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DNA hypermethylation profiles in squamous cell carcinoma of the vulva

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Abstract

Gene silencing through promoter hypermethylation is a growing concept in the development of human cancers. In this study, we examined the contribution of aberrant methylation of promoter regions in methylation-prone tumor suppressors to the pathogenesis of vulvar cancer. Thirteen cell lines from 12 patients with squamous cell carcinoma of the vulva (SCV) were evaluated for aberrant methylation status and gene copy number alterations, concomitantly, using the methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) assay. Of the 22 tumor suppressor genes examined, aberrant methylation was observed for 9 genes: TP73, FHIT, VHL, APC, ESR1, CDKN2B, DAPK1, GSTP1 andI GSF4. The most frequently methylated genes included TP73 in 9 of 13 cell lines, and IGSF4, DAPK1 and FHIT in 3 of 13 cell lines. Methylation specific polymerase chain reaction (MSP) was performed for TP73 and FHIT to confirm aberrant methylation by MS-MLPA. In the context of gene copy number and methylation status, both copies of the TP73 gene were hypermethylated. Loss or decreased mRNA expression of TP73 and IGSF4 by RT-PCR confirmed aberrant methylation. Frequent genetic alterations of loss and gain ofgene copy number included gain of GSTP1 and MEN1, and loss of MFHAS1 and IGSF4 in over 50% of the SCV cell lines. These findings underscore the contribution of both genetic and epigenetic events to the underlying pathogenesis of squamous cell carcinoma of the vulva.

Keywords

Squamous cell carcinoma of vulva (SCV); Epigenetics; Promoter hypermethylation; Methylation; specific Multiplex Ligation; dependent Probe Amplification (MS-MLPA) assay

INTRODUCTION

Chromosome aberrations have served as landmarks in the identification of cancer genes in many tumor types; however, individual genes altered in tumors cannot be deduced solely from the type of chromosome alteration (1). Although the importance of genetic alterations in cancer is well recognized, the appreciation of epigenetic changes is more recent and growing. The term ‘epigenetics’ defines all meiotically and mitotically heritable changes in gene expression

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that are not coded in the DNA sequence itself (2). Establishment and maintenance of epigenetic control (gene silencing) has several aspects, which include promoter region hypermethylation, methyl-binding proteins, DNA methyltransferases, histone deacetylases and chromatin state. CpG islands, which are stretches of DNA with a GC content greater than 55% (3), located in promoter regions of genes are mainly unmethylated in normal tissues except for imprinted genes (4) and X-chromosome genes in females (5). Methylation of these CpG islands causes stable heritable transcriptional silencing (2). Aberrant methylation of CpG islands is a hallmark of human cancers and is found early during carcinogenesis (2). Genes in cellular pathways which are inactivated by promoter region hypermethylation include *MGMT* (DNA repair), $p16^{INK4a}$, $p15^{INK4b}$ (cell cycle), *DAPK* (apoptosis) and *GSTP1* (detoxification) (6–9).

In this study, we focus on aberrant methylation of promoter regions in methylation-prone tumor suppressors in the pathogenesis of vulvar cancer. Vulvar carcinoma accounts for about 4% of all gynecological cancers in the USA (10). It is mainly a disease of elderly women, but has also been reported in some premenopausal women. Squamous cell carcinoma accounts for 90% of vulvar cancers (10). The main risk factors are human papillomavirus infection and chronic vulvar pruritis.

Previous studies from our group(11–13) have reported consistent chromosomal abnormalities in SCV with further characterization of these aberrations at the level of individual genes(1). In this study we examined aberrant promoter methylation of 22 methylation-prone tumor suppressor genes using a high-throughput multi-gene probe panel (41 gene probes, 35 unique genes, including control probes) in 13 SCV cell lines using a modification of the multiplex ligation-dependent probe amplification assay (MLPA) (1,14–16) termed the methylation specific multiplex ligation-dependent probe amplification (MS-MLPA) assay (7–9,17,18).

MATERIALS AND METHODS

Patient histories

The patients' ages, Tumor–Node–Metastasis (TNM) classification of the disease, the histological grade of the tumor, prior therapy at the time of the tissue biopsy for the establishment of the cell line, and the site of the specimen, are noted in Table 1 (11–13). The phenotypic characterization including karyotypic analysis of the two specimens of vulvar carcinoma from the same patient, UM-SCV-1A and UM-SCV-1B has been previously published (11). UM-SCV-1A was obtained from the primary tumor site and UM-SCV-1B from a malignant pleural effusion.

This study performed MS-MLPA on previously cytogenetically characterized cell lines UM-SCV-1A, -1B, -2, -3, -4, -6, -7, UT-SCV-1, -2, -3 and three additional cell lines, UT-SCV-4, -5, and -6, for a total of 13 cell lines.

DNA Extraction

DNA, extracted using the QIAamp Kit (Qiagen Inc, Chatsworth, CA), was obtained from passaged cell lines that originated from fresh tumor samples as described previously(11,19, 20). The number of passages for the cell lines were as follows: UM-SCV-1A – passage(P) 22, UM-SCV-1B – P8, UM-SCV-2 – P22, UM-SCV- 3 – P26, UM-SCV- 4 – P19, UM-SCV-6 – P17, UM-SCV-7 – P13, UT-SCV-1 – P26, UT-SCV-2 and -3 – P27, UT-SCV-4 – P12, UT-SCV-5 – P9, and UT-SCV-6 – P12.

Isolation of total RNA from cultured cells

Cells for tissue culture for isolation of mRNA for gene expression studies using Real Time PCR (RT-PCR) were available only for cell lines UT-SCV-2, -3, -4 and -6. Cultured cells from

these cell lines were grown in a monolayer, rinsed with 1x PBS, trypsinized and collected as a cell pellet. The latter was washed 2 times with PBS, spun, and the supernatant completely removed to obtain cell pellets for immediate RNA extraction. RNA was extracted using the QIAamp RNA mini protocol for isolation of total RNA from cultured cells (Valencia, CA USA).

Reverse Transcriptase (RT-PCR)

Total RNA (1μg) for synthesis was done using the SuperScript First-strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Briefly, RNA, Oligo (dt) primer and dNTP mix were denatured at 65°C for 5 minutes and immediately chilled on ice. This was followed by an annealing step with addition of buffer, DTT, RNaseout inhibitor, and $MgCl_2$ (without SuperScriptII {SSII}) and incubated at 42°C for 2min, followed by the addition of SSII. cDNA synthesis was allowed to continue at 42°C for 50 min. The reaction was terminated at 70°C for 15 minutes and immediately placed on ice. Removal of residual RNA was performed by the addition of 1μl of RNase H incubated at 37°C for 20 min. PCR was performed using 2 μl of RNA free cDNA.

Real-time Reverse Transcription Polymerase Chain Reaction

Expression of *TP73*, *IGSF4* and *DAPK1* was analyzed using a real-time quantification method (LightCycler) according to the manufacturer's recommendations (Roche Diagnostics, Indianapolis, IN). PCR primers were as follows: *TP73*: 5′-GGC TGC GAC GGC TGC AGA G-3′ (forward) and 5′-GCT CAG CAG ATT GAA CTG GGC CAT G-3′ (reverse), giving a product of 256 base pairs; *IGSF4*: 5′-GGC TTC TGC TGT TGC TC-3′ (forward) and 5′-CCC TGA AAT AAA TGG TCT GC-3′ (reverse), producing a product of 183 base pairs, and for *DAPK1*: 5′-TGA CAG TTT ATC ATG ACC GTG TTC AG-3′ (forward) and 5′-GTG CTG GAT CTC CTT CAG GAT-3′ (reverse), for a product size of 231 base pairs (Table 2).

Because the precise amount of total RNA added to each reaction and its quality are difficult to determine, expression of *TP73*, *IGSF4* and *DAPK1* were normalized with an endogenous standard β-actin. Standard curves were used to determine the concentration of *TP73*, *IGSF4*, *DAPK1* and β-actin gene products. Quantification of *TP73*, *IGSF4*, and *DAPK1* expression were obtained by direct comparison with β-actin dilution standards amplified in parallel reactions in the same run (Table 3). After real-time data acquisition, the parameter C_t (threshold cycle/crossing point) was calculated by determining the point at which the fluorescence exceeds an arbitrary threshold limit, which is set to cross the fluorescent signal of all standards in the exponential phase. The target load in the normal and the 4 cell lines, UT-SCV-2, -3, -4 and -6 were quantified by measuring C_t and using a standard curve to determine the starting target message quantity.

For accurate quantification of cDNA targets *TP73, IGSF4,* and *DAPK1*, the amplification efficiency of the target should be similar to that of the internal standard. To obtain this, the slope of the standard curve was converted to amplification efficiency E by the following algorithm: $E=10^{-1/\text{slope}}$. To calculate *TP73*, *IGSF4*, or *DAPK1* expression levels, initially the difference in Ct between *TP73*, *IGSF4*, or *DAPK1* and β-actin, termed CtΔ (delta), was obtained (Table 3). Expression levels of the target were plotted as exponent values $2^{-\Delta Ct}$ (Table 3).

The Methylation Specific Multiplex Ligation Dependent Probe Amplification (MS-MLPA) Assay

The Methylation Specific Multiplex Ligation Dependent Probe Amplification assay allows for the relative quantification of approximately 41 different DNA sequences in a single reaction requiring only 20 ng of human DNA (21). The standard use of the technique to observe quantitative changes in copy number has been outlined in other studies (1,14,15,22).

Modification of the MLPA to detect aberrant methylation (MS-MLPA) has been detailed elsewhere (7,9,17,21).

The probe design in MS-MLPA is similar to ordinary MLPA probes. For 26/41 probes, the recognition sequence detected by the MLPA probe is contained within a restriction site for the methyl-sensitive enzyme, *Hha*I (Figure 1). The 41 gene probe panel (Table 4) interrogates 35 unique genes implicated in cancer for losses and gains in a separate reaction in the absence of the methyl-sensitive enzyme *Hha*I. Because there are two probes each for *VHL*, *CDKN2A*, *BRCA1* and *BRCA2*, and 3 probes for *MLH1*, a normal control DNA sample will generate 41 individual peaks in the absence of *Hha*I (Figures 2 & 3). A concurrently run reaction with the 41 gene probe set in the presence of *Hha*I is designed to detect aberrant promoter hypermethylation by taking advantage of a *Hha*I site in the promoter region of 22 of the 35 unique genes (note that one of the two *BRCA1* probes is designed to recognize a region outside the *Hha*I recognition site, Table 4). Fifteen of the 41 gene probes are designed outside a Hha1 site and serve as undigested controls (Figure 2–5). Upon digestion of the sample DNA with *Hha*I, probes that recognize the unmethylated regions will not generate a signal because these sequences have become cut by *Hha*I and cannot bind to the probe (Figure 1). Conversely, a MLPA probe will bind to an intact methylated site, spared by *Hha*I, and generate an amplification signal (Figures 1, 4 & 5). To detect both aberrant methylation and changes in copy number, each sample requires 2 MLPA reactions. Details of the assay including its interpretation are described elsewhere (21). Aberrant methylation is identified as the appearance of a signal peak that is otherwise absent in normal DNA samples (Figures 2–5). To quantify whether one, both, or more copies of a specific gene locus becomes aberrantly hypermethylated, a previously described mathematical algorithm was employed (17).

Bisulfite Modification and Methylation-Specific Polymerase (MSP) Chain Reaction Assay

MSP was performed for cell lines with sufficient amounts of DNA. Genomic DNA (100ng) from SCV cell line DNA and control universal methylated DNA (Chamicon International, Inc) and control unmethylated DNA (normal genomic DNA) were modified using the EZ DNA methylation kit (Zymo Research, Orange, CA) during which methylated DNA is protected and unmethylated cytosine is converted to uracil (9).

The modified DNA served as a template using primers specific for either the methylated or modified unmethylated *TP73* and *FHIT* sequences. *TP73* methylation specific primers (MS) were sense: 5′-TCGGGGGGCGGTTCGGTTTCGC-3′, anti-sense: 5′-CTCTAACCGCTCCAACCTCTCGCGAACG-3′ (21). Unmethylated DNA specific primers (UMS) were sense: 5′-GGGGGTGGTTTGGTTTTGTGGGT-3′, antisense: 5′-CTAAACTCTAACCACTCCAACCTCTCACAAACA-3′ (21). *FHIT* methylation specific primers (MS) were sense: 5′-TTGGGGCGCGGGTTTGGGTTTTTA CGC-3′, anti-sense: 5′-CGTAAACGACGCCGACCCCACTA-3′ (23). Unmethylated DNA specific primers (UMS) were sense: 5′-TTGGGGTGTGGGTTTGGGTTTTTATG-3′, antisense: 5′-CATAAACAACACCAACCCCACTA-3′ (23).

MSP amplification for *TP73* was performed using 3ul of bisulfite modified DNA in a PCR mix containing 1X PCR buffer, 2mM $MgCl_2$ and 2U Amp gold Taq DNA polymerase, 0.4uM primer followed by 38 cycles at 95°C 50 seconds, 62°C 50 seconds, 72°C 1min. PCR generated a 193bp methylated product and a 195bp unmethylated product (Figure 6).

MSP amplification for *FHIT* was performed using 3ul of bisulfite modified DNA in a PCR mix containing 1X PCR buffer, 2mM $MgCl_2$ and 2U Amp gold Taq DNA polymerase, 0.4uM primer followed by 38 cycles at 95°C 50 seconds, 62°C 50 seconds, 72°C 1min. PCR generated a 74bp methylated and unmethylated product (Figure 7).

The resultant PCR for *TP73* and *FHIT* were separated on 2% agarose gel, stained with ethidium bromide and visualized under UV illumination (Figures 6 & 7).

RESULTS

Aberrant methylation was observed for 9 genes, *APC* (5q21), *CDKN2B* (9p21), *VHL* (3p26), *TP73* (1p36.3), *IGSF4* (11q23.2), *DAPK1* (9q34.1), *ESR1* (6q25.1), *FHIT* (3p14.2) and *GSTP1* (11q13) in 11 of 13 SCV cell lines. The most frequently methylated genes were *TP73* in 9 of 13 cell lines, detected by MS-MLPA in 6/13 and MSP in 3/13, followed by *IGSF4*, *DAPK1* and *FHIT* in 3 of 13 cell lines (Table 5). UM-SCV-3 showed aberrant methylation for 6 of the 9 genes (*APC, VHL, TP73, IGSF4, DAPK1 and ESR1*), all of which had both copies methylated. In UM-SCV-7, hypermethylation was observed for both copies of *APC* and *FHIT*, the sole copy of *IGSF4* and one of two copies of *ESR1*. UT-SCV-2 and UT-SCV-4 showed hypermethylation of 3 of the 9 aberrantly methylated genes: *CDKN2B*, *FHIT* and *GSTP1* in UT-SCV-2 and *TP73*, *IGSF4* and *FHIT* in UT-SCV-4 (Table 5). Promoter hypermethylation of *VHL*, *CDKN2B*, and *GSTP1* was infrequent, occurring in only 1 of 13 cell lines (UM-SCV-3 and UT-SCV-2). Aberrant methylation was not observed in UM-SCV-1B, UM-SCV-6, UT-SCV-1 and UT-SCV-5.

Gene copy number in the context of methylation status, indicated both copies of *TP73*, *FHIT*, *IGSF4*, *APC*, *VHL*, *DAPK1*, and *ESR1* were methylated in 6, 3, 2, 2, 1, 1, and 1 cell lines, respectively (Table 5). For *IGSF4*, in UM-SCV-7, the sole copy was methylated. For *DAPK1* and *ESR1*, one of two copies was methylated in UM-SCV-1A and UM-SCV-2, and UM-SCV-7, respectively (Table 5). Gain of *GSTP1* and *MEN1* was most frequent occurring in 11 of 13 cell lines. Aberrant methylation of *GSTP1*, observed in only UT- SCV-2 indicated hypermethylation of 1 of 3 copies. In 6 of 13 cell lines, the sole *IGSF4* copy was unmethylated. Homozygous loss of *CDKN2B* was noted in UM-SCV-4, loss of one copy in UT-SCV-6, gain of copies in UM-SCV-7 and UT-SCV-4, and methylation of 1 of 3 copies in UT-SCV-2. Five of 13 cell lines with gain of *ESR1* did not show methylation. *FHIT* and *DAPK1* showed gain in 3 of 13 cell lines, none of which were methylated (Table 5). Common losses consisted of *MFHAS1*, *IGSF4*, *VHL*, *BCL2*, *MLH1* and *FANCD2* in 9, 7, 6, 6, 5 and 5 of 13 cell lines, respectively.

Absence or decreased mRNA expression of *TP73* was confirmed by RT-PCR in UT-SCV-3, -4 and -6, supporting aberrant methylation of *TP73* by MS-MLPA. UT-SCV-2 with two unmethylated copies of *TP73* had normal expression of its mRNA transcript (Tables 3 & 6).

Absence of expression of *IGSF4* in UT-SCV-4 was concordant with promoter hypermethylation of both copies. Decreased expression of unmethylated *IGSF4* in UT-SCV-6 was consistent with loss of 1 gene copy. Unmethylated *IGSF4* expression was variable and discordant with gene copy number in UT-SCV-2 (2 copies) and UT-SCV-3 (1 copy) (Table 3). Expression levels of unmethylated *DAPK1* and gene copy number were concordant for UT-SCV-2 (3 copies) and UT-SCV-4 (2 copies), but varied in UT-SCV-3 and UT-SCV-6 (Tables 3 & 6).

MSP was performed for *TP73* and *FHIT*. MSP of *TP73* confirmed aberrant methylation detected by MS-MLPA for UM-SCV-2, UM-SCV-3, UT-SCV-3, -4 and -6. In addition to these cell lines, MSP indicated aberrant methylation of *TP73* in UM-SCV-1a, UM-SCV-6 and UT-SCV-2, not detected by MS-MLPA. MSP did not show methylation of *TP73* in UM-SCV-4, indicated by MS-MLPA. MSP confirmed methylation of *FHIT* detected by MS-MLPA for UT-SCV-2 and UT-SCV-4.

DISCUSSION

Epigenetic alterations produce heritable changes in gene expression without a change in the DNA coding sequence itself. Promoter region hypermethylation is known to be an early event in carcinogenesis(7,8,24,25). The consequence of CpG island hypermethylation, especially for those islands associated with tumor suppressor gene (TSG) promoters is the loss of TSG function, which contributes to tumorigenesis (17).

The Methylation-Specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA) assay is a high throughput quantitative assay permitting the dual detection of genomic alterations of losses and gains and epigenetic events of promoter hypermethylation (7-9,17, 21). In our study using MS-MLPA, a candidate multi-gene approach, we identified, concomitantly, genetic and epigenetic alterations in SCV.

Aberrant methylation was observed for 9 genes in 11 of 13 SCV cell lines. The most frequently methylated gene was *TP73* (9 of 13 cell lines). Decreased *TP73* expression as a consequence of promoter hypermethylation was confirmed by RT-PCR in UT-SCV-3, -4 and -6, supporting aberrant methylation of both copies of *TP73* by MS-MLPA. *TP73*, located at 1p36.3 is involved in cell cycle regulation, and is frequently deleted in many types of human tumors(26–28). *TP73* codes a product which has significant structural homology to the *TP53* gene product in the domains involving transactivation, DNA-binding and oligomerization(29). Functionally, the *TP73* gene product is able to activate the TP53-responsive proteins, inhibit cell growth and induce apoptosis(30).

Evaluation of *TP73* for epigenetic changes has shown aberrant promoter hypermethylation for oligodendroglial tumors, non-hodgkin's lymphomas and nasopharyngeal carcinomas. Hypermethylation of *TP73* in nasopharyngeal carcinomas has been reported with a frequency of 20%(31). In head and neck squamous cell carcinoma (HNSCC) cell lines, hypermethylation of *TP73* occurred as a primary as well as a disease progression event (17).

Expression analysis by RT-PCR of *TP73* corroborated aberrant methylation in the SCV-UT cell lines in this study. Reduced mRNA expression has been reported in methylated lymphomas where *TP73* abnormalities were mainly found in aggressive tumors with bad response to conventional polychemotherapy suggesting a relation between *TP73* inactivation and the aggressiveness of these tumors(32).

IGSF4, *DAPK1* and *FHIT* were aberrantly methylated in 3 of 13 cell lines with concordant loss of expression for *IGSF4* in UT-SCV-4, which supported promoter hypermethylation of both copies. *IGSF4* is a novel immunoglobulin-like intercellular adhesion molecule first characterized as a tumor suppressor of non-small cell lung cancer and termed *TSLC1* (33,34), where silencing was primarily achieved by allelic loss and promoter methylation. The gene is located at 11q23.2 and encodes a transmembrane glycoprotein of 442 amino acids. *TSLC1* (*IGSF4*) silencing through promoter hypermethylation has been suggested as the major mechanism of epigenetic control in several cancers including non-small cell lung cancer, pancreatic cancer and hepatocellular carcinoma(35).

In esophageal squamous cell carcinoma (ESCC), loss of TSLC1 protein expression as a consequence of promoter hypermethylation, a late stage event in ESCC carcinogenesis, has been implicated in invasion and metastasis and aggressive tumor behavior through the disruption of cell-cell interactions(35). Additionally because expression can be restored by a demethylating agent(35), *TSLC1* may offer a promising new therapeutic target in ESCC.

Promoter hypermethylation of the tumor suppressor gene, *TSLC1*, is also a highly frequent event in cervical cancers where epigenetic silencing of *TSLC1* has been implicated in the

progression from high-risk HPV-containing, high-grade CIN lesions to invasive cervical cancer(36). Furthermore, *TSLC1* silencing was accompanied by complete loss or significant decrease of *TSLC1* mRNA expression in these cell lines. In HNSCC, promoter hypermethylation of *IGSF4* was a primary as well as a disease progression event, indicating complete abrogation of tumor suppressor function(17).

Death-associated protein kinase 1, *DAPK1*, located at 9q34.1 encodes a 160-kDa cytoskeletal-associated calcium/calmodulin-dependent serine/threonine kinase which was initially identified as a positive mediator of interferon γ-induced programmed cell death in HeLa cells (37). *DAPK1* expression is commonly lost in urinary bladder, breast, B-cell neoplasms and renal cell carcinoma cell lines due to promoter hypermethylation.

Aberrant promoter methylation of *DAPK1* has been shown to frequently occur in human head and neck cancers(17,38), non-small cell lung carcinomas(39), gastric and colorectal carcinomas(40,41), and uterine cervical carcinomas(42). In HNSCC, *DAPK1* promoter hypermethylation has been associated with metastasis to lymph nodes as well as advanced disease stage(38). A study of *DAPK1* expression in uterine and ovarian carcinomas showed that aberrant promoter methylation of *DAPK1*, which occurred frequently in gynecological carcinomas, led to decreased DAPK protein expression suggesting that *DAPK1* gene silencing is involved in carcinogenesis of female reproductive organs(43). In bladder cancer, the methylation status of *DAPK1* is an important prognostic factor for recurrence(44).

The histidine triad gene, *FHIT*, is a tumor suppressor gene located at 3p14.2 fragile site and is involved in purine metabolism. Loss of heterozygosity of *FHIT* has been associated with esophageal, stomach, colon carcinomas, lung cancers(45) as well as HNSCC(46). Promoter hypermethylation of *FHIT* in squamous cell carcinomas of the esophagus has been reported to be associated with transcriptional inactivation(47). One study of esophageal squamous cell carcinomas detected hypermethylation of *FHIT* in 50% of tumor cell lines and 45% of primary tumors(48). This same study found that hypermethylation of *FHIT* occurred frequently in clinical stages I and II of esophageal squamous cell carcinomas suggesting that *FHIT* hypermethylation may play a role in early carcinogenesis.

The remaining five genes, *APC*, *CDKN2B*, *VHL*, *ESR1* and *GSTP1*, were less frequently methylated, occurring in 2/13, 1/13, 1/13, 2/13 and 1/13 cell lines respectively. Genetic and epigenetic alterations in *APC* (adenomatosis polyposis coli), a tumor suppressor gene originally implicated in colon cancer have been reported in other malignancies including oral squamous cell carcinomas, gastric cancers and esophageal adenocarcinomas. Uesugi et al(49) previously reported *APC* as being mutated and/or deleted in primary oral squamous cell carcinoma (OSCC) tissues and suggested that loss of *APC* function contributes to carcinogenesis in the oral region. Promoter hypermethylation is also an important mechanism of *APC* inactivation in oral cancers occurring in 25% of OSCC cell(49).

Cyclin-dependent kinase inhibitor 2B (*CDKN2B*), which is also known as *p15*, inhibits CDK4 and regulates cell growth by controlling cell cycle G1 progression. The presence of aberrant methylation of *p15* and *p16* in precancerous oral tissues and lesions of the head and neck(7–9,17,50) implicates methylation of *p15* and *p16* as early events in the pathogenesis of these lesions. In undifferentiated nasopharyngeal carcinoma (NPC), preferential methylation of *CDKN2B* has been shown to be a useful tumor marker for NPC(31).

The von Hippel –Lindau (*VHL*) gene is a tumor suppressor gene located at 3p26-p25 and it is responsible for the Von Hippel-Lindau syndrome which is an inherited familial cancer syndrome that makes patients susceptible to a variety of cancers, malignant and benign. Hypermethylation of *VHL* promoter region in clear-cell renal carcinomas is associated with

absence of transcript expression (51). It was also found that treatment of these methylated *VHL* tumors with a demethylating agent resulted in re-expression of the *VHL* transcripts(51).

Estrogen receptor 1 (*ESR1*), mapping to 6q25.1, is important for hormone binding, DNA binding, and activation of transcription(52). *ESR1* has metastasis-suppressor properties in breast cancer cells(53), suggesting a tumor-suppressor role for *ESR1* (54). In esophageal adenocarcinomas (EAC) abnormal methylation patterns were found in premalignant Barrett's tissue in addition to adenocarcinoma tissue suggesting that DNA hypermethylation is an early epigenetic event in the progression of EAC(55).

Glutathione S-transferase pi (*GSTP1*), mapping to 11q13 (56), encodes for the glutathione S-transferase pi enzyme which plays an important role in detoxification and has a role in the susceptibility to cancer and other diseases. The pi-class of glutathione S-transferase enzymes has been associated with preneoplastic and neoplastic changes(57). Inactivation of *GSTP1* by promoter hypermethylation is characteristic of steroid related neoplasms such as breast, liver, and prostate cancers(57,58).

Changes in copy number can influence gene expression, resulting in overexpression or underexpression of a gene product, depending on the function of the gene. Employing a genome-wide strategy, we recently reported several losses and gains of individual genes in this same13 SCV cell line cohort (1). In this study to examine simultaneously DNA methylation and gene copy number alterations in tumor suppressor genes, frequent genetic alterations of loss and gain of gene copy number included gain of *GSTP1*and *MEN1*, and loss of *MFHAS1* and *IGSF4* in over 50% of the SCV cell lines. The most frequent alteration was gain in copy of *GSTP1* and *MEN1*in 11 of the 13 cell lines (Table 5). Gain of *MEN1*, a tumor suppressor (59) associated with the multiple endocrine neoplasia type 1 syndrome located at chromosome 11q13, and loss of the malignant fibrous histiocytoma amplified sequence 1 (*MFHAS1*) gene, an oncogene located at 8p23.1, most likely reflects chromosomal instability with resultant aneuploid subpopulations. The *MEN1* gene encodes the protein menin (60,61), which interacts with a number of proteins that are involved in transcriptional regulation, genome stability and cell division (62). *MFHAS1* expression is enhanced in some malignant fibrous histiocytomas (MFH) (63). Its product is involved in the interaction of proteins related to the cell cycle (64). *MFHAS1*, also known as *MASL1*, is a target gene for genomic amplification as well as chromosomal translocation (63).

Epigenetic events of promoter hypermethylation were validated with RT- PCR for *TP73* and *IGSF4* genes, where reduced mRNA expression corroborated aberrant methylation status. RT-PCR was not in agreement for unmethylated *IGSF4* and *DAPK1* copy number in UT-SCV-2 and -3 and in UT-SCV-3 and -6, respectively. The latter may be due to heterogeneity reflecting subclonal populations.

MSP of *TP73* confirmed aberrant methylation detected by MS-MLPA for UM-SCV-2, UM-SCV-3, UT-SCV-3, -4 and -6. Furthermore, MSP indicated hypermethylation of *TP73* in UM-SCV-1A, UM-SCV-6 and UT-SCV-2, not detected by MS-MLPA. Lack of confirmation by MSP of *TP73* methylation in UM-SCV-4, detected by MS-MLPA may be primarily due to insufficient amounts of DNA for bisulfite conversion. Repeat bisulfite conversion was not done due to depletion of the DNA sample.

While a distinct advantage of MS-MLPA is the ability to examine aberrant promoter methylation in multiple cancer genes in a single assay run, multiplex PCR of a large number of gene probes (22 unique genes) inherently encounters competitive amplification, in contrast to MSP, which examines only one gene at a time (9), and therefore, is more sensitive than MS-MLPA (9). Additionally, MS-MLPA methylation and quantitation detection algorithms may miss hypermethylation events that do not reach the threshold for detection(17). Regardless,

MS-MLPA profiling of multiple genes for aberrantly methylated promoter regions is a valuable screening tool to determine frequency and pattern of gene inactivation in tumorigenesis. These epigenetic signatures, upon subsequent validation as diagnostic or prognostic epigenetic biomarkers, can become reduced to a more definitive candidate gene panel of only a few key genes. The latter would be amenable for increased detection sensitivity by a targeted 3 or 4 MS-MLPA gene probe panel or by MSP.

UMSCV-1A (primary site) and UM-SCV1B (metastasis) cell lines, derived from the same patient had similar if not identical abnormal karyotypes (11) and were concordant for changes in gene copy number as well (1), indicating that genetic events necessary for distant metastasis had evolved at the primary tumor site. There was concordant lack of methylation for 20 of the 22 tumor suppressor genes in the MLPA probe panel in both cell lines, with aberrant methylation of *TP73* and *DAPK1* in UM-SCV-1A only.

The HPV status of cell lines UM-SCV 1-7 and UT-SCV 1-3, previously published (65), indicated that only UM-SCV-6 cell line was positive for HPV 16. UM-SCV-6 did not demonstrate any aberrant methylation. HPV status of cell lines UT-SCV 4-6 is not known.

Our novel genome wide strategy identified several genes with aberrant promoter hypermethylation and alterations in copy number. Recurrent genomic aberrations are good indicators of genes that are causally associated with cancer development or progression and either become or reveal gene targets for therapy. Frequently methylated genes in this SCV cohort included *TP73, IGSF4, DAPK1* and *FHIT*. These findings suggest that epigenetic events of DNA hypermethylation may contribute to the underlying pathogenesis of squamous cell carcinoma of the vulva. As a frequent and consistent target of aberrant promoter hypermethylation, *TP73* may serve as a therapeutic biomarker for gene reactivation studies in SCV.

Promoter methylation-mediated silencing is a hallmark of many established tumor suppressor genes. An important distinction between genetic and epigenetic changes in cancer is that the latter might be more easily reversed using demethylating therapeutic interventions. Because gene silencing, as a consequence of promoter hypermethylation can be partially relieved by demethylation of the promoter region(66,67), the molecules that regulate methylation status of DNA are considered promising targets for new cancer therapies. Consistent and SCV-specific epigenetic alterations would signify important biomarkers relevant to the diagnosis, prognosis, and treatment of squamous cell carcinoma of the vulva.

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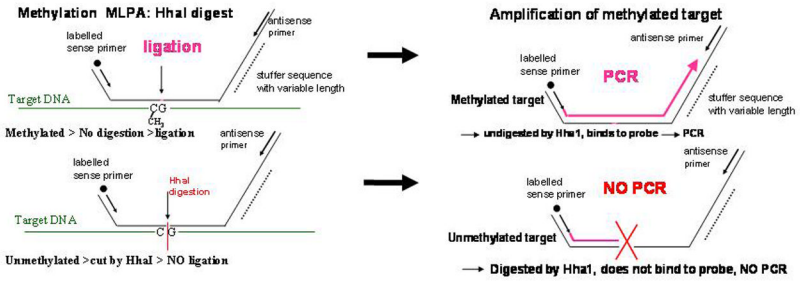


Figure 1.
(17). Methylation Specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA).

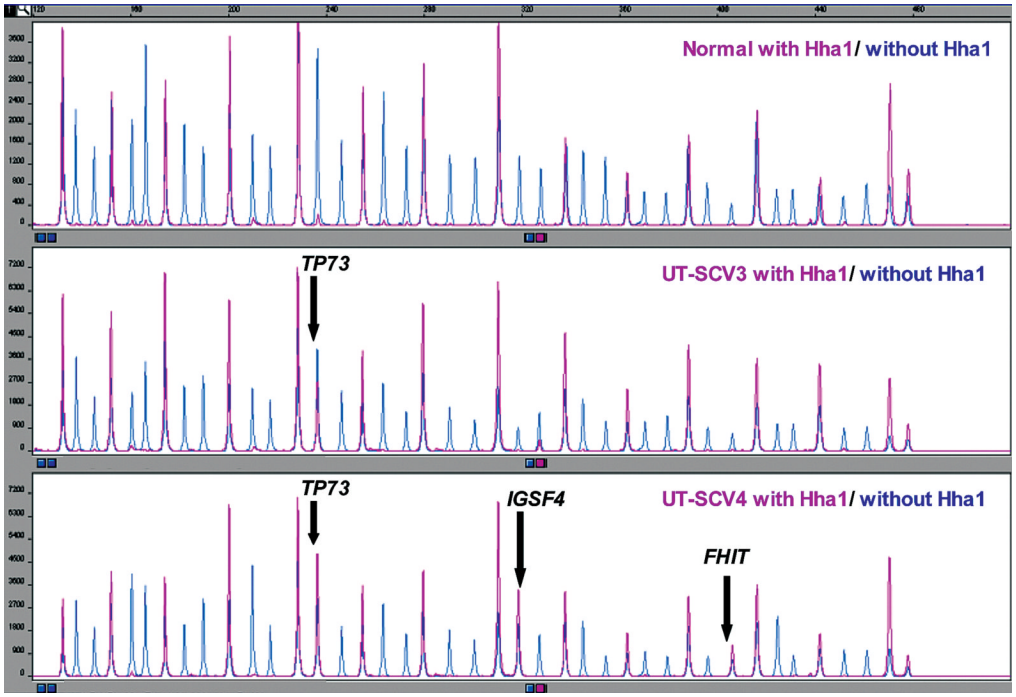


Figure 2.
Normal (control), UT-SCV-3 and UT-SCV-4 MS-MLPA assay results of MS-MLPA probe mix with and without *Hha*I enzyme. Note methylation of both copies of *TP73* in UT-SCV-3 and 4 and methylation of both copies of *IGSF4* and *FHIT* in UT-SCV-4.

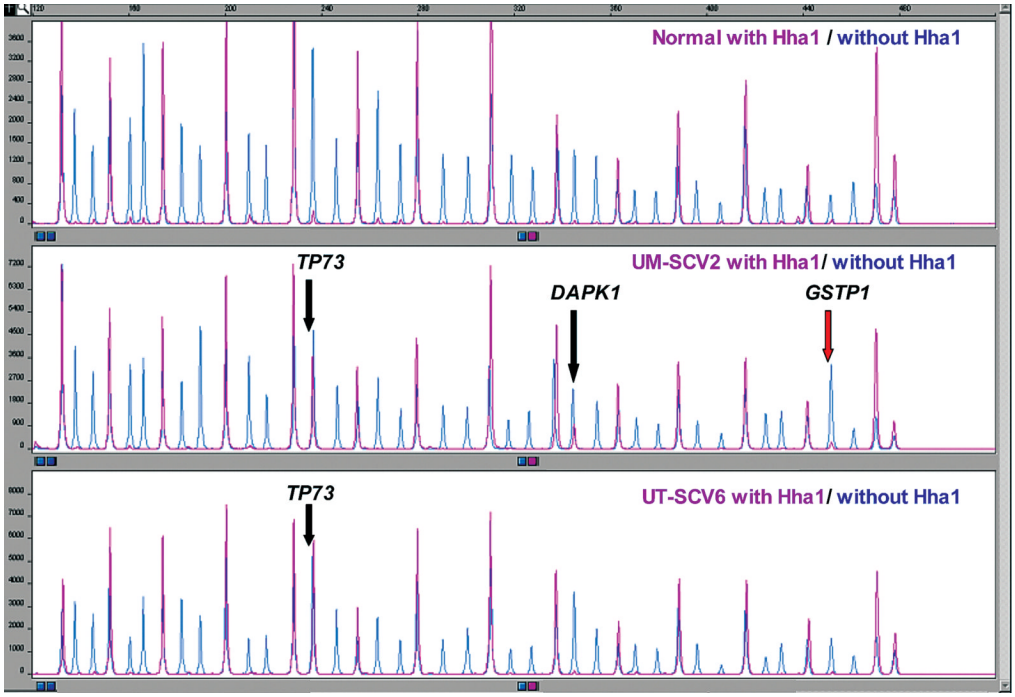


Figure 3.

Normal (control), UM-SCV-2 and UT-SCV-6 MS-MLPA assay results of MS-MLPA probe mix with and without *Hha*I enzyme. Note methylation of both copies of *TP73* in UM-SCV-2 and UT-SCV-6. Methylation of one copy of *DAPK1* and gain of *GSTP1* copy number in UM-SCV-2.

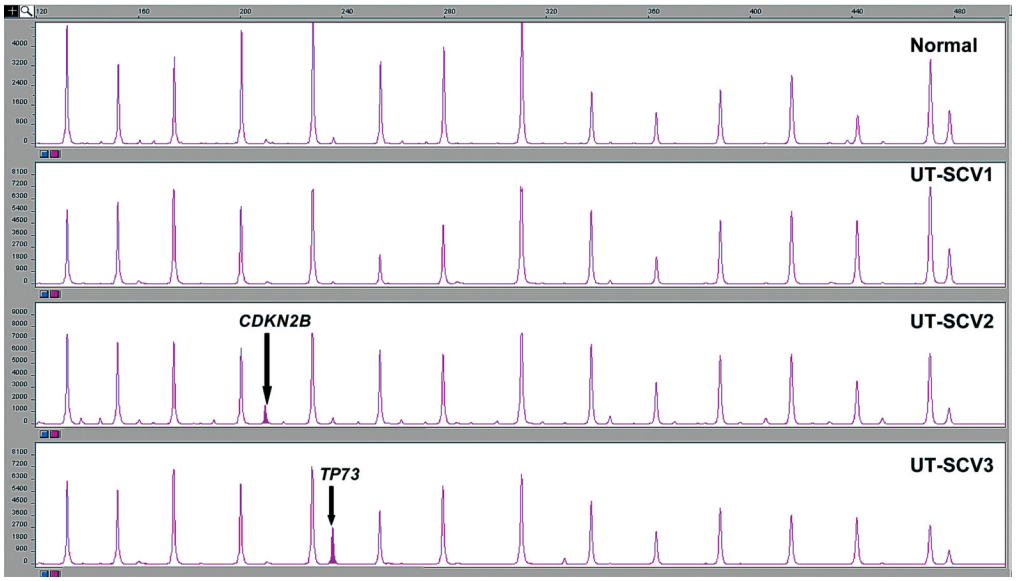


Figure 4.
MS-MLPA assay results showing 15 peaks in the normal (control) with an aberrant methylation peak for *CDKN2B* in UT-SCV-2 and *TP73* in UT-SCV-3.

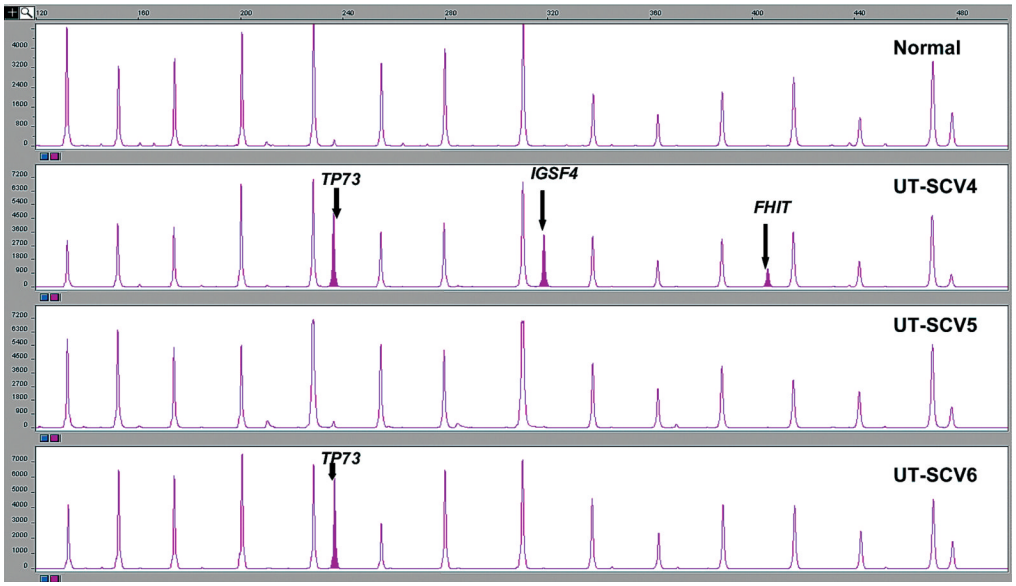


Figure 5.
MS-MLPA assay results showing 15 peaks in the normal (control) with aberrant methylation peaks for *TP73*, *IGSF4 and FHIT* in UT-SCV-4 and *TP73* in UT-SCV-6.

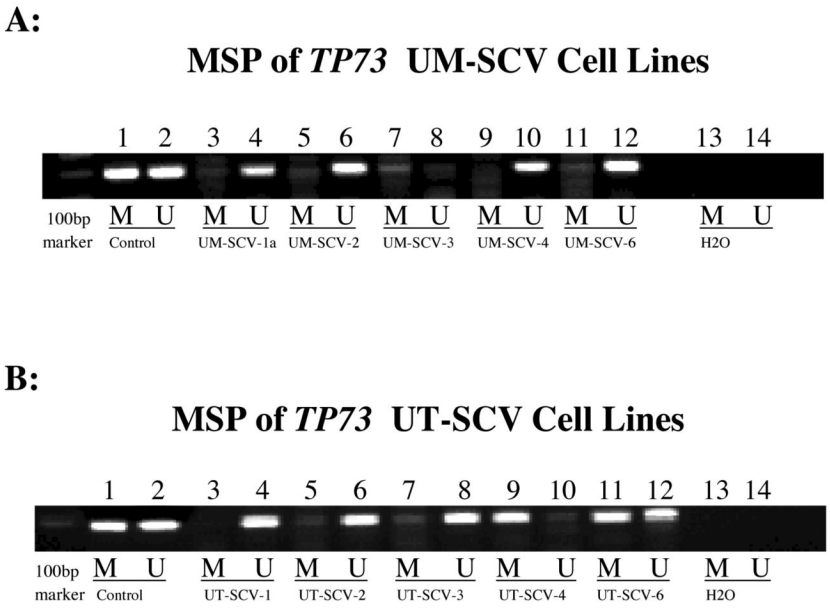


Figure 6.

Methylation Specific PCR (MSP) confirmation of aberrant methylation detected by MS-MLPA for TP73.

Figure 6A: Lanes 1 & 2: universal methylated and unmethylated controls; Lanes 3-12 span UM cell lines 1a, 2-4 and 6. Note presence of methylated product in UM-SCV-1a, -2, -3 and -6. Note absence of methylated product in UM-SCV-4; Lanes 13 & 14: negative control.

Figure 6B: Lanes 1 & 2: universal methylated and unmethylated controls; Lanes 3-12 span UT cell lines 1-4 and 6. Note presence of methylated product in UT-SCV-2, -3, -4, and -6. Note absence of methylated product in UT-SCV-1; Lanes 13 & 14: negative control.

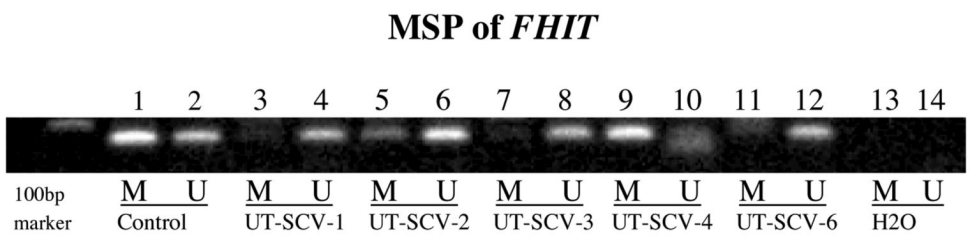


Figure 7.

Methylation Specific PCR (MSP) confirmation of aberrant methylation detected by MS-MLPA for *FHIT*. Lanes 1 & 2: universal methylated and unmethylated controls; Lanes 3-12 span UT cell lines 1-4 and 6. Note presence of methylated product in UT-SCV-2 and UT-SCV-4. Note absence of methylated product in UT-SCV-1, -3 and -6; Lanes 13 & 14: negative control.

Table 1

Patient Clinical and Histopathological Data

Cell line	Age	TNM classification	Grade of the SCC	Prior therapy	Site of the specimen	Survival after initial diagnosis
UM-SCV-1A*	62	T3N2M1	well –poor	none	primary	2 months
UM-SCV-1B*	62	T3N2M1	poor	none	Metastasis	2 months
UM-SCV-2*	86	T3N1M0	poor	surgery	local recurrence	10 months
UM-SCV-3*	66	T2N0M0	mod–well	none	primary	>4, 5 years
UM-SCV-4*	41	T2N2M0	well	none	primary	>4 years
UM-SCV-6*	43	T1N1M0	mod	surgery	primary	15 months
UM-SCV-7*	77	T2N2M0	well –poor	none	primary	1 month
UT-SCV-1*	71	T2N1M0	well	surgery + RT[a]	Metastasis	1 year
UT-SCV-2*	63	T1N0M0	mod	surgery + RT[a] + CT[b]	local recurrence	>13 years
UT-SCV-3*	73	T3N2M0	mod	none	primary	3 months
UT-SCV-4†	31	T2N0M0	mod	none	primary	10 years
UT-SCV-5†	46	T3N2M1	well	none	pleural effusion	5 weeks
UT-SCV-6†	72	T3N2N0	well	none	primary	8 weeks

[a] RT indicates radiotherapy.

[b] CT indicates chemotherapy.

* Raitanen M, et. al. Characterization of 10 vulvar carcinoma cell lines by karyotyping, comparative genomic hybridization and flow cytometry. Gynecol Oncol 2004;93(1):155–63.

† personal communication Dr. Seija Grenman, Turku Central University, Turku, Finland

Table 2

RT-PCR Primer Sequences for SCV

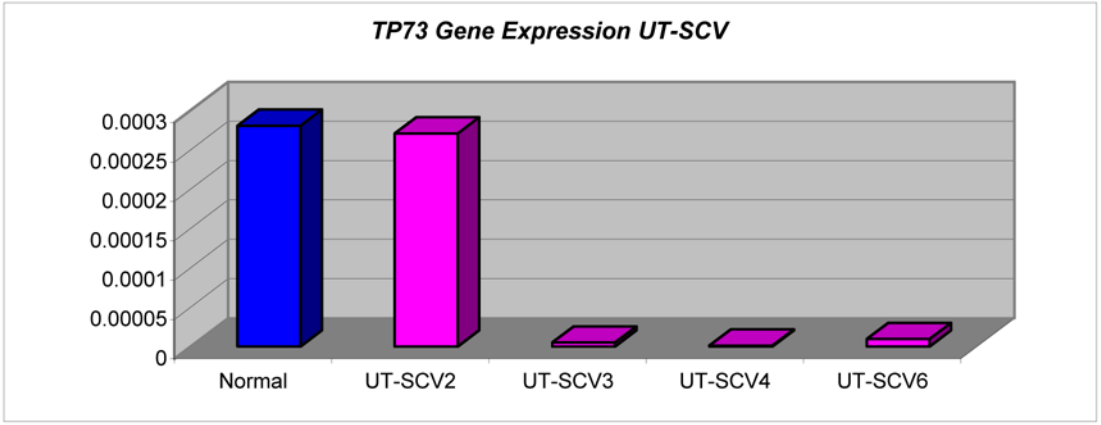
Gene	Forward Primer	Reverse Primer	Product Size
TP73	5′-GGCTGCGACGGCTGCAGAG-3′	5′-GCTCAGCAGATTGAACTGGCCATG-3′	256
IGSF4	Exon 1–2: 5′-GGCTTCTGCTGTTGCTC-3′	Exon 1–2: 5′-CCCTGAAATAAATGGTCTGC-3′	183
DAPK1	Exon 1–2: 5′-TGACAGTTTATCATGACCGTGTTCAG-3′	Exon 1–2: 5′-GTGCTGGATCTCCTTCAGGAT-3′	231

Table 3

RT-PCR results for *TP73*, *IGSF4*, and *DAPK1*

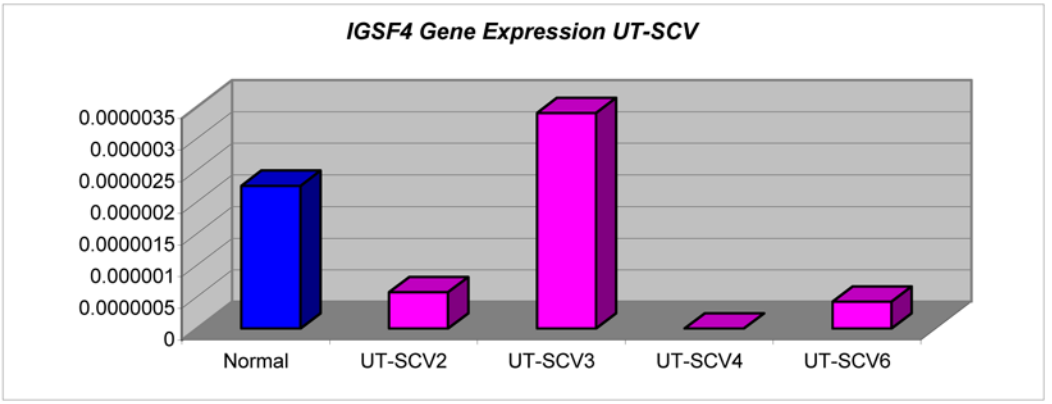
***TP73* Gene expression**

Sample	Ct β-actin	Ct *TP73*	Ct Δ(delta)	$2^{-(Ct\ \Delta)}$
Normal	18.7	30.5	11.8	0.000280444
UT-SCV2	12.49	24.34	11.85	0.000270891
UT-SCV3	10.17	27.7	17.53	5.28377E-06
UT-SCV4	11.9	31.66	19.76	1.12628E-06
UT-SCV6	12.85	29.48	16.63	9.85987E-06



IGSF4 Gene expression

Sample	Ct β-actin	Ct *IGSF4*	Ct Δ(delta)	$2^{-(Ct\ \Delta)}$
Normal	16.18	34.94	18.76	2.25256E-06
UT-SCV2	13.1	33.84	20.74	5.71002E-07
UT-SCV3	11.85	24.09	18.17	3.40244E-06
UT-SCV4	12.63	43.62	30.99	4.689E-10
UT-SCV6	13.87	35.04	21.17	4.23833E-07



DAPK1 Gene expression

***TP73* Gene expression**

Sample	Ct β-actin	Ct *TP73*	Ct Δ(delta)	$2^{-(Ct\ \Delta)}$

Sample	Ct β-actin	Ct *DAPK1*	Ct Δ(delta)	$2^{-(Ct\ \Delta)}$
Normal	16.3	24.95	8.65	0.002489376
UT-SCV2	13.28	20.01	6.73	0.009420374
UT-SCV3	11.87	22	10.13	0.000892414
UT-SCV4	12.81	21.57	8.76	0.002306626
UT-SCV6	14.17	24.54	10.37	0.000755647

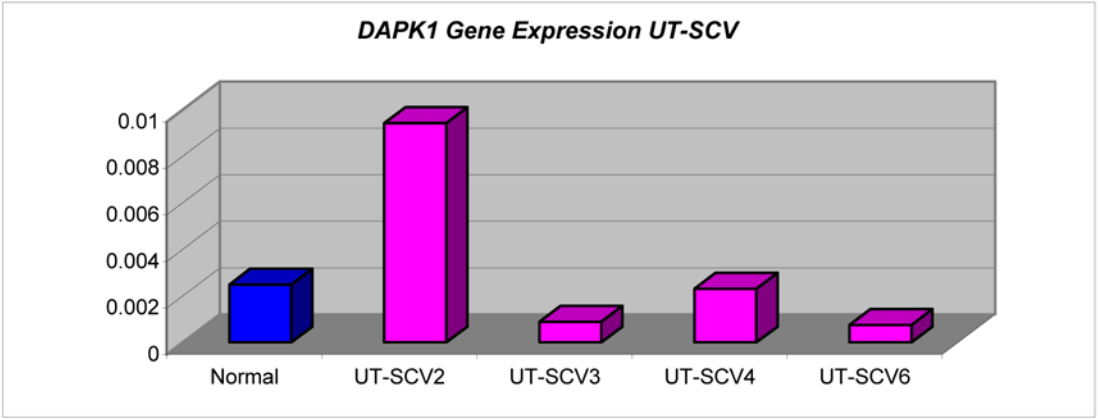


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Table 4

MS-MLPA Probe Panel

#	PCR Product size	Gene probe	Chrom Loc	#	PCR Product size	Gene probe	Chrom Loc	#	PCR Product size	Gene probe	Chrom Loc
1	M-238	*TP73*	01p36		481	*MFHAS1*	08p23.1		310	*RB1*	13q14.3
	472	*NRAS*	01p13.2	12	M-160	*CDKN2A*	09p21		202	*MLH3*	14q24.3
	364	*MSH6*	02p11	13	M-427	*CDKN2A*	09p21		283	*TSC2*	16p13.3
2	M-220	*VHL*	03p26	14	M-211	*CDKN2B*	09p21	21	M-400	*ASC*	16p12
3	M-274	*VHL*	03p26	15	M-346	*DAPK1*	09q34.1	22	M-247	*CDH13*	16q24.2
4	M-265	*FANCD2*	03p26		136	*CREM*	10p12.1	23	M-355	*HIC1*	17p13.3
5	M-166	*MLH1*	03p21.3	16	M-193	*MEN1*	11q13		337	*BRCA1*	17q21
6	M-292	*MLH1*	03p21.3	17	M-454	*GSTP1*	11q13	24	M-436	*BRCA1*	17q21
7	M-463	*MLH1*	03p21.3	18	M-319	*IGSF4*	11q23		256	*BCL2*	18q21.3
8	M-328	*RASSF1*	03p21.3		175	*TNFRSF1A*	12p13		391	*KLK3*	19q13
9	M-409	*FHIT*	03p14.2		445	*TNFRSF7*	12p13	25	M-184	*KLK10*	19q13.3
10	M-148	*APC*	05q21	19	M-382	*CDKN1B*	12q13.1		229	*NF2*	22q12
	154	*IL4*	05q31.1	20	M-301	*BRCA2*	13q12	26	M-142	*TIMP3*	22q12.3
11	M-373	*ESR1*	06q25.1		418	*BRCA2*	13q12.3				

probes with Hha I sites in CpG rich promoter regions

genes represented by more than one gene probe

Table 5

SCV Aberrant gene methylation and copy number

	APC	CDKN2B	VHL	TP73	IGSF4	DAPK1	ESR1	FHIT	GSTP1
UM-SCV-1A	2	2	2	*2	1	n=2; m=1	2	2	2
UM-SCV-1B	2	2	2	2	1	2	3	3	4
UM-SCV-2	2	2	2	n=2; m=2	1	n=2; m=1	2	2	7
UM-SCV-3	n=2; m=2	2	n=2; m=2	n=2; m=2	n=2; m=2	n=2; m=2	n=2; m=2	2	3
UM-SCV-4	2	0	2	n=2; m=2	3	2	2	3	3
UM-SCV-6	2	2	2	*2	1	2	3	2	4
UM-SCV-7	n=2; m=2	3	2	2	n=1; m=1	3	n=2; m=1	N=2; m=2	4
UT-SCV-1	2	2	2	2	2	2	3	3	3
UT-SCV-2	2	n=3; m=1	2	*2	2	3	3	N=2; m=2	n=3; m=1
UT-SCV-3	2	2	2	n=2; m=2	1	2	3	2	2
UT-SCV-4	2	3	2	n=2; m=2	n=2; m=2	2	2	N=2; m=2	3
UT-SCV-5	2	2	2	2	2	2	2	2	3
UT-SCV-6	2	1	2	n=2; m=2	1	3	2	1	3

n = copy number without Hha1; m = methylated copy number after Hha1 digestion detected by MS-MLPA

* = unmethylated by MS-MLPA but methylated by MSP

Table 6

Gene expression by RT-PCR and Copy Number in UT-SCV Cell lines

Sample	*TP73*			*IGSF4*			*DAPK1*		
	Expression	**Copy**	**Methylated**	**Expression**	**Copy**	**Methylated**	**Expression**	**Copy**	**Methylated**
UT-SCV-2	Normal	2	0	L	2	0	H	3	0
UT-SCV-3	L	2	2	H	1	0	L	2	0
UT-SCV-4	L	2	2	absent	2	2	normal	2	0
UT-SCV-6	L	2	2	L	1	0	L	3	0