362_HOXA7	1.51675	0.845497	0.55744	4	2.861457
opHsV0400010984	1.51375	0.37722	0.249196	4	2.855513
H200002104_NP_116195.2	1.51275	0.902294	0.59646	4	2.853534
H300002650_LSM5	1.511	0.465699	0.308206	4	2.850075
H200020265_KIAA1914	1.5055	0.458942	0.304844	4	2.839231
H200015533_GMNN	1.504	0.840186	0.558635	4	2.83628
H200001910_RHOBTB3	1.50375	0.422485	0.280954	4	2.835789
H300017088_Q9P2H4_HUMAN	1.50275	0.634124	0.421976	4	2.833824
H200008732_C9orf76	1.50125	0.473563	0.315446	4	2.830879

Table 15 continued

Name of the upregulated gene in HN cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
H300021781_CENPA	1.499	0.448031	0.298886	4	2.826467
H300001620_HLA-DRA	1.49575	0.567848	0.379641	4	2.820107
H200012628_Q9NW34_HUMAN	1.49475	0.546389	0.365539	4	2.818153
H200000353_CSF2	1.49475	1.097309	0.734109	4	2.818153
H200007874_UBE2L6	1.494	0.280316	0.187628	4	2.816688
H200014214_80	1.492	0.366445	0.245607	4	2.812786
H300000884	1.48925	0.503138	0.337846	4	2.80743
opHsV0400001961_CRIP1	1.4865	0.428895	0.288527	4	2.802084
opHsV0400006256_ATP6V0A2	1.4855	0.946856	0.637399	4	2.800142
opHsV0400006563_CTHRC1	1.47725	0.686588	0.464774	4	2.784175
opHsV0400006261_ZNF180	1.4765	0.488886	0.331112	4	2.782728
opHsV0400001268_LCP1	1.47475	0.467856	0.317244	4	2.779355
opHsV0400006299_MRPS18C	1.474	0.239612	0.162559	4	2.77791
H300006103_NP_872297.1	1.4725	0.534455	0.362957	4	2.775024
H200006020_NP_001009567.1	1.46925	1.162508	0.791225	4	2.768779
opHsV0400000242_RSAD2	1.46725	0.436163	0.297266	4	2.764943
opHsV0400012866	1.4665	0.795741	0.542612	4	2.763506
opHsV0400001371_PTE2B_HUMAN	1.46375	0.887962	0.606635	4	2.758244
H200003290_DEPDC1B	1.462	0.88802	0.607401	4	2.7549
H200018907_TRIM22	1.46125	0.430851	0.294851	4	2.753468
opHsV0400000079_Q6NZ63HUMAN	1.4525	0.568938	0.391696	4	2.736819
H200003131_PPM2C	1.447	0.64873	0.448327	4	2.726405
H200014355_NP_945185.1	1.44525	1.123801	0.777582	4	2.7231
H200009537_RBP7	1.44425	0.729837	0.50534	4	2.721213
H300004315_XP_372200.1	1.44375	0.204927	0.141941	4	2.72027
H300013841_RAD51AP1	1.441333	1.307693	0.90728	3	2.715717
H200013413_LRP3	1.43925	0.238458	0.165682	4	2.711799
H300020946_BRIX_HUMAN	1.438	0.890683	0.61939	4	2.70945
H200006095_90	1.4365	0.413611	0.287929	4	2.706634
opHsV0400002785_Q86TT0HUMAN	1.4365	1.357682	0.945132	4	2.706634
opHsV0400005875_NP_705611.1	1.43375	0.530447	0.369972	4	2.70148

Table 16: 262 downregulated genes in HN cell line. The gene list is from the least downregulated gene to most downregulated gene.

Name of the downregulated gene in HN cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
H300019074_WRNIP1	-1.59875	1.383307	-0.86524	4	0.330163
H200016212_DLL1	-1.5995	0.75427	-0.47157	4	0.329991
opHsV0400002581_C14orf78	-1.6	1.305228	-0.81577	3	0.329877
H300014158_DOCK9	-1.603	1.419751	-0.88568	3	0.329192
opHsV0400004066	-1.60625	0.469854	-0.29252	4	0.328451
H300019058_ANXA6	-1.609	0.960446	-0.59692	4	0.327826
H200006821_IL1RN	-1.60925	1.012012	-0.62887	4	0.327769
opHsV0400002604_Q96FP4_HUMAN	-1.61175	0.725436	-0.45009	4	0.327201
H200017253_PLXNB1	-1.6135	0.858486	-0.53206	4	0.326805
opHsV0400003246_XP_497922.1	-1.61725	0.884012	-0.54661	4	0.325956
H200006140_CCND2	-1.61975	0.396532	-0.24481	4	0.325392
H200019789_NP_001060.1	-1.6245	0.419842	-0.25844	4	0.324322
H200002453_SRPX	-1.62525	0.903225	-0.55575	4	0.324154
H300003388_ETS2	-1.6265	1.082986	-0.66584	4	0.323873
H200002000_RHBDL2	-1.62775	0.806054	-0.49519	4	0.323592
opHsV0400004958_KLF10_HUMAN	-1.62925	1.289555	-0.7915	4	0.323256
H200006008_PRSS11	-1.62925	0.384313	-0.23588	4	0.323256
H200002536_CDYL	-1.63625	0.844779	-0.51629	4	0.321692
H200017078_TUBB6	-1.63775	0.438952	-0.26802	4	0.321357
H300002496_NOTCH2NL	-1.63825	0.768268	-0.46896	4	0.321246
H200006868_DAB2	-1.64125	1.419207	-0.86471	4	0.320579
opHsV0400009653	-1.64167	1.064244	-0.64827	3	0.320486
H200002949_C6orf117	-1.64175	1.555972	-0.94775	4	0.320468
H300012687_IPYR2_HUMAN	-1.6435	0.991842	-0.60349	4	0.320079
H200006008_PRSS11	-1.64775	0.37658	-0.22854	4	0.319137
H200012241_NP_689893.1	-1.64875	0.668634	-0.40554	4	0.318916
opHsV0400011304	-1.64975	0.543631	-0.32952	4	0.318695
opHsV0400002608	-1.657	1.091706	-0.65885	3	0.317098
H200011608_CRYL1	-1.66075	1.226167	-0.73832	4	0.316275
H200008160_NP_001001701.1	-1.66075	0.452508	-0.27247	4	0.316275
opHsV0400005657	-1.66125	0.922962	-0.55558	4	0.316165
opHsV0400012140	-1.66675	1.337455	-0.80243	4	0.314962
opHsV0400011469	-1.66925	1.384516	-0.82942	4	0.314417
opHsV0400002357_GBP6	-1.66925	1.006323	-0.60286	4	0.314417
H200012925	-1.67225	0.50195	-0.30016	4	0.313764

Table 16 continued

Name of the downregulated gene in HN cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
H300014718_DUSP6	-1.6765	0.836957	-0.49923	4	0.312841
H200010650_DSG2	-1.67675	0.840799	-0.50145	4	0.312786
H300003254_TM4SF1	-1.679	0.469498	-0.27963	4	0.312299
H200000493_DSG3	-1.68525	0.600096	-0.35609	4	0.310949
H200001205_NP_057659.1	-1.68675	0.780043	-0.46245	4	0.310626
H200004941_LPXN	-1.68675	0.865594	-0.51317	4	0.310626
H200001079_EGFL5	-1.68875	0.893202	-0.52891	4	0.310196
H300022632_PAM	-1.6895	0.908499	-0.53773	4	0.310034
H200001094_NP_057702.1	-1.694	0.666506	-0.39345	4	0.309069
opHsV0400005724	-1.702	1.596677	-0.93812	3	0.30736
H300020881_PTPN13	-1.7025	1.469151	-0.86294	4	0.307253
H200007830_80	-1.703	1.316424	-0.773	4	0.307147
H300009483_NHLH2	-1.70525	1.646993	-0.96584	4	0.306668
H200005895_NQO2	-1.70675	1.421316	-0.83276	4	0.306349
H200012177_RAB4A	-1.7155	0.919008	-0.53571	4	0.304497
H300019976_IPYR2_HUMAN	-1.71875	0.370455	-0.21554	4	0.303812
H300009845_RNASE7	-1.72025	0.897532	-0.52174	4	0.303496
opHsV0400000833_NET1	-1.7255	1.398363	-0.81041	4	0.302394
H300022102_AMPH	-1.72975	1.302425	-0.75296	4	0.301504
H200004702_SLC6A15	-1.733	0.668861	-0.38596	4	0.300826
H300000194_CSPG2	-1.73325	0.690035	-0.39812	4	0.300774
opHsV0400012025	-1.735	0.736238	-0.42434	4	0.300409
H300017847_FN1	-1.73575	0.744819	-0.4291	4	0.300253
opHsV0400002147_Q86XE2HUMAN	-1.73625	1.403075	-0.80811	4	0.300149
H200003613_NLGN2	-1.7445	1.690551	-0.96907	4	0.298437
opHsV0400004135_NOTCH2	-1.7465	0.867778	-0.49687	4	0.298024
opHsV0400006289	-1.75025	0.524869	-0.29988	4	0.29725
opHsV0400000971_WBSCR1	-1.751	1.682545	-0.96091	4	0.297096
H300014817_LAMA3	-1.752	0.985253	-0.56236	4	0.29689
opHsV0400010474	-1.75625	1.164768	-0.66321	4	0.296017
H200005804_CDKN2B	-1.763	1.059426	-0.60092	3	0.294635
H200013653_PADI3	-1.7655	1.54012	-0.87234	4	0.294125
opHsV0400012141	-1.768	0.323393	-0.18291	4	0.293615
H300014940_B3GNT5	-1.76825	1.385017	-0.78327	4	0.293565
H200003299_SLC16A4	-1.7685	0.985443	-0.55722	4	0.293514
H200007001_IL18	-1.77075	0.600791	-0.33929	4	0.293056
H200015568_NP_060945.2	-1.771	0.833682	-0.47074	4	0.293006

Table 16 continued

Name of the downregulated gene in HN cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
H200000289 LTBR	-1.7725	1.539506	-0.86855	4	0.292701
H200009840 VIM	-1.773	1.472988	-0.83079	4	0.2926
H300013119 NP_065682.2	-1.78775	0.722342	-0.40405	4	0.289623
H300021562 FN1	-1.79	0.725466	-0.40529	4	0.289172
opHsV0400001891 ALDH1A3	-1.793	1.146318	-0.63933	4	0.288571
H300020771 SQSTM1	-1.79875	0.724315	-0.40268	4	0.287424
H200008177 IL1RAP	-1.799	0.359881	-0.20004	4	0.287374
opHsV0400006351	-1.804	1.021688	-0.56635	4	0.286379
H300017962 CTBP2	-1.8085	1.727057	-0.95497	4	0.285488
H300005700 KRT8	-1.81075	0.937817	-0.51792	4	0.285043
H300006159 OR10Z1	-1.81125	1.342866	-0.7414	4	0.284944
H300003817 NP_006207.1	-1.816	0.369494	-0.20347	4	0.284007
H200009720 GDF15	-1.8165	1.713194	-0.94313	4	0.283909
opHsV0400007052 DMRTA2	-1.817	1.027746	-0.56563	4	0.283811
H200006151 Q5T9X8 HUMAN	-1.8195	1.323231	-0.72725	4	0.283319
H200011042 S100A8	-1.82025	0.464048	-0.25494	4	0.283172
opHsV0400001839	-1.82375	0.613236	-0.33625	4	0.282486
H300014334 NP_079082.2	-1.82625	1.394609	-0.76365	4	0.281997
H300021172 HOP HUMAN	-1.8305	1.707345	-0.93272	4	0.281167
H300009457 GADD45B	-1.8385	0.788505	-0.42888	4	0.279612
H200006750 NP_067651.2	-1.84025	0.677885	-0.36837	4	0.279273
opHsV0400005248 FAM46B	-1.84425	0.621962	-0.33724	4	0.2785
H300010985 XP_056455.4	-1.84475	1.191614	-0.64595	4	0.278404
H200007913 GLUL	-1.84525	0.727058	-0.39402	4	0.278307
opHsV0400005180	-1.84875	1.707691	-0.9237	4	0.277633
H300008877 IRX1	-1.85325	1.157741	-0.62471	4	0.276768
H200014313 BEXL1	-1.85625	0.813239	-0.43811	4	0.276193
opHsV0400000674 SPRE1 HUMAN	-1.864	1.535201	-0.82361	4	0.274714
H300011977 UGP2	-1.867	0.531943	-0.28492	4	0.274143
H300012294 NP_996918.1	-1.86775	1.234636	-0.66103	4	0.274
opHsV0400007640	-1.87125	1.308335	-0.69918	4	0.273336
H200011819 NP_002956.1	-1.8715	0.452331	-0.24169	4	0.273289
opHsV0400012111	-1.874	0.467187	-0.2493	4	0.272816
H200009653 RAB4A	-1.8775	1.208294	-0.64357	4	0.272155
H300013341 ARI5B HUMAN	-1.88067	1.431925	-0.76139	3	0.271558
H200003061 SLC22A15	-1.8835	0.345235	-0.18329	4	0.271025
H200015766 CLCA2	-1.89025	1.41579	-0.749	4	0.26976

Table 16 continued

Name of the downregulated gene in HN cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
H300019778_PFKP	-1.8905	0.441144	-0.23335	4	0.269714
H300002182_FEZ1	-1.89725	0.352279	-0.18568	4	0.268455
H300015033_MAGED2	-1.9015	1.104153	-0.58067	4	0.267665
opHsV0400000309_PRKX	-1.90175	1.079995	-0.5679	4	0.267619
H300003420_XP_114621.3	-1.906	0.795925	-0.41759	4	0.266831
H300015331_CALD1	-1.9095	0.600979	-0.31473	4	0.266185
H200001479_Q8IW73_HUMAN	-1.9105	0.81507	-0.42663	4	0.266
H200000578_SGNE1	-1.9115	1.342315	-0.70223	4	0.265816
H300018512_DDX3Y	-1.91325	1.374225	-0.71827	4	0.265494
H200006912_TRIM29	-1.9135	0.46742	-0.24428	4	0.265448
H200010727_IRS1	-1.9155	0.956011	-0.49909	4	0.26508
H200016641_MN1	-1.9165	1.734132	-0.90484	4	0.264896
H200001041_CA12	-1.92375	1.209104	-0.62851	4	0.263569
opHsV0400008763	-1.92525	1.671196	-0.86804	4	0.263295
opHsV0400012807	-1.9255	1.44655	-0.75126	4	0.263249
H300002442_SPAG4L	-1.926	1.575548	-0.81804	3	0.263158
H300019695_BICD2_HUMAN	-1.93333	1.714992	-0.88706	3	0.261824
opHsV0400000862	-1.937	0.627197	-0.3238	4	0.261159
H200013276_SERPINB7	-1.9395	1.250102	-0.64455	4	0.260707
H200011747_MMP2	-1.94133	1.266238	-0.65225	3	0.260376
H300006466_SGCB	-1.94775	0.48478	-0.24889	4	0.25922
H200006031_IPKA_HUMAN	-1.95125	1.027964	-0.52682	4	0.258592
H200013147_NP_079045.1	-1.95125	1.613092	-0.8267	4	0.258592
H200009840_VIM	-1.95225	1.347477	-0.69022	4	0.258413
opHsV0400000719_KRT16	-1.954	0.677366	-0.34666	4	0.2581
opHsV0400000067_Q8WWJ9_HUMAN	-1.964	1.440904	-0.73366	3	0.256317
H300011685	-1.96925	0.791119	-0.40174	4	0.255386
H200015680_DSC2	-1.97375	0.82822	-0.41962	4	0.25459
H300020530_SERPINB3	-1.974	1.412462	-0.71553	4	0.254546
H200011421_CDKN1C	-1.97425	1.74466	-0.88371	4	0.254502
H200008269_CD99	-1.979	0.568187	-0.28711	4	0.253666
opHsV0400005505_Q8N1M2_HUMAN	-1.97975	0.916015	-0.46269	4	0.253534
H200009233_SIA7B_HUMAN	-1.9845	0.563657	-0.28403	4	0.2527
H300004503_NP_076415.2	-1.98675	1.388697	-0.69898	4	0.252307
H300002800_AHNAK	-1.9885	1.011638	-0.50874	4	0.252001
H200005431_DIXDC1	-1.9885	1.306747	-0.65715	4	0.252001
opHsV0400000907	-1.9915	0.91352	-0.45871	4	0.251477

Table 16 continued

Name of the downregulated gene in HN cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
opHsV0400003043_MYADM	-1.993	1.074113	-0.53894	4	0.251216
H300015953_SH3TC1	-1.99525	0.816961	-0.40945	4	0.250824
opHsV0400003766_NP_710154.1	-1.997	0.569637	-0.28525	4	0.25052
H200010435_SPAG7	-1.99725	1.183802	-0.59272	4	0.250477
H200000006_FECH	-2	1.136739	-0.56837	3	0.25
H300002958_JAG1	-2.0005	0.980535	-0.49014	4	0.249913
H200017303_THIC_HUMAN	-2.0045	1.227046	-0.61215	4	0.249221
H200017197_HSD11B1	-2.008	1.799181	-0.89601	4	0.248618
opHsV0400000692_Q96HF5_HUMAN	-2.0175	1.505647	-0.74629	4	0.246986
opHsV0400004098_S100A14	-2.0225	0.647593	-0.32019	4	0.246131
H300008020_XP_294590.1	-2.023	1.021751	-0.50507	4	0.246046
H200014140_PTPNS1	-2.0295	0.915507	-0.4511	4	0.24494
opHsV0400005956	-2.03025	1.987607	-0.979	4	0.244813
H300010309_MYADM	-2.03125	0.793007	-0.3904	4	0.244643
H200017469_RHCG	-2.0325	1.47413	-0.72528	4	0.244431
opHsV0400006431_SNURF	-2.035	1.122016	-0.55136	4	0.244008
opHsV0400009072	-2.04975	0.847366	-0.4134	4	0.241526
H200006884_SERPINE1	-2.05525	0.878614	-0.4275	4	0.240607
opHsV0400013047	-2.077	1.65584	-0.79723	4	0.237007
opHsV0400002303_NDN	-2.102	1.514516	-0.72051	4	0.232935
opHsV0400011802	-2.11075	0.990842	-0.46943	4	0.231527
opHsV0400003761_SAT	-2.12375	0.674588	-0.31764	4	0.22945
H200020570_SESN3	-2.1265	1.558735	-0.733	4	0.229013
H200004216_PLXDC2	-2.13375	1.498274	-0.70218	4	0.227865
opHsV0400000655_GJB2	-2.13575	1.589741	-0.74435	4	0.227549
opHsV0400007539	-2.13667	1.170169	-0.54766	3	0.227405
H300019732_PRKY	-2.14125	1.288663	-0.60183	4	0.226683
H300008679_EGLN3	-2.14575	1.567392	-0.73046	4	0.225977
H300006003_SLC25A6	-2.15325	0.568798	-0.26416	4	0.224806
opHsV0400000393_COL16A1	-2.1535	0.408445	-0.18967	4	0.224767
H300009738_Q14922_HUMAN	-2.159	1.707737	-0.79099	4	0.223911
opHsV0400011805	-2.16475	1.242039	-0.57376	4	0.223021
H200012019_AKR1B10	-2.1675	1.019482	-0.47035	4	0.222596
opHsV0400002365_PTPRM	-2.16825	0.521267	-0.24041	4	0.22248
H200001587_NP_996918.1	-2.169	0.508558	-0.23447	4	0.222365
H200006902_KLF10_HUMAN	-2.16925	0.651168	-0.30018	4	0.222326
H200006867_SHC1	-2.18975	0.62549	-0.28564	4	0.219189

Table 16 continued

Name of the downregulated gene in HN cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
opHsV0400012682	-2.1915	1.25688	-0.57353	4	0.218924
H300016644_WASPIP	-2.20725	1.987152	-0.90028	4	0.216547
H300002193_ZFP36L2	-2.21575	0.47565	-0.21467	4	0.215275
H200008152_ODZ2	-2.216	0.481832	-0.21743	4	0.215237
H300002481_ZBTB34	-2.23325	1.45323	-0.65072	4	0.212679
H200010061_RPS6KA2	-2.23625	1.708891	-0.76418	4	0.212237
opHsV0400001284	-2.24625	1.297053	-0.57743	4	0.210771
opHsV0400007647	-2.25667	1.907378	-0.84522	3	0.209255
opHsV0400000170_MT1L	-2.25975	0.362426	-0.16038	4	0.208808
H300000923_POSTN	-2.26175	1.625951	-0.71889	4	0.208519
H300014335_KRT6B	-2.2635	0.877822	-0.38782	4	0.208266
H300020178	-2.26375	0.710005	-0.31364	4	0.20823
H200012731_F2R	-2.273	1.46346	-0.64385	4	0.206899
H200007876_SLC2A1	-2.2955	0.738457	-0.3217	4	0.203697
H200015364_GSTP1	-2.301	0.589462	-0.25618	4	0.202922
H300021749_FOLR3	-2.30675	0.871734	-0.37791	4	0.202115
H200014031_HSD17B2	-2.30675	0.762819	-0.33069	4	0.202115
H300000043_RGS17	-2.31825	1.010659	-0.43596	4	0.200511
H200000430_COL7A1	-2.36225	0.65418	-0.27693	4	0.194488
opHsV0400005864_SEPP1	-2.3655	1.345137	-0.56865	4	0.19405
H300022249_GP110_HUMAN	-2.37275	2.22949	-0.93962	4	0.193077
H300022236_Q9BSP9_HUMAN	-2.37325	0.970672	-0.40901	4	0.19301
H200006097_ID1	-2.40875	1.092811	-0.45368	4	0.188319
H300012916_MAOA	-2.4185	1.992456	-0.82384	4	0.187051
H200001408_SFRP1	-2.43325	1.071149	-0.44021	4	0.185148
H200008006_SEMA3C	-2.4605	0.55147	-0.22413	4	0.181684
H300021145_ANGPTL4	-2.475	0.780793	-0.31547	4	0.179867
H200018876_IL24	-2.4765	1.466241	-0.59206	4	0.17968
H200000153_Q9BSP9_HUMAN	-2.48525	1.071613	-0.43119	4	0.178593
H200011925_COL8A1	-2.498	1.49758	-0.59951	4	0.177022
opHsV0400006127_TNFRSF6B	-2.5015	0.365641	-0.14617	4	0.176593
H300003213_GOLPH3L	-2.50675	1.333089	-0.5318	4	0.175952
H200015098_IL1F9	-2.51975	1.041925	-0.4135	4	0.174373
H200004912_GJB6	-2.52425	1.61561	-0.64004	4	0.17383
H200005918_SYK	-2.52775	1.763101	-0.6975	4	0.173409
H200006177_SERPINB2	-2.559	0.795586	-0.3109	4	0.169693
H300005637_NP_689532.1	-2.5665	1.81099	-0.70563	4	0.168813

Table 16 continued

Name of the downregulated gene in HN cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
H200006699_FBN2	-2.58725	0.790933	-0.3057	4	0.166403
opHsV0400012721	-2.61325	2.010573	-0.76938	4	0.163431
H300013272_DDX3Y	-2.6735	1.555192	-0.58171	4	0.156746
H300010096_AKR1C2	-2.6785	0.993972	-0.37109	4	0.156204
H200014446_P2RY5	-2.68125	1.943099	-0.7247	4	0.155906
H300013882_AES	-2.6825	0.454182	-0.16931	4	0.155771
opHsV0400011550	-2.686	2.445253	-0.91037	3	0.155394
H200016719_AREG	-2.70425	0.555952	-0.20558	4	0.15344
opHsV0400011776	-2.7105	2.555899	-0.94296	4	0.152777
H200005781_NP_056234.1	-2.71325	1.705237	-0.62849	4	0.152486
H200000280_SPRR1B	-2.735	1.000044	-0.36565	4	0.150205
H200005198_GALNT5	-2.76025	1.335756	-0.48393	4	0.147599
opHsV0400002884	-2.76625	2.725509	-0.98527	4	0.146986
opHsV0400010653	-2.79075	1.439192	-0.5157	4	0.144511
H200003227_CMKOR1	-2.8075	0.905171	-0.32241	4	0.142843
H200012184_COL4A1	-2.82025	0.634544	-0.225	4	0.141586
H200002398_HCLS1	-2.8465	1.290239	-0.45327	4	0.139033
H300006645_Q8IYY5_HUMAN	-2.85	0.85245	-0.29911	4	0.138696
H200012016_EVA1	-2.8505	0.68078	-0.23883	4	0.138648
H200011635_LXN	-2.86425	2.020295	-0.70535	4	0.137333
opHsV0400006073_CFHL1	-2.86475	0.87589	-0.30575	4	0.137285
H200007909_Q86XJ2_HUMAN	-2.872	0.595409	-0.20731	4	0.136597
H200014027_CA2	-2.93825	1.069223	-0.3639	4	0.130466
opHsV0400010774	-2.9685	1.306912	-0.44026	4	0.127759
H200000301_CDKN2A	-2.97475	1.792289	-0.6025	4	0.127207
H200011169_TCF7L1	-2.99475	1.037295	-0.34637	4	0.125456
H200006236_CDH11	-3.0245	1.237091	-0.40902	4	0.122895
H200006995	-3.052	0.953395	-0.31238	4	0.120575
H200004653_SULF2	-3.06	1.158361	-0.37855	4	0.119908
H300003117_Q8N4W5_HUMAN	-3.06767	2.914295	-0.95	3	0.119272
H200016561_NP_060910.2	-3.071	1.721832	-0.56067	4	0.118997
H200020242_C9orf26	-3.0795	1.212845	-0.39384	4	0.118298
H300006196_MAF	-3.124	1.538379	-0.49244	4	0.114705
H200007907_FOXO1A	-3.13275	1.02355	-0.32673	4	0.114011
H200014307_FADS2	-3.1405	0.463705	-0.14765	4	0.113401
opHsV0400011317	-3.20067	3.195774	-0.99847	3	0.108769
H300004073_AKR1C1	-3.28425	0.748321	-0.22785	4	0.102646

Table 16 continued

Name of the downregulated gene in HN cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
H300004901_RPS4Y1	-3.3215	1.307476	-0.39364	4	0.10003
H200014029_EIF1AY	-3.58	1.302563	-0.36384	4	0.08362
H300021913_AKR1C3	-3.734	1.248393	-0.33433	4	0.075154
H200005947_GJA1	-3.869	1.115312	-0.28827	4	0.068441
opHsV0400010227_Q96NA9_HUMAN	-3.90075	0.856613	-0.2196	4	0.066951

Table 17: 128 upregulated genes in HSC-3 cell line. The gene list is from the most upregulated gene to least upregulated gene.

Name of the upregulated gene in HSC-3 cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
H200004378_MAGEA8	3.152	0.849621	0.26955	4	8.88887
H200005801_MAGEA1	3.03575	0.936701	0.308557	4	8.200717
H200011948_EREG	2.597	0.780137	0.300399	4	6.050272
H200011983_Q9BT26_HUMAN	2.59275	1.084013	0.418094	4	6.032475
opHsV0400002734_NP_848664.1	2.547	0.915678	0.359512	4	5.844178
H200004597_XM_374020.2	2.44275	0.932021	0.381546	4	5.436771
H200002223_TXNRD1	2.39975	0.65304	0.272128	4	5.277117
opHsV0400008521	2.36525	1.704137	0.720489	4	5.152419
H200017455_NP_060582.1	2.2765	0.326759	0.143536	4	4.845011
opHsV0400003034_KLK10	2.27275	0.695054	0.305821	4	4.832434
H200004485_HOXC9	2.267	1.211413	0.534369	4	4.813212
H200009720_GDF15	2.19	0.475102	0.216942	4	4.563055
opHsV0400003019_NP_115749.2	2.1475	0.334409	0.15572	4	4.430594
H300004818_MAGEA9	2.14725	1.140736	0.531254	4	4.429826
opHsV0400001562_IGSF4	2.10425	0.735674	0.349613	4	4.299742
H300006703_LY6K	2.0865	0.680104	0.325955	4	4.247165
H300022245_PHF15	2.05875	0.78175	0.379721	4	4.166252
opHsV0400000415_NP_149024.1	2.045	0.503042	0.245986	4	4.126733
opHsV0400004777_NP_998889.1	2.01425	1.010318	0.501585	4	4.039705
opHsV0400001984_XP_377060.1	1.9955	0.737407	0.369535	4	3.987543
H300015028_NMNAT3	1.9795	0.698805	0.353021	4	3.943564
H300022040_SH3YL1	1.97	0.476095	0.241673	4	3.917681
H300017088_Q9P2H4_HUMAN	1.96975	0.513645	0.260766	4	3.917002
opHsV0400006083_MAGEA4	1.9685	0.824239	0.418714	4	3.91361
H300006491_NP_940887.1	1.95075	0.90601	0.464442	4	3.865754
opHsV0400003953_HIST1H1C	1.9215	0.423402	0.22035	4	3.788167
opHsV0400007760	1.92075	0.563209	0.293223	4	3.786198
H300008288_NP_443726.1	1.91625	0.43419	0.226583	4	3.774407
H200014299_RHPN2	1.9135	0.454259	0.237397	4	3.767219
H200011351_AIM2	1.90825	1.427721	0.748183	4	3.753535
opHsV0400010848	1.88475	0.534614	0.283653	4	3.692889
H200006887_PSIP1	1.88475	0.326691	0.173334	4	3.692889
H300001921_PKD1L2	1.87575	1.677794	0.894466	4	3.669924
opHsV0400000818_IL8	1.8655	0.570452	0.30579	4	3.643942
H200006441_OLR1	1.862	1.144727	0.614783	4	3.635112

Table 17 continued

Name of the upregulated gene in HSC-3 cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
H300005599 NP 705835.1	1.86175	0.966071	0.518905	4	3.634483
H300021371 Q9P2H4 HUMAN	1.8485	0.707264	0.382615	4	3.601256
H200013441 KCNJ14	1.846	1.040101	0.563435	4	3.595021
H300020946 BRIX HUMAN	1.8405	0.397678	0.216071	4	3.581341
H300021118 BBC3	1.824	0.587338	0.322006	4	3.540615
opHsV0400002065 Q86YU6 HUMAN	1.81675	0.672504	0.370169	4	3.522867
H200000454 INHBB	1.80425	1.785077	0.989374	4	3.492476
H200014850 STK39	1.79125	0.621751	0.347105	4	3.461146
H200013135 UCN	1.78225	1.208734	0.678207	4	3.439622
H200006680 EMP1	1.7745	0.571015	0.321789	4	3.421194
H200011423 PKIB	1.764	0.507071	0.287455	4	3.396385
H200010852 ITCH HUMAN	1.7505	0.444094	0.253696	4	3.364752
opHsV0400003702 COX8C	1.75025	1.599525	0.913884	4	3.364169
H200007980 USMG5	1.74175	0.568977	0.32667	4	3.344406
opHsV0400011949	1.7305	0.339008	0.195902	4	3.318428
H300003162 ANKRD18A	1.7195	0.567176	0.32985	4	3.293223
H300010199 EIF5A2	1.7175	0.63642	0.37055	4	3.28866
H300011213 Q9NSQ0 HUMAN	1.70825	1.174061	0.687289	4	3.267642
opHsV0400003313 NP 003513.1	1.6915	0.457245	0.270319	4	3.229924
opHsV0400011821	1.686	0.751921	0.445979	4	3.217633
H200006097 ID1	1.68225	0.546952	0.325131	4	3.209281
H200004662 PELI2	1.682	1.000514	0.594836	4	3.208725
H200000169 TACSTD1	1.68	0.297876	0.177307	4	3.20428
H200021283 NANOS1	1.6795	0.536871	0.319661	4	3.203169
H200017773 GTPBP1	1.6745	1.163442	0.6948	4	3.192087
opHsV0400004721 HIST1H2BH	1.6675	0.606325	0.363613	4	3.176636
H300004810 PTENP1	1.6675	0.556886	0.333964	4	3.176636
H200006754 KRT15	1.653	0.513669	0.310749	4	3.144869
H200004708 PNN	1.641	1.23787	0.754339	4	3.118819
H200010772 GPR160	1.6405	0.936346	0.570768	4	3.117739
opHsV0400010379	1.63925	0.657288	0.400969	4	3.115039
H200017587 RIOK1	1.639	0.569017	0.347173	4	3.114499
H300004419 HSC20 HUMAN	1.630333	0.374844	0.229919	3	3.095845
H300010303 TMEM41B	1.62875	0.279295	0.171478	4	3.092449
H200008677 SERPINB1	1.623667	0.414045	0.255006	3	3.081572
H200000756 XCL1	1.6205	1.11755	0.689633	4	3.074816
opHsV0400002545 C9orf75	1.6185	0.569555	0.351903	4	3.070556

Table 17 continued

Name of the upregulated gene in HSC-3 cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
H200006453_RNF38	1.6165	0.338344	0.209306	4	3.066302
opHsV0400006608_NP_689497.1	1.6135	0.691395	0.428506	4	3.059933
H300020977_PSIP1	1.606	0.371028	0.231026	4	3.044067
opHsV0400008140	1.6015	0.515305	0.321764	4	3.034587
H300019484_MRPS18B	1.601	0.471496	0.294501	4	3.033535
H200006623_SEMA4D	1.60025	1.241406	0.775758	4	3.031958
H200000524_SLC25A4	1.599	0.420158	0.262763	4	3.029333
opHsV0400003565_NP_006638.1	1.5965	0.829345	0.519477	4	3.024088
opHsV0400011837	1.59475	0.943297	0.591501	4	3.020422
H300002440	1.56625	0.361304	0.230681	4	2.96134
H300000318_HIST1H2BK	1.55725	0.41875	0.268904	4	2.942923
H300001749_AQP11	1.55375	1.13034	0.727492	4	2.935792
H200020534_TRIM6	1.5485	0.425175	0.274572	4	2.925128
H200002977_NP_065726.1	1.5465	0.770006	0.497903	4	2.921076
H200002273_NP_620134.2	1.54575	0.342185	0.221371	4	2.919558
H200011237_C10orf45	1.54325	0.454406	0.294448	4	2.914503
H300013839_Q8N601_HUMAN	1.5355	0.402652	0.262229	4	2.898889
H300018428_BID	1.5315	0.627854	0.40996	4	2.890863
opHsV0400001983	1.531	0.47726	0.311731	4	2.889861
H300005107_C10orf116	1.53025	0.548939	0.358725	4	2.888359
H300018167_AMID	1.5295	0.785779	0.513749	4	2.886858
H200007656_ELF3	1.51475	0.340044	0.224489	4	2.857493
H300008564_RASSF6	1.51125	0.362357	0.239773	4	2.850569
opHsV0400001907	1.51075	0.501022	0.331638	4	2.849581
H200014940_SYCP2	1.502	1.161495	0.773299	4	2.832351
opHsV0400005403_HIST1H2BM	1.5	0.980374	0.653583	4	2.828427
opHsV0400003794_Q71RB1_HUMAN	1.49575	0.399363	0.266999	4	2.820107
H200019082_TIMM9	1.4935	0.359623	0.240792	4	2.815712
H300010984_PHF15	1.4935	0.450442	0.301601	4	2.815712
opHsV0400002715_Q68G75_HUMAN	1.49275	0.562713	0.376964	4	2.814249
H300007931_NP_057385.2	1.4915	0.450681	0.302167	4	2.811812
H300020380_TATDN1	1.491	0.640439	0.429536	4	2.810837
opHsV0400003875_Q8NHD1_HUMAN	1.48975	0.663412	0.445317	4	2.808403
opHsV0400001186_NP_254280.1	1.48925	0.294015	0.197425	4	2.80743
H200010529_AMOTL2	1.489	0.369736	0.248312	4	2.806943
H200003398_CWF19L2	1.481	1.349314	0.911083	4	2.791422
H200004072_SIRT1_HUMAN	1.48025	0.515023	0.347929	4	2.789971

Table 17 continued

Name of the upregulated gene in HSC-3 cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
H200006199_EDG2	1.48	0.40603	0.274345	4	2.789487
H200003664_C20orf100	1.4785	0.933017	0.631056	4	2.786589
H200005619_DIPA_HUMAN	1.4765	0.563093	0.38137	4	2.782728
H200001173_NP_932062.1	1.47275	0.496299	0.336988	4	2.775504
H200010289_C6orf139	1.46625	0.579713	0.395371	4	2.763028
H200000560_CEBPG	1.464	0.409896	0.279984	4	2.758722
H300021980_RHOT1	1.46075	0.371909	0.254601	4	2.752514
H200009826_KRTHA4	1.45975	0.9836	0.673814	4	2.750607
H200006824_FZD2	1.4565	0.584051	0.400996	4	2.744418
opHsV0400013404	1.44975	0.433341	0.298907	4	2.731607
H300000063_ANKRD22	1.44725	0.878237	0.606832	4	2.726878
H200020107_PSMA3	1.4455	0.455472	0.315096	4	2.723572
H200001621_STRBP	1.445	0.783382	0.542133	4	2.722628
H300001211_ARVCF	1.44375	0.753391	0.521829	4	2.72027
H200003385_PARP2	1.44275	0.926379	0.642093	4	2.718385
H300005845	1.44225	0.643212	0.445978	4	2.717443
H300006484_CYP1A1	1.44	0.984089	0.683395	4	2.713209
H300001097	1.4365	0.434289	0.302324	4	2.706634
opHsV0400004914	1.43125	0.553855	0.386973	4	2.696803

Table 18: 117 downregulated genes in HSC-3 cell line. The gene list is from the least downregulated gene to most downregulated gene.

Name of the downregulated gene in HSC-3 cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
H300004618_LGALS7	-1.59825	0.652359	-0.40817	4	0.330277
H300001203_GLIPR1	-1.6	0.633926	-0.3962	4	0.329877
H300009606_ISG20_HUMAN	-1.60025	0.636049	-0.39747	4	0.32982
opHsV0400001923_IDH2	-1.60425	0.757475	-0.47217	4	0.328907
H200013160_CARD15	-1.60725	1.224737	-0.76201	4	0.328223
opHsV0400003766_NP_710154.1	-1.60875	0.487525	-0.30305	4	0.327882
H200000491_PRG1	-1.60975	0.736354	-0.45743	4	0.327655
H300003923_DIAPH2	-1.6105	0.696324	-0.43237	4	0.327485
opHsV0400003043_MYADM	-1.61525	0.581445	-0.35997	4	0.326408
H200005275_KLK11	-1.6155	1.168752	-0.72346	4	0.326352
H200000280_SPRR1B	-1.6335	1.10972	-0.67935	4	0.322305
H200011042_S100A8	-1.64	1.123603	-0.68512	4	0.320856
H300014335_KRT6B	-1.64675	0.782004	-0.47488	4	0.319359
opHsV0400002054_RARB	-1.647	1.398813	-0.84931	4	0.319303
H200006117_TP53INP1	-1.66175	0.708937	-0.42662	4	0.316056
H300020154_TUBB3	-1.668	0.663706	-0.39791	4	0.314689
H200005378_NP_612398.1	-1.669	0.449427	-0.26928	4	0.314471
H200005918_SYK	-1.66975	1.210319	-0.72485	4	0.314308
H300007287_GCNT1	-1.6715	1.245413	-0.74509	4	0.313927
opHsV0400005920_ITGB6	-1.67825	0.791641	-0.47171	4	0.312461
H200003227_CMKOR1	-1.68625	0.819594	-0.48605	4	0.310734
H200006265_EFEMP1	-1.688	0.832564	-0.49322	4	0.310357
H300016617_THY1	-1.693	1.424375	-0.84133	4	0.309283
H200012016_EVA1	-1.69325	0.522671	-0.30868	4	0.30923
H200001136_NP_775925.1	-1.69675	0.554422	-0.32676	4	0.30848
H200000047_IGFBP2	-1.6995	0.581298	-0.34204	4	0.307893
H300010309_MYADM	-1.6995	0.748583	-0.44047	4	0.307893
opHsV0400006248_SCMH1	-1.70275	1.414602	-0.83077	4	0.3072
H200014262_DSCR1	-1.7075	0.495285	-0.29006	4	0.30619
H300010992_RBPMS	-1.715	0.429382	-0.25037	4	0.304603
H200011819_NP_002956.1	-1.72275	1.028266	-0.59687	4	0.302971
H300010137_CALML3	-1.7315	1.122493	-0.64828	4	0.301139
opHsV0400005864_SEPP1	-1.74075	0.803666	-0.46168	4	0.299214
opHsV0400011936_	-1.747	1.360383	-0.7787	3	0.297921
H200005431_DIXDC1	-1.756	1.022538	-0.58231	4	0.296068

Table 18 continued

Name of the downregulated gene in HSC-3 cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
H300000194_CSPG2	-1.757	0.801492	-0.45617	4	0.295863
H300000923_POSTN	-1.7605	0.694177	-0.39431	4	0.295146
H300018412_MGST1	-1.7615	0.822925	-0.46717	4	0.294941
opHsV0400010804	-1.76175	0.760455	-0.43165	4	0.29489
H200013413_LRP3	-1.7665	0.715098	-0.40481	4	0.293921
H200014313_BEXL1	-1.771	0.709235	-0.40047	4	0.293006
H200017501_MAGEH1	-1.77275	0.725866	-0.40946	4	0.29265
H300003004_NP_006465.3	-1.788	0.671364	-0.37548	4	0.289573
H200000850_FKBP10	-1.80325	0.670965	-0.37209	4	0.286528
H300003809_CTGF	-1.818	0.659317	-0.36266	4	0.283614
opHsV0400002995_BCL2A1	-1.8235	1.294393	-0.70984	4	0.282535
opHsV0400004303_MXD4	-1.82425	0.722652	-0.39614	4	0.282388
H300003568_CPS1	-1.82675	1.062375	-0.58157	4	0.281899
H200004216_PLXDC2	-1.82975	0.755352	-0.41282	4	0.281313
H200001726_KRT23	-1.834	0.87163	-0.47526	4	0.280486
opHsV0400012721	-1.85175	1.48234	-0.80051	4	0.277056
H200013065	-1.85225	0.9005	-0.48617	4	0.27696
opHsV0400010233	-1.8585	1.76167	-0.9479	4	0.275763
H200011416_A4GALT	-1.8655	0.72525	-0.38877	4	0.274428
H200000073_GALC	-1.87525	1.044675	-0.55709	4	0.27258
H200010239_CTSL2	-1.8785	0.774282	-0.41218	4	0.271966
H200000789_NP_056359.1	-1.88175	0.858455	-0.4562	4	0.271354
H200011433_SNX10	-1.88875	0.754188	-0.39931	4	0.270041
H200010620_IL6	-1.8895	1.383369	-0.73214	4	0.269901
H200013672_ZNF569	-1.895	1.521567	-0.80294	4	0.268874
H200014737_PTGS2	-1.8985	0.67565	-0.35589	4	0.268222
H200008429_Q8TAW3_HUMAN	-1.901	1.625141	-0.85489	4	0.267758
opHsV0400005248_FAM46B	-1.9065	0.885208	-0.46431	4	0.266739
H200009421_NP_775823.1	-1.93	0.606021	-0.314	4	0.262429
opHsV0400012118	-1.9465	0.605055	-0.31084	4	0.259445
opHsV0400000655_GJB2	-1.95175	0.743374	-0.38088	4	0.258502
H200002119_ADCY6	-1.9525	0.916307	-0.4693	4	0.258368
H200014431_Y0961_HUMAN	-1.9755	0.760543	-0.38499	4	0.254282
opHsV0400011667	-1.99475	0.714675	-0.35828	4	0.250911
H200011635_LXN	-2.00975	1.066807	-0.53082	4	0.248316
H200009088_ZNF606	-2.012	1.487884	-0.73951	4	0.247929
H200004653_SULF2	-2.01375	0.747939	-0.37142	4	0.247629

Table 18 continued

Name of the downregulated gene in HSC-3 cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
H300013721 TPM4 HUMAN	-2.021	0.612298	-0.30297	4	0.246387
H300003220 THBS1	-2.0215	1.036309	-0.51264	4	0.246302
H300021562 FN1	-2.022	0.948529	-0.4691	4	0.246217
H200005569 LIPG	-2.04625	0.550896	-0.26922	4	0.242113
H300006196 MAF	-2.0505	0.813085	-0.39653	4	0.2414
H200012876 HYAL3	-2.0525	0.589862	-0.28739	4	0.241066
opHsV0400010566	-2.0745	1.480604	-0.71372	4	0.237418
H200002091 PGBD5	-2.07575	1.316248	-0.63411	4	0.237212
opHsV0400006127 TNFRSF6B	-2.09875	0.750611	-0.35765	4	0.23346
H200006334 ID3	-2.1125	0.668089	-0.31626	4	0.231246
H200006031 IPKA HUMAN	-2.13125	0.959348	-0.45013	4	0.22826
H200005423 Q9H7B6 HUMAN	-2.133	1.512657	-0.70917	4	0.227983
H200007909 Q86XJ2 HUMAN	-2.14075	0.764061	-0.35691	4	0.226762
H200011334 DER7 HUMAN	-2.14425	1.85577	-0.86546	4	0.226212
H300021749 FOLR3	-2.15275	0.651404	-0.30259	4	0.224884
H200005198 GALNT5	-2.15775	0.771067	-0.35735	4	0.224106
H300018293 SLC38A5	-2.16125	0.50926	-0.23563	4	0.223562
H200016573 ADPN	-2.16325	0.577262	-0.26685	4	0.223253
opHsV0400010774	-2.16425	0.597593	-0.27612	4	0.223098
H300021145 ANGPTL4	-2.20475	0.76283	-0.34599	4	0.216922
opHsV0400012764	-2.23	0.580492	-0.26031	4	0.213159
H200006995	-2.2485	0.791391	-0.35196	4	0.210443
H200006600 PTPRK	-2.2635	0.981011	-0.4334	4	0.208266
H200004031 NP 945120.1	-2.264	0.729965	-0.32242	4	0.208194
H200004612 PDGFC	-2.283	0.625134	-0.27382	4	0.20547
H200001686 TM4SF10	-2.30975	1.356076	-0.58711	4	0.201695
H200018197 ZNF83	-2.31775	0.474314	-0.20464	4	0.20058
opHsV0400012111	-2.356	0.525024	-0.22285	4	0.195332
H200015098 IL1F9	-2.38625	1.008139	-0.42248	4	0.191279
H200009642 ITGAV	-2.3925	0.630469	-0.26352	4	0.190452
H200004832 C3orf14	-2.4275	0.846863	-0.34886	4	0.185887
H300015331 CALD1	-2.43825	0.623359	-0.25566	4	0.184507
H200012184 COL4A1	-2.46775	0.999759	-0.40513	4	0.180773
opHsV0400000212 SH3BGRL	-2.47625	0.893669	-0.3609	4	0.179711
H300016644 WASPIP	-2.58325	1.607772	-0.62238	4	0.166865
H200005947 GJA1	-2.6245	1.273362	-0.48518	4	0.162161
H200020242 C9orf26	-2.63075	1.189428	-0.45213	4	0.16146

Table 18 continued

Name of the downregulated gene in HSC-3 cell line	Average value of 4 slides	STD	CV	Count of slides	Ratio
H300000043_RGS17	-2.63425	0.842874	-0.31997	4	0.161069
opHsV0400010227_Q96NA9_HUMAN	-2.65575	1.140632	-0.4295	4	0.158686
H300017162_Q9H7B6_HUMAN	-2.658	0.852331	-0.32067	4	0.158439
H200006236_CDH11	-2.69325	1.264642	-0.46956	4	0.154615
opHsV0400010653	-2.7575	1.222115	-0.4432	4	0.14788
H200008006_SEMA3C	-2.841	0.770015	-0.27104	4	0.139564
H200014307_FADS2	-3.011	1.080029	-0.35869	4	0.124051
H300008702_ITGB6	-3.28575	0.967254	-0.29438	4	0.102539

Table 19: The most upregulated and downregulated 20 genes in BHY cell line and the expression levels of these 20 genes in the other two OSCC cell lines.

Name of the most upregulated 20 gene in BHY cell line. The gene list is from the more upregulated gene to less upregulated gene.	Expression ratio of the gene in BHY	Expression ratio of the gene in HN cell	Expression ratio of the gene in HSC-3 cell
H300004818 MAGEA9	7.909026	2.070171	4.429825
H200001373 Q96AL8 HUMAN	7.86529	10.51407	2.0145
H200005842 GFI1	6.768096	3.717931	1.04215
opHsV0400005829 OAS1	6.37533	7.281572	0.71254
H200007111 NP_689700.1	6.087079	3.100498	1.2456
H200005801 MAGEA1	5.877693	11.27847	1.8964
opHsV0400002611 PRAME	5.8665	6.631108	8.2007
opHsV0400006088 NOV	5.862435	2.455471	0.94251
H200011702 HOXA10	5.820931	4.453686	0.76598
H200012712 BIRC3	5.181968	5.754737	0.5627
H200004378 MAGEA8	5.143499	2.806943	8.88886
H200000112 DLX2	5.112397	1.306312	2.20014
H200003290 DEPDC1B	5.092945	2.7549	1.46598
H200000421 DPYD	4.930555	0.622437	1.00124
H200000404 IRK2 HUMAN	4.917755	0.53016	2.01245
opHsV0400006060	4.916051	3.857056	0.8854
H200011805 NMES1 HUMAN	4.798222	12.48826	0.7645
H300010220 ID2	4.76673	1.236704	1.0235
H200005149 NP_116262.2	4.735445	1.304503	0.450547
H200006112 NP_006811.1	4.64684	4.524474	0.96325
Name of the most downregulated 20 gene in BHY cell line. The gene list is from the less downregulated gene to more downregulated gene.	Expression ratio of the gene in BHY	Expression ratio of the gene in HN	Expression ratio of the gene in HSC-3 cell
H300018293 SLC38A5	0.203803	0.742905	0.223562
H200001823 ARMCX1	0.203204	0.508299	0.373907
H200011330 RKHD3	0.200337	0.195095	1.235689
H300021749 FOLR3	0.198162	0.202115	0.224884
H200001408 SFRP1	0.195106	0.185148	0.366148
H300021145 ANGPTL4	0.190584	0.179867	0.216922
H200016276 ESPN	0.186791	0.726734	0.342043
H300016617 THY1	0.186242	0.872665	0.309283
H200018944 KLF8	0.18621	0.272013	0.434522

Table 19 continued

Name of the most downregulated 20 gene in BHY cell line. The gene list is from the less downregulated gene to more downregulated gene.	Expression ratio of the gene in BHY	Expression ratio of the gene in HN	Expression ratio of the gene in HSC-3 cell
H200005423_Q9H7B6_HUMAN	0.184891	0.449845	0.227983
H200001686_TM4SF10	0.165913	0.607413	0.201695
H200004832_C3orf14	0.160012	2.255192	0.185887
H200011635_LXN	0.155071	0.137333	0.248316
opHsV0400010653_	0.150335	0.144511	0.14788
H200005947_GJA1	0.129588	0.068441	0.162161
H200017869_	0.125318	0.634635	1.469785
H200014307_FADS2	0.123044	0.113401	0.124051
H300017162_Q9H7B6_HUMAN	0.118339	0.498011	0.158439
opHsV0400010227_Q96NA9_HUMAN	0.101005	0.066951	0.158686
H300000043_RGS17	0.093121	0.200372	0.161069

Table 20: The most upregulated and downregulated 20 genes in HN cell line and the expression levels of these 20 genes in the other two OSCC cell lines.

Name of the most upregulated 20 gene in HN cell line. The gene list is from the more upregulated gene to less upregulated gene.	Expression ratio of the gene in HN	Expression ratio of the gene in BHY cell	Expression ratio of the gene in HSC-3 cell
H200011805 NMES1 HUMAN	12.2461	4.798222	0.98746
H200005801 MAGEA1	11.27847	5.877693	8.200717
H200001373 Q96AL8 HUMAN	10.51407	7.86529	2.04345
opHsV0400013404	9.04738	1.330529	2.731607
H200004165 FSTL5	7.823152	2.39828	2.18635
opHsV0400005829 OAS1	7.281572	6.37533	1.90835
H200010620 IL6	7.206256	4.452143	0.269901
H200010289 C6orf139	7.156479	3.922436	2.763028
H300005969 COX7B2	6.699252	1.24387	1.82643
opHsV0400002611 PRAME	6.631108	5.8665	1.02386
H300006342 NP_775969.1	6.463206	1.7436	1.19476
H200010916 NP_114156.1	6.040843	1.03434	1.90345
H200012712 BIRC3	5.754737	5.181968	1.56723
H300006447 MT1K	5.66863	0.212237	1.87352
H200007081 SLCO2A1	5.634353	2.721213	1.90032
H200002977 NP_065726.1	5.514576	3.700576	2.921076
H200004279 ID4	5.36378	1.00232	2.28734
H200008845 STMN3	5.135483	4.018062	1.78253
H300022894 NP_002116.2	5.088534	1.84334	1.1872
opHsV0400000818 IL8	5.066537	4.259695	3.643942
Name of the most downregulated 20 gene in HN cell line. The gene list is from the less downregulated gene to more downregulated gene.	Expression ratio of the gene in HN	Expression ratio of the gene in BHY	Expression ratio of the gene in HSC-3 cell
H200014027 CA2	0.130466	0.53432	0.724973
opHsV0400010774	0.127759	0.317483	0.223098
H200000301 CDKN2A	0.127207	0.6617	1.600475
H200011169 TCF7L1	0.125456	0.307253	0.398527
H200006236 CDH11	0.122895	0.65293	0.154615
H200006995	0.120575	0.309337	0.210443
H200004653 SULF2	0.119908	0.493116	0.247629
H300003117 Q8N4W5 HUMAN	0.119272	2.763506	0.4214
H200016561 NP_060910.2	0.118997	2.204192	0.600714

Table 20 continued

Name of the most downregulated 20 gene in HN cell line. The gene list is from the less downregulated gene to more downregulated gene.	Expression ratio of the gene in HN	Expression ratio of the gene in BHY	Expression ratio of the gene in HSC-3 cell
H200020242_C9orf26	0.118298	0.225156	0.16146
H300006196_MAF	0.114705	0.227313	0.2414
H200007907_FOXO1A	0.114011	0.4355	0.67354
H200014307_FADS2	0.113401	0.123044	0.124051
opHsV0400011317	0.108769	0.63243	0.71256
H300004073_AKR1C1	0.102646	2.305373	2.441893
H300004901_RPS4Y1	0.10003	0.43224	0.39214
H200014029_EIF1AY	0.08362	0.817052	1.464847
H300021913_AKR1C3	0.075154	0.6213	2.628974
H200005947_GJA1	0.068441	0.129588	0.162161
opHsV0400010227_Q96NA9_HUMAN	0.066951	0.101005	0.158686

Table 21: The most upregulated and downregulated 20 genes in HSC-3 cell line and the expression levels of these 20 genes in the other two OSCC cell lines.

Name of the most upregulated 20 gene in HSC-3 cell line. The gene list is from the more upregulated gene to less upregulated gene.	Expression ratio of the gene in HSC-3	Expression ratio of the gene in BHY cell	Expression ratio of the gene in HN cell
H200004378_MAGEA8	8.88887	5.143499	2.806943
H200005801_MAGEA1	8.200717	5.877693	11.27847
H200011948_EREG	6.050272	4.004161	1.490969
H200011983_Q9BT26_HUMAN	6.032475	1.2334	4.322153
opHsV0400002734_NP_848664.1	5.844178	3.118819	3.088165
H200004597_XM_374020.2	5.436771	4.13389	3.868435
H200002223_TXNRD1	5.277117	3.269908	2.587843
opHsV0400008521	5.152419	4.235405	1.431969
H200017455_NP_060582.1	4.845011	2.378002	3.319003
opHsV0400003034_KLK10	4.832434	3.113959	1.801252
H200004485_HOXC9	4.813212	2.113304	1.42652
H200009720_GDF15	4.563055	1.647467	3.522257
opHsV0400003019_NP_115749.2	4.430594	1.642052	2.900899
H300004818_MAGEA9	4.429826	7.909026	2.070171
opHsV0400001562_IGSF4	4.299742	1.313576	2.159579
H300006703_LY6K	4.247165	3.564007	4.538608
H300022245_PHF15	4.166252	1.7834	0.777007
opHsV0400000415_NP_149024.1	4.126733	2.03424	0.80413
opHsV0400004777_NP_998889.1	4.039705	1.819762	1.706452
opHsV0400001984_XP_377060.1	3.987543	1.834962	1.318594
Name of the most downregulated 20 gene in HSC-3 cell line. The gene list is from the less downregulated gene to more downregulated gene.	Expression ratio of the gene in HSC-3	Expression ratio of the gene in BHY	Expression ratio of the gene in HN cell
H200001686_TM4SF10	0.201695	0.165913	0.607413
H200018197_ZNF83	0.20058	0.543085	0.598221
opHsV0400012111	0.195332	0.379258	0.272816
H200015098_IL1F9	0.191279	0.40669	0.174373
H200009642_ITGAV	0.190452	0.270509	0.330908
H200004832_C3orf14	0.185887	0.160012	2.255192
H300015331_CALD1	0.184507	0.402902	0.267479
H200012184_COL4A1	0.180773	0.493116	0.141586
opHsV0400000212_SH3BGRL	0.179711	0.383421	0.427427

Table 21 continued

Name of the most downregulated 20 gene in HSC-3 cell line. The gene list is from the less downregulated gene to more downregulated gene.	Expression ratio of the gene in HSC-3	Expression ratio of the gene in BHY	Expression ratio of the gene in HN cell
H300016644_WASPIP	0.166865	0.204121	0.216547
H200005947_GJA1	0.162161	0.129588	0.068441
H200020242_C9orf26	0.16146	0.225156	0.118298
H300000043_RGS17	0.161069	0.093121	0.200511
opHsV0400010227_Q96NA9_HUMAN	0.158686	0.101005	0.066951
H300017162_Q9H7B6_HUMAN	0.158439	0.118339	0.498011
H200006236_CDH11	0.154615	0.65293	0.122895
opHsV0400010653_	0.14788	0.150335	0.144511
H200008006_SEMA3C	0.139564	0.259175	0.181684
H200014307_FADS2	0.124051	0.123044	0.113401
H300008702_ITGB6	0.102539	0.281948	0.512811

APPENDIX B: FIGURES

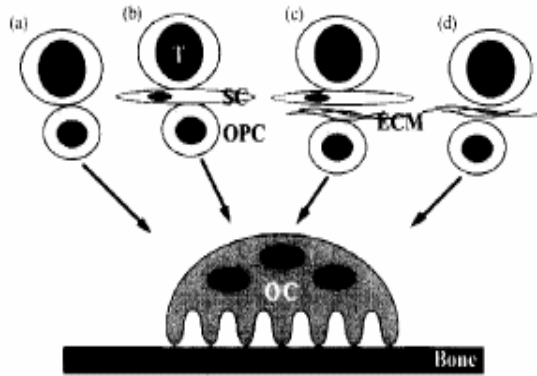


Figure 1: Schematic drawing showing the relationship between tumor cells, osteoclast precursor cells, stromal cells, and extracellular matrix. Osteoclast precursor cells are apposed to tumor cells in some instances (a), but stromal cells and/or extracellular matrix usually exists between the two cells (b), (c), (d). (T, tumor cells; SC, stromal cells; ECM, extracellular matrix; OPC, osteoclast precursor cells.) (7).

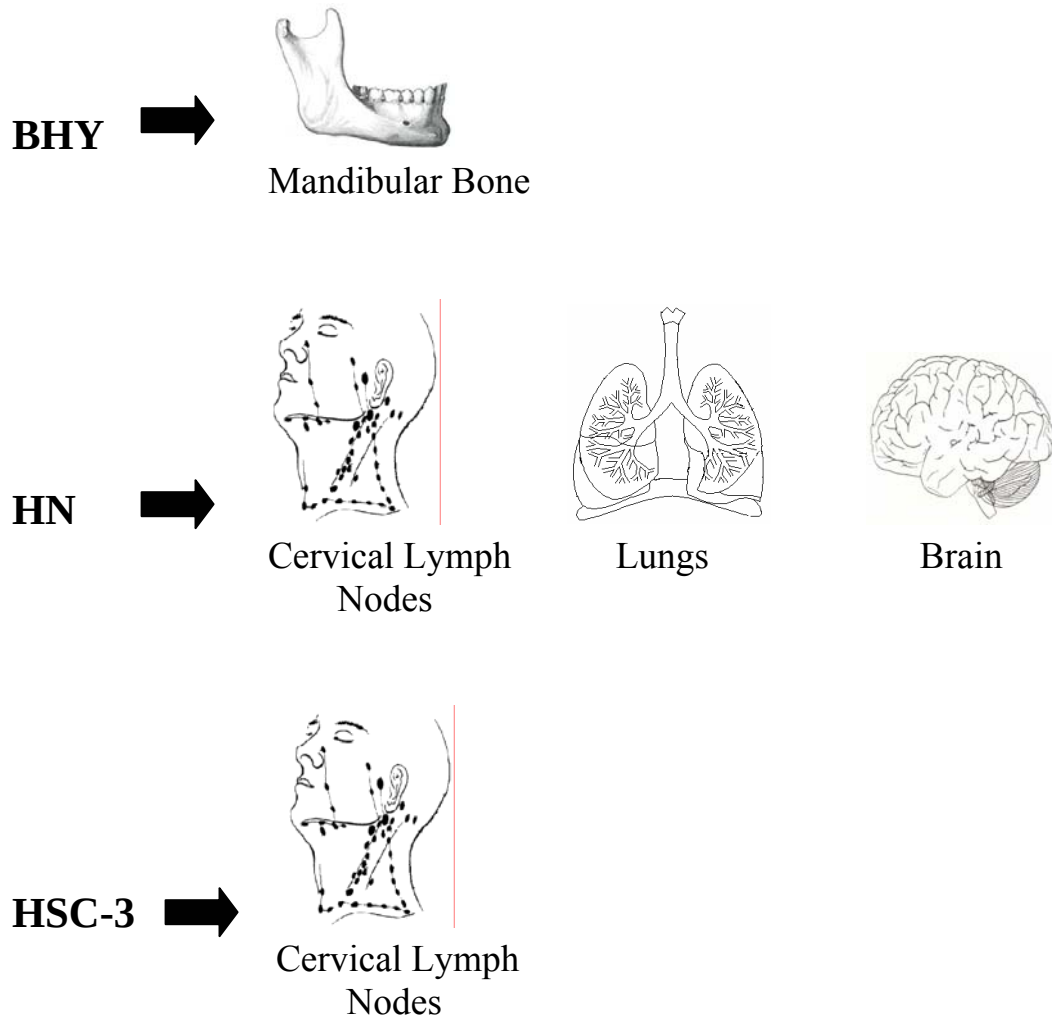


Figure 2: The organs to which BHY, HN, and HSC-3 cells invade and/or metastasize are shown in this schematic drawing.

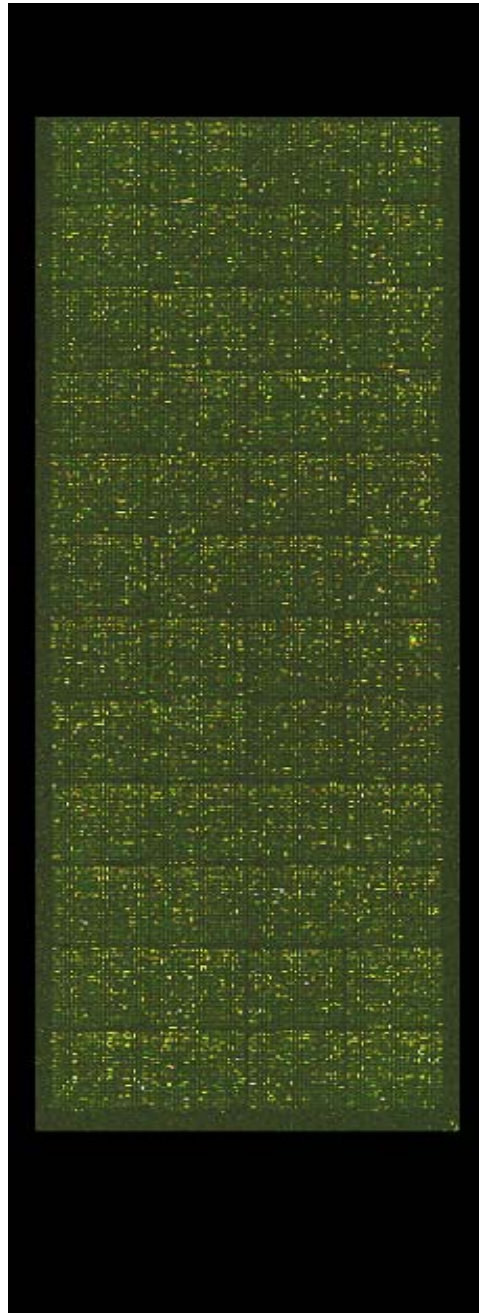


Figure 3: Operon Human Version 3.0 microarray slides

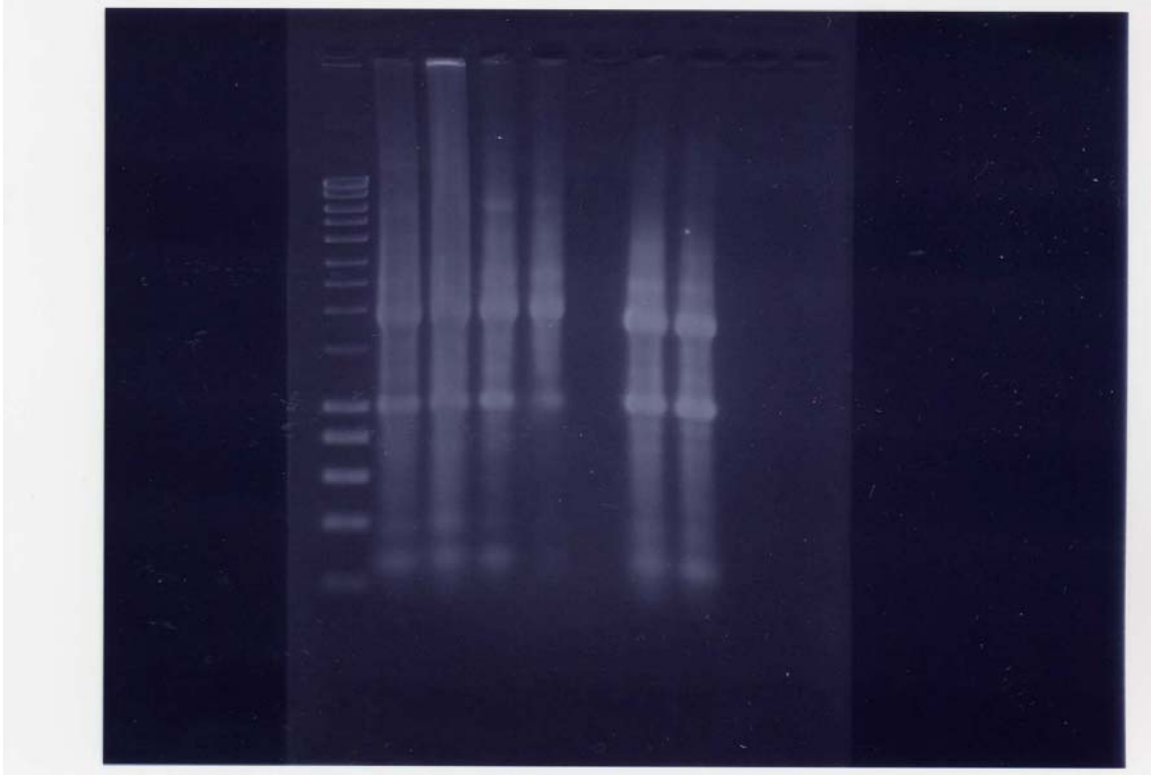


Figure 4a: Total RNA samples before the synthesis of cDNA for Real Time PCR. Column 1: Marker, 2: HNEC, 3: BHY, 4: HN, 5: HSC-3, 6: blank, 7: JP1, 8: JP2.

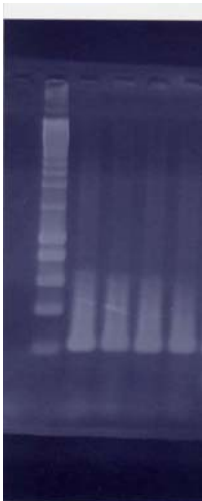


Figure 4b: Glucose-6-phosphate gene expression levels of cDNA samples used for Real Time PCR. Column 1:Marker, Column 2: HNEC, Column 3: BHY, Column 4: HN, Column 5: HSC-3.

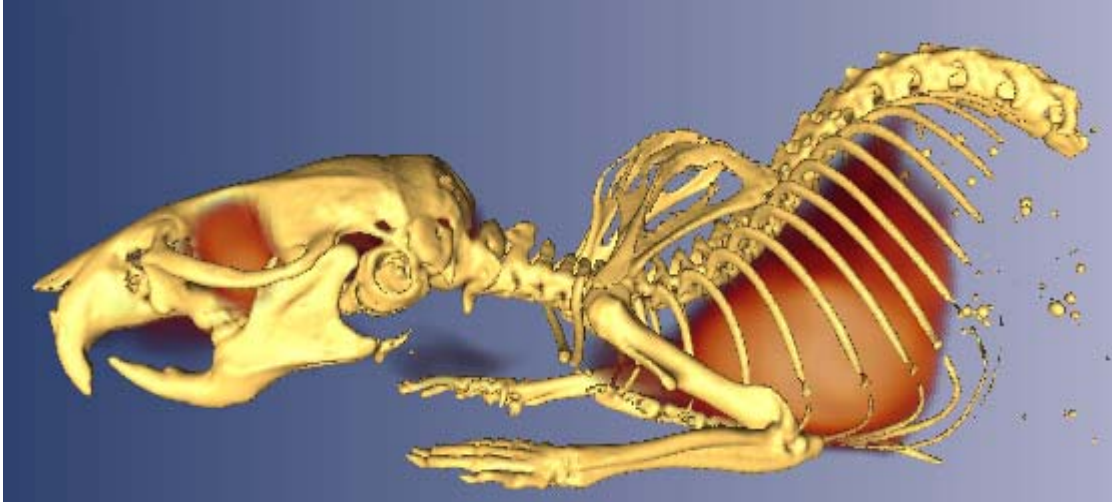


Figure 5: A left sagittal PET image of a mouse with HNEC cell line injection. No malignancy was observed.

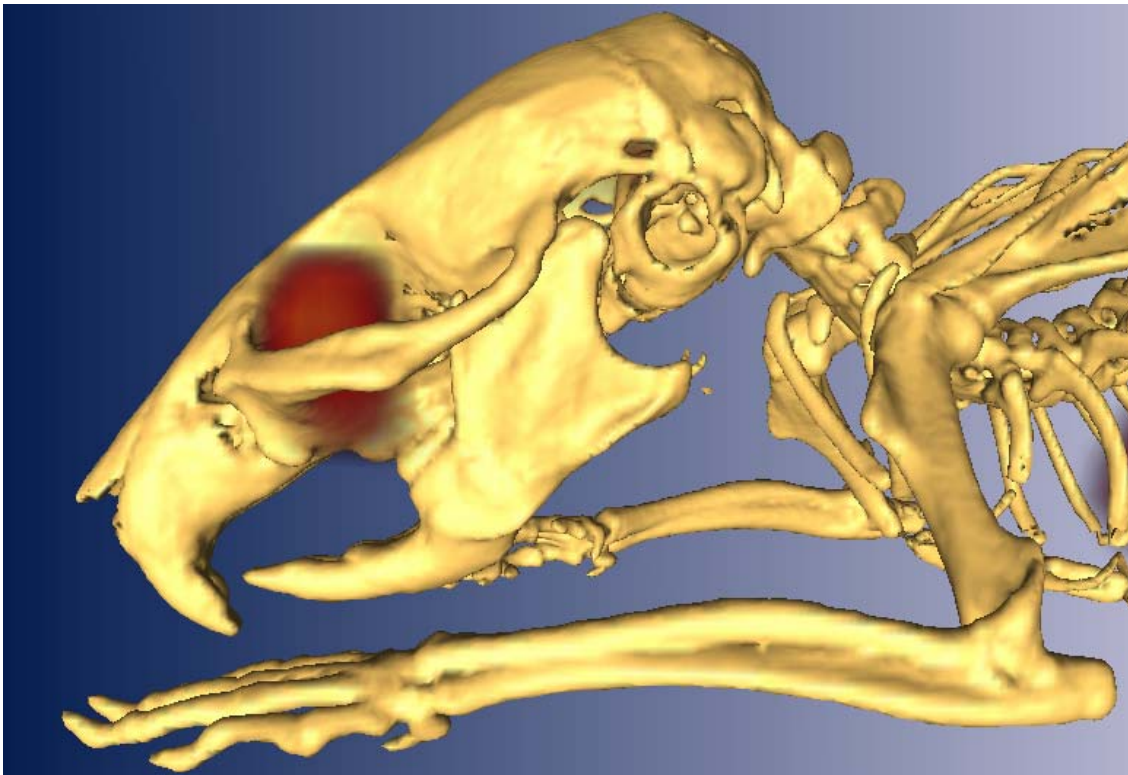


Figure 6: A left sagittal PET image of a mouse with Matrigel injection. No malignancy was observed.



Figure 7: An axial PET/CT image of a mouse with HNEC cell line injection. No malignancy was observed. All structures look normal.

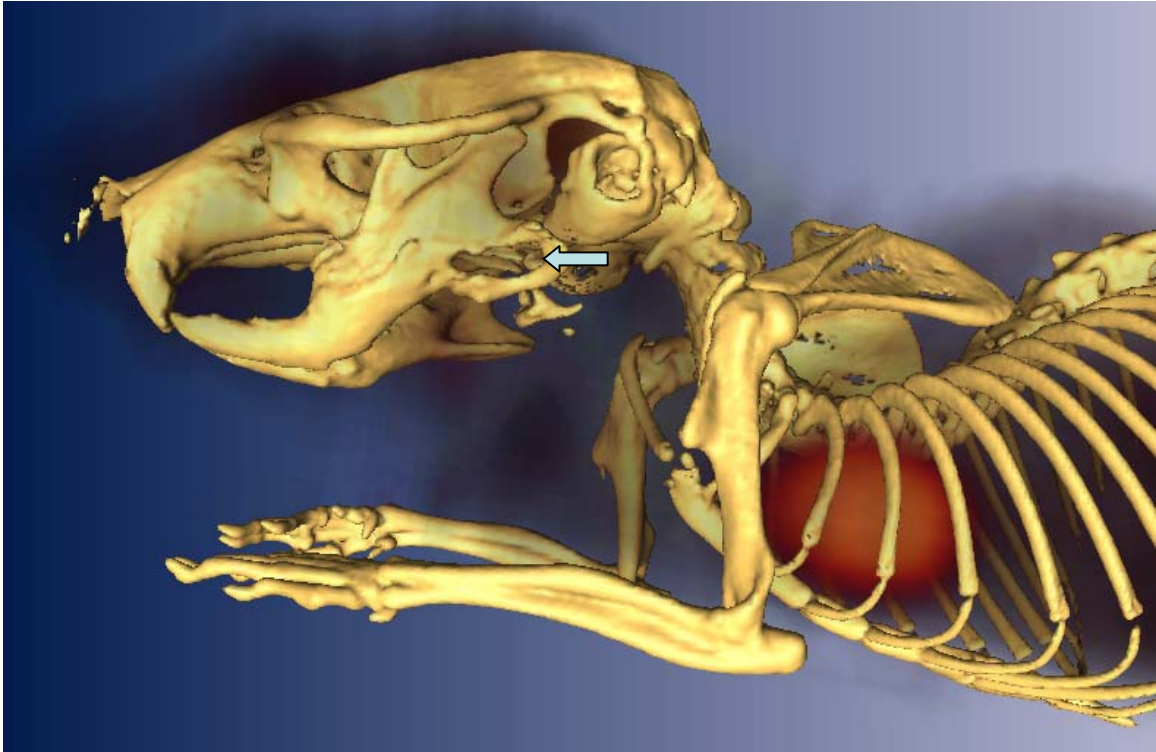


Figure 8: A left sagittal PET image of a mouse with BHY cell line injection. Arrow shows the mandibular bone resorption due to invasion of OSCC to the bone.

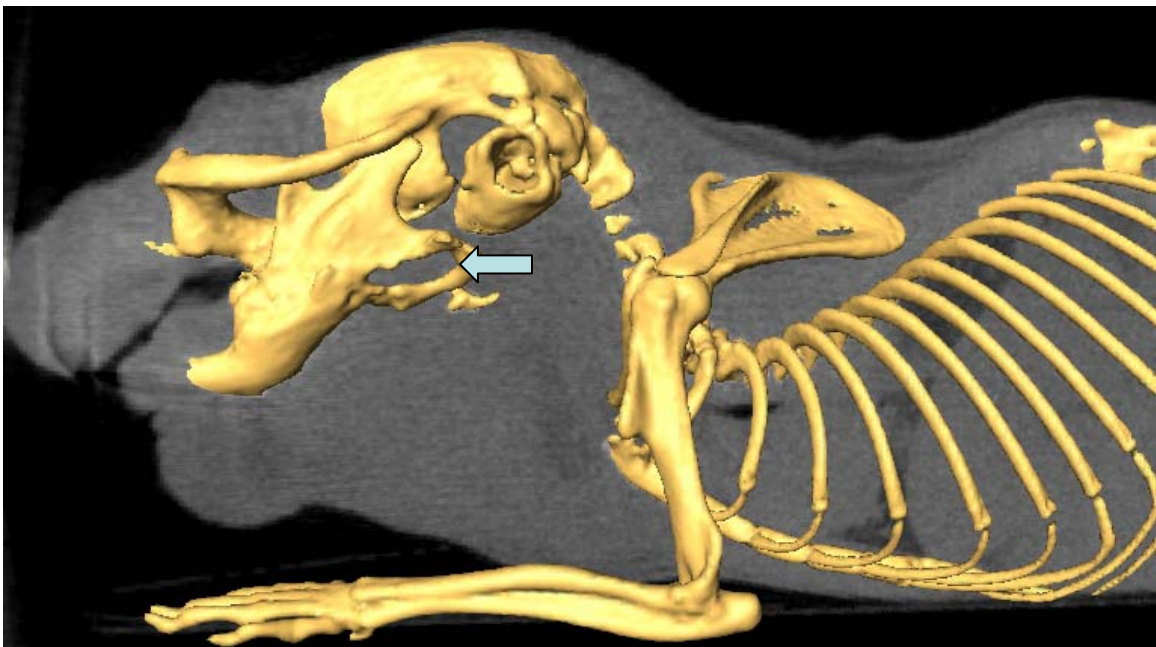


Figure 9: A left sagittal PET/CT image of a mouse with BHY cell line injection.

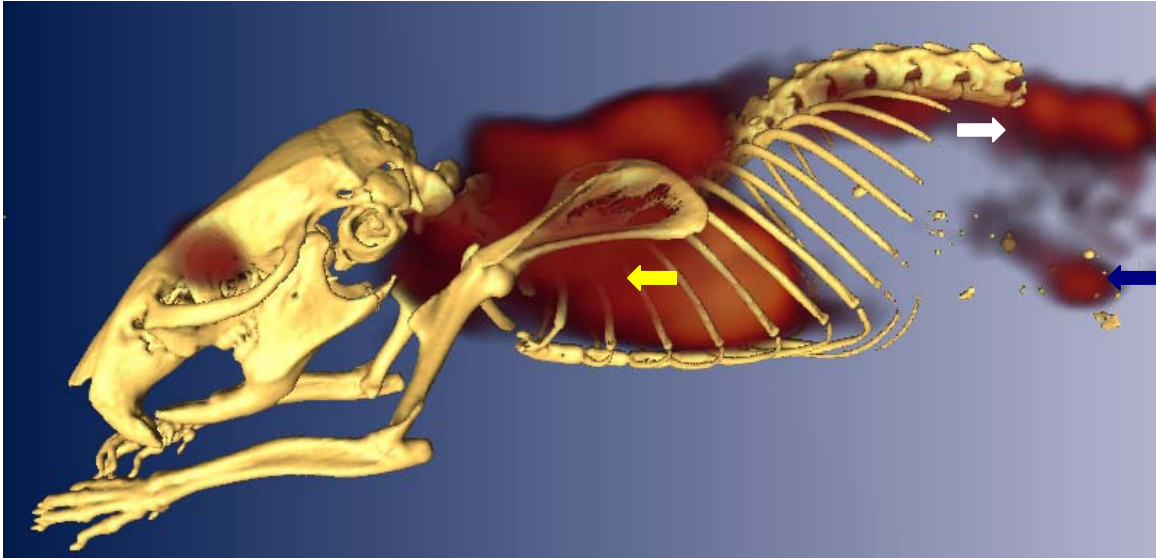


Figure 10: A left sagittal PET image of a mouse with HN cell line injection. Yellow arrow shows lung metastasis, blue arrow shows kidney metastasis and white arrow shows the distant lymph node metastasis of OSCC.

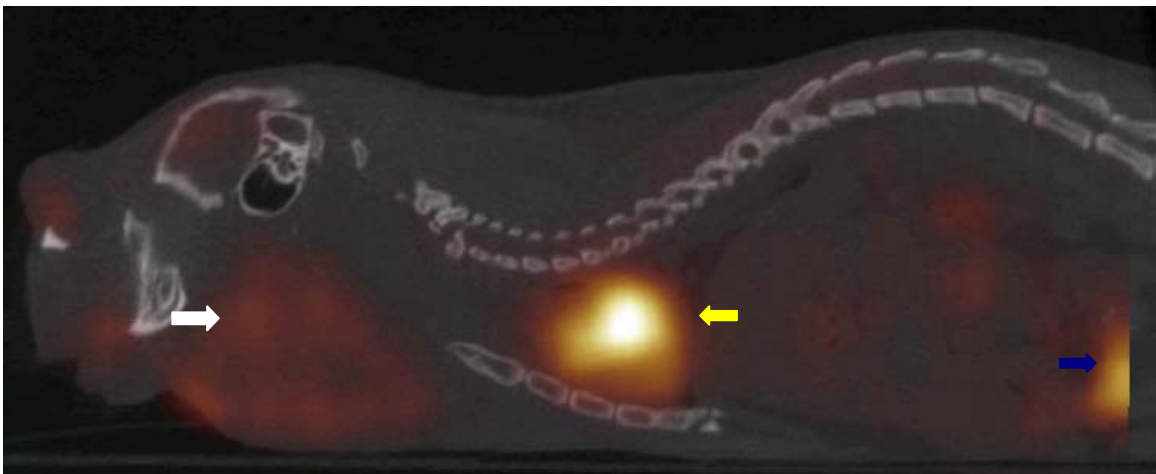


Figure 11: A left sagittal PET/CT image of a mouse with HN cell line injection. Yellow arrow shows lung metastasis, blue arrow shows kidney metastasis and white arrow shows the cervical lymph node metastasis of OSCC.

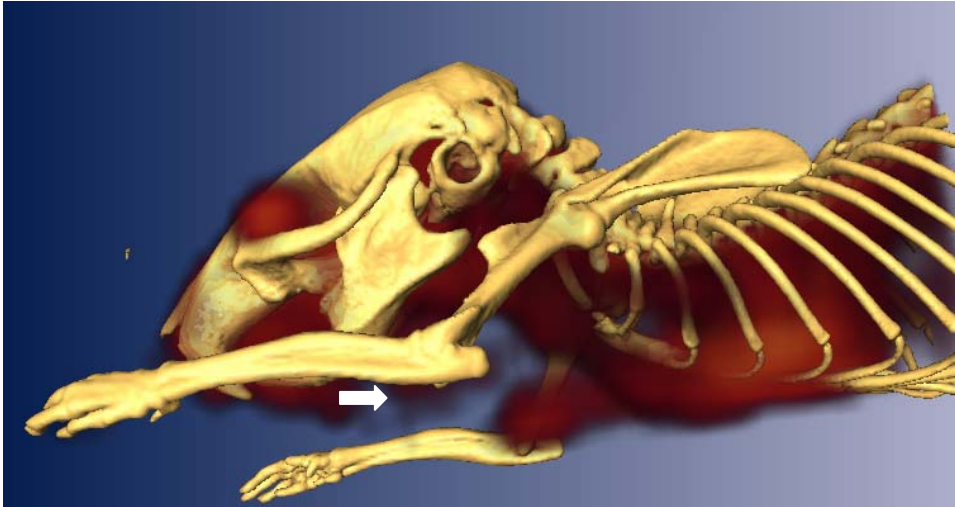


Figure 12: A left sagittal PET image of a mouse with HSC-3 cell line injection. White arrow shows cervical lymph node metastasis.

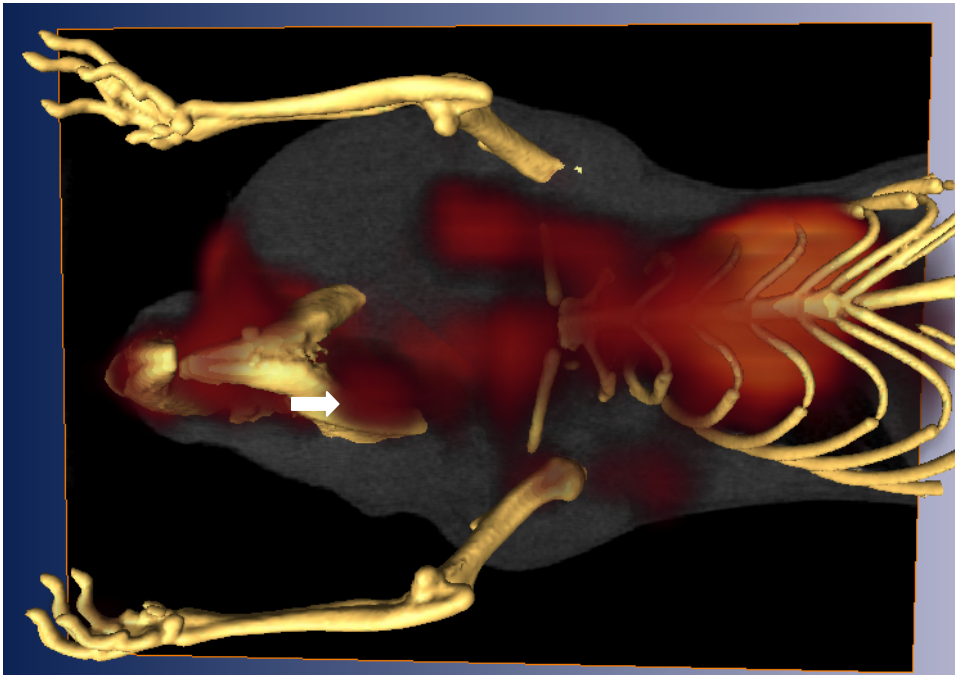


Figure13: An axial PET/CT image of a mouse with HSC-3 cell line injection. White arrow shows cervical lymph node metastasis.

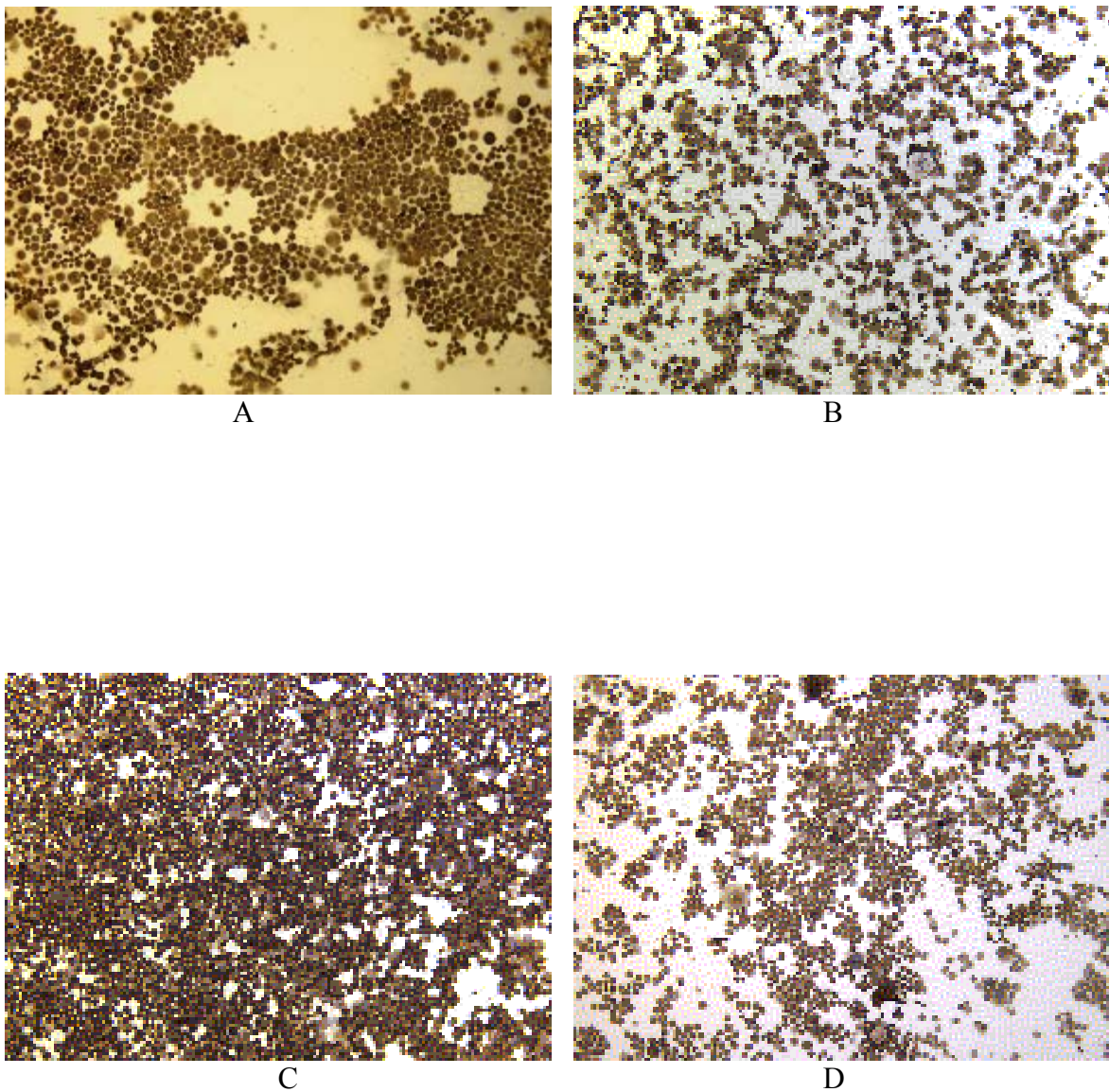


Figure 14: Immunocytochemistry images showing MMP-1 expression. A: HNEC, B: BHY, C: HN, D: HSC-3. Original magnification is 100x.

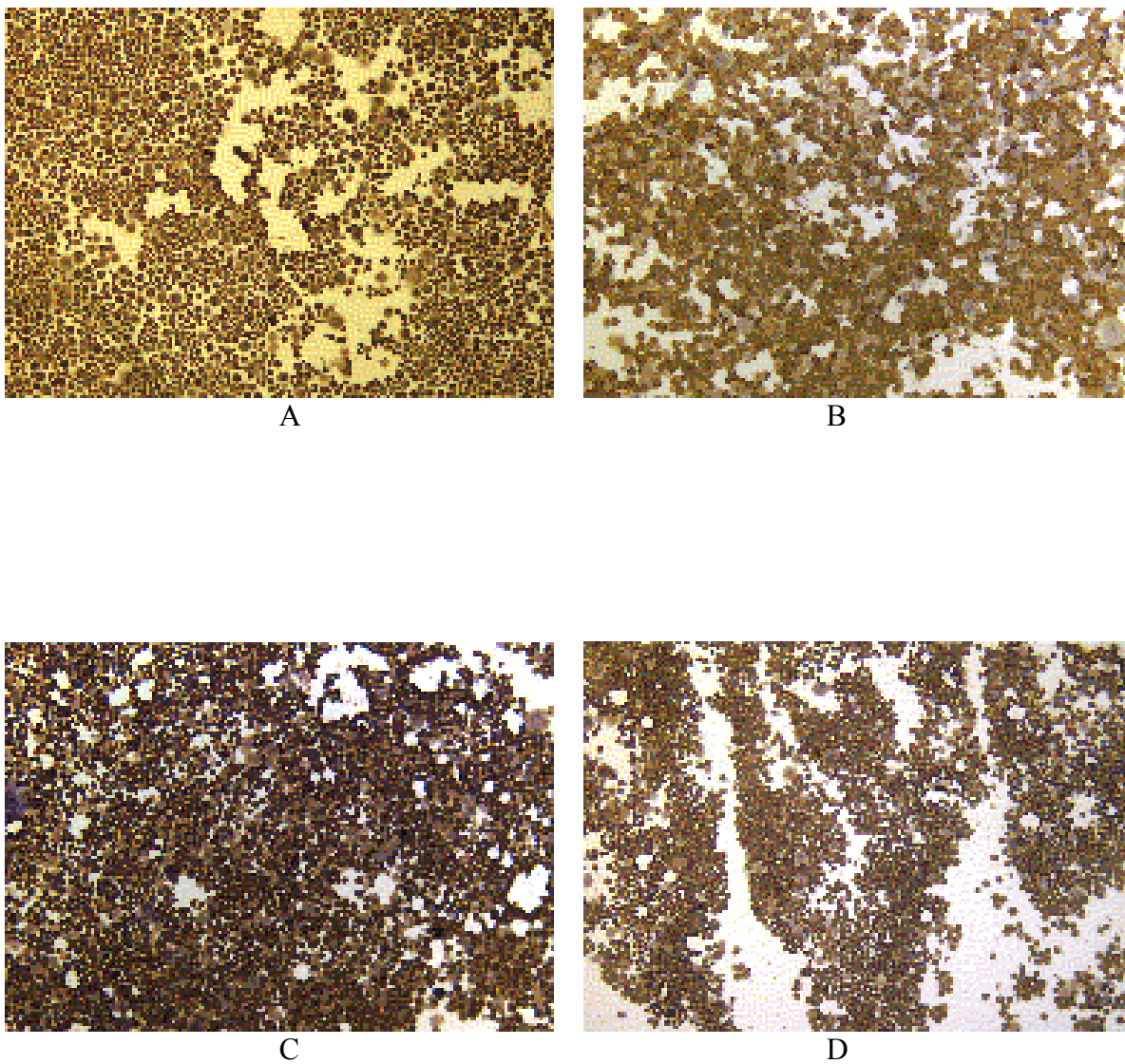
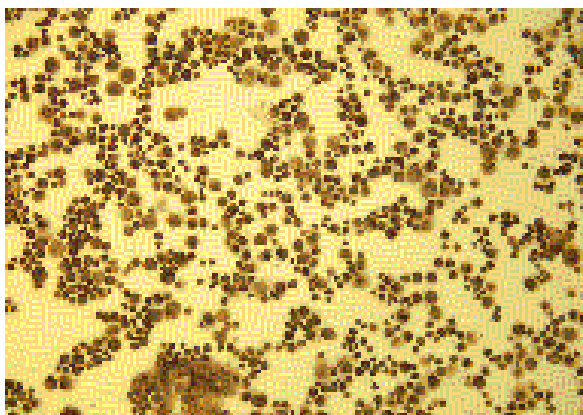
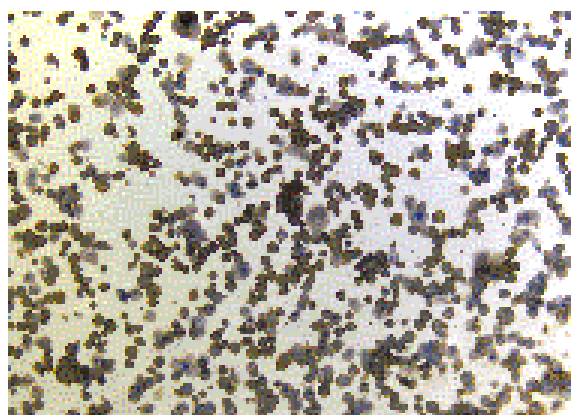


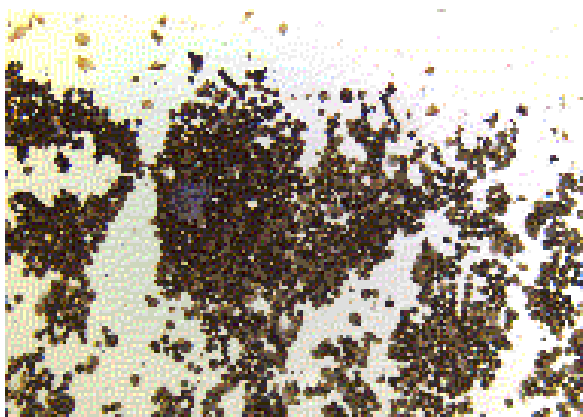
Figure 15: Immunocytochemistry images showing MMP-2 expression. A: HNEC, B: BHY, C: HN, D: HSC-3. Original magnification is 100x.



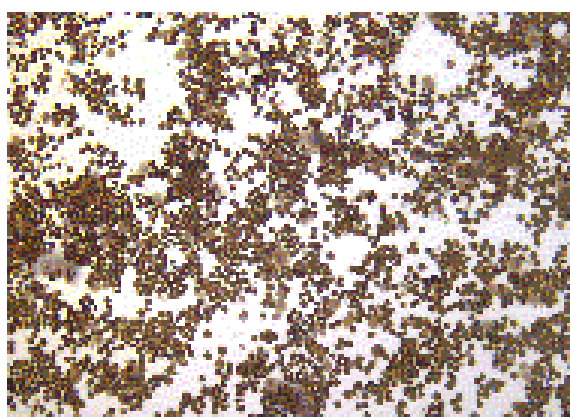
A



B

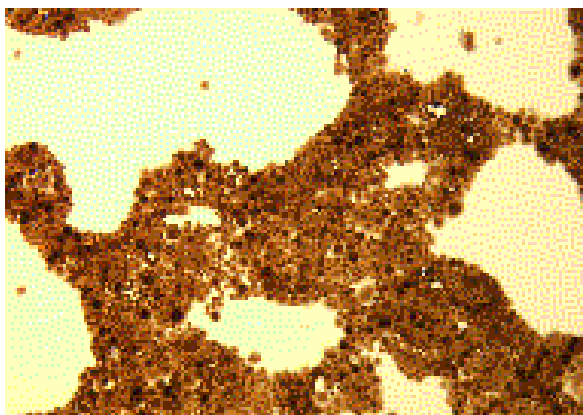


C

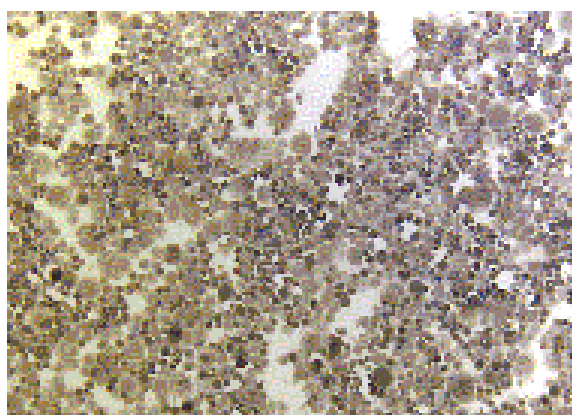


D

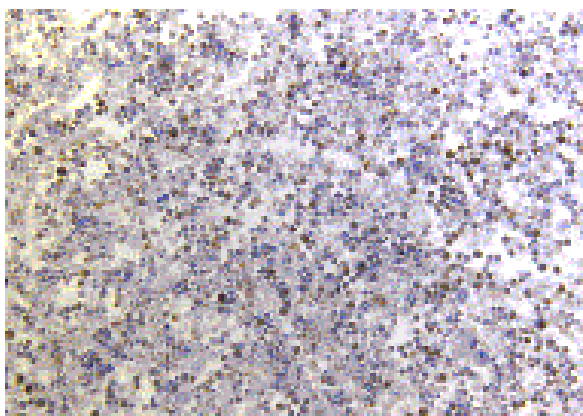
Figure 16: Immunocytochemistry images showing MMP-3 expression. A: HNEC, B: BHY, C: HN, D: HSC-3. Original magnification is 100x.



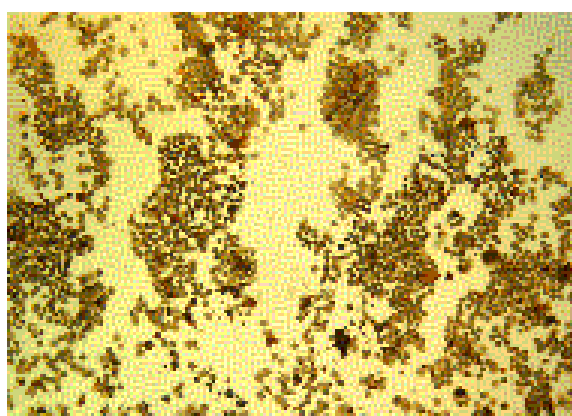
A



B



C



D

Figure 17: Immunocytochemistry images showing MMP-9 expression. A: HNEC, B: BHY, C: HN, D: HSC-3. Original magnification is 100x.

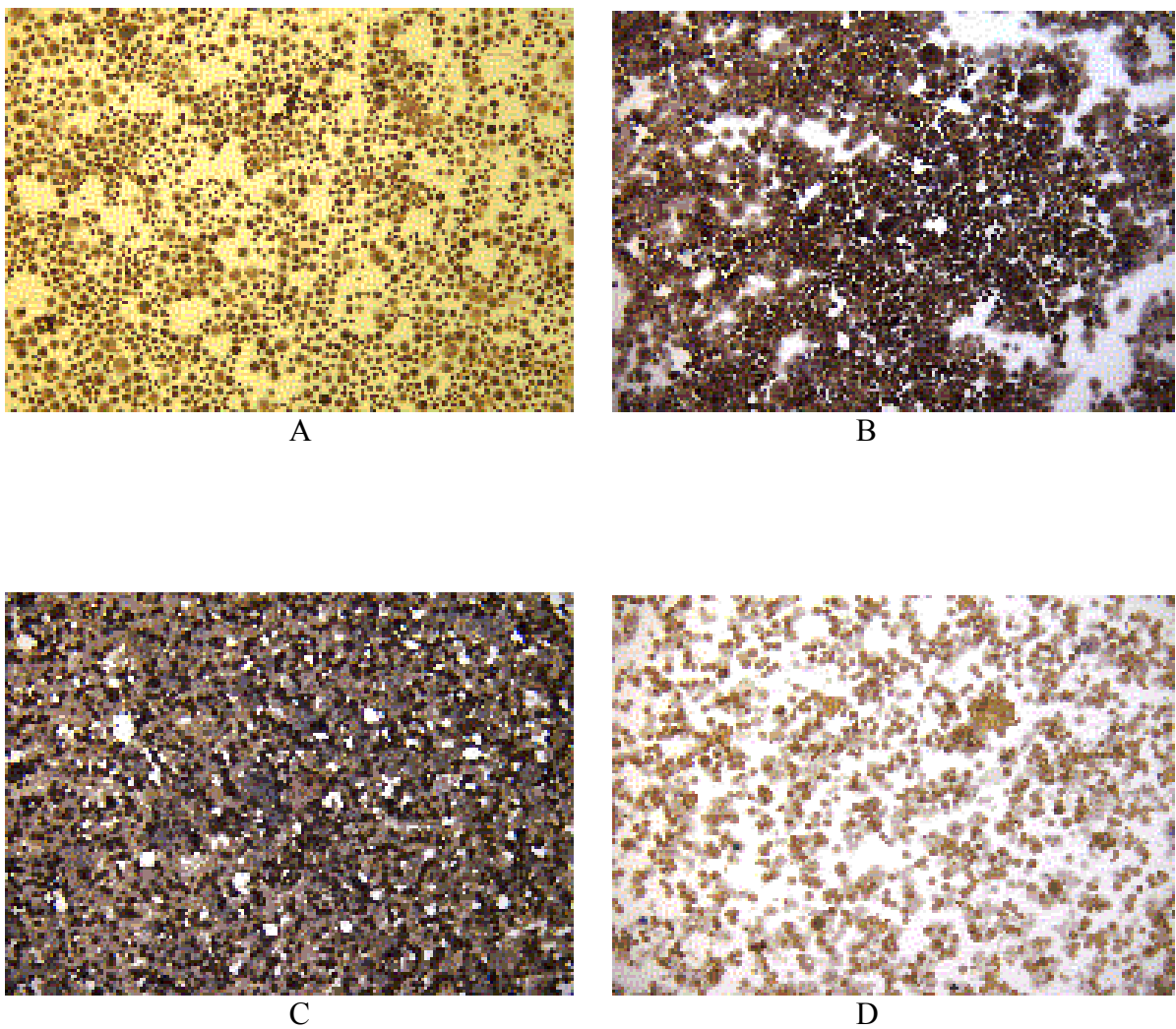
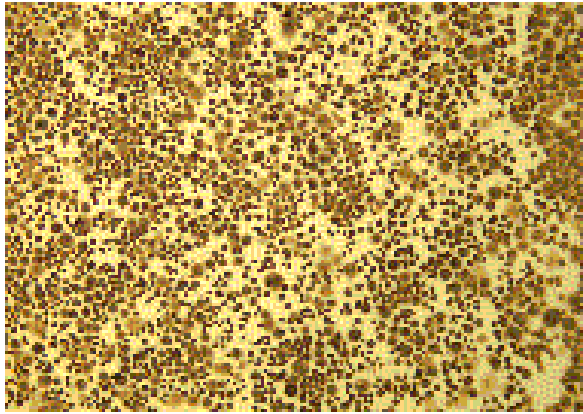
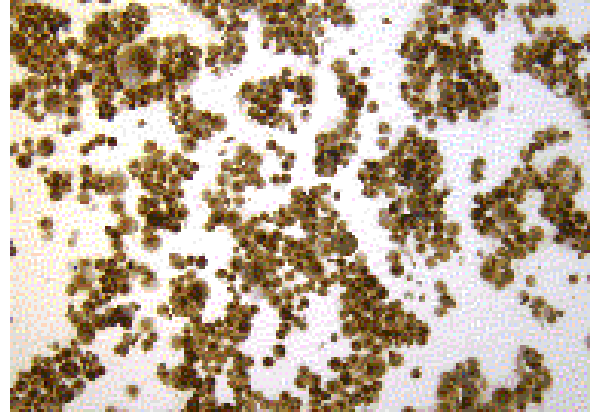


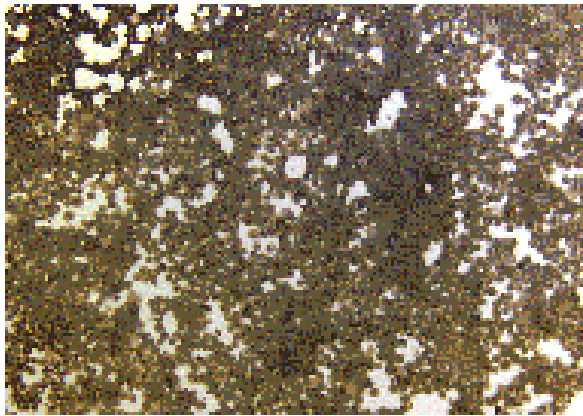
Figure 18: Immunocytochemistry images showing EMMPRIN expression. A: HNEC, B: BHY, C: HN, D: HSC-3. Original magnification is 100x.



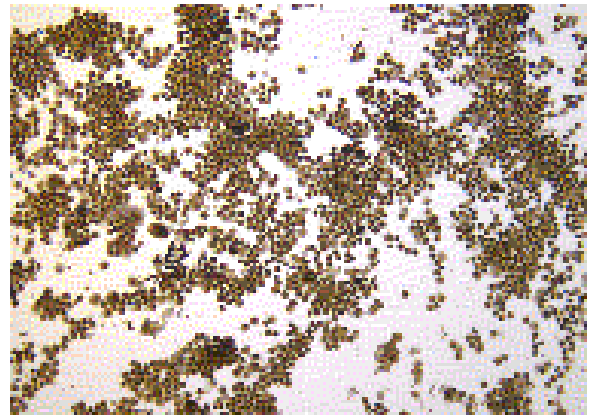
A



B

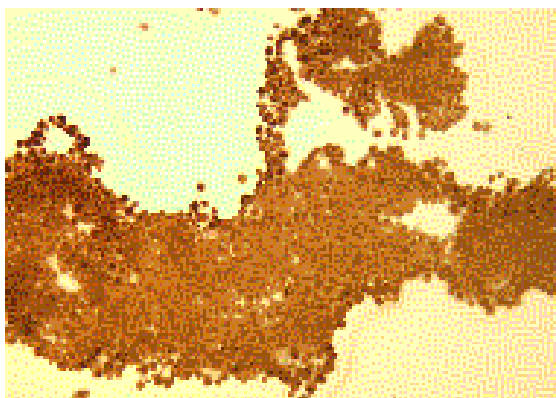


C

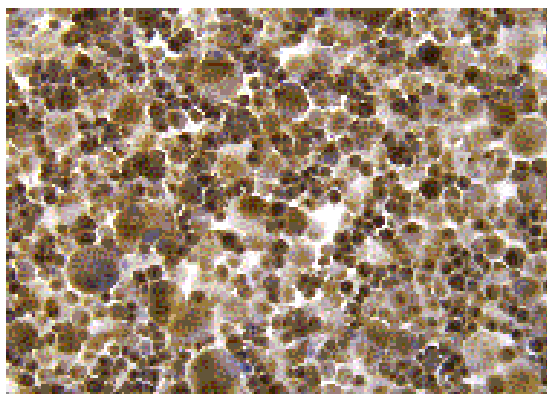


D

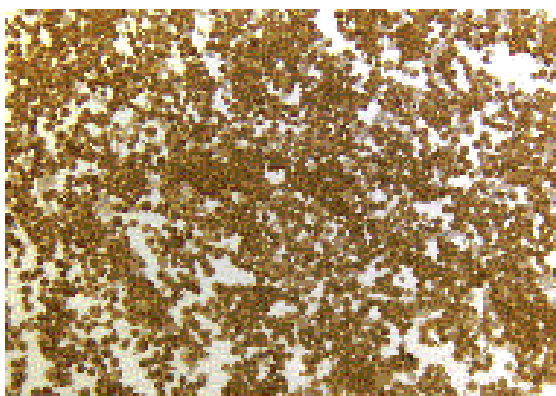
Figure 19: Immunocytochemistry images showing cathepsin B expression. A: HNEC, B: BHY, C: HN, D: HSC-3. Original magnification is 100x.



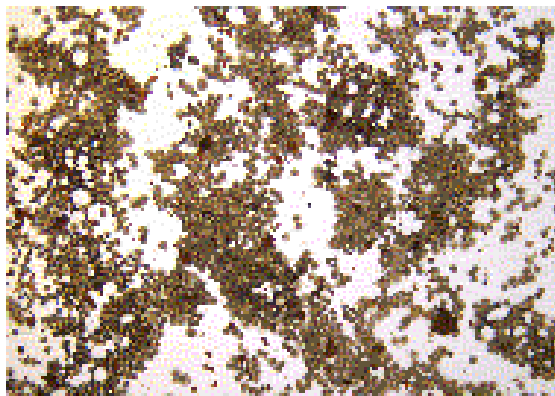
A



B



C



D

Figure 20: Immunocytochemistry images showing cathepsin L expression. A: HNEC, B: BHY, C: HN, D: HSC-3. Original magnification is 100x.

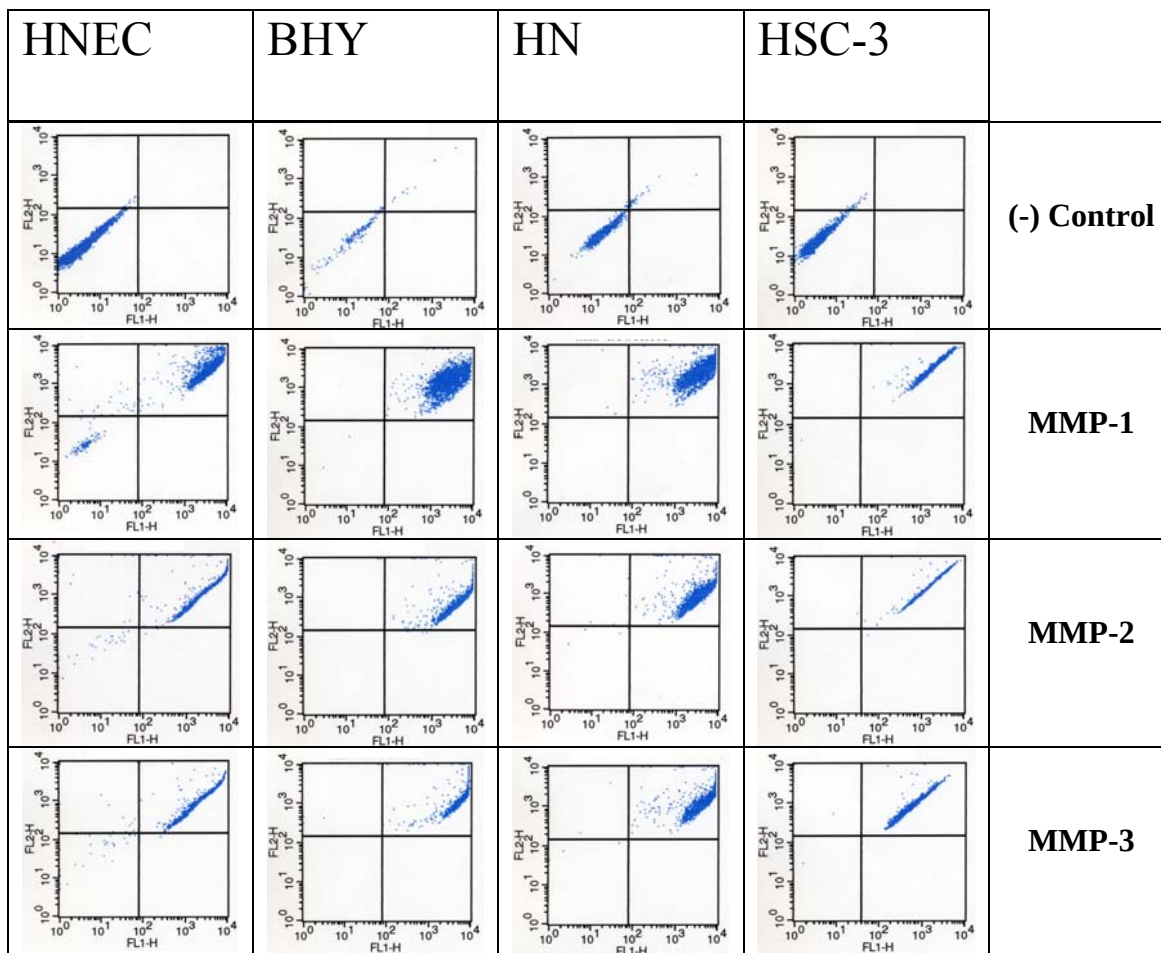


Figure 21: Flow cytometry results of (-) control, MMP-1, MMP-2, and MMP-3 expression in HNEC, BHY, HN and HSC-3 cell lines.

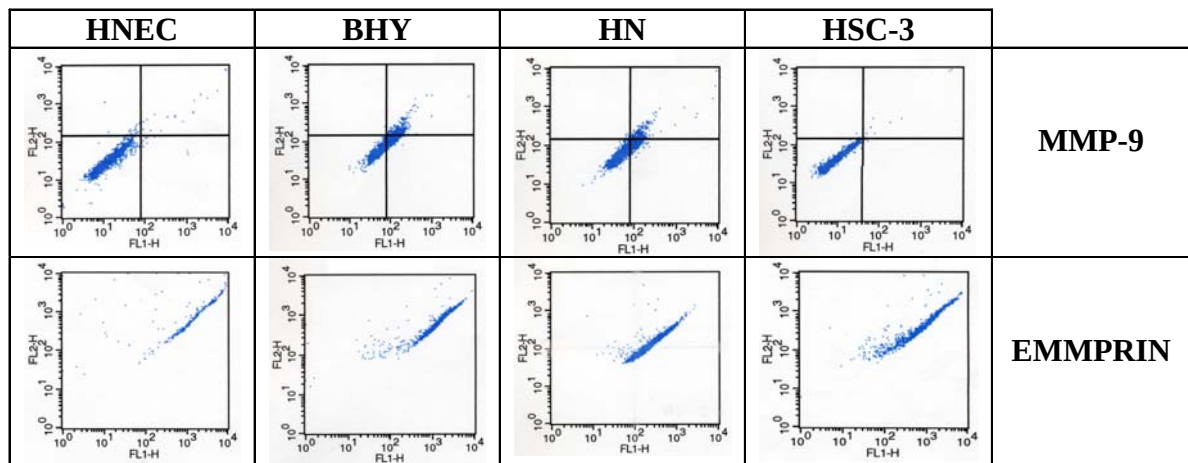


Figure 22: Flow cytometry results of MMP-9 and EMMPRIN expression in HNEC, BHY, HN and HSC-3 cell lines.

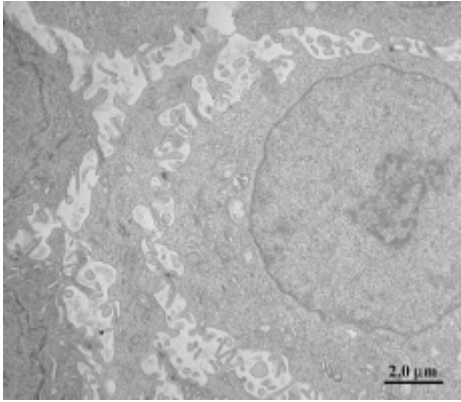


Figure 23: Electron microscopy picture of BHY cells. Original magnification is 5,000x.

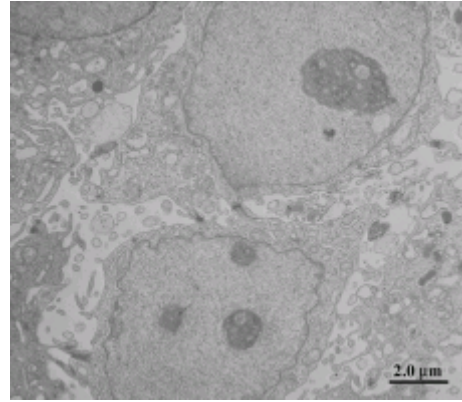


Figure 24: Electron microscopy picture of HN cells. Original magnification is 5,000x.

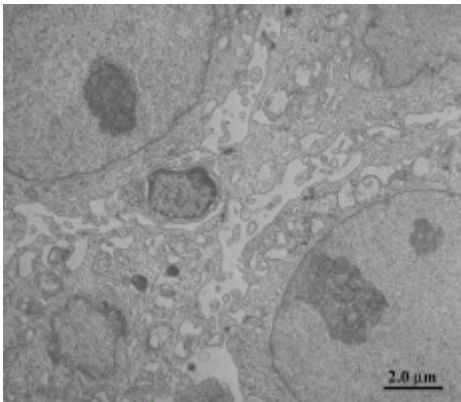


Figure 25: Electron microscopy picture of HSC-3 cells. Original magnification is 5,000x.

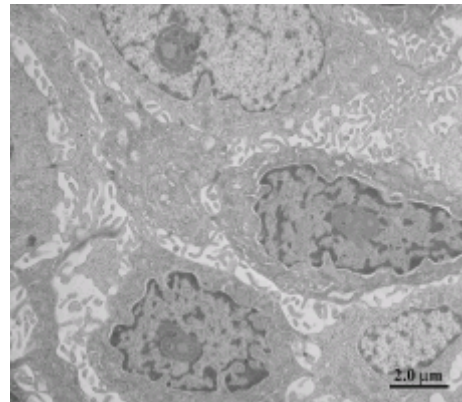


Figure 26: Electron microscopy picture of HNEC cells. Original magnification is 5,000x.

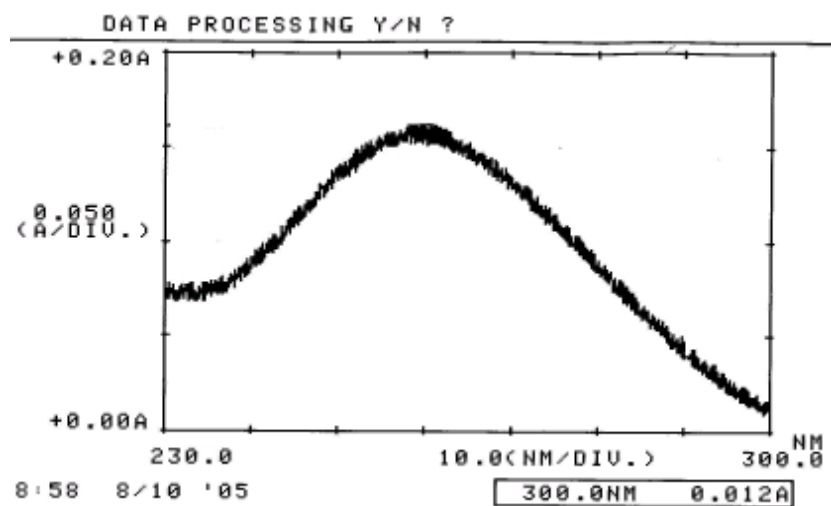


Figure 27: The quantity measurement of total RNA of the BHY cell line.

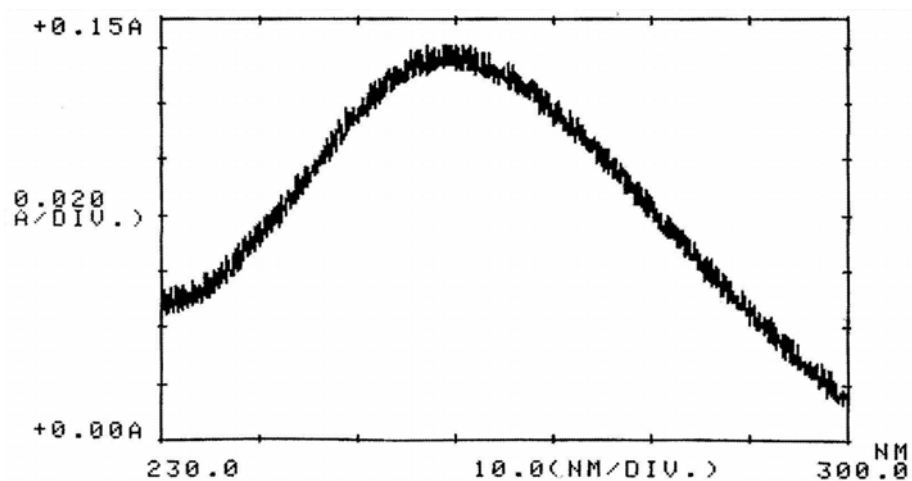


Figure 28: The quantity measurement of total RNA of the HN cell line.

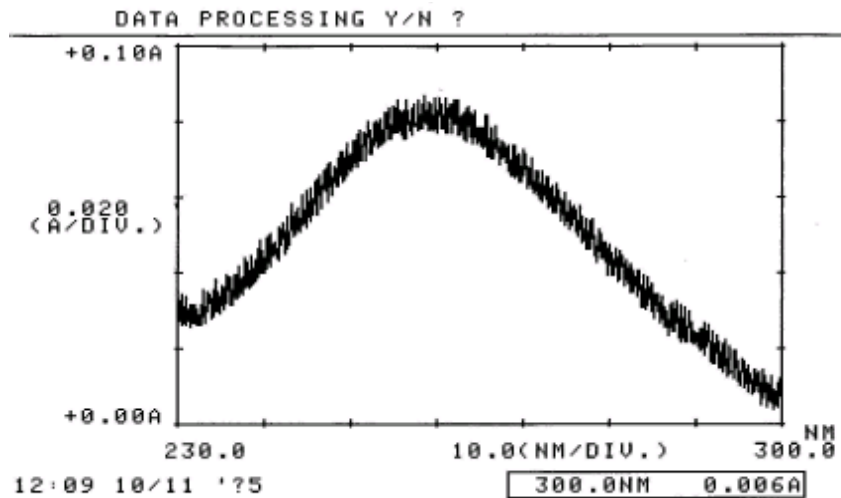


Figure 29: The quantity measurement of total RNA of the HSC-3 cell line.

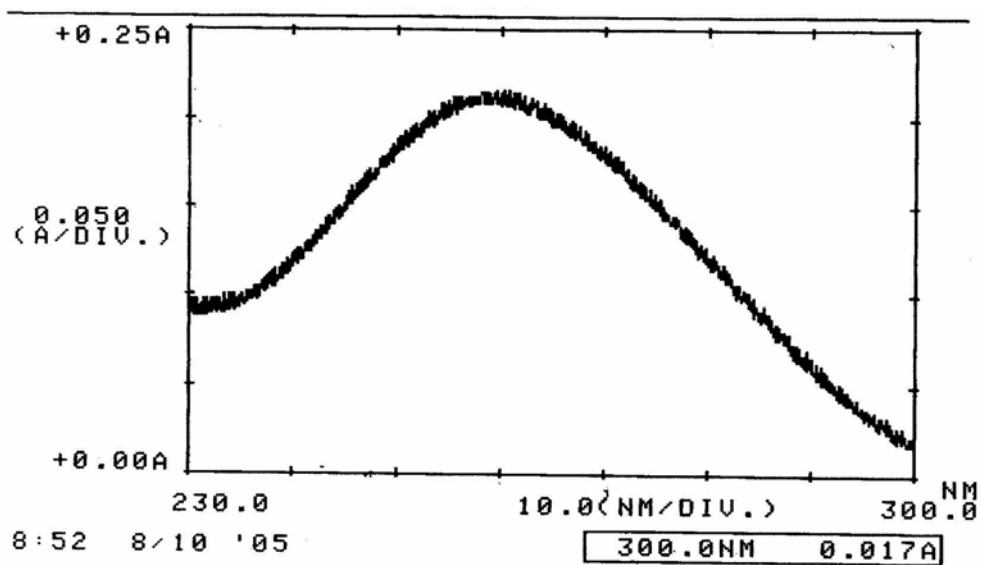


Figure 30: The quantity measurement of total RNA of the HNEC cell line.

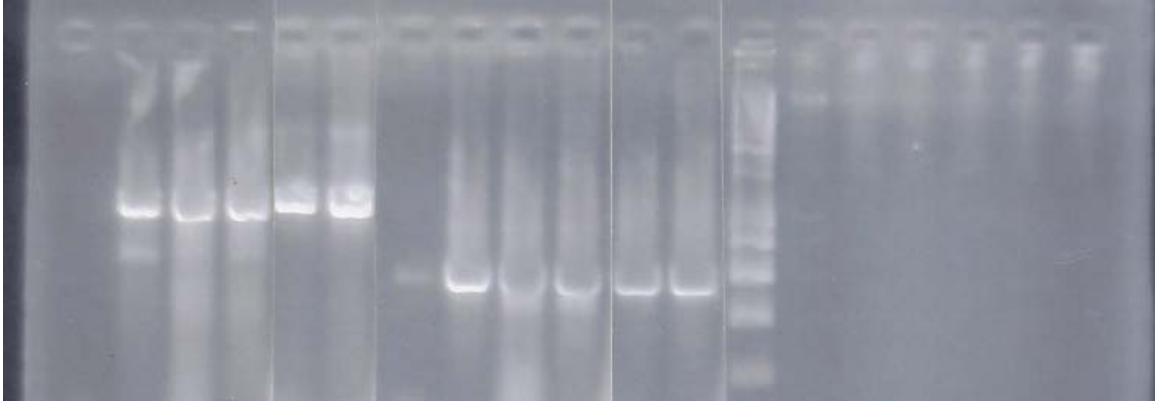


Figure 31: Aldolase and β -actin genes expression on three OSCC cell lines and HNEC cell line. Column 1: Aldolase (-) control; Column 2: Aldolase (+) control; Column 3: Aldolase in BHY cell line; Column 4: Aldolase in HNEC cell line; Column 5: Aldolase in HN cell line; Column 6: Aldolase in HSC-3 cell line; Column 7: β -actin (-) control; Column 8: β -actin (+) control; Column 9: β -actin in BHY; Column 10: β -actin in HNEC; Column 11: β -actin in HN; Column 12: β -actin in HSC-3; Column 13: IH marker.

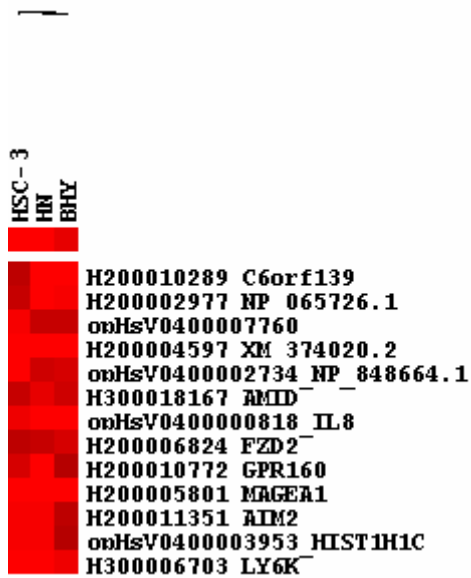


Figure 32: Clusterization of the 13 genes that are upregulated in all three OSCC cell lines.

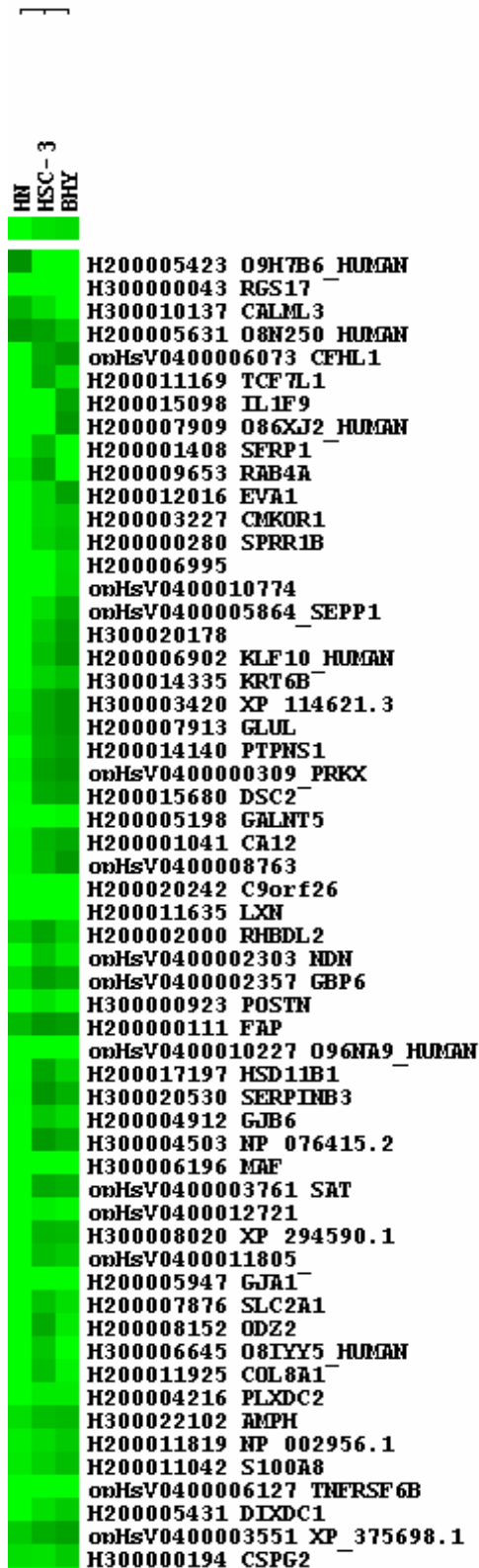


Figure 33: Clusterization of 83 downregulated genes in all three OSCC cell lines.



H200014307 FADS2
onHsV0400010653_
H300019058 ANXA6
H200002453 SRPX
H300021145 ANGPTL4
H200003299 SLC16A4
onHsV0400005248 FAM46B
H200008154 SERPINF1
onHsV0400001923 IDH2
H200018126 RAB40B
H300021562 FN1
H300011907 POU2F2
H300016644 WASPIP
H200006265 EFEMP1
H200001595 HADHSC
H200001136 NP_775925.1
H200008006 SEMA3C
onHsV0400012688
H300015331 CALD1
onHsV0400012111
onHsV0400010566
H200009642 ITGAV
onHsV0400012118
onHsV0400000212_SH3BGRL

Figure 33 continued.

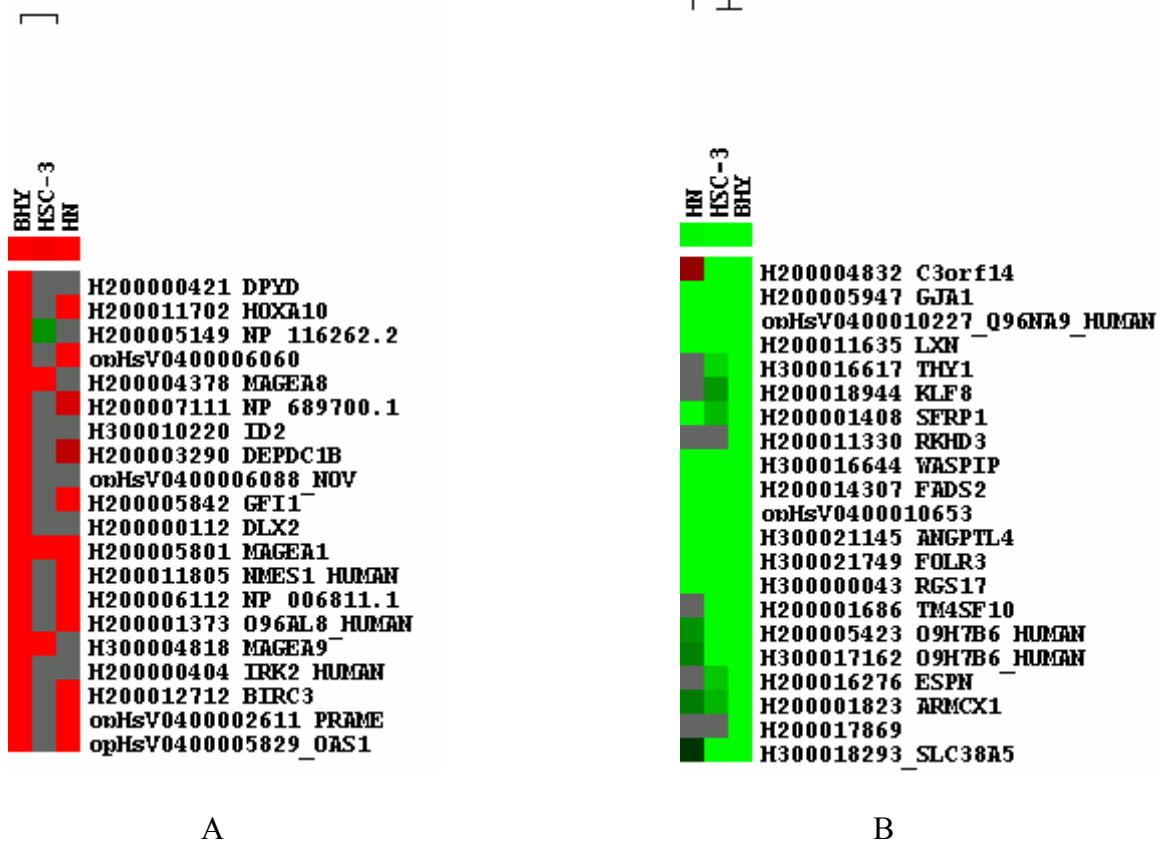


Figure 34: Clusterization of the top 20-upregulated (A) and downregulated (B) genes of BHY cell line with other two OSCC cell lines, HN and HSC-3.

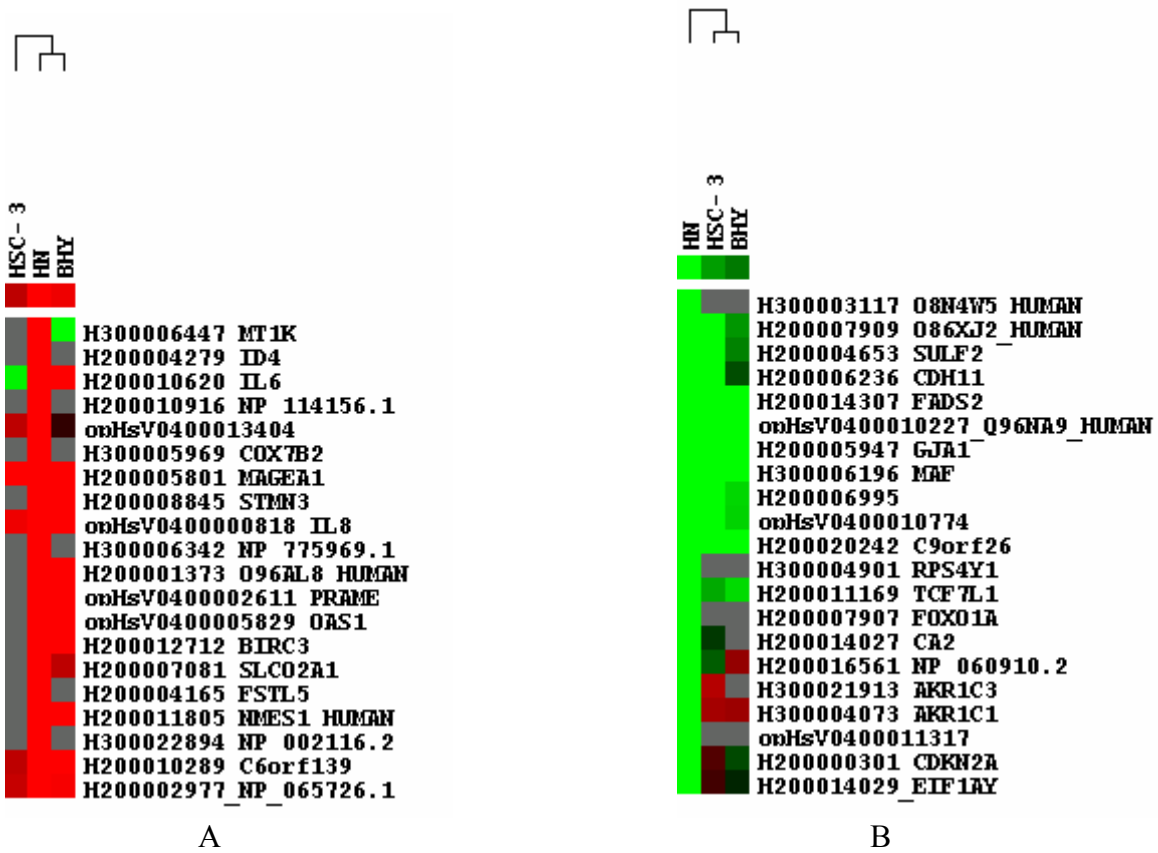


Figure 35: Clusterization of the top 20-upregulated (A) and downregulated (B) genes of HN cell line with other two OSCC cell lines, BHY and HSC-3.

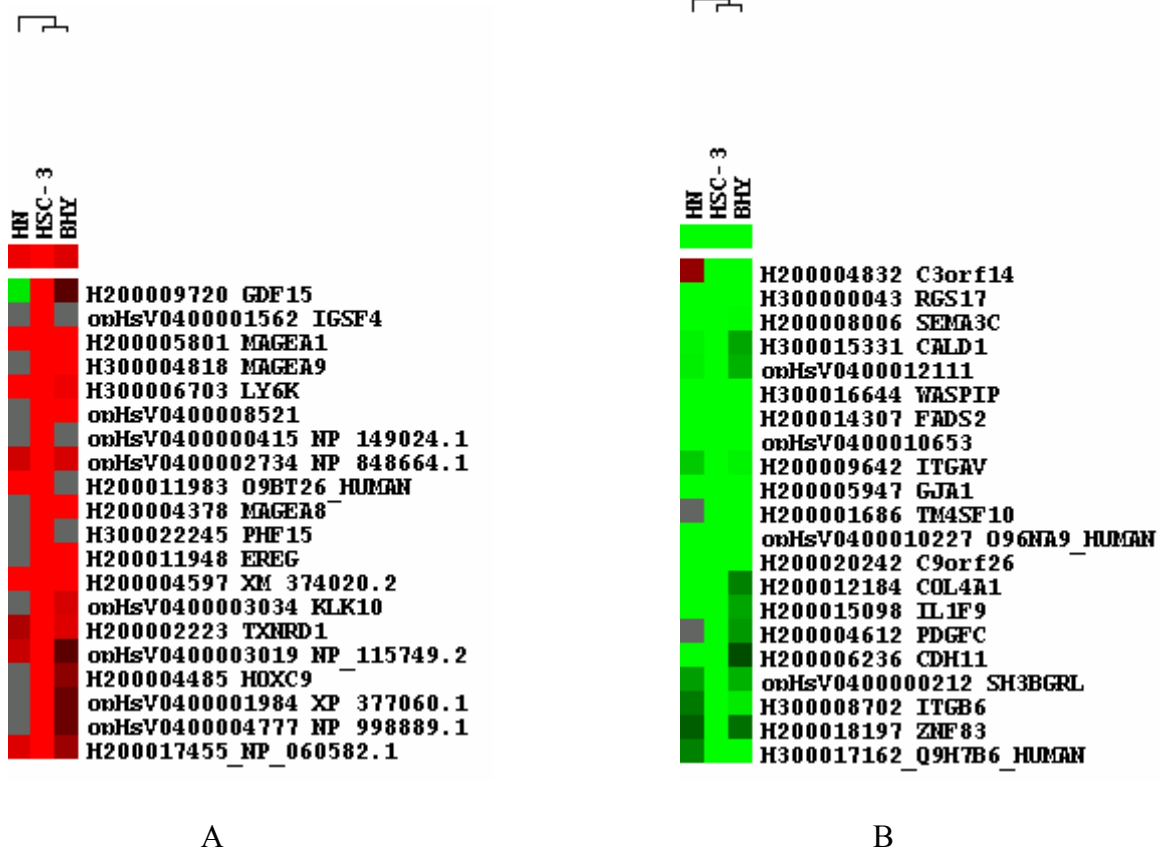


Figure 36: Clusterization of the top 20-upregulated (A) and downregulated (B) genes of HSC-3 cell line with other two OSCC cell lines, BHY and HN.

7

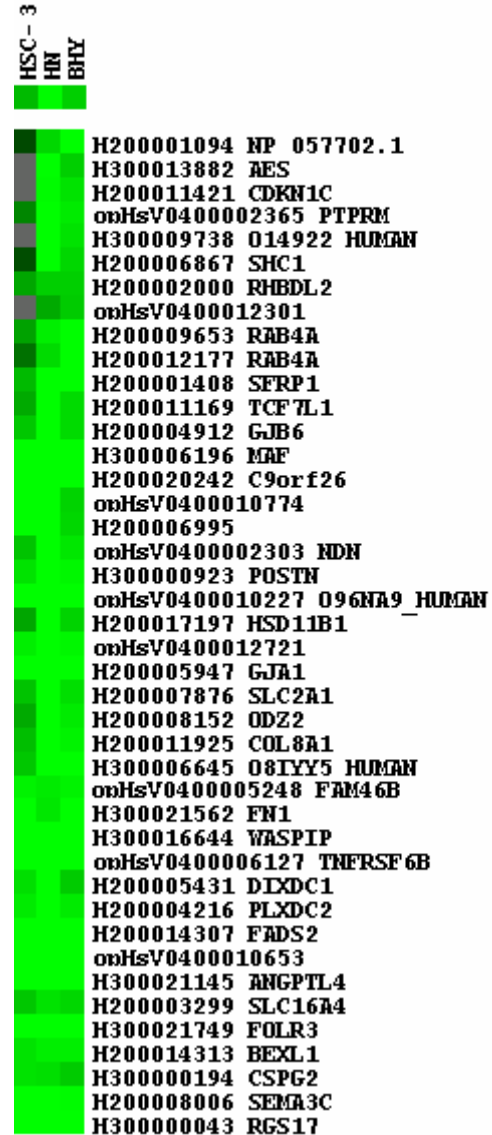
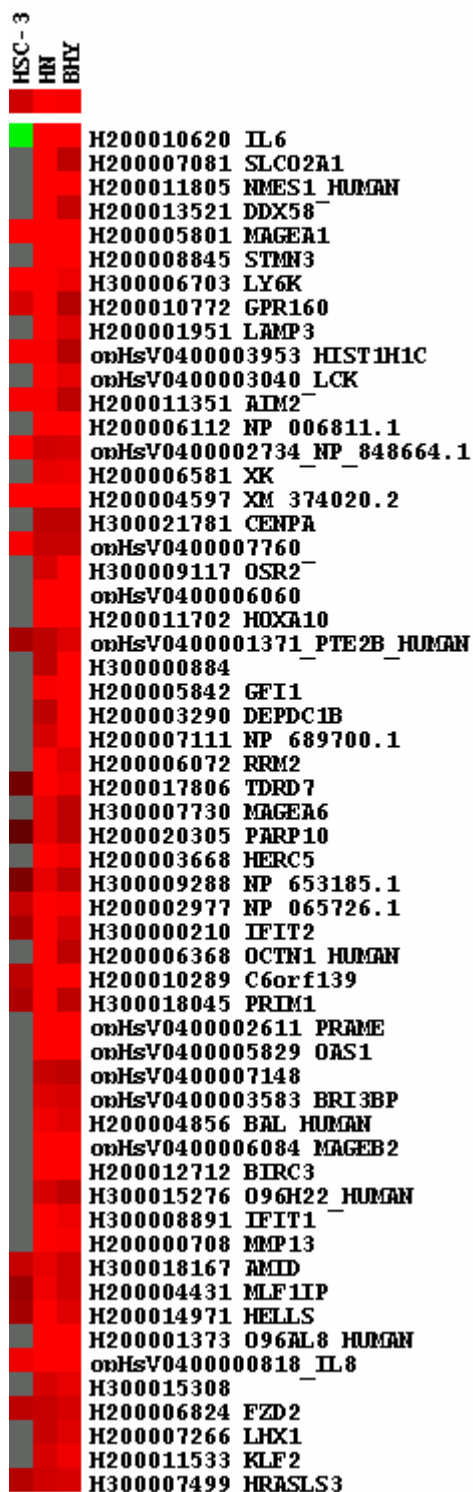


Figure 37: 57 upregulated genes and 42 downregulated genes on both BHY and HN cell lines.

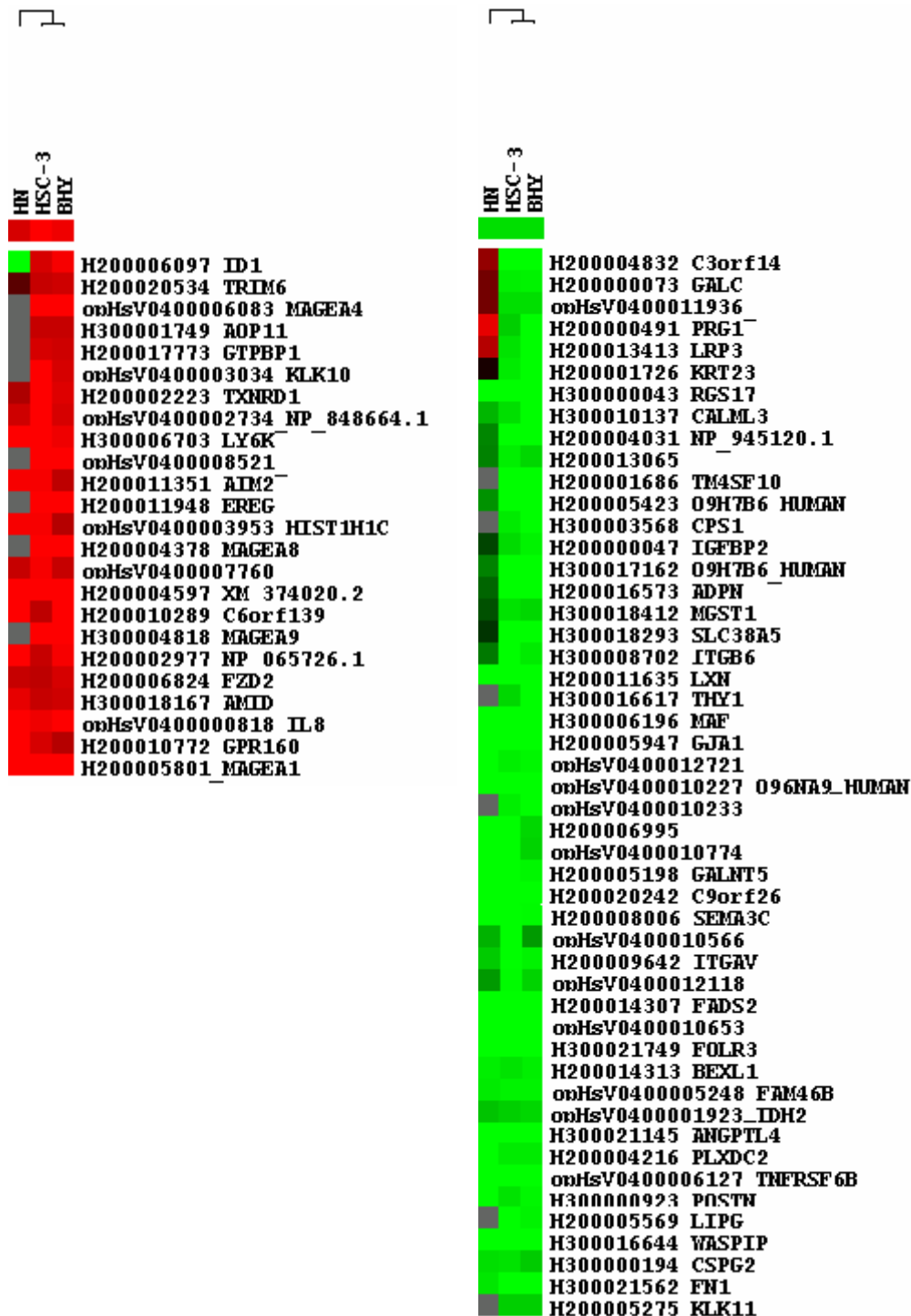


Figure 38: 24 upregulated and 49 downregulated genes on both BHY and HSC-3 cell lines.

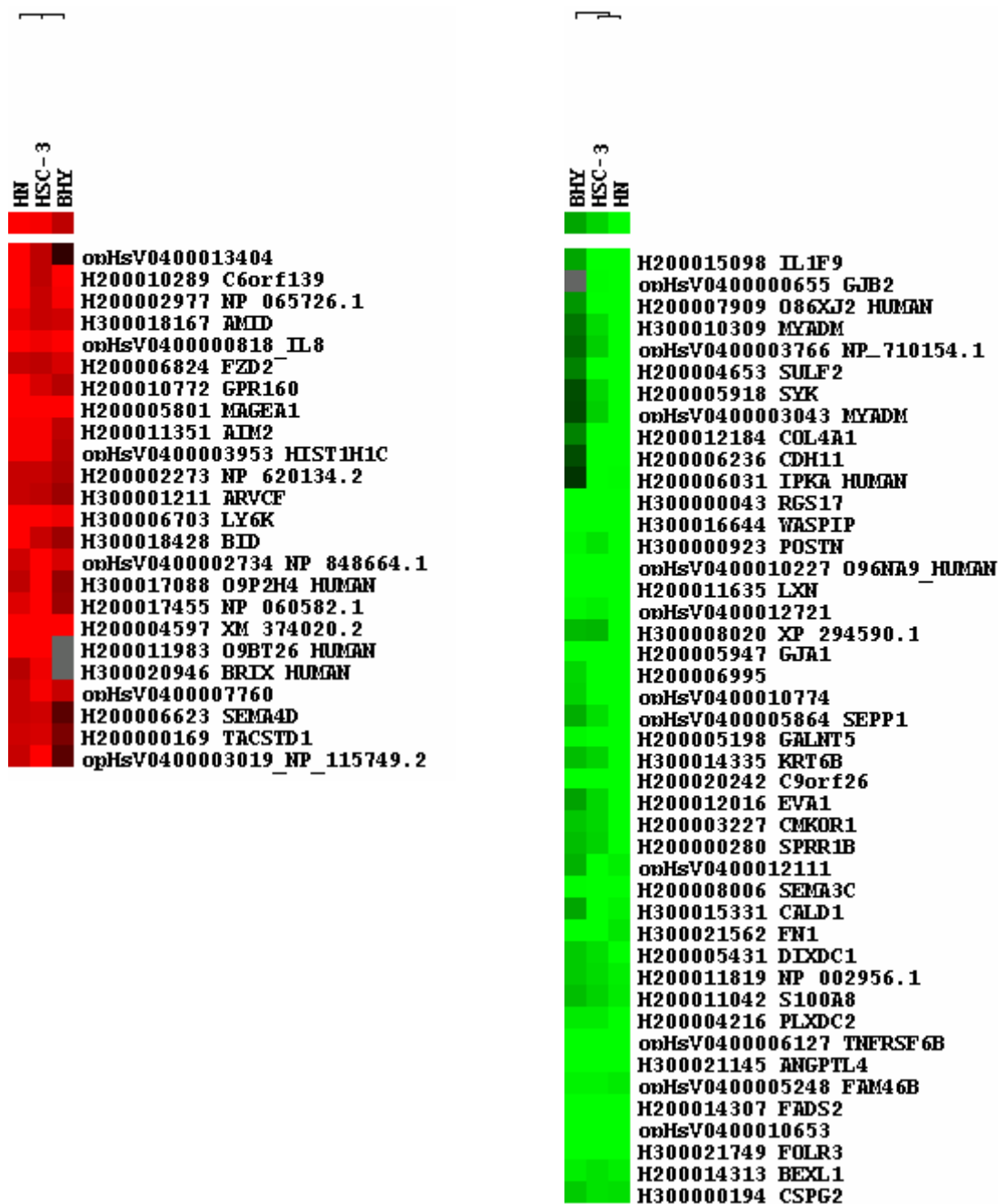


Figure 39: 24 upregulated and 44 downregulated genes on both HN and HSC-3 cell lines.

Input genes /			
Pathway genes	p-value	Impact factor	
5 / 273	9.0813E-4	22.396	MAPK signaling pathway - KEGG
5 / 206	4.1569E-3	26.322	Regulation of actin cytoskeleton - KEGG
2 / 130	1.1611E-2	6.947	Axon guidance - KEGG
14 / 256	2.1493E-2	29.306	Cytokine-cytokine receptor interaction - KEGG
4 / 57	3.7354E-2	7.752	Hedgehog signaling pathway - KEGG
1 / 75	4.4807E-2	3.812	Long-term depression - KEGG
1 / 75	4.4807E-2	2.501	Fc epsilon RI signaling pathway - KEGG
1 / 69	5.0397E-2	2.386	Complement and coagulation cascades - KEGG
2 / 53	5.2528E-2	4.695	Taste transduction - KEGG
3 / 31	6.1429E-2	10.344	Olfactory transduction - KEGG
4 / 119	6.3326E-2	27.301	Tight junction - KEGG
2 / 15	7.6046E-2	3.237	Dentatorubropallidoluysian atrophy (DRPLA) - KEGG
5 / 176	1.0895E-1	11.572	Calcium signaling pathway - KEGG
1 / 63	1.1251E-1	42.064	B cell receptor signaling pathway - KEGG
7 / 84	1.1649E-1	14.686	TGF-beta signaling pathway - KEGG
3 / 30	1.2367E-1	9.594	Huntington's disease - KEGG
1 / 16	1.3149E-1	1.997	Parkinson's disease - KEGG
2 / 79	1.4264E-1	28.3	Phosphatidylinositol signaling system - KEGG
8 / 153	1.5561E-1	16.888	Jak-STAT signaling pathway - KEGG
1 / 46	1.5668E-1	4.987	Epithelial cell signaling in Helicobacter pylori infection - KEGG
8 / 87	1.7071E-1	19.218	ECM-receptor interaction - KEGG
7 / 99	1.7821E-1	26.229	Gap junction - KEGG
3 / 77	1.7852E-1	12.918	Adherens junction - KEGG
10 / 194	1.8656E-1	19.894	Focal adhesion - KEGG
5 / 117	2.0722E-1	20.254	Leukocyte transendothelial migration - KEGG
1 / 44	2.1602E-1	5.169	Type II diabetes mellitus - KEGG
2 / 69	2.2525E-1	3.444	Adipocytokine signaling pathway - KEGG
3 / 93	2.6433E-1	7.122	T cell receptor signaling pathway - KEGG
6 / 135	2.7596E-1	20.388	Insulin signaling pathway - KEGG
3 / 67	3.5291E-1	8.859	Long-term potentiation - KEGG
1 / 44	3.5855E-1	2.017	Type I diabetes mellitus - KEGG
7 / 112	3.7399E-1	9.185	Cell cycle - KEGG
6 / 128	3.7820E-1	12.202	Natural killer cell mediated cytotoxicity - KEGG
4 / 97	3.8098E-1	11.186	GnRH signaling pathway - KEGG
1 / 22	3.8582E-1	1.796	Alzheimer's disease - KEGG
1 / 46	3.9466E-1	1.567	Notch signaling pathway - KEGG

Figure 40: Pathway analysis of the BHY cell line based on both upregulated and downregulated gene data by using Kyoto Encyclopedia of Gene and Genomes (KEGG).

Input genes / Pathway genes	p-value	Impact factor	
1 / 206	8.2871E-3	4.299	Regulation of actin cytoskeleton - KEGG
6 / 84	1.1013E-2	13.341	TGF-beta signaling pathway - KEGG
2 / 15	1.2520E-2	4.021	Dentatorubropallidolusian atrophy (DRPLA) - KEGG
3 / 57	1.4325E-2	6.402	Hedgehog signaling pathway - KEGG
2 / 194	2.8455E-2	6.051	Focal adhesion - KEGG
6 / 153	3.3102E-2	12.607	Jak-STAT signaling pathway - KEGG
1 / 16	3.8213E-2	2.534	Parkinson's disease - KEGG
3 / 273	3.8891E-2	9.922	MAPK signaling pathway - KEGG
8 / 112	5.3308E-2	8.27	Cell cycle - KEGG
1 / 176	5.6943E-2	2.477	Calcium signaling pathway - KEGG
7 / 256	6.7702E-2	15.875	Cytokine-cytokine receptor interaction - KEGG
1 / 130	8.9412E-2	2.164	Axon guidance - KEGG
3 / 91	1.2145E-1	9.005	Toll-like receptor signaling pathway - KEGG
1 / 87	1.6263E-1	5.052	ECM-receptor interaction - KEGG
1 / 302	1.7146E-1	2.111	Neuroactive ligand-receptor interaction - KEGG
4 / 128	1.7423E-1	6.916	Natural killer cell mediated cytotoxicity - KEGG
2 / 45	1.7510E-1	3.048	Ubiquitin mediated proteolysis - KEGG
2 / 135	2.1090E-1	5.814	Insulin signaling pathway - KEGG
1 / 77	2.2234E-1	2.343	Colorectal cancer - KEGG
1 / 97	2.4613E-1	3.073	GnRH signaling pathway - KEGG
1 / 30	2.6143E-1	1.631	Huntington's disease - KEGG
3 / 84	2.7128E-1	9.49	Apoptosis - KEGG
3 / 99	3.0164E-1	11.263	Gap junction - KEGG
1 / 79	3.1530E-1	26.012	Phosphatidylinositol signaling system - KEGG
1 / 75	3.1530E-1	2.965	Long-term depression - KEGG
1 / 75	3.1530E-1	1.654	Fc epsilon RI signaling pathway - KEGG
1 / 35	3.3679E-1	1.521	Neurodegenerative disorders - KEGG
1 / 67	3.4712E-1	2.924	Long-term potentiation - KEGG
3 / 119	3.7892E-1	23.111	Tight junction - KEGG
3 / 132	3.9404E-1	75.209	Cell adhesion molecules (CAMs) - KEGG
1 / 44	4.0975E-1	4.891	Type II diabetes mellitus - KEGG
4 / 147	4.3261E-1	9.326	Wnt signaling pathway - KEGG
2 / 93	4.4768E-1	5.134	T cell receptor signaling pathway - KEGG
3 / 117	4.5826E-1	11.754	Leukocyte transendothelial migration - KEGG
1 / 46	4.7859E-1	4.502	Epithelial cell signaling in Helicobacter pylori infection - KEGG

Figure 41: Pathway analysis of the BHY cell line based on upregulated gene data by using Kyoto Encyclopedia of Gene and Genomes (KEGG).

Input genes / Pathway genes	p-value	Impact factor	
3 / 31	5.8335E-3	11.366	Olfactory transduction - KEGG
2 / 53	7.9599E-3	5.514	Taste transduction - KEGG
7 / 87	1.1533E-2	20.018	ECM-receptor interaction - KEGG
7 / 256	6.4107E-2	14.126	Cytokine-cytokine receptor interaction - KEGG
2 / 30	7.0210E-2	8.791	Huntington's disease - KEGG
1 / 119	7.0472E-2	8.956	Tight junction - KEGG
1 / 130	9.2928E-2	4.928	Axon guidance - KEGG
1 / 112	1.3568E-1	2.629	Cell cycle - KEGG
1 / 22	1.3571E-1	2.25	Alzheimer's disease - KEGG
4 / 99	1.3675E-1	18.289	Gap junction - KEGG
4 / 206	1.7843E-1	22.593	Regulation of actin cytoskeleton - KEGG
4 / 302	1.9106E-1	5.627	Neuroactive ligand-receptor interaction - KEGG
1 / 77	2.2802E-1	3.063	Colorectal cancer - KEGG
8 / 194	2.3007E-1	15.298	Focal adhesion - KEGG
1 / 57	2.5706E-1	2.356	Hedgehog signaling pathway - KEGG
1 / 46	2.5706E-1	1.753	Notch signaling pathway - KEGG
2 / 117	2.5765E-1	8.745	Leukocyte transendothelial migration - KEGG
3 / 97	2.6439E-1	8.881	GnRH signaling pathway - KEGG
1 / 84	2.7818E-1	2.925	TGF-beta signaling pathway - KEGG
1 / 44	2.8201E-1	2.121	Type I diabetes mellitus - KEGG
1 / 93	2.9209E-1	2.294	T cell receptor signaling pathway - KEGG
3 / 77	2.9426E-1	12.7	Adherens junction - KEGG
2 / 69	3.1284E-1	3.302	Adipocytokine signaling pathway - KEGG
1 / 79	3.2163E-1	2.701	Phosphatidylinositol signaling system - KEGG
1 / 35	3.3161E-1	1.862	Neurodegenerative disorders - KEGG
1 / 69	3.3729E-1	1.56	Complement and coagulation cascades - KEGG
2 / 67	3.6794E-1	6.907	Long-term potentiation - KEGG
4 / 135	3.8270E-1	16.872	Insulin signaling pathway - KEGG
3 / 132	3.8551E-1	33.013	Cell adhesion molecules (CAMs) - KEGG
2 / 153	3.9707E-1	5.814	Jak-STAT signaling pathway - KEGG
4 / 147	4.2257E-1	15.621	Wnt signaling pathway - KEGG
2 / 128	4.2712E-1	5.993	Natural killer cell mediated cytotoxicity - KEGG
4 / 176	4.4891E-1	9.725	Calcium signaling pathway - KEGG
1 / 63	4.5413E-1	41.448	B cell receptor signaling pathway - KEGG
2 / 84	4.9198E-1	6.264	Apoptosis - KEGG

Figure 42: Pathway analysis of theBHY cell line based on downregulated gene data by using Kyoto Encyclopedia of Gene and Genomes (KEGG).

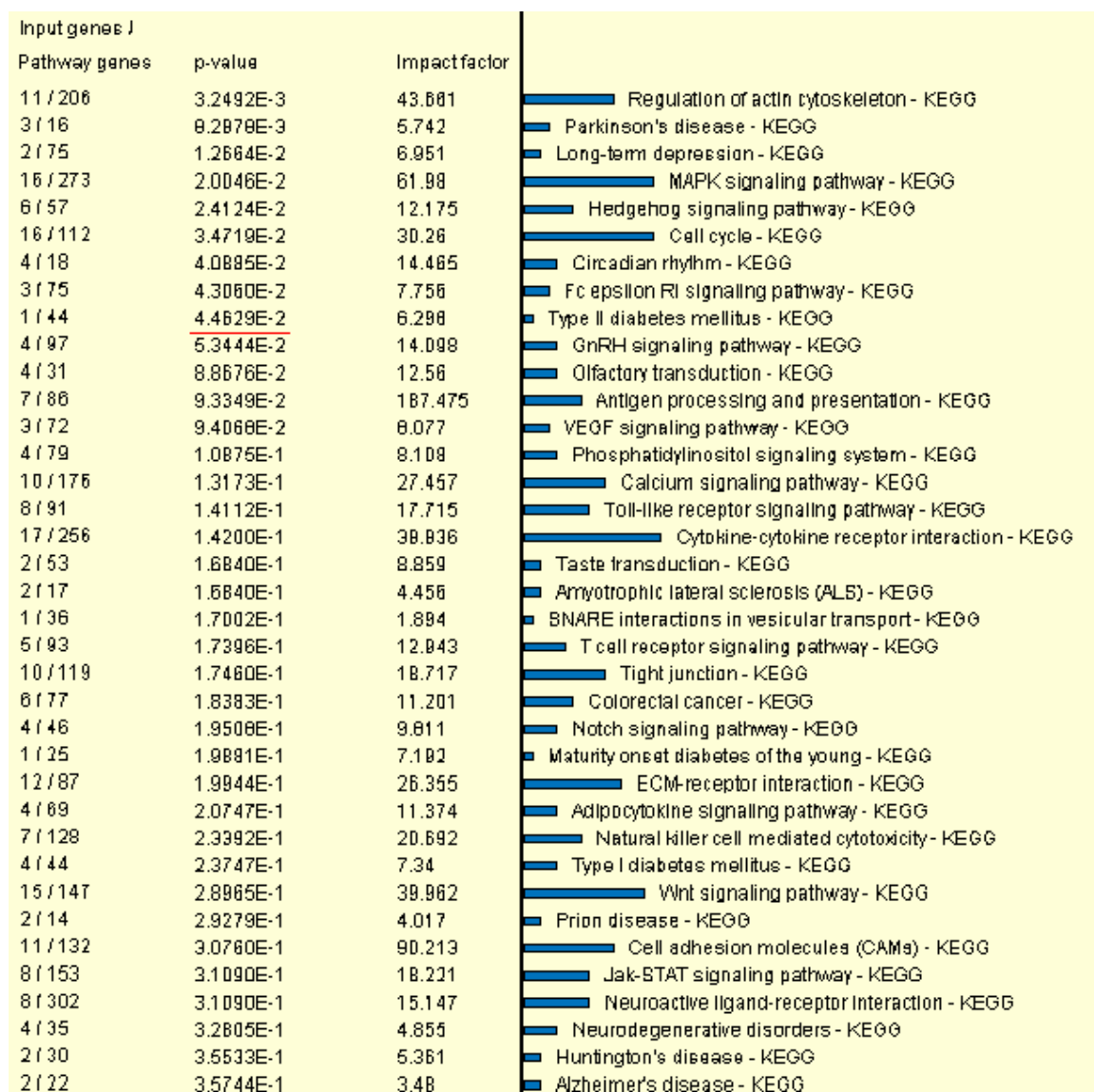


Figure 43: Pathway analysis of the HN cell line based on both upregulated and downregulated gene data by using Kyoto Encyclopedia of Gene and Genomes (KEGG).

Input genes / Pathway genes	p-value	Impact factor	
1 / 273	2.6740E-4	7.88	■ MAPK signaling pathway - KEGG
1 / 206	9.3037E-4	4.158	■ Regulation of actin cytoskeleton - KEGG
6 / 86	2.2387E-3	204.978	■ Antigen processing and presentation - KEGG
2 / 16	5.3560E-3	4.925	■ Parkinson's disease - KEGG
2 / 194	5.4708E-3	6.108	■ Focal adhesion - KEGG
4 / 44	8.5943E-3	8.782	■ Type I diabetes mellitus - KEGG
9 / 112	1.0802E-2	15.209	■ Cell cycle - KEGG
1 / 176	1.8052E-2	4.111	■ Calcium signaling pathway - KEGG
1 / 130	3.3302E-2	4.559	■ Axon guidance - KEGG
1 / 25	3.8557E-2	7.904	■ Maturity onset diabetes of the young - KEGG
2 / 147	7.5761E-2	7.244	■ Wnt signaling pathway - KEGG
4 / 91	9.4527E-2	10.269	■ Toll-like receptor signaling pathway - KEGG
1 / 99	1.1595E-1	4.516	■ Gap junction - KEGG
1 / 77	1.1595E-1	2.5	■ Colorectal cancer - KEGG
2 / 117	1.1741E-1	13.846	■ Leukocyte transendothelial migration - KEGG
3 / 46	1.4166E-1	8.51	■ Epithelial cell signaling in Helicobacter pylori infection - KEGG
1 / 79	1.8820E-1	25.236	■ Phosphatidylinositol signaling system - KEGG
2 / 87	2.1161E-1	3.094	■ ECM-receptor interaction - KEGG
5 / 132	2.2013E-1	17.843	■ Cell adhesion molecules (CAMs) - KEGG
1 / 72	2.6211E-1	2.96	■ VEGF signaling pathway - KEGG
1 / 63	3.1741E-1	37.195	■ B cell receptor signaling pathway - KEGG
2 / 84	3.2598E-1	9.498	■ Apoptosis - KEGG
4 / 128	3.3551E-1	9.735	■ Natural killer cell mediated cytotoxicity - KEGG
2 / 84	3.6076E-1	3.877	■ TGF-beta signaling pathway - KEGG
4 / 153	3.7040E-1	10.718	■ Jak-STAT signaling pathway - KEGG
2 / 93	3.7910E-1	8.472	■ T cell receptor signaling pathway - KEGG
2 / 75	4.1763E-1	3.058	■ Fc epsilon RI signaling pathway - KEGG
3 / 302	4.2414E-1	6.451	■ Neuroactive ligand-receptor interaction - KEGG
2 / 69	4.3780E-1	9.456	■ Complement and coagulation cascades - KEGG
1 / 35	4.6063E-1	1.497	■ Neurodegenerative disorders - KEGG
5 / 256	4.9583E-1	16.808	■ Cytokine-cytokine receptor interaction - KEGG

Figure 44: Pathway analysis of the HN cell line based on upregulated gene data by using Kyoto Encyclopedia of Gene and Genomes (KEGG).

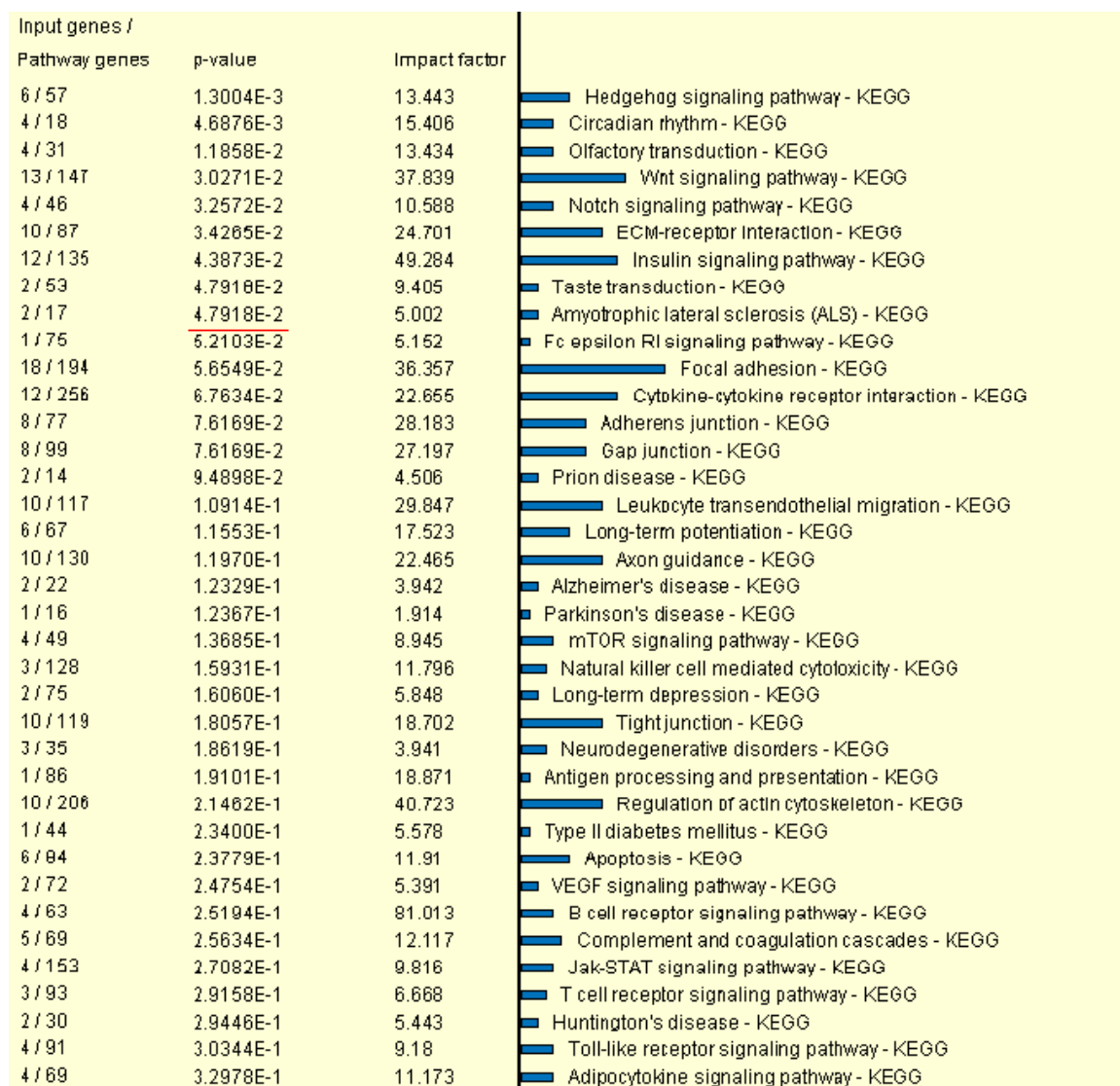


Figure 45: Pathway analysis of the HN cell line based on downregulated gene data by using Kyoto Encyclopedia of Gene and Genomes (KEGG).

Input genes /			
Pathway genes	p-value	Impact factor	
5 / 273	5.4807E-4	16.92	MAPK signaling pathway - KEGG
3 / 119	1.8160E-2	17.519	Tight junction - KEGG
7 / 206	1.9679E-2	36.944	Regulation of actin cytoskeleton - KEGG
2 / 302	3.5037E-2	3.945	Neuroactive ligand-receptor interaction - KEGG
1 / 75	3.7814E-2	3.978	Fc epsilon RI signaling pathway - KEGG
1 / 75	3.7814E-2	3.828	Long-term depression - KEGG
4 / 176	3.8240E-2	10.42	Calcium signaling pathway - KEGG
2 / 128	4.3665E-2	6.021	Natural killer cell mediated cytotoxicity - KEGG
2 / 53	5.7921E-2	5.754	Taste transduction - KEGG
4 / 135	6.0693E-2	15.56	Insulin signaling pathway - KEGG
3 / 31	6.9089E-2	7.883	Olfactory transduction - KEGG
4 / 130	7.9288E-2	13.755	Axon guidance - KEGG
9 / 194	8.7229E-2	17.945	Focal adhesion - KEGG
3 / 153	9.9141E-2	7.701	Jak-STAT signaling pathway - KEGG
1 / 63	9.9398E-2	51.095	B cell receptor signaling pathway - KEGG
2 / 79	1.2469E-1	27.235	Phosphatidylinositol signaling system - KEGG
3 / 30	1.3741E-1	6.553	Huntington's disease - KEGG
3 / 57	1.3741E-1	6.542	Hedgehog signaling pathway - KEGG
1 / 46	1.4079E-1	4.583	Epithelial cell signaling in Helicobacter pylori infection - KEGG
4 / 112	1.4315E-1	5.976	Cell cycle - KEGG
2 / 67	1.5191E-1	6.523	Long-term potentiation - KEGG
3 / 77	1.5535E-1	7.904	\$hsa05210\$ - KEGG
1 / 86	1.5777E-1	1.951	Antigen processing and presentation - KEGG
1 / 45	1.7658E-1	2.504	Ubiquitin mediated proteolysis - KEGG
11 / 256	1.8127E-1	25.572	Cytokine-cytokine receptor interaction - KEGG
3 / 97	1.8442E-1	10.025	GnRH signaling pathway - KEGG
5 / 91	1.9515E-1	7.72	Toll-like receptor signaling pathway - KEGG
8 / 132	1.9999E-1	117.73	Cell adhesion molecules (CAMs) - KEGG
8 / 87	1.9999E-1	21.899	ECM-receptor interaction - KEGG
7 / 99	2.0628E-1	29.84	Gap junction - KEGG
1 / 17	2.4585E-1	2.827	Amyotrophic lateral sclerosis (ALS) - KEGG
1 / 35	2.7331E-1	1.571	\$hsa01510\$ - KEGG
3 / 69	2.9863E-1	6.991	Complement and coagulation cascades - KEGG
1 / 15	2.9996E-1	2.273	Dentatorubropallidolusian atrophy (DRPLA) - KEGG
6 / 117	3.0428E-1	20.147	Leukocyte transendothelial migration - KEGG
7 / 147	3.2057E-1	20.742	Wnt signaling pathway - KEGG
2 / 44	3.7630E-1	2.777	Type I diabetes mellitus - KEGG

Figure 46: Pathway analysis of the HSC-3 cell line based on both upregulated and downregulated gene data by using Kyoto Encyclopedia of Gene and Genomes (KEGG).

Input genes /			
Pathway genes	p-value	Impact factor	
1 / 206	4.6297E-3	3.361	■ Regulation of actin cytoskeleton - KEGG
2 / 273	8.7742E-3	7.763	■ MAPK signaling pathway - KEGG
4 / 91	4.6297E-2	6.456	■ Toll-like receptor signaling pathway - KEGG
1 / 135	6.4090E-2	4.625	■ Insulin signaling pathway - KEGG
2 / 57	8.0448E-2	4.851	■ Hedgehog signaling pathway - KEGG
1 / 15	1.0144E-1	2.744	■ Dentatorubropallidoluysian atrophy (DRPLA) - KEGG
2 / 176	1.4636E-1	4.452	■ Calcium signaling pathway - KEGG
1 / 153	1.5246E-1	4.552	■ Jak-STAT signaling pathway - KEGG
2 / 119	1.6892E-1	15.348	■ Tight junction - KEGG
1 / 128	1.7038E-1	2.3	■ Natural killer cell mediated cytotoxicity - KEGG
1 / 99	2.0081E-1	6.73	■ Gap junction - KEGG
1 / 77	2.0081E-1	3.079	■ Adherens junction - KEGG
1 / 77	2.0081E-1	2.154	■ Colorectal cancer - KEGG
2 / 130	2.1309E-1	6.441	■ Axon guidance - KEGG
2 / 117	2.2299E-1	9.372	■ Leukocyte transendothelial migration - KEGG
2 / 112	2.9043E-1	2.913	■ Cell cycle - KEGG
1 / 79	2.9094E-1	25.353	■ Phosphatidylinositol signaling system - KEGG
3 / 84	2.9920E-1	6.944	■ Apoptosis - KEGG
1 / 44	3.0532E-1	1.643	■ Type I diabetes mellitus - KEGG
3 / 147	3.3075E-1	9.637	■ Wnt signaling pathway - KEGG
2 / 132	3.4345E-1	70.946	■ Cell adhesion molecules (CAMs) - KEGG
2 / 302	3.5774E-1	2.936	■ Neuroactive ligand-receptor interaction - KEGG
1 / 44	4.3273E-1	5.382	■ Type II diabetes mellitus - KEGG
1 / 45	4.5678E-1	2.091	■ Ubiquitin mediated proteolysis - KEGG
1 / 86	4.8024E-1	1.468	■ Antigen processing and presentation - KEGG
4 / 256	4.8608E-1	10.217	■ Cytokine-cytokine receptor interaction - KEGG
2 / 84	4.9594E-1	5.595	■ TGF-beta signaling pathway - KEGG
1 / 46	4.9694E-1	4.035	■ Epithelial cell signaling in Helicobacter pylori infection - KEGG

Figure 47: Pathway analysis of the HSC-3 cell line based on upregulated gene data by using Kyoto Encyclopedia of Gene and Genomes (KEGG).

Input genes / Pathway genes	p-value	Impact factor	
8187	4.1202E-3	23.585	ECM-receptor interaction - KEGG
3131	6.6104E-3	8.909	Olfactory transduction - KEGG
2153	8.6743E-3	6.579	Taste transduction - KEGG
3130	1.5288E-2	7.507	Huntington's disease - KEGG
6199	2.0665E-2	28.75	Gap junction - KEGG
61132	4.0045E-2	47.947	Cell adhesion molecules (CAMs) - KEGG
11119	8.2890E-2	2.404	Tight junction - KEGG
71256	7.3972E-2	16.057	Cytokine-cytokine receptor interaction - KEGG
11117	7.6030E-2	3.336	Amnionotrophic lateral sclerosis (ALS) - KEGG
3189	1.4017E-1	6.259	Adipocytokine signaling pathway - KEGG
1122	1.4274E-1	1.96	Alzheimer's disease - KEGG
4177	1.5004E-1	11.088	Adherence junction - KEGG
91194	1.5443E-1	17.696	Focal adhesion - KEGG
21176	1.5918E-1	6.184	Calcium signaling pathway - KEGG
11128	1.8183E-1	3.87	Natural killer cell mediated cytotoxicity - KEGG
3169	1.9362E-1	7.179	Complement and coagulation cascades - KEGG
21130	2.2849E-1	7.526	Axon guidance - KEGG
1157	2.6871E-1	2.495	Hedgehog signaling pathway - KEGG
3197	2.8286E-1	9.839	GnRH signaling pathway - KEGG
1144	2.9444E-1	1.756	Type I diabetes mellitus - KEGG
1175	3.0496E-1	3.071	Fc epsilon RI signaling pathway - KEGG
1175	3.0496E-1	2.921	Long-term depression - KEGG
1179	3.0496E-1	2.247	Phosphatidylinositol signaling system - KEGG
21112	3.0766E-1	3.268	Cell cycle - KEGG
1135	3.4542E-1	1.469	Neurodegenerative disorders - KEGG
41117	3.5344E-1	21.072	Leukocyte transendothelial migration - KEGG
21153	3.7576E-1	3.388	Jak-STAT signaling pathway - KEGG
2167	3.8873E-1	6.118	Long-term potentiation - KEGG
31135	3.8897E-1	11.317	Insulin signaling pathway - KEGG
1191	4.0759E-1	2.279	Toll-like receptor signaling pathway - KEGG
1144	4.1937E-1	3.788	Type II diabetes mellitus - KEGG
61206	4.3975E-1	34.568	Regulation of actin cytoskeleton - KEGG
1163	4.4706E-1	50.442	B cell receptor signaling pathway - KEGG
41147	4.4928E-1	11.49	Wnt signaling pathway - KEGG
2177	4.5344E-1	5.982	Colorectal cancer - KEGG
2184	4.7819E-1	6.476	TGF-beta signaling pathway - KEGG

Figure 48: Pathway analysis of the HSC-3 cell line based on downregulated gene data by using Kyoto Encyclopedia of Gene and Genomes (KEGG).

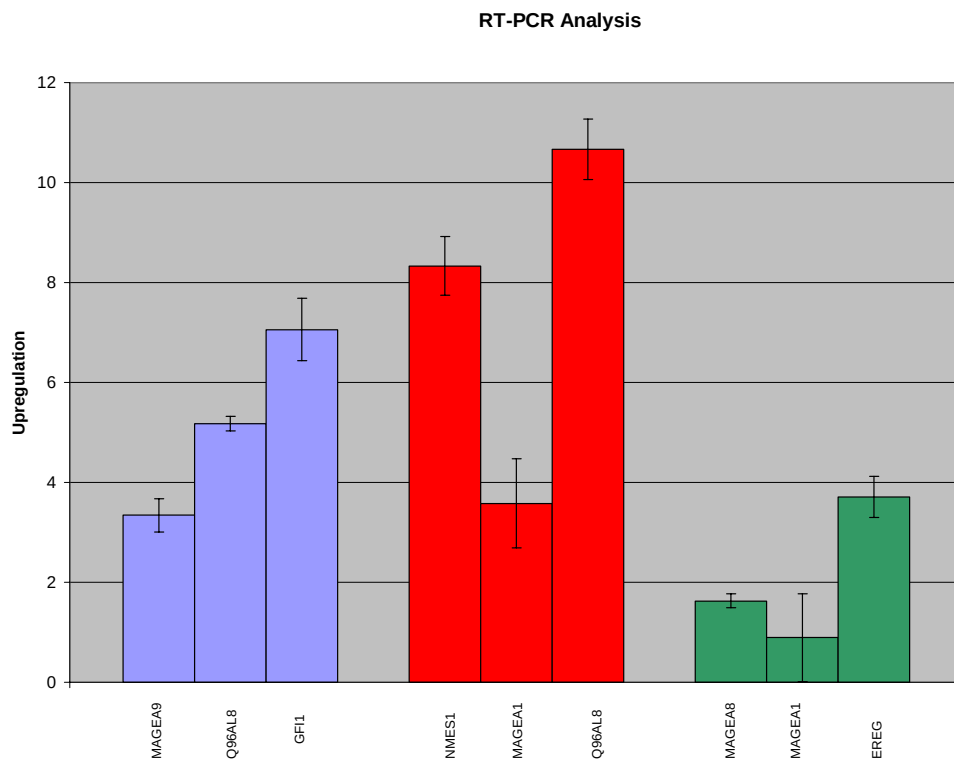


Figure 49: RT-PCR Analysis results. Blue columns represent the BHY, red columns represent the HN and green columns represent the HSC-3 cell lines.

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Vita

N. Fazil Erdem was born on January 05, 1973 in Ordu, Turkey. He received his dentistry degree from School of Dentistry, Marmara University, Turkey in June 1998. He got his Masters Degree in Oral Surgery in July 2002 from School of Dentistry, Yeditepe University. He started his Doctorate program in Department of Oral and Maxillofacial Surgery, Comparative and Experimental Medicine Program, at The University of Tennessee in January 2003. His primary research work was in the mechanism of invasion and metastasis of oral squamous cell carcinoma.