Detection of Micrometastases of Squamous Cell Carcinoma Tumor Cells in Muscle Tissue

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Abstract. *The aim of this study was to evaluate microarray technology in the detection of micrometastases of head and neck squamous cell carcinoma (HNSCC) in muscle tissue. Three hundred SCCVII tumor cells were injected intramuscularly into the right flank of ten C3H/Km mice. One week later, the animals were euthanized and the muscle tissue was taken out. Histology (H&E staining), microarray and reverse transcriptase polymerase chain reaction analysis (RT-PCR) of the tissue was performed. Histology showed a few tumor cells between the muscle fibers. Microarray technology showed the different gene expression pattern of the muscle tissue with micrometastases in comparison to normal muscle tissue. Only genes with a fold change difference of 10 or greater were considered. Gene expression analysis revealed changes in the expression levels of 91 genes of micrometastases in muscle tissue. RT-PCR confirmed gene up-regulation. Significant differences in gene expression between micrometastases in muscle tissue and pure muscle tissue were found. The genes found to be up-regulated could be used to detect micrometastases in muscle tissue.*

Head and neck malignancies account for 6% of all cancers diagnosed in the United States and result in an estimated 14,000 deaths annually (1). Although improvements in local control and survival have been achieved with the use of combined-modality therapies, 5-year survival rates for patients with head and neck cancer have not improved significantly over the past 20 years (2, 3). Local relapse is in most cases due to micrometastases, either in lymph nodes or the surrounding muscle tissue. The limitations of routine pathology for detecting micrometastatic disease (4, 5) have made it necessary to explore molecular means of diagnosis that can detect disease through tissue sampling. Molecular detection of head and neck squamous cell carcinoma (HNSCC) cells in a background of surrounding muscle tissue demands highly specific and sensitive biomarkers. Ideally, these biomarkers would be abundantly, yet exclusively, expressed in squamous epithelium, whereas having negligible expression in muscle tissue. One method for the molecular detection of these biomarkers that has shown promise in recent studies is microarray technology. It allows qualitative and quantitative analysis of biomarkers to be performed with great sensitivity and from minute amounts of starting material. Because this technology is sensitive, it offers the potential to improve clinical decision making (6-9). SCCVII is a syngeneic squamous cell carcinoma cell line of C3H mice and has been used as a model for human head and neck cancer (10). In this study, we applied gene expression microarray technology to muscle tissue after injection of squamous cell carcinoma cells in order to test if this method is a sensitive method of proving or excluding the presence of micrometastases in muscle tissue.

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Materials and Methods

All animal experiments were performed in compliance with institutional animal care committee guidelines and with the approval of the Animal Care Committee.

Tumor cell implantation into muscle tissue. Ten C3H/Km (Stanford animal facility, CA, USA) male mice aged 12 weeks were anesthetized with intraperitoneal Nembutal (pentobarbital, Abbott Laboratories, CA, USA) (58 mg/kg) and their right flank was shaved and prepared with isopropyl alcohol. An average of 300 tumor cells (mouse SCC VII) in Hanks` solution were injected intramuscularly into the right flank of each mouse. The total volume of injection was 100 µl. One week later, the animals were euthanized and the muscle tissue taken out. As control, muscle tissue of the contralateral side of five animals was used and muscle tissue after injection of 100 µl saline. In addition, in five animals tumor was grown subcutaneously until a size of 500 mm^3.

Histology. In order to reveal micrometastases in muscle tissue, hematoxylin and eosin (H&E) staining was performed. For the H&E staining, tissue samples were preserved in 10% formalin solution for 96 h. Afterwards, they were embedded in paraffin, sectioned, stained with H&E, and then mounted on glass slides.

Microarray analysis. For microarray analysis, samples of the muscle tissue with micrometastases, muscle tissue after injection of 100 µl saline, muscle tissue alone and tumor tissue alone was used. The tissue samples for microarray analysis were deep-frozen at a temperature of –80˚C. In total, 10 muscle tissue samples with micrometastases, five muscle tissue samples and five tumor tissue samples were analyzed. The total RNA was isolated using TRIzol Reagent® (GibcoBRL Life Technologies, Rockville, MD, USA) and double-stranded cDNA was created using the SuperScript Choice system (Life Technologies, Rockville, MD, USA). In further steps, the cDNA was extracted and precipitated. Biotinylated cRNA was synthesized using Enzo Bio Array High Yield RNA Transcript Labelling Kit (Enzo Diagnostics Inc., Farmingdale, NY, USA). After incubation the labeled cRNA was cleaned-up according to the RNeasy Mini kit protocol (Qiagen, Valencia, CA, USA). The cRNA was fragmented and hybridized on the murine Genome U74Av2 set array. The chips were washed and stained with streptavidin phycoerythrin (SAPE; Molecular Probes, Eugene, OR, USA). To amplify staining, streptavidin phycoerythrin solution was added twice with an anti-streptavidin biotinylated antibody (Vector Laboratories, Burlingame, CA, USA) staining step in between. The probe array was scanned on a Hewlett-Packard confocal microscope scanner (Hewlett Packard Gene Array Scanner; Hewlett Packard Corporation, Palo Alto, CA, USA) at the excitation wavelength of 488 nm. The amount of light emitted at 570 nm was proportional to the target bound at each location on the probe array. All samples were prepared as described and hybridized onto the Affymetrix Murine Genome U74Av2 Set array (Affymetrix, Santa Clara, CA, USA).

Quantitative reverse transcriptase-polymerase chain reaction analysis (qRT-PCR). To validate the results of the microarray experiment quantitative real-time polymerase chain reaction assays on genes of interest was performed. qRT-PCR was performed according to standard procedures using the muscle tissue samples containing micrometastases. RNA was extracted from the tissue with the following steps: Tissue was homogenized in 5 ml of lysis buffer (6 mol/l urea, 3 mol/L lithium chloride, 50 mmol/l sodium acetate, 200 µg/ml heparin, and 0.1% sodium dodecyl sulphate). The homogenized tissue was centrifuged at 16000 g for 20 minutes, extracted twice with an equal volume of phenol and chloroform, and precipitated with ethanol. The RNA pellet was air dried and dissolved in water treated with diethylpyrocarbonate. RT-PCR was then performed (DNA Thermal Cycler 480; Perkin-Elmer, Oak Brook, IL, USA). For qRT-PCR the following genes were used in this study found to be highly up-regulated in the microarray experiment: Complement component 1, q subcomponent, c polypeptide, S100 calcium-binding protein A4, Cytokeratin (endoB) gene, Epithelial membrane protein 3, Epithelial membrane protein 1, Colony-stimulating factor 1 receptor, Apolipoprotein E, Stromal cell-derived factor, CD14 antigen. The primer for the different genes were as followed: for Complement component 1, q subcomponent, c polypeptide forward, *5'-GCTTGTA GTACACCAGCGTGTT-3'*, and reverse, *5'-AAGGTGCCCGGTCTCTA CTA-3'*, for S100 calcium-binding protein A4 forward, *5'-GCTTGT AGTACACCAG CGTGTT-3'*, and reverse, *5'-AAGGTGCCCGGTCTC TACTA-3'*, for Cytokeratin (endoB) forward 5'-CTTGTGGAGTGGG TGGCTAT -3', and reverse, 5'-CCACTTGGTGTCCAGAACCT-3', for Epithelial membrane protein 3 forward 5'-CTTGTGGAGTG GGTGGCTAT -3', and reverse, 5'-CCACTTGGTGTCCAGAACCT -3', for Epithelial membrane protein 1 forward 5'-ATTGCCAATGT CTGGTTGGTTT-3', and reverse, 5'-AGAACGCCGATGATGA AGCT-3', for Colony-stimulating factor 1 receptor forward 5'-AGATA TTCGAGCAGGGTCTAC-3', and reverse, 5'-GGGATATCAGTC AGAAAGGTT -3', for Apolipoprotein E forward 5'-GTT GCTGG TCACATTCCTGG-3-3', and reverse, 5'-GCAG GTAATCCC AAAAGCGAC -3', for Stromal cell-derived factor forward *5'-AGGCTACCTGGATCAGGCTTC-3*', and reverse, *5'-ACATTCTTTT CAGCCTACCTCC-3'*, for CD14 antigen forward 5'- AGAGG CAGCCGAAGAGTTCAC-3', and reverse, 5'-GCGCTC CATGG TCGATAAGT -3'. GAPDH was used as an internal control for normalization. GAPDH-forward: *5'-TGCACCACCAACTGCT TAGC-3'*; GAPDH-reverse: *5'-GGCATGGACTGTGGTCATGAG-3'*. The cycling conditions used for the amplification were as follows: 5 min at 94˚C followed by 40 cycles of 20 sec at 94˚C, 20 sec at 59˚C, and 30 sec at 72˚C with a final extension at 72˚C for 10 min. The products were checked in 2% agarose gel, along with a 100-base pair ladder (Promega, Madison, WI, USA). PCR amplification and quantitation was performed using ABI SYBR Green Master Mix (Applied Biosystems, Foster City, CA) and Stratagene MX3000P™ (Cedar Creek, Texas). The expression values of investigated genes compared with that of GAPDH were calculated using the $2^{-\Delta\Delta Ct}$ method. The mean value and standard deviation of each analyzed tissue sample group was calculated. All reactions were conducted in triplicate.

Analysis of microarray data. Pre-processing of the Affymetrix arrays was carried out using GeneData Refiner 3.06 software (Genedata, Lexington, MA, USA). Each tissue sample was analyzed once, producing one result of fold change by comparing the samples with micrometastases with those of muscle tissue alone, and with tumor tissue samples. The mean value and standard deviation of each analyzed tissue sample group was calculated. Expression intensity values for each gene were derived using Refiner (Genedata, Lexington, MA, USA) by applying the Microarray Suite 5.0 algorithm (Affymetrix, Santa Clara, CA, USA).

Statistical analysis. Genes differentially expressed between the muscle tissue containing micrometastases compared to normal muscle tissue and tumor tissue were identified using a Satterthwaite *t*-test to robustly estimate significance despite unequal variance among groups ($p<0.001$). Only genes having a mean fold difference in expression of 10.0 or more were considered.

Results

Histology. In the muscle tissue after injection of tumor cells, no tumor cell nests were found however, a few tumor cells (Figure 1A, arrow) between the muscle fibers were detected, otherwise histology showed normal muscular tissue structure (H&E; Figure 1B).

Microarray analysis. Gene expression analysis revealed up-regulation of the expression of 91 genes in micrometastases in muscle tissue compared to pure muscle tissue and in 21 genes compared to pure tumor tissue.

The genes up-regulated in micrometastases from muscle tissue compared to pure muscle tissue are related to different functional groups. They belong to genes involved in immune response, protein binding, receptor activity, membrane function, cell matrix, cell growth, cell core, calcium binding, enzyme activity, lipid metabolism and nucleotide activity.

In the immune response group, the most up-regulated gene was complement component 1, q subcomponent, c polypeptide and complement component 1, q subcomponent, alpha polypeptide, with a fold increase of 108±14 and 103±14, respectively. In the protein binding group, the most specific up-regulated genes were the S100 calcium binding protein A4, calpactin I light chain and the S100 calcium-binding protein A6, with a fold increase of 108±7.6, 24±2.2 and 23±2.9, respectively. Different receptors were up-regulated by 11±4 to 17±2 fold, for example the mRNA for 4F2/CD98 light chain receptor, peptidylprolyl isomerase C-associated protein and the mannose receptor, C type 1. Two genes with important membrane function were up-regulated, the retinoic acid-inducible E3 protein and the proteolipid protein 2. The most up-regulated cell matrix gene was that for the cytokeratin (endoB), with a fold increase of 68±12. Genes affected which are related to cell growth were epithelial membrane protein 3, thymic shared antigen-1 (*Tsa-1*) gene and epithelial membrane protein 1 with a fold increases of 29±2.7, 29±2.5 and 16±1.5, respectively. In the group of genes for cell core and nucleotide activity, the Nsp-like 1 protein (Nspl1) (28±6.9-fold), mouse beta-tubulin (isotype Mß 5) (21±2.2- fold) and dynamin (18±1.7- fold) were up-regulated. An important function in calcium binding is held by endothelial monocyte-activating polypeptide I (29±3.9 fold increase). Up-regulated genes with a high enzyme activity were cathepsin S, mouse lysozyme M gene, Tyro protein tyrosine kinase binding protein, colony-stimulating factor 1 receptor and (cpp32) apoptotic protease mRNA with a fold increase of between 26±2.5 and 54±6.1. Genes involved in lipid metabolism were those for apolipoprotein E, phospholipid transfer protein and annexin III (Tables I-III). Genes up-regulated in micrometastases in muscle tissue compared to tumor tissue are those genes which are specifically up-regulated in muscle tissue. They belong to groups related to calcium metabolism, muscle contraction and development, energy supply, general metabolism, receptor activity and molecule transport and tissue regulation. These genes are specifically related to muscle tissue and muscle tissue metabolism (Table IV). Comparing the gene expression profile of the muscle tissue after injection of 100 µl saline and the contralateral muscle tissue, no significant gene expression differences were observed.

qRT-PCR. To validate gene expression profiling by microarray we performed qRT-PCR for genes highly up-regulated in micrometastases in muscle tissue. In micrometastases in muscle tissue we chose the complement component 1, q subcomponent, c polypeptide; S100 calcium-binding protein A4; cytokeratin (endoB); epithelial membrane protein 1 and 3; colony-stimulating factor 1 receptor; apolipoprotein E; stromal cell-derived factor; and CD14 antigen. Although the degree of up-regulation detected by the two methods varied, direct comparison of values of differentially expressed genes showed an overall pattern concordant between RT-PCR and Affymetrix cDNA array experiments the same trend for induction was detected by both methods for each target gene. No mismatches between the RT-PCR and the Affymetrix results were found. The overall gene expression changes obtained by RT-PCR were greater with smaller standard deviations (Table V).

Discussion

The sensitivity of pathological analysis by H&E staining for the detection of small tumor deposits in muscle tissue has been improved by the addition of immunohistochemical staining, which has been demonstrated to up-stage disease in many patients who were classified as having no clinically measurable metastatic disease (11), however there is always the possibility of missing very small micrometastases. Early studies focused on the detection of clonal genetic changes that were specific for HNSCC cells, such as mutations in *p53* (12). In recent years, researchers have shifted focus from tumor-specific towards tissue specific markers, as they seek to take advantage of the differential gene expression between HNSCC cells and other tissues (13, 14). DNA microarray analysis of human tumor specimens to identify metastasis-related genes has been reported for several types of cancer (15-17). Roepman *et al*. identified 102 genes in primary tumors as an expression profile for the prediction of lymph node metastasis from primary HNSCC (18). Chung *et al*. also used DNA microarray to classify HNSCC and predict lymph node metastasis (19). Due to heterogeneity, HNSCC cells may utilize different gene products to achieve similar functions. Therefore, it is difficult to validate expression of a large number of genes at the protein level in tissue specimens, and to validate their biological relationship and functional pathways in metastasis. DNA microarray data mining analysis has provided important information for understanding the biological behaviors of metastatic HNSCC cells.

The gene expression pattern of micrometastases in muscle tissue in our experiment was completely different to that of pure muscle tissue and muscle tissue after injection of saline. The gene expression patterns of pure muscle tissue and muscle tissue after injection of saline did not differ significantly. This is an indication that the damage of muscle tissue due to the injection needle did not cause significant gene expression changes. In our experiment, we chose a fold difference of 10 or more in order to select only highly up-regulated genes in order to derive a very high specificity for

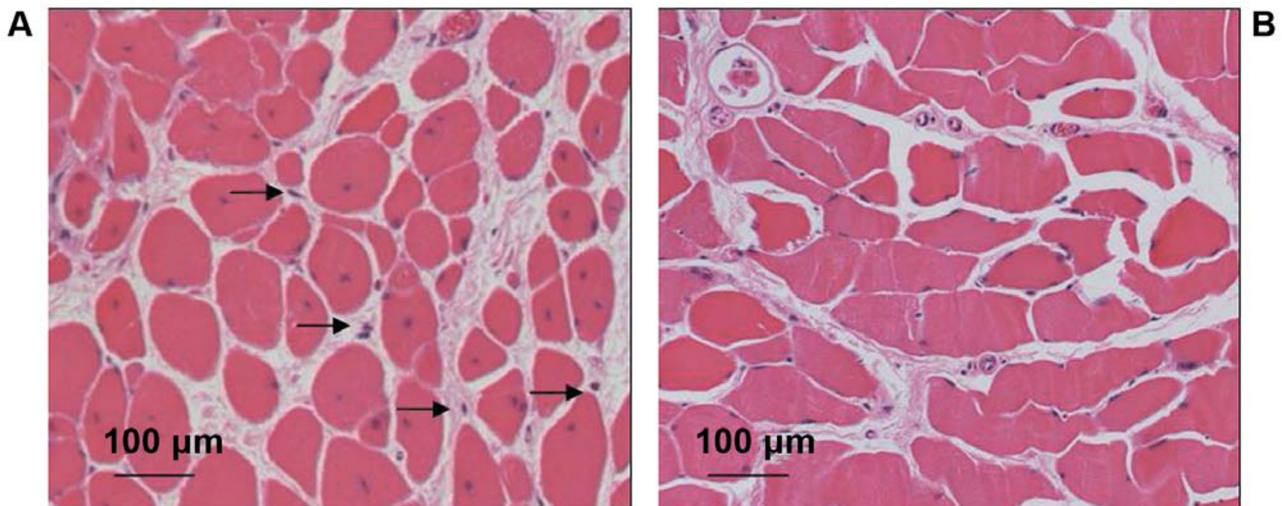

Figure 1. *A: Hematoxylin and eosin staining of muscle tissue after injection of tumor cells. The arrows indicate a few tumor cells between the muscle fibers. B: Hematoxylin and eosin staining of normal muscle tissue.*

the up-regulated genes. In other studies, gene expression was considered as significantly altered, if there was a fold difference of two or more (20, 21).

The up-regulated genes in micrometastases in muscle tissue found in our experiment belong to different physiological functional groups from those up-regulated in pure tumor tissue. The genes are important for immune response, protein binding, receptor activity, membrane function, cell matrix, cell growth, cell core, calcium binding, enzyme activity, lipid metabolism and nucleotide activity. In our experiment qRT-PCR was performed for nine of the highly up-regulated genes from the different physiological functional groups, and the results were consistent with the results of oligonucleotide microarray analysis. In the group of immune response genes the most up-regulated genes were complement component 1, q subcomponent, c polypeptide and complement component 1, q subcomponent, alpha polypeptide. C1q is the target recognition protein of the classical complement pathway that is crucial for the clearance of pathogens and apoptotic cells (22). It is involved in a number of immunological processes, such as phagocytosis of bacteria, neutralization of retroviruses, cell adhesion, modulation of dendritic cells, B-cells and fibroblasts, and maintenance of immune tolerance by clearance of apoptotic cells (23). In the group of genes for binding protein the most specific up-regulated gene was the S100 calcium-binding protein A4. S100A was shown to promote metastasis in several experimental animal models, and S100A4 and A6 protein expression is associated with patient outcome for a number of tumor types. These proteins have a wide range of biological functions, such as regulation of angiogenesis, cell survival, motility, and invasion (24). Different receptors were up-regulated, for example the mRNA for 4F2/CD98 light chain receptor. The precise function of the 4F2 molecule remains unknown. However, a role for 4F2 in the regulation of cell growth and activation has been suggested by the finding that 4F2 is expressed at low levels in most quiescent cells *in vivo*, but it is expressed at high levels on all dividing human tissue culture cells and most, if not all, malignant human cells (25). Two genes having an important membrane function were up-regulated the retinoic acid-inducible E3 protein mRNA and the proteolipid protein 2. Retinoic acid-inducible gene-I (Rig-I) is an intracellular pattern recognition receptor that plays important roles during innate immune responses. The mechanisms and signaling molecules that participate in the downstream events that follow activation of Rig-I are incompletely characterized. In addition, the factors that define the intracellular availability of Rig-I and determine the steady-state levels of this protein are only partially understood but are likely to play a major role during innate immune responses (26). Proteolipid protein 2 is a protein up-regulated in tumors, especially in oligodendrogliomas, and important for the development of tumors (27). The most up-regulated cell matrix gene was cytokeratin (endoB). Cytokeratin is a cytoskeletal intermediate filament protein. At present, there are 20 subtypes expressed in various types of human epithelial cells. The cytokeratin isotype depends on the cell type and the localization of cytokeratin in the cytoplasm (28). The most up-regulated gene related to cell growth was epithelial membrane protein 3. Epithelial membrane proteins are expressed in many tissues, and

Table I. *Comparison of the gene expression profile of micrometastases in muscle tissue compared to muscle tissue. Tumor specific genes related to immune response, protein binding and receptor activity found to be significantly up-regulated in micrometastases. Data are expressed as mean of the fold change (FC) and standard deviation (SD).*

Gene name	Acc. No	FC±SD
Immune response		
Complement component 1, q subcomponent, c polypeptide	X66295	108±14
Complement component 1, q subcomponent, alpha polypeptide	X58861	103±14
major histocompatibility locus class III regions Hsc70t gene	AF109905	25±3.1
Granulin	D16195	25±3.5
Mouse MHC (Qa) Q2-k gene for class I antigen, exons 1-3	X58609	17±1.4
Histocompatibility 2, K region locus 2	M27134	17±1.4
Major histocompatibility locus class III regions Hsc70t gene	AF109905	12±3
Major histocompatibility complex region	AF110520	12±2.5
Mouse mRNA with a set 1 repetitive element for a class I major histocompatibility complex(MHC) antigen	X00246	11±1.2
CD14 antigen	X13333	11±1.0
Protein binding		
S100 calcium-binding protein A4	M36579	108±7.6
Calpactin I light chain	M16465	24±2.2
S100 calcium-binding protein A6	X66449	23±2.9
Lipocortin 1	M69260	21±1.7
HSP47 mRNA	X60676	21±3.1
Procollagen C-proteinase enhancer protein	X57337	20±1.8
Mac-2 antigen	X16834	20±3.3
Mouse mRNA for I-E (beta-b) gene	X00958	17±3.5
Mouse calpactin I heavy chain (p36) mRNA	M14044	17±1.8
Procollagen, type VI, alpha 2	Z18272	14±1.0
Mouse mRNA for 14-3-3 zeta	D83037	14±2.5
Lamin A	D49733	11±5
Immunosuperfamily protein Bl2 mRNA	AF061260	10±4.1
Heparan sulfate proteoglycan 1, cell surface-associated (fibroglycan)	U00674	10±4
Interferon-activated gene 204	M31419	10±3.1
Receptor activity		
mRNA for 4F2/CD98 light chain	AB017189	17±2
Peptidylprolyl isomerase C-associated protein	X67809	14±1.4
Mannose receptor, C type 1	Z11974	14±1.2
Vascular cell adhesion molecule 1	M84487	13±1.9
Mouse Ia-associated invariant chain (Ii) mRNA fragment	X00496	12±2.3
Solute carrier family 4 (anion exchanger), member 2	J04036	11±5
Interleukin 10 receptor, beta	U53696	11±4

FC: Fold change; SD: standard deviation.

Table II. *Comparison of the gene expression profile of micrometastases in muscle tissue compared to muscle tissue. Tumor-specific genes related to the cell membrane, cell matrix, cell growth and cell core and nucleotide activity found to be significantly up-regulated in micrometastases.*

Gene name	Acc. No	FC±SD
Membrane		
Retinoic acid-inducible E3 protein mRNA,	U29539	12±2.0
Plp2 mRNA for proteolipid protein 2	AB031292	11±3
Cell matrix		
Cytokeratin (endoB) gene	M22832	68±12
mRNA for PGI (biglycan)	X53928	29±2.7
Cofilin 1	D00472	24±2.6
Procollagen, type XVIII, alpha 1	U03715	24±4
Mouse fibronectin (FN) mRNA	M18194	21±2.1
mbh1 gene for Myc basic motif homologue-1 (mbh1)	X54511	20±2.5
Transforming growth factor, beta induced, 68 kDa	L19932	20±2.5
Mouse tropomyosin isoform 2 mRNA	M22479	18±2.0
Mouse procollagen type V alpha 2 (Col5a-2) mRNA	L02918	13±1.1
Type VI collagen alpha 3 subunit mRNA	AF064749	11±3
Procollagen, type VI, alpha 1	X66405	11±1.0
Cell growth		
Epithelial membrane protein 3	U87948	29±2.7
thymic shared antigen-1 (Tsa-1) gene	U47737	29±2.5
Epithelial membrane protein 1	X98471	16±1.5
Cyclin B2	X66032	12±1.9
Stromal cell derived factor 1	L12029	11±1.1
F52 mRNA for a novel protein	X61399	10±3
Cell core and nucleotide activity		
Nsp-like 1 protein (Nspl1) gene, complete cds; tRNA-Sec gene, complete sequence; and FosB protein (Fosb) gene	AF093624	28±6.9
Mouse mRNA for beta-tubulin (isotype Mbeta 5)	X04663	21±2.2
Dynamin	L31397	18±1.7
Proliferating cell nuclear antigen	X57800	16±1.0
Mouse histone H2A.1 gene	M33988	15±1.7
Adenylate kinase isozyme 2	AB020202	15±1.5
RA70	AB014485	12±1.1
Sec61 mRNA	AB032902	12±1.1
eIF3 p66	AB012580	12±1.1
Small nuclear ribonucleoprotein D1	M58558	10±4
Mouse alpha-tubulin isotype M-alpha-6 mRNA	M13441	10±4
High mobility group protein 14	X53476	10±3.5

FC: Fold change; SD: standard deviation.

functions in cell growth, differentiation, and apoptosis have been reported. Epithelial membrane protein 1 and 3 are highly up-regulated during squamous differentiation and in certain tumors, and a role in tumorigenesis has been proposed. They are also highly up-regulated during squamous cell differentiation and in certain tumor types, and

Table III. *Comparison of the gene expression profile of micrometastasis containing muscle tissue compared to pure muscle tissue. Tumor specific genes related to calcium binding, enzym activity and lipid metabolism found to be significantly up-regulated in micrometastases.*

Gene name	Acc. No	FC±SD
Calcium-binding		
Endothelial monocyte-activating polypeptide I mRNA	U41341	29±3.9
Follistatin-like	M91380	15±1.1
Matrix gamma-carboxyglutamate (gla) protein	D00613	12±1.0
Mouse mRNA for annexin V	D63423	11±1.0
Enzyme activity		
Cathepsin S	AJ223208	54±6.1
Mouse lysozyme M gene	M21050	43±5.5
TYRO protein tyrosine kinase binding protein	AF024637	31±3.7
Colony stimulating factor 1 receptor	X06368	27±3.2
Cpp32 apoptotic protease mRNA	U63720	26±2.5
Cystatin B	U59807	19±1.5
Large multifunctional protease 7	U22033	18±2.2
Antioxidant enzyme AOE372 mRNA	U96746	16±1.7
Mouse cytochrome beta-558 mRNA, 3 end	M31775	16±1.6
Mouse MHC class I D-region cell surface antigen (D2d) gene	M27034	15±1.6
Fumarylacetoacetate hydrolase	Z11774	15±1.3
Legumain	AJ000990	15±1.1
Putative steroid dehydrogenase (KIK-I) mRNA	AF064635	14±2.3
Pigment epithelium-derived factor (PEDF) mRNA	AF036164	12±1.3
Spermidine/spermine N1-acetyl transferase	L10244	12±1.0
Protein-tyrosine phosphatase mRNA	AF013490	11±6
Protein inhibitor of nitric oxide synthase (PIN) mRNA	AF020185	11±1.3
Exostoses (multiple) 1	X96639	11±1.2
Alpha-mannosidase II	X61172	10±3
Lipid metabolism		
Apolipoprotein E	D00466	16±4.8
Phospholipid transfer protein	U28960	11±1.4
Annexin III	AJ001633	11±1.1

FC: Fold change; SD: standard deviation.

a role in tumorigenesis has been proposed (29). Of genes related to cell core and nucleotide activity, the most up-regulated gene was *Nspl1*. *Nspl1* contributes to integrin and receptor tyrosine kinase signaling (30). An important function in calcium binding is played by endothelial monocyte-activating polypeptide I, which has a pro-coagulant activity (31). The most up-regulated gene with a high enzyme activity was that for cathepsin S. Cathepsins have been found to participate in apoptosis, and also play a role in the promotion of tumors during cancer progression. In addition, it has been suggested that the expression of lysosomal cathepsins are substantially increased in malignant tumors (32).

Table IV. *Comparison of the gene expression profile of micrometastases containing muscle tissue compared to pure tumor tissue. Specific genes are related to muscle tissue could be found to be significantly up-regulated in micrometastases.*

Gene name	Acc. No	FC±SD
Calcium		
ATPase, Ca 2+ transporting, cardiac muscle, fast twitch 1	X67140	309±12
Parvalbumin	X59382	47±6
Muscle contraction		
Alpha-actinin 3 (Actn3) mRNA	AF093775	131±9
Skeletal muscle calsequestrin mRNA	U93291	115±13
Murine MLC1F/MLC3F gene for myosin alkali light chain (exon 1) (fast skeletal muscle isoform)	X12973	104±19
mRNA for myosin heavy chain 2X	AJ002522	41±3
Nebulin-related anchoring protein	U76618	23±6
Phospholemman precursor, gene	AF091390	22±8
Mouse skeletal muscle beta tropomyosin mRNA	M81086	13±
Muscle development		
Mus musculus myosin light chain 2 mRNA	U77943	78±8
Actin, alpha 1, skeletal muscle	M12347	74±13
RBP associated molecule RAM14-1 mRNA	U41739	20±3
Desmin	L22550	17±3
Myelodysplasia/myeloid leukemia factor 1 (Mlf1) mRNA	AF100171	15±2
Energy		
Cytochrome C oxidase	U15541	48±4
Metabolism		
Mouse adenylosuccinate synthetase mRNA	M74495	40±4
mRNA for pyruvate dehydrogenase kinase-like protein	AJ001418	26±9
Stearoyl-coenzyme A desaturase 1	M21285	11±3
Receptor activity		
CD24a antigen	M58661	24±9
Integral membrane protein 2	L38971	21±4
Transport		
Mouse RyR1 mRNA for skeletal muscle ryanodine receptor	D38216	47±6
Regulation		
Mouse DNA for tob family	D78382	13±3
Eukaryotic translation elongation factor 1 alpha 2	L26479	13±3

FC: Fold change; SD: standard deviation.

A highly up-regulated gene active in the lipid metabolism was apolipoprotein E. This and its gene product are involved in cholesterol transport, lipid metabolism and protein synthesis, by mediating the binding of the low-density lipoprotein (LDL) receptor, and the apolipoprotein E

Table V. *RT-PCR confirmed the up-regulation of selected genes when comparing micrometastases in muscle tissue to muscle tissue and micrometastases containing muscle tissue to tumor tissue.*

		Microarray	RT-PCR
Gene name	Acc. No.	FC±SD	FC±SD
Complement component 1, q subcomponent, c polypeptide	X66295	108±14	132.6±12.8*
S100 calcium-binding protein A4	M36579	108±7.6	116.3±8.4*
Cytokeratin (endoB) gene	M22832	68±12	75.9±11*
Epithelial membrane protein 3	U87948	29±2.7	37.1±4.1*
Epithelial membrane protein 1	X98471	16±1.5	26.1±3.2*
Colony-stimulating factor 1 receptor	X06368	27±3.2	29.5±6.1*
Apolipoprotein E	D00466	16±4.8	19.1±2.3*
Stromal cell-derived factor	L12029	11±1.1	11.1±3.2*
CD14 antigen	X13333	11±1.0	14±2.0

FC: Fold change; SD: standard deviation; *Significantly different from control, *t*-test ($p<0.05$).

receptor of lipids to specific lipoprotein receptors. It is also involved in numerous other functions, including tissue repair, immune response and regulation, as well as cell growth and differentiation (33). The differentially expressed genes here play crucial roles in the development, differentiation, and functioning of tumor tissues, and because they display remarkable tissue specificity (34, 35), the different patterns of gene expression are ideal for use as tissue classifiers. For example, the epithelial membrane protein 1 and 3 are highly up-regulated in squamous cell differentiation. This helps differentiate squamous cell tumor from other tumor types. The different gene expression patterns found in our study hold potential for assisting in the determination of the primary tumor site for metastases of unknown origin. Our demonstration of this highly discriminatory assay for the detection of small tumor deposits not detected by histology will hopefully supply the pilot data needed to incorporate this technique into a clinically-relevant application to improve staging of patients with metastatic HNSCC. The further clinical relevance of this study might be that it is helpful to apply microarray technology to surgical margins for molecular analysis. Several samples of the surrounding muscle tissue should be taken in order to assess more sensitively the possibility of micrometastasis to deeper tissue layers, however with the risk of missing nests of micrometastasis.

Conclusion

Our animal model of metastatic HNSCC plus DNA microarray analysis provided valuable information on the unique biological behaviors of SCCVII cells. We identified the epithelial membrane protein 1 and 3 in the tumor cells, confirmed by qRT-PCR, and put forward putative molecular bases leading to these behaviors.

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Author Contributions

Silke Steinbach, conception and design, acquisition of data, analysis and interpretation of data, drafting the article, final approval of the version to be published; Esther L. Yuh, conception and design, acquisition of data, analysis and interpretation of data, drafting the article, final approval of the version to be published; Mykhaylo Burbelko, analysis and interpretation of data, drafting the article, final approval of the version to be published; Walter Hundt, conception and design, acquisition of data, analysis and interpretation of data, drafting the article, final approval of the version to be published.

Disclosures

Competing interests: None.

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